FMD RESEARCH: BRIDGING THE GAPS WITH NOVEL TOOLS

EDITED BY: Alejandra Victoria Capozzo, Cyril Gerard Gay, Wilna Vosloo and Mariano Pérez-Filgueira

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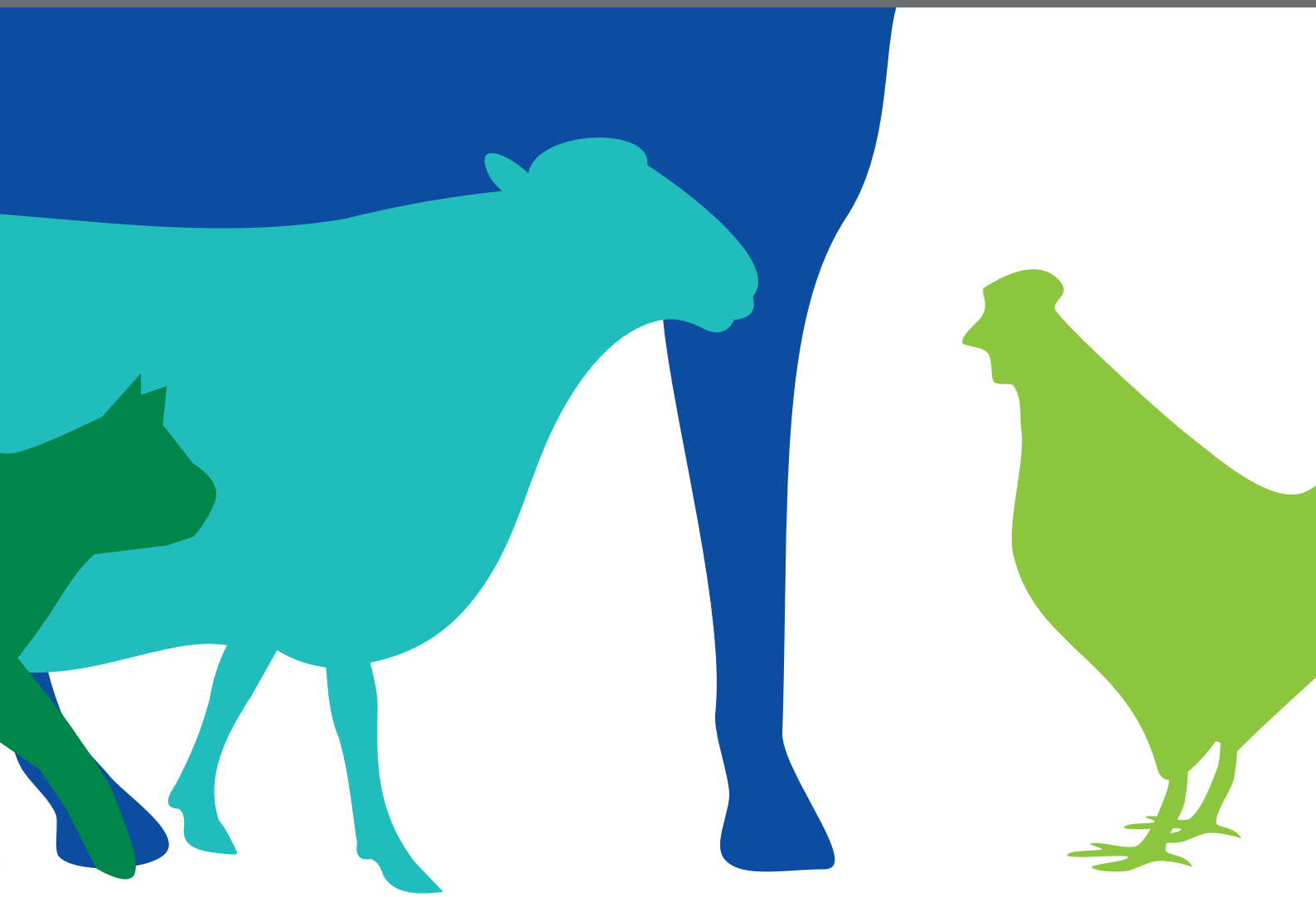

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FMD RESEARCH: BRIDGING THE GAPS WITH NOVEL TOOLS

Topic Editors:
Alejandra Victoria Capozzo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Cyril Gerard Gay, United States Department of Agriculture, United States
Wilna Vosloo, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia
Mariano Pérez-Filgueira, Instituto Nacional de Tecnología Agropecuaria (Argentina), Argentina

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EDITORIAL
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Editorial: FMD Research: Bridging the Gaps With Novel Tools

Alejandra V. Capozzo[1], Mariano Pérez-Filgueira[1], Wilna Vosloo[2] and Cyril G. Gay[3]*

[1] Instituto de Virología e Innovaciones Tecnológicas (IVIT), Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA)- Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina, [2] Australian Centre for Disease Preparedness (Formerly Australian Animal Health Laboratory), Commonwealth Scientific and Industrial Research Organisation (CSIRO)-Health and Biosecurity, Geelong, VIC, Australia, [3] Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, NY, United States

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Editorial on the Research Topic

FMD Research: Bridging the Gaps With Novel Tools

Foot and mouth disease (FMD) remains a major threat for livestock industries, affecting large numbers of cloven-hoofed animal species worldwide with an estimated annual global economic loss of between US$6.5 and 21 billion and ∼US$ 5 billion related to production losses and vaccination alone (1). The devastating effects of FMD affects all countries around the world impacting from smallholders' farms in low-income countries suffering reduced productivity (2), to middle-to-high income countries affected by the cost of prevention, surveillance, and control measures in domestic species as well as the severe restrictions imposed on international trade (3).

FMD's etiological agent is a small non-enveloped positive sense single-stranded RNA virus (FMDV) belonging to the *Picornaviridae* family, genus *Aphthovirus* (4). Considered as one of the most infectious amongst human or animal disease agents known, the virus is recognized for its high antigenic variability and efficient transmission among a wide range of susceptible animal species (5). Although progress in the global control of FMD is ongoing and supported by international organizations such as the World Organisation for Animal Health (OIE) and United Nations Food and Agriculture Organisation, the disease is still endemic in many parts of the world, circulating in over 75% of the global livestock population (2).

Scientists working on FMD research around the world are networking through the Global Foot and Mouth Disease Research Alliance (GFRA—https://www.ars.usda.gov/gfra/). The GFRA continually assesses research gaps and priorities, shares the latest scientific advances, and enables and promotes collaborations and networking among the different laboratories conducting FMD research worldwide. This Research Topic focuses on recent studies that address FMD knowledge gaps, comprising 24 original manuscripts covering priority areas such as diagnostics, field surveillance, evolution, molecular epidemiology, immunopathogenesis, vaccine development, immunology, and antiviral therapy.

Strategies for FMD control vary between regions depending on their epidemiological situation. To manage the risk, it is essential that governments, farmers, veterinarians, and industries engage in significant surveillance, prevention, control, and preparedness programs.

Field surveillance is a critical component of any disease control program. Singanallur et al. demonstrated circulation of FMDV in goat populations in Lao, using two serological tests on a set of samples collected from several provinces and analyze different factors related to seropositivity. Using a different strategy, Ularamu et al. collected tissue samples from 27 outbreaks of FMD in different states of Nigeria to gain more knowledge on FMDV circulation in this country. FMDV isolates obtained were serotyped and further characterized by VP1 sequencing and phylogenetic

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Salome Dürr,
University of Bern, Switzerland

****Correspondence:***
Alejandra V. Capozzo
alejandra_capozzo@yahoo.com.ar

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analysis. Velazquez-Salinas et al. provided a collection of VP (viral protein) 1 and P1 (complete capsid coding region) protein sequences from 29 different districts in Uganda, which combined with geographic information, may be used to perform phylogenetic analyses and antigenic characterization of the FMDV variants circulating in this region. Finally, aiming to improve the serotype-independent FMDV detection, Mishu et al. analyzed the comparative evolutionary divergence of VP2 and VP1 nucleotide sequences to determine the level of conservation in VP2 at different hierarchical levels of three FMDV serotypes (O, A, and Asia1) currently circulating in Asia.

For many years, FMD diagnostic tests have evolved to analyze different aspects of the disease and vaccination, however many of them still need to be assessed and validated for both sensitivity and specificity. Gray et al. analyzed the comparative performance for FMDV isolation between a highly sensitive primary cell culture (BTY) and two continuous cell lines derived from goat (ZZ-R 127) and swine (LFBK-αVβ6). Also, new approaches aim to improve the efficacy of the surveillance programs. A phage display library was explored by Chitray et al. to identify antigenic determinants for recombinant vaccines and for the generation of reagents for improved diagnostic enzyme-linked immunosorbent assays (ELISA) specific for the FMDV serotypes A, Southern-African Territories (SAT) 1 and SAT3. In a different approach, the article by Armson et al. discussed the use of pooled milk and rRT-PCR for active large-scale dairy farm surveillance. With this strategy, the authors provided evidence of subclinical virus infection in vaccinated herds that could be important in the epidemiology of FMD in endemic countries where vaccination is used. Also discussing the implementation of alternative surveillance strategies, Eschbaumer et al. reviewed benefits and limitations of empowering veterinarians to perform rapid diagnostic testing in the field. The need for point-of-care (pen-side) diagnostic test kits, such as lateral flow devices and mobile versions of RT-PCR and RT-LAMP, was also highlighted by Wong et al. in a review that analyzed some of the advances in FMD diagnostic tools.

Simultaneously, novel FMD vaccines and formulations are being developed. A new fully DIVA-compatible vaccine platform is presented, based on highly attenuated virus containing negative antigenic markers in conserved non-structural proteins to enable the differentiation of vaccinated from infected animals (Hardham et al.). Efforts are also moving toward strategies that eliminate growing live FMDV during the manufacturing process of vaccines. This topic includes two approaches: Cañas-Arranz et al., studied the antibody neutralizing and cellular immune responses elicited in swine by synthetic antigens based on dendrimer peptides harboring the major FMDV antigenic B-cell site and a T-cell epitope from 3D polymerase protein, while Mignaqui et al. reported on the optimization of the production strategies for virus like particle (VLP)-based FMD vaccine antigens generated by transient gene expression in mammalian cells. A comparative performance between VLP-based and conventional FMD vaccines on the humoral response and *ex-vivo* activation of dendritic cells in cattle is also presented by Quattrocchi et al.. Finally, Bidart et al. studied the use of particle adjuvants as immunostimulants in experimental vaccines formulated with inactivated whole FMDV antigens in mice and cattle.

In direct relation with the generation of novel prophylactic and therapeutic tools, fundamental studies on the immunological responses to the infection and vaccination can provide scientific bases for such developments. Marrero Diaz De Villegas et al. analyzed the feasibility of using computational methods based on the side chain optimizations to predict neutralizing interactions between antibodies and FMDV antigenic sites. Also using genetic and structural data, Maake et al. predicted naturally occurring amino acid positions that correlate with antigenic changes among different FMDV SAT3 isolates, previously characterized by their *in vitro* cross-neutralizing capacity against a reference serum. The mechanisms of induction of innate immune responses by non-coding synthetic RNA mimicking structural domains in the FMDV genome was also analyzed by Rodriguez-Pulido et al. using a cell line derived from wild boar lung cells. In the same way, Medina et al. reviewed the use of interferon-based biotherapeutics to boost the innate immunity and block FMDV replication in natural hosts.

In close relation with the immunity induced in natural hosts after infection, fundamental studies on the FMDV pathogenesis are paramount to design new intervention strategies and evaluate actual risks related to managing and trading of animals and animal-derived products. Muthukrishnan et al. provided novel information on the FMD pathogenesis and humoral immune responses elicited in small ruminants after experimental infection with a serotype O virus strain. Zhu et al. presented new information on the differential expression in a set of bovine genes previously identified in transcriptomic studies performed on nasopharyngeal tissue samples from FMDV carriers and non-carriers. Viral persistence in cattle and buffalos was also investigated by Bertram et al. by developing statistical models to describe the extinction of FMDV persistent infection using data from primary longitudinal studies of naturally infected animals. A mathematical model is presented by Cabezas et al. to estimate the potential FMDV transmission in cattle, according to the livestock production methods in the US. Dekker et al. also used mathematical modeling, applied to previous *in vivo* experiments, to quantify the effect of vaccination and physical distancing on the FMDV transmission in pigs. Also working in swine, Stenfeldt et al. produced experimental data on infection associated with FMDV-infected pigs, showing results on the *in vivo* transmission between infected and naïve animals, and the persistence of FMDV infectivity in refrigerated carcasses.

This wide collection of original manuscripts undoubtedly contributes to the understanding of the disease and the development of suitable tools and methods for FMD control. However, there are still research gaps that need to be fulfilled to provide the knowledge and tools required to advance the progressive global control of FMD. It is noteworthy that no research papers related to vaccine selection and protection against heterologous FMDV strains were submitted. This subject, together with basic immunity, constitute one of the areas where there is still room for improvement. More work is needed to expand our understanding of vaccine cross-protection, especially in heterologous systems, information that is critical to select

suitable vaccines to respond to FMD outbreaks and in the designing of antigen and vaccine banks.

The eradication of FMD is complex and must involve the integration of new approaches as control strategies. Successful health management policies to contain and eradicate FMD must combine diverse intervention and outbreak mitigation approaches. In this context, GFRA will continue promoting multidisciplinary scientific research among its partners aiming to develop comprehensive responses to the numerous challenges still posed by FMD at the global scale.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA REPORT
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Genetic Diversity of Circulating Foot and Mouth Disease Virus in Uganda Cross-Sectional Study During 2014–2017

Lauro Velazquez-Salinas[1,2], Frank Norbert Mwiine[3†], Zaheer Ahmed[1], Sylvester Ochwo[3], Anna Munsey[2], Julius J. Lutwama[4], Andres M. Perez[2], Kimberly VanderWaal[2] and Elizabeth Rieder[1*]*

[1] Foreign Animal Disease Research Unit, USDA/ARS Plum Island Animal Disease Center, Greenport, NY, United States, [2] College of Veterinary Medicine, University of Minnesota, Minnesota, MN, United States, [3] College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Kampala, Uganda, [4] Department of Emerging and Re-emerging Diseases, Uganda Virus Research Institute, Entebbe, Uganda

Keywords: FMDV, VP1, Uganda, Cattle, Sequences

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Mariano Pérez-Filgueira, National Agricultural Technology Institute (Argentina), Argentina

Reviewed by:
Shankar Yadav, European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy
Brianna R. Beechler, Oregon State University, United States

***Correspondence:**
Lauro Velazquez-Salinas
lauro.velazquez@usda.gov
Elizabeth Rieder
elizabeth.rieder@usda.gov

†ORCID:
Frank Norbert Mwiine
orcid.org/0000-0002-8434-4710

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INTRODUCTION

Foot and mouth disease (FMD) is one of the most economically devastating animal diseases, threatening the livestock industry around the world (1). FMD is caused by foot and mouth disease virus (FMDV), an RNA virus in the *Picornaviridae* viral family, genus *Aphthovirus*, from which seven different serotypes have been described (A, O, C, Asia 1, SAT 1, SAT 2, and SAT 3) (2). The existence of multiple topotypes and the lack of cross protection between serotypes are just some of the factors limiting the control and eradication of FMDV (3). Thus, it is imperative to continuously characterize FDMV genetic diversity in affected countries.

In Uganda, factors like uncontrolled animal movments, the existence of wildlife reservors, and poor vaccine performance have created conditions for FMDV to maintain endemicity since it was first reported there in 1953 (4–6). In terms of genetic diversity, recent reports demonstrate the presence of at least five out of the seven serotypes (A, O, SAT 1, SAT 2, and SAT 3) and multiple topotypes, affecting livestock across the country (4, 7–10). Historically, FMDV O has been one of the most prevalent serotypes in Uganda, the most recently report indicates the circulation of at least five different lineages (11).

In this context, the implementation of quarantines and vaccination programs have failed to control FMD in this country (12). Reports indicate that FMD clinical cases increased in Uganda during the 2000's relative to the 1990's (13). A recently risk analysis study showed the complexity involving the epidemiology of FMD in Uganda, being the proximity with international borders one of the most important factors associated with the circulation of FMDV in this country (14). Based on the sanitary conditions in east Africa, officially the export and import trade activities of livestock in Uganda is limited (1.5% all export values), but should be taken into account as a potential factor to favor the circulation of FMDV in the region, being Burundi, Democratic Republic of Congo, Kenya, Rwanda, Southern Sudan and Tanzania the major export markets (http://www.fao.org/3/a-at589e.pdf). The rapid evolution of FMDV in Uganda might be explained by a combination of evolutionary mechanisms characteristic of RNA viruses (recombination, positive, and negative selection, and random drift constraints), which all shape the quasispecies dynamics of endemic populations, thereby increasing the ability of this virus to rapidly adapt to different conditions in nature (15, 16). In this context, the continuous genetic characterization of circulating FMDV variants could support the development of more effective control strategies in this country (13).

Herein, we are reporting the availability of a valuable collection of a VP1 and P1 (complete capsid coding) protein coding region sequences in the GenBank database, representing the genetic diversity of FMDV from 29 districts representing different geographical regions in Uganda between 2014 and 2017 (**Supplementary File 1**).

The VP1 protein coding region is the genetic marker typically used to perform phylogenetic analyses and to group FMDV into specific genotypes, also referred as topotypes (17). The VP1 protein contains relevant antibody neutralizing sites and T and B-cell epitopes which have been the subject of multiple studies aimed at understanding the evolution of FMDV in response to immunological pressures (18–22)

METHODS

Esophageal-pharyngeal ("Probang") sampling was part of a cross sectional study conducted in cattle herds in Uganda between 2014 and 2017 during a multidisciplinary research project supported by the Cooperative Biological Engagement Program of the U.S. Department of Defense Threat Reduction Agency, Defense Threat Reduction Agency. The research was conducted by experts from Plum Island Animal Disease Center (PIADC), University of Minnesota in the United States, University of Makerere, and the Virus Research Institute in Uganda.

After collection, probang samples were snap-frozen, and stored at −70°C at University of Makerere, until samples were sent to PIADC for testing. Sequencing work was conducted at PIADC in the United States. All viral sequences were obtained from viral isolations on cell monolayers of LFPKαVβ6 (one passage) (23). Isolates were from oropharyngeal fluid samples (probang samples) collected from naturally infected FMD cattle herds in Uganda between 2014 and 2017. (For more details about the location of each isolate see **Supplementary File 1**).

Viral RNA was isolated from cell culture supernatants using the RNeasy MiniKit (QIAGEN) and sequencing work was performed by the Sanger method following a protocol previously described, which includes the use of universal FMDV primers (24). Final consensus VP1 coding region sequences were obtained using Sequencher v4.8 (Gene codes, Ann Arbor, MI, USA). Based on the nucleotide homology, different sequences were classified into specific serotypes using the Blastin algoritm (25). Based on the nucleotide variability, for some of the viral isolations, the entire P1 coding region was obtained using a methodology previously described (26).

The viral sequence collection reported here is currently being analyzed in combination with sequences previously reported in East Africa in order to establish the phylogenetic relationships of recent viral lineages in this region. The aim of our work is to support the Ugandan authorities for the development of a risk-based approach to mitigate the impact of FMD in this country. Interestingly, for more than 25 years, Ugandan authorities have used a trivalent FMD vaccine containing serotypes O, SAT 1, and SAT 2, which is manufactured in Kenya (KEVEVAPI) (10). Information on the quality and potency of the vaccine is not available. Additionally, the vaccine is manufactured with fairly historic viral strains (GenBank access: *O* = K77/78; HM756588, SAT1 = T155/71; HQ267519, and SAT2 = K52/84; HM623685).

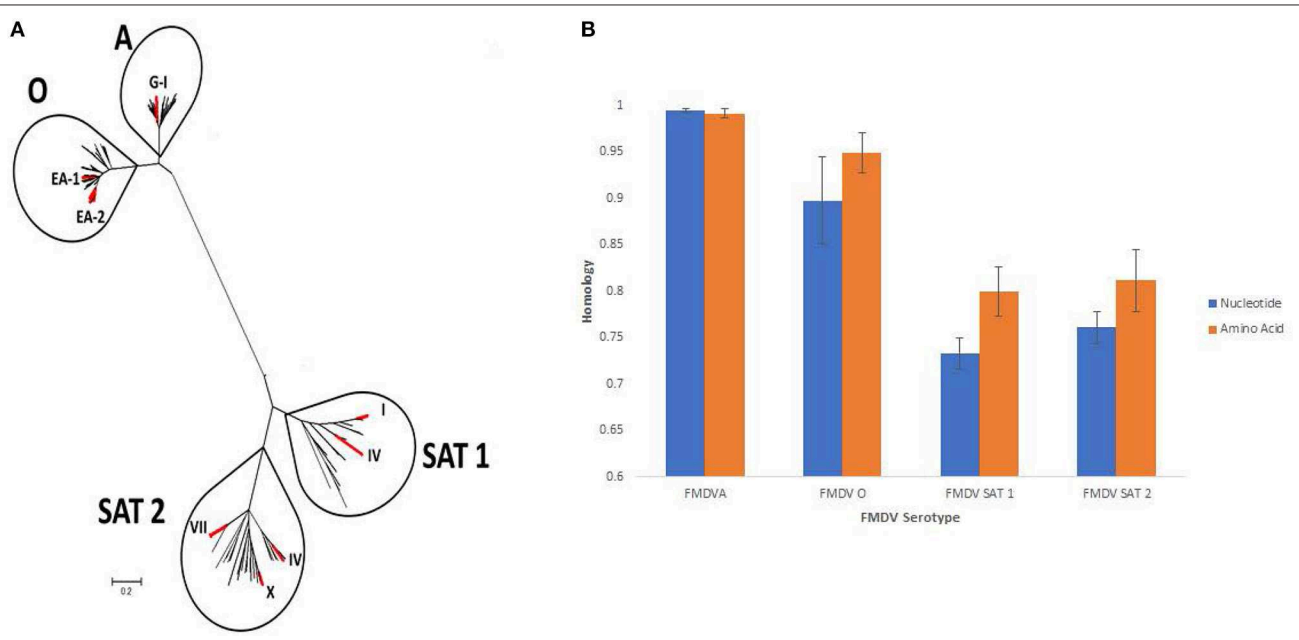

FIGURE 1 | FMDV sequencing dataset from Uganda (2014–2017). **(A)** phylogenetic analysis conducted by maximum likelihood method, showing the genetic diversity of the FMDV sequencing dataset reported in this article. Multiple reference sequences from each serotype previously described by Knowles et al. (2) were included for this analysis. Branches in red represents specific topotypes associated with the sequences reported in this database. **(B)** Homology from each serotype at nucleotide and amino acid levels was deduced by pairwise distance analysis. In case of serotypes 0, SAT 1, and SAT 2 pairwise distance was calculated between different topotypes, thus explaining the disparate amino acid homology displayed between these serotypes. Analysis were conducted on the software MEGA 10.0.5.

The serotype O strain included in the vaccine is characterized as topotype EA-1, however recent reports have demonstrated inefficacy of the vaccine against FMDV serotype O, topotype East Africa two (EA-2), one of the most prevalent genetic lineages in Uganda (13, 27). In this context, our collection of viral sequences might support the selection of potential vaccine candidate strains to reformulate the current trivalent vaccine, and ultimately improve FMD control strategies in Uganda.

Furthermore, since very little is known about the evolutionary dynamics of different serotypes circulating in Uganda, this sequence collection is currently being used to identify specific sites in the capsid protein evolving under positive selection using a codon-based phylogenetic framework (28).

These results will help to choose appropriate viral lineages to support further work by next generation sequencing, which will increase our understanding about the contribution of different viral proteins in the evolution of different viral lineages in Uganda. Also, these extensive collection of viral sequences will represent an important reference for future phylogenetic analyses conducted in Uganda.

Collectively, the purpose of this report is to announce the availability of this sequence dataset, which represents the genetic variability of FMDV in Uganda during 2014–2017, in public databases. The entire VP1 sequence datset collection from this project comprises a total of 258 sequences including serotypes A ($n = 4$) (topotype G-I), O ($n = 148$) (topotypes EA-1 and EA-2), SAT 1 ($n = 70$) (topotypes I and IV), and SAT 2 ($n = 36$) (topotypes IV, VII, and X). Information about the genetic diversity and homology at nucleotide and amino acid levels among the sequences within each serotipe contained in this data set is shown in **Figures 1A,B**, respectively. However, part of the collection ($n = 117$) was already used for initial phylogenetic analysis, and these sequences were reported elsewhere (11). To avoid possible duplications, here we are reporting the remaining sequences, comprised of 141 previously unpublished VP1 sequences representing serotypes O ($n = 102$) and SAT 1 ($n = 50$), as well as a total of 36 P1 sequences including serotypes O ($n = 30$) and SAT 2 ($n = 6$).

Genbank accession numbers and corresponding sequences are available in **Supplementary File 1**.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the the Accession Genbank; number information for the sequence dataset collection is detailed in the **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Commitee of the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Kampala, Uganda. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ER, FM, JL, KV, and AP conceived the study. ER, FM, and JL obtained funding. LV-S and ZA performed virus sequencing/genomic analysis. AM and KV perform data interpretation. FM and JL performed sampling activities. LV-S and ER wrote the manuscript. All authors read and approved the manuscript content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00162/full#supplementary-material

Supplementary File 1 | Accession Genbank number information form the entire VP1 and P1 sequence dataset collection representing the FMDV genetic diversity in Uganda between 2014 and 2017. Rows colored in green represent unreported sequences, while colored in blue represent sequences used for phylogenetic analysis reported in Mwiine et al. (11). Codes for vaccination status are represented by *N* = non-vaccinated, *Y* = vaccinated, and *U* = unknown. It is important to consider that majority of the sequences come from viral isolations conducted on cattle vaccinated most likely after being exposed to FMDV. To visualize potential FMDV serotype or topotype affinity, information was stratified by breed.

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Extinction Dynamics of the Foot-and-Mouth Disease Virus Carrier State Under Natural Conditions

Miranda R. Bertram[1,2], *Shankar Yadav*[1,2,3], *Carolina Stenfeldt*[4], *Amy Delgado*[3]* and *Jonathan Arzt*[1]*

[1] Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, ARS, USDA, Orient Point, NY, United States,
[2] Oak Ridge Institute for Science and Education, PIADC Research Participation Program, Oak Ridge, TN, United States,
[3] Monitoring and Modeling, Center for Epidemiology and Animal Health, APHIS, USDA, Fort Collins, CO, United States,
[4] Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, United States

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***Correspondence:**
Amy Delgado
amy.h.delgado@usda.gov
Jonathan Arzt
Jonathan.Arzt@ars.usda.gov

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Foot-and-mouth disease (FMD) is one of the most economically important livestock diseases worldwide. Following the clinical phase of FMD, a large proportion of ruminants remain persistently infected for extended periods. Although extinction of this carrier state occurs continuously at the animal and population levels, studies vary widely in their estimates of the duration of persistent infection. There is a need for robust statistical models to capture the dynamics of persistent infection for the sake of guiding FMD control and trade policies. The goal of the current study was to develop and assess statistical models to describe the extinction of FMD virus (FMDV) persistent infection using data from primary longitudinal studies of naturally infected cattle and Asian buffalo in Vietnam and India. Specifically, accelerated failure time (AFT) models and generalized linear mixed models (GLMM) were developed to predict the probability of persistent infection in seropositive animals and identified carriers at the individual animal level at sequential time points after outbreaks. The primary studies were analyzed by country and combined using an individual-participant data meta-analysis approach. The models estimated similar trends in the duration of persistent infection for the study/species groups included in the analyses, however the significance of the trends differed between the models. The overall probabilities of persistent infection were similar as predicted by the AFT and GLMM models: 6 months: 99% (AFT) /80% (GLMM), 12 months: 51% (AFT) /32% (GLMM), 18 months: 6% (AFT) /5% (GLMM), 24 months: 0.8% (AFT) /0.6% (GLMM). These models utilizing diverse and robust data sets predict higher probabilities of persistence than previously published, suggesting greater endurance of carriers subsequent to an outbreak. This study demonstrates the utility of statistical models to investigate the dynamics of persistent infection and the importance of large datasets, which can be achieved by combining data from several smaller studies in meta-analyses. Results of this study enhance current knowledge of the FMDV carrier state and may inform policy decisions regarding FMDV persistent infection.

Keywords: foot-and-mouth disease, FMDV, persistent infection, extinction dynamics, Vietnam, India, meta-analysis, carrier

INTRODUCTION

Foot-and-mouth disease virus (FMDV; *Aphthovirus*, *Picornaviridae*) is the causative agent of foot-and-mouth disease (FMD), one of the most economically important diseases of livestock worldwide. Classical FMD is characterized by fever, loss of appetite, and formation of characteristic vesicles on feet, udders, and in the oral cavity (1, 2). Although mortality is usually low, the high morbidity has an important economic impact due to decreased production, regional quarantine practices, and trade restrictions (3, 4). The existence of prolonged asymptomatic persistent infection (carrier state) in ruminants has practical implications in FMDV-endemic regions that are distinct from management practices in regions striving to regain FMD-free status after an outbreak (5). Appropriate practices for management of carriers have not been established in either context.

Subsequent to acute infection, a substantial proportion of infected ruminants become persistently infected, which has traditionally been defined by detection of FMDV in oropharyngeal fluid (OPF) 28 days or more after infection (6, 7). However, more recent studies have indicated that persistently infected animals can be identified as early as 10 days post-infection (8). Vaccination with a homologous virus strain protects against clinical disease, but does not prevent subclinical or persistent infection (8–10). The virus persists in the epithelium of the nasopharynx (8, 11) or associated lymphoid tissue (12) of cattle and buffalo. Transmission from persistently infected cattle to naïve animals via direct contact has not been demonstrated (10, 13, 14), however deposition of oropharyngeal fluid from persistently infected cattle into the nasopharynx of naïve cattle has been demonstrated to cause disease (15). Transmission via direct contact in the persistent phase has only been demonstrated to occur from African Cape buffalo (*Syncerus caffer*) (16, 17), and the role of persistently infected animals in FMDV epidemiology remains unclear. However, concerns over the potential risk of transmission from persistently infected animals have prompted authorities to implement trade restrictions for animals and animal products for extended periods following FMD outbreaks and from FMD-endemic regions (18).

FMDV is reported to persist for up to 2 years in cattle (14, 19, 20), 5–12 months in sheep and goats (21), and up to 5 years in African buffalo (22). However, the virus is cleared from carriers at variable times by mechanisms which have been described (23, 24). The rate of decrease in the proportion of persistently infected animals has been reported as 0.03–0.11 per month (13, 14, 19). A meta-analysis of experimental studies reported that most infected cattle clear the infection within 6 months (13); however recent field studies indicated that approximately half of infected cattle remain persistently infected 12 months after infection (19), and some cattle maintain persistent infection for more than 24 months (14). The variability among distinct studies and analytical approaches impedes development of effective control measures to account for FMDV persistent infection. Most importantly there is a need for robust methods to describe and predict the duration of persistent infection at the individual and population levels.

Recent longitudinal field studies have utilized survival analysis to describe the dynamics of extinction of persistent infection in cattle under natural endemic conditions (14, 19). These studies demonstrated gradual clearing of the infection over time at the population level; however, they did not predict the probability of persistent infection at specific time points in the study populations. A recent analytical approach proposed defining extinction of persistent infection based on a probability function to better reflect the dynamic state of persistent infection (25). These authors used cross-sectional data to develop a statistical model to estimate the probability of persistent infection based on an animal's age, whether the animal had antibodies against FMDV, and the time since the most recent outbreak in the herd. This approach to predicting the probability of the presence of persistently infected animals in a herd at a defined time(s) following an outbreak may be beneficial for developing FMD control policies. However, the very low probability (0.7%) of persistent infection across all animals more than 12 months after an outbreak reported in that study is inconsistent with the data from recent longitudinal field studies of FMDV-infected animals (14, 19).

Longitudinal studies offer the advantage of directly observing the dynamics of persistent infection in individuals over time. Disadvantages of longitudinal studies are that they are highly labor-intensive, time-consuming, expensive, and logistically challenging under field conditions in endemic regions. In contrast, cross-sectional studies often have larger sample sizes and can be completed more rapidly and economically than longitudinal studies. However, it is unclear whether cross-sectional data is appropriate for modeling the dynamics of persistent infection. Alternatively, meta-analyses could be used to mitigate some of the challenges of small sample sizes by combining data across several longitudinal studies (26). Meta-analysis approaches incorporating data from multiple studies have the additional advantage of incorporating diverse field conditions (viral strain, host factors, husbandry, environmental factors) into a more holistic output.

The goals of the current study were to assess the utility of two distinct statistical models for predicting the probability of persistent FMDV infection post-outbreak at the individual animal level, and to compare different analytical methods to assess extinction of the carrier state. The current study incorporated and analyzed three primary longitudinal studies of FMDV persistent infection in Vietnam and India using both generalized linear mixed models and accelerated failure time models to predict the probability of persistent infection in cattle and Asian buffalo (*Bubalus bubalis*) at various times following an FMD outbreak. Additionally, data from all three studies were combined using an individual participant data meta-analysis approach (26) to further assess the dynamics of persistent infection across the three study populations. Results of this study will help to inform FMD surveillance and control efforts in Vietnam, India, and other FMD-endemic countries as well as FMD-free countries, and will help to inform policy decisions concerning FMDV persistent infection.

METHODS

To qualify for inclusion in these analyses, studies had to take place following a natural outbreak in an FMD-endemic country, OPF samples had to be collected from the same individual animals at least twice, and sampling had to occur 28 days or more post-outbreak. Additionally, raw data had to be available for each animal. Three studies from our laboratory met the inclusion criteria—one in Vietnam and two in India. The primary datasets incorporated in the analyses herein were derived within the scope of long-term, longitudinal projects on endemic FMD in India and Vietnam between 2010 and 2015 (14, 19, 27–29). The included studies are described below. For the current study, persistent infection was defined as the detection of FMDV RNA in OPF.

Ethics Approval

The work described herein was performed by federal staff of the Department of Animal Health, Ministry of Agriculture, and Rural Development, Government of Vietnam or the Directorate of Foot and Mouth Disease, Indian Council of Agricultural Research, Ministry of Agriculture, Government of India. The work occurred and the animals were maintained within facilities that were owned, maintained, or overseen by these divisions of the federal governments; thus, no permits or approvals were required. All cases described herein occurred spontaneously in domestic cattle or buffalo with no experimentation, inoculation, or treatment of live animals.

Vietnam

Study Description

Cattle and Asian buffalo were sampled as part of a targeted surveillance study in areas with a recent history of FMD outbreaks in Long An and Son La provinces in Vietnam as previously described (27, 29). Briefly, serum samples were collected in March 2012, and subsequently oropharyngeal fluid (OPF) samples were collected using a probang cup (30) from 323 animals that were seropositive for FMDV anti-NSP antibodies using a 3ABC ELISA kit (PrioCheckR, Prionics, Netherlands). OPF samples were collected every 1–2 months between April—October 2012 for up to 4 samples per animal (**Figure 1**). OPF samples were analyzed by real-time reverse transcription PCR (rRT-PCR) targeting the 3D region of the FMDV genome as previously described (31). Briefly, RNA was extracted using the MagMax Viral RNA Isolation Kit (Ambion), and extracted RNA was subjected to rRT-PCR using a previously described probe (32) and primers (33). As previously reported, 10.8% of seropositive animals were carriers, based on FMDV RNA detection in OPF, and beef cattle were more likely to be carriers than buffalo or dairy cattle (27). A subset of 155 cattle and 49 buffalo from 93 herds for which the owner reported the animal's history of clinical FMD (yes or no) were analyzed in the current study. Vaccination status was reported for ∼70% of animals, a majority (97%) of which had been previously vaccinated. However, the date of vaccination was not available for most animals, and the effect of vaccination on the duration of persistent infection could not be evaluated in this study.

FMD Outbreak History

For animals for which the owner reported a history of clinical FMD, the year of infection was reported by the owner. The date (month and year) of the outbreak which occurred in the Commune of the herd during the reported year of infection was retrieved from records of the Department of Animal Health (DAH), Vietnam. The midpoint of the month was used as the outbreak date in subsequent analyses. For animals for which the owner reported no history of clinical FMD, the outbreak date reported for other animals in the herd was used as the outbreak date. If no previous FMD infection was reported in the herd, the date of the most recent outbreak in the Commune prior to sampling was used as the outbreak date. For the purposes of this study, it was assumed that animals were infected during the reported outbreak and no reintroduction or subclinical circulation of the virus occurred on farms included in the study. This assumption was based upon documentation by farm-level questionnaire and by reviewing official records of the Department of Animal Health that no cases of FMD were observed or reported in the intervening period. The virus strain was not identified for all animals; however, all carrier animals from which sequence data were obtained were infected with strains from lineage O/ME-SA/PanAsia (27).

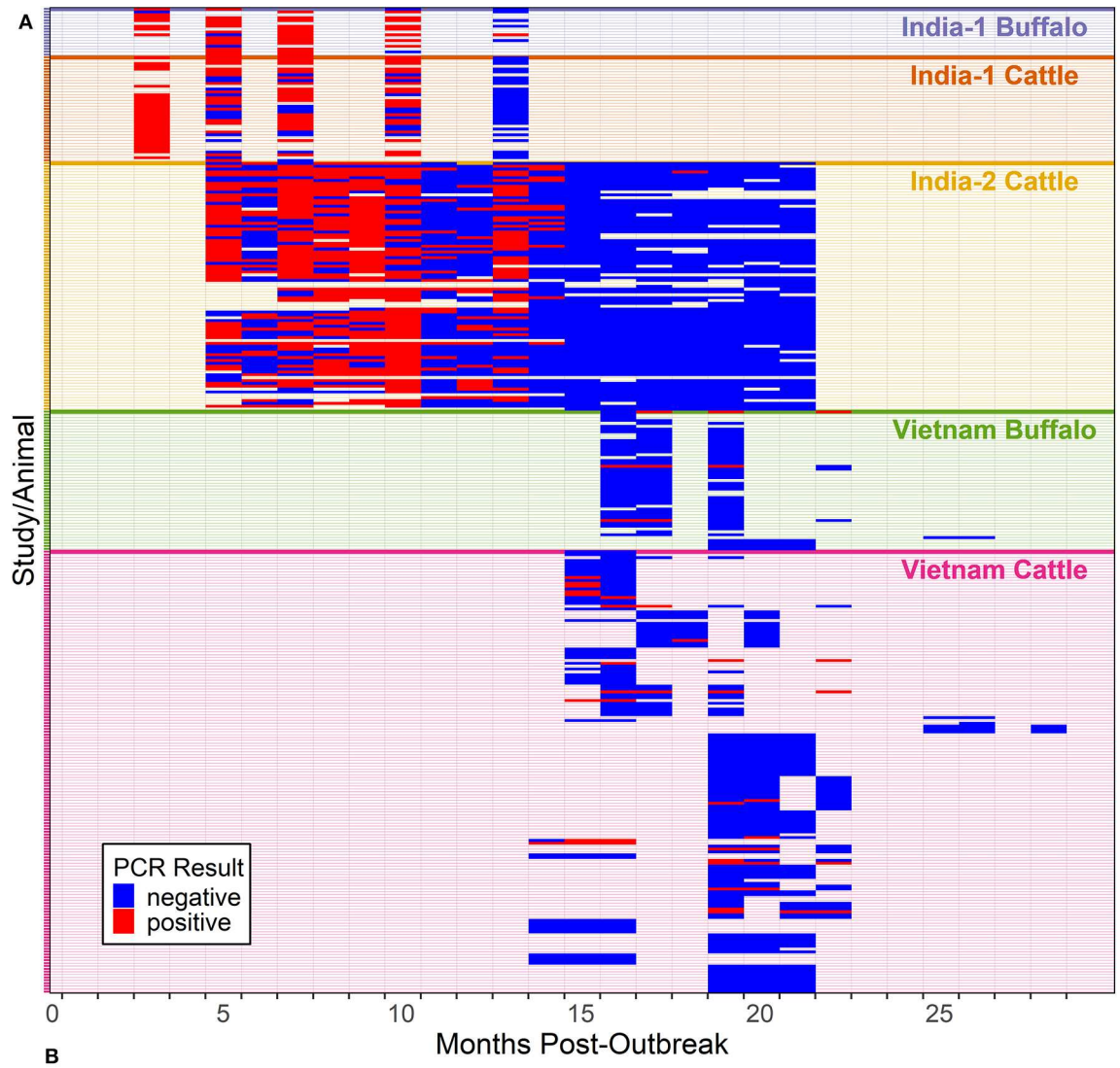
India

Study Descriptions

Cattle and Asian buffalo were sampled following two independent outbreaks in large dairy herds in India as previously described (19, 28, 34). Despite biosecurity practices in place, both premises had vulnerabilities to FMDV incursion, including unvaccinated cattle in the surrounding areas, and personnel moving between the farm and their personal livestock. The source of the virus was not identified in either outbreak.

Briefly, one study (India-1) investigated persistent infection in cattle and Asian buffalo following an FMD outbreak on a large dairy farm in India, which occurred in January 2014 (28, 34). Animals were vaccinated 3–4 times a year with a trivalent (A, O, Asia-1) vaccine, and had most recently been vaccinated ∼50 days prior to the outbreak. A convenience sample of 37 cattle and 17 buffalo, identified as carriers based on FMDV RNA detection in OPF, were sampled at 2–3 months intervals from 3–13 months post-outbreak (**Figure 1**). FMDV RNA was detected by rRT-PCR as described for Vietnam samples. The sampling included animals that were clinically or subclinically infected during the outbreak. As previously reported, all study animals were seropositive for FMDV anti-NSP antibodies by r3AB3 I-ELISA (35). Additionally, all study animals were persistently infected 3 months post-outbreak, and 7–17% of cattle and buffalo, respectively, remained persistently infected 13 months post-outbreak. The duration of persistent infection was not significantly different between clinically and subclinically affected animals, nor between cattle and buffalo (34).

The second study (India-2) investigated persistent infection in dairy cattle on one management unit in India following an FMD outbreak which occurred in October 2013 (19). Animals were vaccinated twice yearly with a trivalent (A, O, Asia-1) vaccine, and had most recently been vaccinated



Study/Species	Study Duration (mo. post-outbreak)	Sampling Frequency	No. Animals	No. Samples	No. Carriers
India-1 Buffalo	3-13 months	2-3 months	17	51	17
India-1 Cattle	3-13 months	2-3 months	37	140	36
India-2 Cattle	5-21 months	1 month	87	1268	79
Vietnam Buffalo	14-28 months	1-2 months	49	129	3
Vietnam Cattle	14-28 months	1-2 months	155	418	23

FIGURE 1 | FMDV RNA detection in oropharyngeal fluid by rRT-PCR. Data are presented from two studies in India and one in Vietnam, and include samples from cattle and Asian buffalo. **(A)** Each row represents one animal and the grouping of rows is colored according to study/species group. Study/species group is labeled on the right side of the figure and colored corresponding to the study/species in the table in **(B)**. Columns represent sampling times (months post-outbreak), and samples are represented by red (positive) and blue (negative) bars. **(B)** A summary of the data is provided in the table.

3–4 days prior to the outbreak. A convenience sample of 47 juvenile and 31 adult cattle, identified as carriers based on FMDV RNA detection in OPF as described for Vietnam samples, were sampled monthly from 5–21 months post-outbreak (**Figure 1**). The sampling included animals that were clinically or subclinically affected during the recent outbreak. As previously reported, all study animals were seropositive for FMDV anti-NSP antibodies by r3AB3 I-ELISA (35). The average duration of persistent infection was 13.1 months, and all animals cleared the infection by 19 months post-outbreak. There was no significant difference in the duration of persistent infection between clinically and subclinically affected animals (19). An

additional 9 animals that entered the study late were included in the current analyses.

Analyses of the duration of persistent infection have been reported previously for each of the India studies (19, 28, 34). Additionally, both outbreaks were caused by the same strain of FMDV (O/ME-SA/Ind2001d), and the studies had similar study designs. Therefore, the two India studies were combined in the current analyses by country as well as in the meta-analyses of all three studies, and estimates were produced for each study/species group. For the purposes of this study, it was assumed that animals were infected during the reported outbreak and no novel incursion or subclinical circulation of the virus occurred on the farms included in the study. This assumption was based upon documentation by the herd veterinarians that no cases of FMD were observed or reported during the period of the study and the finding that all carrier viruses from which sequence data was acquired were phylogenetically closely related to the outbreak strains (34).

Statistical Analysis

Accelerated Failure Time Model

For each country separately and for all three studies combined, the probability of persistent infection was investigated using interval-censored survival analysis, in which the time to event is modeled as an interval rather than an exact time. Failure to detect FMDV RNA in an OPF sample was the event of interest. The elapsed time between the outbreak date and the date of the last positive sample (rounded to the nearest month) was used as the low end of the interval, and the elapsed time between the outbreak date and the date of the next negative sample (rounded to the nearest month) was used as the high end of the interval in which the event occurred. For animals without a positive sample (i.e., left-censored), the low end of the interval was set to "NA," and for animals that remained persistently infected throughout the study (i.e., right-censored), the high end of the interval was set to "NA," as recommended by the software package authors. For the initial analysis, the Kaplan-Meier estimator was used to create a survival curve (data not shown). Due to small sample sizes and violations of the proportional hazards assumption, accelerated failure time (AFT) analyses were used, since they perform better than Cox proportional hazard analyses under these conditions (36, 37). To account for species differences and other study-site related variability, a combined study and species variable was created (study/species). Preliminary models were fit using the Weibull, exponential, log-logistic, and log-normal distributions, and the distribution which minimized the Akaike information criterion (AIC) was selected as the appropriate distribution for the final model. Analyses were implemented in R v3.5.3 using the *survival* base package (38). Goodness of fit of the final models was evaluated by visualization of the qq-plots of times of survival percentiles, Cox-Snell residuals, and comparison of predicted survival curves to Kaplan-Meier curves, as implemented in the *AFTtools* package (39). The final models were used to predict the duration of FMDV RNA detection at percentiles from 0.01 to 0.99 using the *predict* function in the *survival* package, and the results were subsequently used to estimate the probability of FMDV RNA detection at 6, 12, 18, and 24 months post-outbreak. Figures were created using the *ggplot2* package (40).

Generalized Linear Mixed Model

For each country separately and for all three studies combined, the probability of persistent infection was investigated using Generalized Linear Mixed Model (GLMM). Detection of FMDV RNA in OPF (yes/no) was the outcome variable, and the main independent variable was the elapsed time (rounded to the nearest month) between the outbreak date and the sample collection date. The combined study/species variable was also included as a fixed effect to account for variability among studies and species. Additionally, individual ID was included as a random variable to account for repeated measures on the same animals. GLMMs were built including time post-outbreak (in months) with and without the combined study and species variable, and the best fit model was selected considering the statistical and biological relevance. The model building and analyses were performed in R v3.5.2 using the *lme4* package (41). The final model equations were used to predict the probability of FMDV RNA detection in OPF at 6, 12, 18, and 24 months post-outbreak in Microsoft Excel 2019. Figures were created using the *ggplot2* package in R v3.5.2 (40).

RESULTS

Observed Extinction Dynamics (All Primary Studies)

The final dataset used to investigate the dynamics of extinction of persistent infection consisted of 2,006 samples from 345 seropositive animals or identified carriers, across the 3 studies (**Figures 1**, **4A**). All farms included in the analyses reported no FMD cases during the timeframe of the study or within 28 days prior to the start of the study. As a result, animals from which FMDV RNA was detected in OPF during the study were considered persistently infected.

In Vietnam, FMDV RNA was detected in ∼8% of samples at the first sampling time, 14 months post-outbreak, which increased to 22% at 15 months post-outbreak, then gradually decreased. No FMDV RNA was detected in samples collected after 25 months post-outbreak (**Figure 2A**). In the India-1 study, FDMV RNA was detected in all cattle samples and ∼90% of buffalo samples at the first sampling time 3 months post-outbreak, and the proportion decreased gradually until 10 months post-outbreak, with a rapid decrease between 10 and 13 months post-outbreak. Approximately 15% of buffalo samples were persistently infected at the last sample 13 months post-outbreak, whereas no FMDV RNA was detected in any cattle samples at that time (**Figure 3A**). In the India-2 study, FMDV RNA was detected in ∼70% of cattle samples at the first sample 5 months post-outbreak, and the proportion tended to decrease until 15 months post-outbreak. No FMDV RNA was detected after 15 months post-outbreak, with the exception of one animal at 18 months post-outbreak (**Figure 3A**).

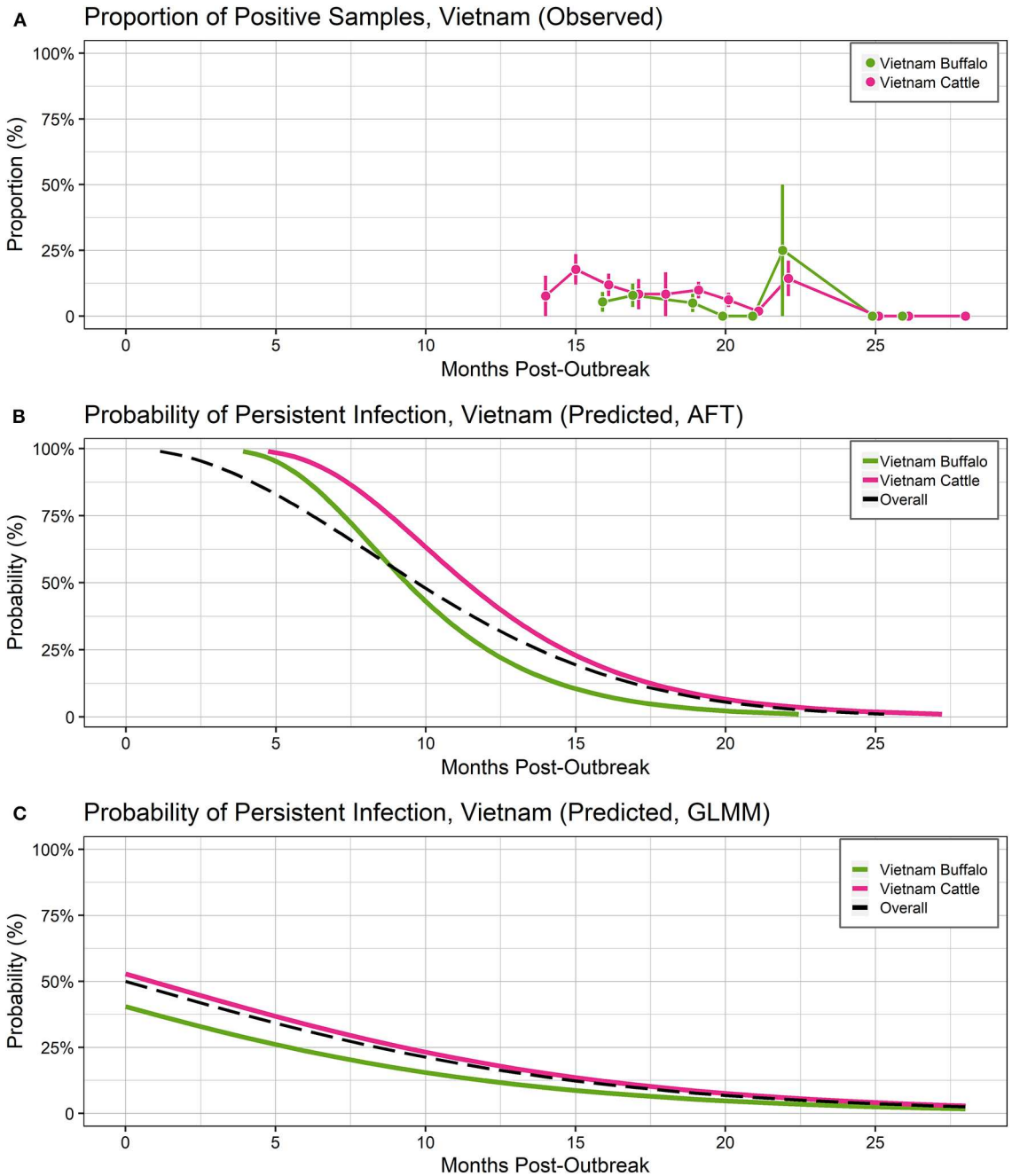


FIGURE 2 | Probability of persistent infection, Vietnam data only. All study animals were seropositive for FMDV anti-NSP antibodies by 3ABC-ELISA. **(A)** Observed proportion of OPF samples positive for FMDV RNA. Mean and standard error are shown. **(B)** Predicted probability of persistent infection, AFT model. **(C)** Predicted probability of persistent infection, GLMM model.

Modeled Dynamics of Persistent Infection in Vietnam

Accelerated Failure Time Model (Vietnam)

Overall, 26/204 (12.7%) animals were persistently infected in the Vietnam study. Based on AIC, the log-normal distribution was most appropriate for the final model (AIC = 215.14), and the model assumptions were appropriate based on evaluation of goodness of fit. In the final model, the duration of FMDV RNA detection in OPF for cattle and buffalo did not differ significantly ($p = 0.1$) (**Table 1**).

Using the AFT final model, the predicted probability of persistent infection in seropositive animals in Vietnam 6 months post-outbreak was 88% for buffalo and 95% for cattle, which decreased to 25 and 44%, respectively, 12 months post-outbreak,

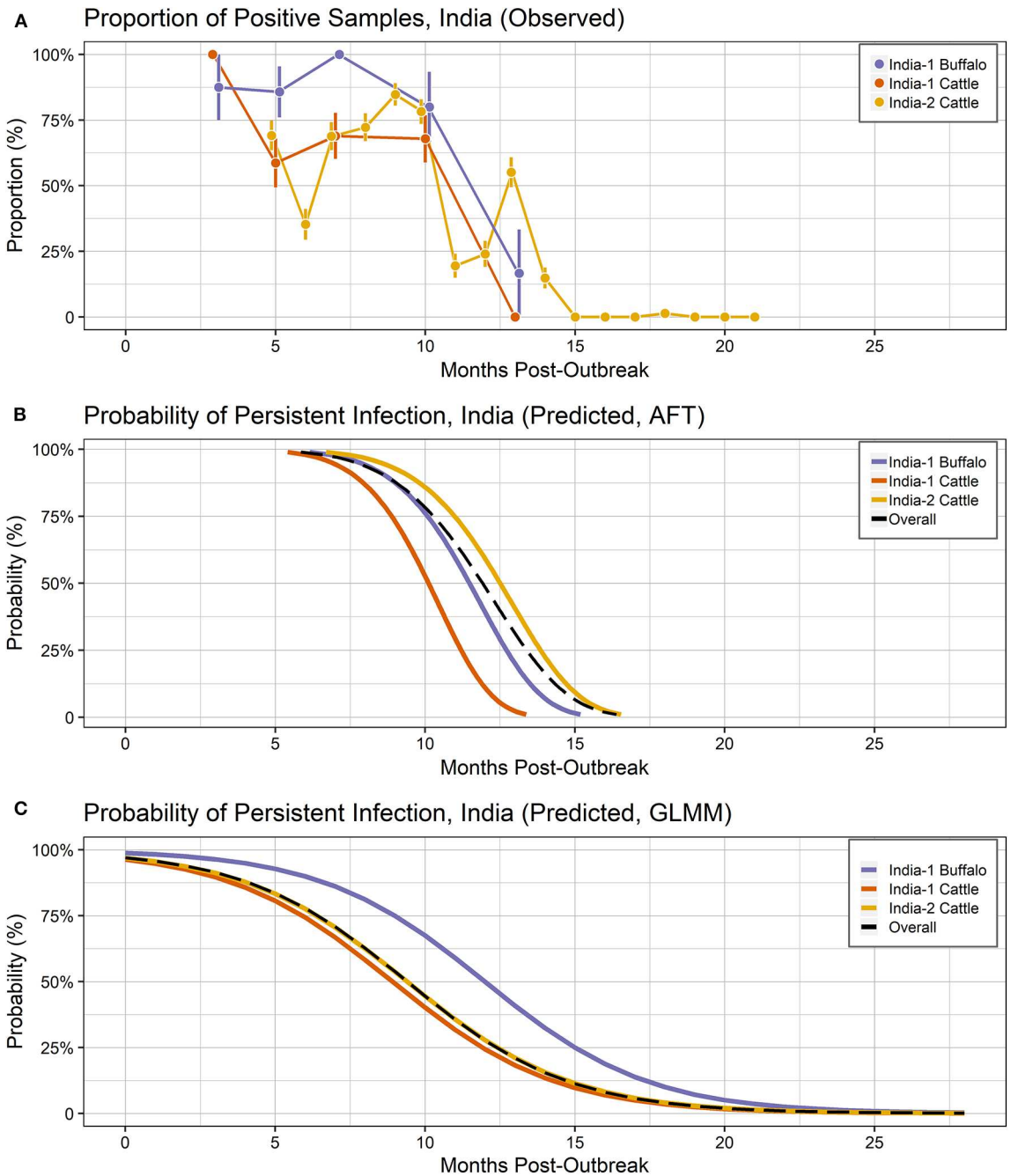


FIGURE 3 | Probability of persistent infection, India data only. All study animals were seropositive for FMDV anti-NSP antibodies by r3AB3 I-ELISA. **(A)** Observed proportion of OPF samples positive for FMDV RNA. Mean and standard error are shown. **(B)** Predicted probability of persistent infection, AFT model. **(C)** Predicted probability of persistent infection, GLMM model.

and <1% for buffalo and 2% for cattle at 24 months post-outbreak (**Table 2**, **Figure 2B**).

Generalized Linear Mixed Model (Vietnam)

Overall, FMDV RNA was detected in 46/547 (8.4%) total samples from the Vietnam study. The best fit model included time post-outbreak and the study/species variable. In the final model, the time post-outbreak was a significant variable ($p = 0.04$), and the odds of persistent infection decreased by 12% with each month post-outbreak (**Table 3**). Similar to the AFT model, the odds of persistent infection did not differ significantly between cattle and buffalo ($p = 0.2$).

Using the GLMM final model, the predicted probability of persistent infection in seropositive animals in Vietnam 6 months

post-outbreak was 23% for buffalo and 34% for cattle, which decreased to 12 and 19%, respectively, 12 months post-outbreak, and 3 and 5% at 24 months post-outbreak (**Table 2**, **Figure 2C**).

Dynamics of Persistent Infection in India

Accelerated Failure Time Model (India)

Overall, 132/141 (93.6%) animals were persistently infected in the two India studies. Based on AIC, the Weibull distribution was most appropriate for the final model (AIC = 470.96), and the model assumptions were appropriate based on evaluation of goodness of fit. In the final model, the duration of FMDV RNA detection in OPF for India-1 buffalo and India-2 cattle did not differ significantly ($p = 0.2$) (**Table 4**). In contrast, the duration was 0.8 times shorter for India-1 cattle than for India-2 cattle, and the difference was significant ($p < 0.0001$).

Using the AFT final model, the predicted probability of persistent infection in identified carriers in India 6 months post-outbreak was >99% for India-1 buffalo and India-2 cattle, and 98% for India-1 cattle. At 12 months post-outbreak, the probability decreased to 39% for India-1 buffalo and 59% for India-2 cattle, whereas the decrease was even greater for India-1 cattle (11% probability at 12 months post-outbreak). At 18 months post-outbreak, the probability of persistent infection was <0.05% for all groups (**Table 2**, **Figure 3B**).

Generalized Linear Mixed Model (India)

Overall, FMDV RNA was detected in 511/1,459 (35.0%) total samples from the two studies in India. The best fit model included time post-outbreak and the study/species variable. The odds of persistent infection decreased by 31% with each month post-outbreak (**Table 5**). In contrast to the AFT model, the odds of persistent infection were 2.6 times higher in India-1 buffalo compared to India-2 cattle, and the difference was significant ($p = 0.01$). Contrastingly, the odds of persistent infection were not significantly different between India-1 cattle and India-2 cattle ($p = 0.4$).

Using the GLMM final model, the predicted probability of persistent infection in identified carriers in India 6 months post-outbreak was 90% for India-1 buffalo, 74% for India-1 cattle, and 78% for India-2 cattle. The GLMM predicted higher probability of persistent infection at later timepoints compared to the AFT:

TABLE 1 | Accelerated failure time model for the duration of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak in Vietnam.

Variable	Coef*	Std. Err**	*P*-value	Time ratio	95% CI
Intercept	2.24	0.16	<0.0001		
Vietnam buffalo	(ref)				
Vietnam cattle	0.19	0.12	0.1	1.21	0.96, 1.53
log(scale)	−0.98	0.20	<0.0001		

**Coef, Model coefficient.*
***Std. Err, standard error.*
The model was fitted using the log-normal distribution.

TABLE 3 | Generalized linear mixed model for the probability of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak in Vietnam.

Variable	Coef*	Std. Err**	*P*-value	Odds ratio	95% CI
Intercept	−0.38	1.21	0.75		
Month post-infection	−0.13	0.06	**0.04**	**0.88**	0.78, 0.99
Vietnam buffalo	(ref)				
Vietnam cattle	0.5	0.4	0.21	1.65	0.75, 3.61

**Coef, Model coefficient.*
***Std. Err, standard error.*
Significant values are indicated in bold.

TABLE 2 | Predicted probability of persistent infection following an FMD outbreak.

Study	Species	AFT predictions				GLMM predictions			
		Months post-outbreak				Months post-outbreak			
		6	12	18	24	6	12	18	24
Vietnam only	Vietnam buffalo	88.18%	25.44%	4.09%	0.61%	23.63%	12.36%	6.04%	2.84%
	Vietnam cattle	95.52%	44.15%	11.01%	2.32%	33.78%	18.86%	9.58%	4.60%
	Vietnam overall	76.48%	34.81%	9.53%	1.57%	31.30%	17.19%	8.64%	4.13%
India only	India-1 buffalo	99.15%	39.28%	0%	0%	89.97%	49.95%	9.99%	1.22%
	India-1 cattle	98.01%	11.03%	0%	0%	74.35%	24.38%	3.46%	0.40%
	India-2 cattle	99.52%	59.24%	0.03%	0%	77.63%	27.85%	4.12%	0.48%
	India overall	98.84%	48.6%	0.03%	0%	77.90%	27.69%	4.00%	0.45%
India and Vietnam	India-1 buffalo	99.5%	40.88%	2.47%	0.24%	91.05%	51.75%	10.16%	1.18%
	India-1 cattle	98.26%	16.49%	0.72%	0.07%	75.21%	24.23%	3.26%	0.35%
	India-2 cattle	99.75%	58.51%	4.9%	0.49%	77.56%	26.70%	3.70%	0.40%
	Vietnam buffalo	99.58%	45.61%	2.98%	0.29%	75.77%	24.79%	3.36%	0.36%
	Vietnam cattle	99.86%	71.08%	8.25%	0.85%	87.21%	41.82%	7.04%	0.79%
	Overall	99.23%	50.75%	5.76%	0.82%	80.38%	32.08%	5.17%	0.6%

Predictions were made from three longitudinal studies in Vietnam and India using an accelerated failure time model (AFT) and a generalized linear mixed model (GLMM).

TABLE 4 | Accelerated failure time model for the duration of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak in India.

Variable	Coef*	Std. Err**	*P*-value	Time ratio	95% CI
Intercept	2.58	0.02	<0.0001		
India-1 buffalo	−0.09	0.07	0.19	0.92	0.81, 1.04
India-1 cattle	−0.21	0.04	**<0.0001**	**0.81**	0.75, 0.87
India-2 cattle	(ref)				
log(scale)	−1.91	0.08	<0.0001		

**Coef, Model coefficient.*
***Std. Err, standard error.*
The model was fitted using the Weibull distribution.
Significant values are indicated in bold.

TABLE 5 | Generalized linear mixed model for the probability of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak in India.

Variable	Coef*	Std. Err**	*P*-value	Odds ratio	95% CI
Intercept	3.44	0.20	0.0		
Month post-infection	−0.37	0.01	**0.0**	**0.69**	0.68, 0.71
India-1 buffalo	0.95	0.37	**0.01**	**2.59**	1.25, 5.34
India-1 cattle	−0.18	0.21	0.41	0.84	0.55, 1.26
India-2 cattle	(ref)				

**Coef, Model coefficient.*
***Std. Err, standard error.*
Significant values are indicated in bold.

4–10% at 18 months post-outbreak and 0.5–1% at 24 months post-outbreak (**Table 2**, **Figure 3C**).

Dynamics of Persistent Infection Across All Studies

In order to achieve the most robust estimates possible, the probability of persistent infection across all primary studies was modeled in a similar approach, which examined the duration of persistent infection (AFT) and the effect of time on the probability of persistent infection (GLMM). The goal of this combined meta-analysis was to benefit from the breadth of variability of study design, viral, host, and environmental factors included across the three primary studies. The study/species variable was included in all models to account for species and study-site variability. Estimates were generated for each study/species group. Estimates are reported for a target population of seropositive animals, but may be considered biased upward due to the inclusion of only identified carriers in the India studies.

Accelerated Failure Time Model (Combined Studies)

In total, 158/345 (45.8%) animals were carriers across the three studies. For the analysis of all primary studies combined, the log-logistic distribution was most appropriate for the final model (AIC = 722.71), and the model assumptions were appropriate based on evaluation of goodness of fit. In the final model, the duration of persistent infection was 0.8 times shorter for India-1 cattle compared to India-2 cattle ($p < 0.0001$). For all other groups, the duration of persistent infection was not significantly different from India-2 cattle (**Table 6**).

For all groups combined, the overall probability of persistent infection in seropositive animals predicted using the AFT final model was 99.23% (range: 98.26–99.86%) at 6 months after an outbreak, 50.75% (range: 16.49–71.08%) at 12 months post-outbreak, 5.76% (range: 0.72 – 8.25%) at 18 months post-outbreak, and at 24 months post-outbreak was 0.82% (range: 0.07–0.85%) (**Table 2**, **Figure 4B**).

Generalized Linear Mixed Model (Combined Studies)

For the analysis of all studies combined, the best fit model included time post-outbreak and the study/species variable. In the final model, the odds of persistent infection decreased by 31% with each month post-outbreak (**Table 7**). In contrast to the AFT model, the odds of persistent infection at any given time were three times higher in India-1 buffalo and two times higher in Vietnam cattle compared to India-2 cattle ($p = 0.02$ and $p = 0.01$, respectively). Additionally, the odds of persistent infection were not significantly different for India-1 cattle compared to India-2 cattle ($p = 0.7$).

TABLE 6 | Accelerated failure time model for the duration of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak across three studies.

Variable	Coef*	Std. Err**	*P*-value	Time ratio	95% CI
Intercept	2.53	0.02	<0.0001		
India-1 buffalo	−0.09	0.08	0.26	0.92	0.79, 1.07
India-1 cattle	−0.24	0.05	**<0.0001**	**0.79**	0.71, 0.87
India-2 cattle	(ref)				
Vietnam buffalo	−0.06	0.08	0.43	0.94	0.80, 1.10
Vietnam cattle	0.07	0.04	0.08	1.07	0.99, 1.15
log(scale)	−2.10	0.08	<0.0001		

**Coef, Model coefficient.*
***Std. Err, standard error.*
The model was fitted using the log-logistic distribution.
Significant values are indicated in bold.

For all groups combined, the overall probability of persistent infection in seropositive animals predicted using the GLMM final model was 80.38% (range: 75.21–91.05%) at 6 months after an outbreak, 32.08% (range: 24.23–51.75%) at 12 months post-outbreak, 5.17% (range: 3.26–10.16%) at 18 months post-outbreak, and at 24 months post-outbreak was 0.6% (range: 0.35–1.18%) (**Table 2**, **Figure 4C**).

DISCUSSION

Persistent infection with foot-and-mouth disease virus is a challenge for FMD control and eradication in endemic regions. Similarly, FMD-free regions must consider the existence of carriers when responding to incursions. Additionally, neoteric subclinical infection is indistinguishable from persistence under field conditions, and may pose a greater threat of transmission (5, 42). Although the role of persistently infected animals in FMDV epidemiology remains controversial, persistently infected animals are known to carry virus in a form that is directly infectious to susceptible animals (15). This is consistent with the demonstrated very low, but non-zero, quantitative risk of

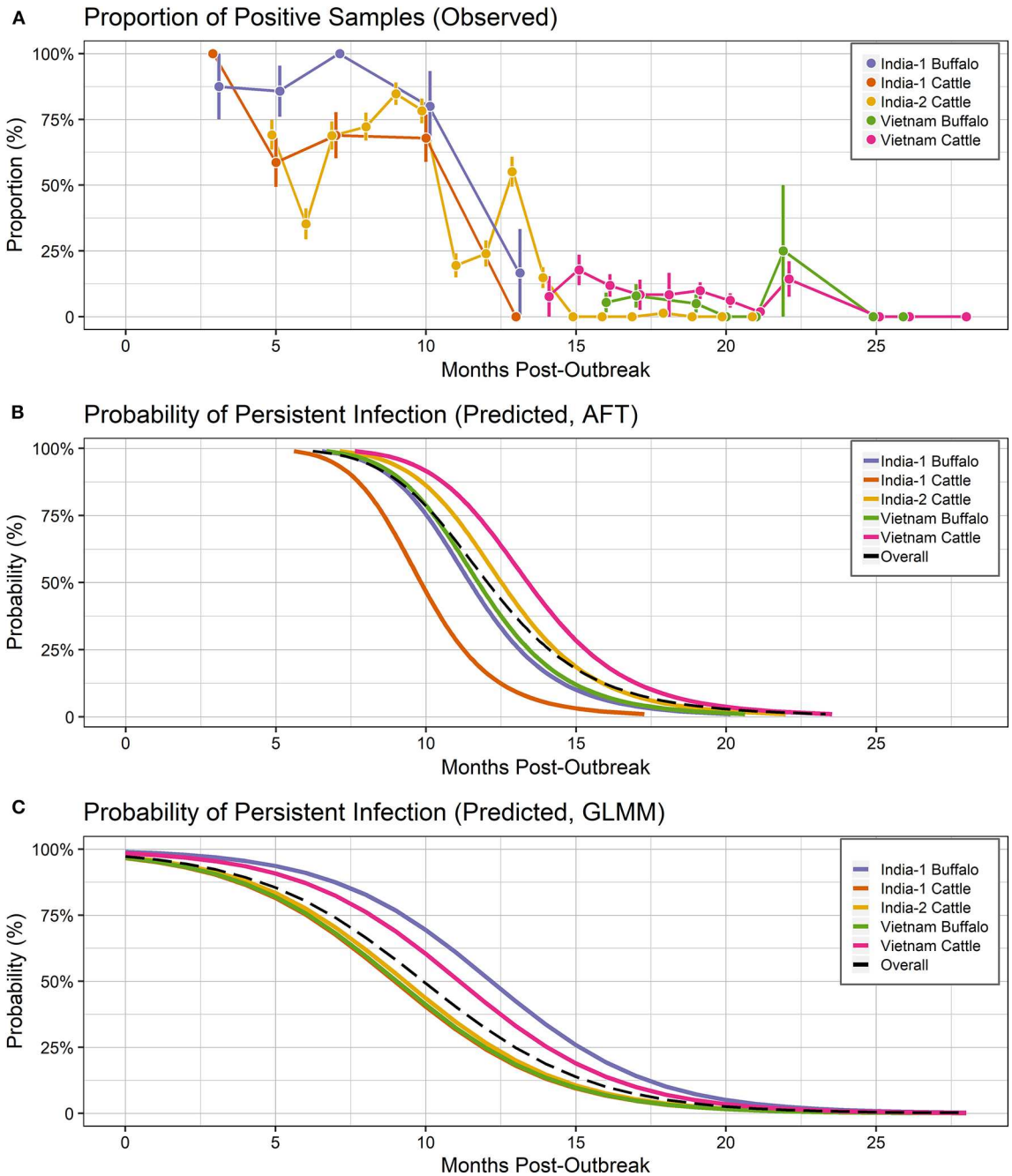


FIGURE 4 | Probability of persistent infection, Vietnam and India data combined. All study animals were seropositive for FMDV anti-NSP antibodies by 3ABC-ELISA (Vietnam) or by r3AB3 I-ELISA (India). **(A)** Observed proportion of OPF samples positive for FMDV RNA. Mean and standard error are shown. **(B)** Predicted probability of persistent infection, AFT model. **(C)** Predicted probability of persistent infection, GLMM model.

transmission (13, 14). Because of this, global FMD control policies must consider persistent as well as acute infection. Quantitative estimates of the probability of persistent infection at specified times post-outbreak may provide a tool to more accurately assess the potential risks posed by persistently infected animals, which will help to guide control efforts. The current study compared two statistical modeling approaches, a survival analysis model (AFT) and generalized linear model (GLMM), for estimating the probability of persistent infection, while evaluating the benefits of meta-analytical approaches to leverage primary datasets that span a wider range of conditions including study design and duration, virus-specific factors, host-specific factors, and environmental factors.

In the by-country analyses of the Vietnam primary study, the AFT and GLMM models generated similar assessment of the impact of species on the duration of persistent infection. In

TABLE 7 | Generalized linear mixed model for the probability of FMDV RNA recovery from oropharyngeal fluid following an FMD outbreak across three studies.

Variable	Coef*	Std. Err**	*P*-value	Odds ratio	95% CI
Intercept	3.49	0.22	0.0		
Month post-infection	−0.38	0.02	**0.0**	**0.68**	0.66, 0.71
India-1 buffalo	1.08	0.47	**0.02**	**2.94**	1.17, 7.40
India-1 cattle	−0.13	0.3	0.66	0.88	0.49, 1.58
India-2 cattle	(ref)				
Vietnam buffalo	−0.10	0.42	0.81	0.90	0.40, 2.06
Vietnam cattle	0.68	0.25	**0.007**	**1.97**	1.21, 3.22

**Coef, Model coefficient.*
***Std. Err, standard error.*
Significant values are indicated in bold.

both modeling approaches, the duration of persistent infection did not differ significantly between cattle and buffalo. Similarly, a previous study reported that the odds of persistent infection did not differ significantly between dairy cattle and buffalo, although the odds were significantly higher for beef cattle being carriers in that study (27). It is possible that either distinct host genetics or the different management practices for beef vs. dairy cattle influence viral extinction. Overall, our results indicate that species is not a significant factor affecting persistent infection in cattle and buffalo in Vietnam.

For the Vietnam analyses, the predicted probabilities of persistent infection among seropositive animals were similar between the modeling approaches at 18 and 24 months post-outbreak. Both models predicted <5% probability of persistent infection at 24 months post-outbreak. In contrast, a previous study in Vietnam estimated that the mean duration of persistent infection was 27 months (14). This discrepancy may be partially explained by the small sample size ($n = 10$) and large uncertainty (±6 months) in the outbreak dates used in the previous study. The sample collection period may also influence the estimated duration of persistent infection. Due to the logistics of field sampling, we were unable to collect samples earlier than 14 months post-outbreak. Although model predictions were similar at later timepoints, predictions were widely different at 6 and 12 months post-outbreak, likely due to the lack of data at these timepoints and differences in assumptions between models. These results highlight the need for robust datasets to develop accurate models. Further studies in Vietnam or other endemic settings should include earlier timepoints to more accurately describe the dynamics of the extinction of the FMDV carrier state.

In contrast to the Vietnam study, the models differed in their estimates of duration and significance for the by-country analyses of the India studies. In the AFT model, the duration of persistent infection did not differ for India-1 buffalo compared to India-2 cattle, whereas the GLMM estimated a significantly longer duration for India-1 buffalo. Similarly, a previous analysis of the India-1 primary study reported that a higher proportion of buffalo were persistently infected compared to cattle; however the difference was not significant in that study (34). Although both models herein estimated a shorter duration of persistent infection for India-1 cattle compared to India-2 cattle, the difference was only significant in the AFT model. Differences between model results for India-1 buffalo may be due to the relatively small ($n = 17$) number of buffalo included in the study. Differences in results may also be due to differences in data handling between the two models. The input unit for the AFT was animal, whereas the input unit for GLMM was sample, and the difference in number of samples between the India-1 and India-2 studies was much greater than the difference in number of animals.

Despite the differences between models, the predicted probabilities of persistent infection among identified carriers were largely similar between the models at 6 and 12 months post-infection for the India analyses. The predicted probabilities of persistent infection at 12 months post-outbreak were consistent with previous analyses of the India data, which used the Kaplan-Meier estimator to estimate the duration of persistent infection. Previously, 14% of cattle were reported to be persistently infected at 10.5 months post-outbreak in the India-1 study (28), and the average duration of persistent infection was 13 months in the India-2 study (19). Similar to the Vietnam analyses, model predictions were more divergent at timepoints beyond the sample collection period for the India studies (18 and 24 months post-outbreak).

Although model predictions were similar in the by-country analyses for the timepoints within the collection period for the studies, predictions were widely different when the models extrapolated beyond the range of the data. These differences in predictions are due to differences in data handling between the models. AFT utilizes a single data point for each animal—the interval in which the animal cleared the infection—and assumes every animal is persistently infected until that interval. The model assumes the initial proportion of persistently infected animals is 100% and can only decrease over time (43). Additionally, AFT models are constrained by the distribution specified when building the model. In contrast, GLMM utilizes multiple data points per animal—each individual sample—and animals may have intermittent negative samples due to imperfect tests for detection of FMDV in OPF (32). The model does not assume an initial proportion of persistently infected animals, and the proportion is allowed to vary over time (43). These differences in the models result in more divergent predictions outside the data range, particularly when a limited range of timepoints is used to build the models. To overcome the limited ranges of timepoints in the individual studies in our analyses, we combined all three studies in an additional analysis to further assess the dynamics of FMDV persistent infection.

In the combined meta-analyses of all three studies, the AFT and GLMM models had the same direction of change compared to India-2 cattle for all groups except India-1 buffalo; however the significance of the change was different for all groups except Vietnam buffalo. These results suggest the trends of the associations between study/species group and duration of persistence are reliable; however the significance of the associations should be interpreted cautiously and in consideration of the model used in the analysis. As expected, model predictions were more similar for the combined analyses than for the analyses by country, highlighting the benefits of

increased sample size and range of collection times in the combined analyses. However, based on data availability, the current analyses were limited to studies in Asia and included mostly vaccinated animals as well as relatively low diversity of FMDV strains. Vaccination does not protect against persistent infection, and previous studies have shown no difference in the proportion of carriers among vaccinated and naïve animals (8, 44, 45). Therefore, vaccination likely does not affect the duration of persistent infection, however this should be investigated in future studies. Due to the potential variability across viral strains, environmental conditions, and host genetic backgrounds, future studies should assess FMDV persistent infection across a wider geographical area and virus diversity.

Additionally, both models assume no reintroductions or incursions of novel FMDVs during the primary studies. This assumption was based upon official records from DAH, Vietnam and herd health records for the India studies that indicated no new outbreaks of FMD occurred in the herds contributing to the primary studies (19, 28). Additionally, viral sequences obtained from consecutive samples from a subset of the animals indicated these animals were not re-infected with a different FMDV strain (27, 34). Neoteric subclinical infection (5) was largely ruled out by lack of detection of sequences of novel strains within the geotemporal space of the primary studies (27, 34), but cannot be completely excluded since sequence data could not be obtained from every sample from which FMDV RNA was detected.

In the meta-analyses, the AFT and GLMM models predicted >98% and 75–91% probability of persistent infection in seropositive animals at 6 months post-outbreak, respectively. In contrast, a previous meta-analysis of four experimental studies reported only 52% of animals were persistently infected at 6 months post-outbreak (13). The longer duration of persistent infection in the current study may reflect differences in infection dynamics between natural and experimental conditions, including controlled exposure and small sample sizes in experimental studies. Model predictions at 12 months post-outbreak were consistent with a previous field study which reported 20% of animals were persistently infected 12 months post-outbreak (46). In contrast, a previous probability analysis reported substantially lower (0.7%) probability of persistent infection at 12 months post-outbreak (25). The wide discrepancy between this previous analysis and the current analyses may be due in part to differences in study design. For example, the three primary studies in the current analyses were longitudinal studies, specifically intended to monitor viral extinction in carriers, and included only animals with previous exposure to FMDV (Vietnam) or identified carriers (India). By contrast, the Bronsvoort et al. (25) study used a cross-sectional study design and included unexposed animals as well as animals previously exposed to FMDV. Furthermore, the previous analysis was also limited to a single study setting, with accompanying limitations on sampling times post-outbreak and host, viral, and environmental factors. The current study used detection of viral RNA in OPF to determine carrier status. RNA detection by rRT-PCR may be more sensitive than virus isolation (VI) under some circumstances (47, 48), which would result in a higher apparent prevalence of carriers in these analyses. However, in a previous analysis of the India-1 study herein, the duration of persistent infection was not different as determined by rRT-PCR or VI (34). Similarly, other experimental studies have reported comparable sensitivities for rRT-PCR and VI (8), suggesting that viral RNA detection similarly reflects the true carrier status of an animal. The current study attempted to improve upon previous analyses of the extinction of the FMDV carrier state by combining data from three distinct primary studies to develop mathematical models that represent the dynamics of persistent infection, and then use the models to predict the probability of persistent infection at specified times post-outbreak. These analyses provide a more tailored approach to the development of control measures to minimize the risk posed by persistently infected animals.

Overall, the two models produced similar predictions in the combined analyses, suggesting that either model may be satisfactory for describing the dynamics of FMDV carrier state extinction. Researchers should consider which model is more appropriate for a particular study based on the study design, data structure, and whether model assumptions are biologically appropriate. Additionally, results should be interpreted in consideration of the model used for analyses. Our results suggest that when only cross-sectional data are available, a GLMM approach may be suitable to model the probability of FMDV persistent infection in the study population. Cross-sectional studies are less expensive and can be completed faster than longitudinal studies, offering advantages in risk assessment relating to persistently infected animals in an endemic population. However, detection of virus in OPF is inconsistent (6, 14, 19, 49), and cross-sectional studies may, therefore, underestimate the proportion of persistently infected animals at any given time post-outbreak. Additionally, a wide range of times post-outbreak is needed to more accurately model persistent infection dynamics, and repeated cross-sections may be needed to achieve this. As demonstrated in the current study, meta-analysis of several longitudinal studies can overcome some limitations of individual studies, such as small sample size and limited sampling frequency, while providing a more robust view of viral dynamics within animals over time. Furthermore, this approach can help researchers and disease control experts better understand how persistent infection varies across populations.

CONCLUSION

FMDV persistent infection causes a substantial economic burden on endemic countries due to trade restrictions, which have traditionally treated persistent infection as a binary state (present/absent) with a fixed duration. However, persistent infection is a dynamic process, and statistical models can be useful to assess the decreasing probability of persistent infection at increasing times post-outbreak. Additionally, meta-analysis reduces the impact of limitations in individual studies. In the current study, the AFT and GLMM models predicted similar probabilities of persistent infection at 18 and 24 months post-outbreak, while the probability of detection of persistent infection was higher using the AFT model at 6 and 12 months

post-outbreak. Because it may over-estimate probabilities at earlier timepoints, the AFT model is the more cautious approach for designing policies to reduce or eliminate the potential risk presented by persistently infected animals following an FMD outbreak. Additional studies with larger sample sizes and expanded meta-analyses, including more primary studies, are likely to provide more nuance and depth to our understanding of this dynamic process, leading to an improved understanding of FMDV persistent infection after outbreaks and how to predict and manage this disease state.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the authors upon reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the work described herein was performed by federal staff of the Department of Animal Health, Ministry of Agriculture and Rural Development, Government of Vietnam or the Directorate of Foot and Mouth Disease, Indian Council of Agricultural Research, Ministry of Agriculture, Government of India. The work occurred and the animals were maintained within facilities that were owned, maintained, or overseen by these divisions of the federal governments; thus, no permits or approvals were required. All cases described herein occurred spontaneously in domestic cattle or buffalo with no experimentation, inoculation, or treatment of live animals.

AUTHOR CONTRIBUTIONS

MB performed the statistical analysis and drafted the manuscript. SY contributed to the statistical analysis, and helped to draft the manuscript. AD participated in the design and coordination, and helped to draft the manuscript. CS contributed to study design of primary studies and drafting and revising the manuscript. JA conceived the study, participated in the design and coordination, and helped to draft the manuscript. All authors read and approved of the final manuscript.

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Foot-and-Mouth Disease Surveillance Using Pooled Milk on a Large-Scale Dairy Farm in an Endemic Setting

Bryony Armson[1,2], Simon Gubbins[1], Valérie Mioulet[1], Ibrahim A. Qasim[3], Donald P. King[1] and Nicholas A. Lyons[1,4]*

[1] *Vesicular Disease Reference Laboratory, The Pirbright Institute, Surrey, United Kingdom,* [2] *Boyd Orr Centre for Population and Ecosystem Health, Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom,* [3] *Directorate of Animal Resources Services, Ministry of Environment, Water and Agriculture, Riyadh, Saudi Arabia,* [4] *European Commission for the Control of Foot-and-Mouth Disease (EuFMD), Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome, Italy*

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***Correspondence:**
Bryony Armson
b.armson@surrey.ac.uk

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Pooled milk is used for the surveillance of several diseases of livestock. Previous studies demonstrated the detection of foot-and-mouth disease virus (FMDV) in the milk of infected animals at high dilutions, and consequently, the collection of pooled milk samples could be used to enhance FMD surveillance. This study evaluated pooled milk for FMDV surveillance on a large-scale dairy farm that experienced two FMD outbreaks caused by the A/ASIA/G-VII and O/ME-SA/Ind-2001d lineages, despite regular vaccination and strict biosecurity practices. FMDV RNA was detected in 42 (5.7%) of the 732 pooled milk samples, and typing information was concordant with diagnostic reports of clinical disease. The FMDV positive milk samples were temporally clustered around reports of new clinical cases, but with a wider distribution. For further investigation, a model was established to predict real-time RT-PCR (rRT-PCR) C_T values using individual cattle movement data, clinical disease records and virus excretion data from previous experimental studies. The model explained some of the instances where there were positive results by rRT-PCR, but no new clinical cases and suggested that subclinical infection occurred during the study period. Further studies are required to investigate the effect of vaccination on FMDV excretion in milk, and to evaluate more representative sampling methods. However, the results from this pilot study indicate that testing pooled milk by rRT-PCR may be valuable for FMD surveillance and has provided evidence of subclinical virus infection in vaccinated herds that could be important in the epidemiology of FMD in endemic countries where vaccination is used.

Keywords: foot-and-mouth disease, surveillance, pooled milk, subclinical infection, vaccination, real-time RT-PCR

INTRODUCTION

Milk has been exploited for the surveillance of several pathogens of livestock including bovine viral diarrhea virus (1, 2), Schmallenburg virus (3), *Coxiella burnetti* (4), bovine respiratory syncytial virus (5), and *Neospora caninum* (6). The use of pooled milk samples has also been validated as a rapid, cost-effective approach for the routine surveillance of diseases such as brucellosis (7) and mastitis caused by *Mycoplasma* spp. (8).

Previous experiments have shown that the mammary gland is an organ that is highly susceptible to foot-and-mouth disease virus (FMDV) replication, and FMDV can be detected in milk from experimentally infected animals before, during and after the appearance of clinical signs (9–13). Additionally, it has been demonstrated that FMDV can be detected and typed by real-time reverse transcription polymerase chain reaction (rRT-PCR) assays in milk from naturally infected cattle in endemic scenarios and during an outbreak in a normally FMD-free country (9, 14). Previous studies (9, 13) have suggested that it could be possible to identify one acutely-infected milking cow in a typical-sized dairy herd (100–1,000 individuals) using milk from bulk tanks or milk tankers. This theory was based on the detection of FMDV RNA in milk samples, collected from infected cattle, that had been highly diluted over 10,000-fold in negative milk. Simulation modeling using these data (13, 15, 16) support the requirement for further research to assess the use of pooled milk as a useful tool to enhance FMD surveillance.

Collection of pooled milk at the herd level could offer a representative sampling framework for FMD surveillance on large-scale dairy farms in endemic countries. Milk is routinely collected and has several advantages over vesicular material or serum by being non-invasive and potentially less susceptible to selection bias in targeted (risk-based) surveillance. For example, the use of milk does not rely on disease reporting by farmers or veterinary professionals, and sub-clinically may be confirmed using milk which would otherwise go undetected (14).

Results from the studies mentioned above have motivated further investigations using pooled milk from different production systems in endemic settings. Saudi Arabia is an FMD endemic country in which a range of production systems exist, including nomadic and small-scale herds containing small ruminants and cattle, and large-scale dairy production systems (17). Large-scale dairy farms can house in excess of 20,000 cattle, and often keep detailed records of individual cattle health, movements, milk yields and vaccination status (18–20). In recent years, Saudi Arabia has experienced outbreaks due to viral lineages that are not normally present in this region, including the A/ASIA/G-VII and O/ME-SA/Ind-2001 lineages (21, 22). These FMD outbreaks also affected large-scale dairy farms, despite regular vaccination and strict biosecurity practices, where milk was being routinely collected as part of a herd health monitoring program (18, 20).

The aim of this study was to validate the use of pooled milk for the surveillance of FMD in large-scale dairy production systems in Saudi Arabia which would also inform potential targeted/risk-based surveillance in FMD-free countries in the event of an outbreak. The specific objectives were to (i) validate the use of pooled milk collected from a large scale dairy farm in Saudi Arabia for the detection and characterization of FMDV by real-time rRT-PCR; (ii) compare the results obtained by FMDV rRT-PCR with clinical incidence; (iii) model the predicted C_T values of pooled milk samples based on detailed epidemiological data available from the farm; (iv) estimate the sensitivity and specificity of this surveillance approach to assess the usefulness of pooled milk as a cost-effective, non-invasive surveillance tool.

MATERIALS AND METHODS

Study Area and Population

The study area was a large-scale dairy farm located in central Saudi Arabia. The farm housed approximately 4,000 Holstein Friesian cattle and was organized into management houses (H). Lactating groups ($n = 17$) were milked four times a day. The farm had a fenced outer perimeter and there were no other FMD susceptible livestock or wildlife present on the farm. The study population was all cattle on the farm that were in lactating groups during the study period (10/09/2015 to 25/02/2016). The farm had electronic recording systems for monitoring individual animal health and movements. Lactating cattle were regularly vaccinated every 105 days with a killed, aqueous adjuvanted (aluminum hydroxide and saponin), NSP purified FMD vaccine (containing O Manisa, O-3039, O-PanAsia2, A Iran-05, A Saudi-95, Asia-1 Shamir, and SAT-2 virus strains) (Aftovaxpur, Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany) (20).

In September 2015, the farm had clinical cases of FMD due to the then emerging A/ASIA/G-VII viral lineage (21), confirmed by the OIE/FAO World Reference Laboratory for foot-and-mouth disease (WRLFMD) at The Pirbright Institute, UK. In February 2016, 3 months after the last clinical case (on 12/11/2015), new clinical cases were observed and confirmed as serotype O (ME-SA/Ind-2001d lineage), with the last recorded clinical case on 07/03/2016. All recording of clinical cases was done by farm staff supervised by veterinary surgeons employed by the farms and entered into an electronic farm recording system. The FMD case definition was any individual bovine seen with increased salivation and any of the following additional clinical signs: mouth lesions, feet lesions, teat lesions, fever, reduced feed intake, and lameness. The farm policy was to isolate new cases of FMD in a dedicated isolation facility. If the isolation facility was full, or the number of observed cases in the group exceeded ∼5%, cases remained within groups. Milk from clinical cases continued to be collected along with that of the other cows in the house. Animals were moved from isolation back to the main herd either after complete recovery, or when sufficiently recovered, depending on available space in the isolation facility.

Pooled Milk Sampling

As part of routine herd health surveillance, milk samples were collected using a proportional in-line milk sampler, designed to pull a representative sample from each house, and delivered to the farm laboratory. Throughout the study period (10/09/2015 to 25/02/2016), milk samples ($n = 732$) were collected twice weekly (between 10/09/2015 and 03/12/2015), and then weekly or on an *ad-hoc* basis (between 10/12/2015 and 25/02/2016) due to the infrequency of clinical cases, until the presumed end of the outbreak. Milk samples were collected from 17 management houses that contained lactating cows and on an *ad-hoc* basis from two houses containing cows separated due to various diseases including FMD (the "sick-cow pen"). All milk samples were labeled with the date and house identification number and were stored in a freezer at −20°C until they were shipped to The Pirbright Institute (TPI, UK) for FMDV detection.

Laboratory Testing of Pooled Milk Samples

Viral Isolates

FMDV cell culture isolates were obtained from archival stocks held in the WRLFMD repository. Cell culture isolate O/SAU/1/2016 was diluted in unpasteurized whole milk, and used as a positive control for the pan-serotypic rRT-PCR assay and the serotype specific O (ME-SA/Ind-2001d lineage) rRT-PCR assay. For the serotype specific A (ASIA/G-VII lineage) rRT-PCR assay, cell culture isolate A/SAU/6/2015 was diluted in unpasteurized whole milk and used as a positive control.

FMDV Detection Assays

RNA extraction and the pan-serotypic rRT-PCR were carried out as previously described using an optimized method (9). Briefly, RNA extractions were carried out using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) using a sample input of 200 μL on a MagMAX™ Express 96 Extraction Robot (Applied Biosystems®) according to manufacturer's instructions. VetMAX™ Xeno™ Internal Positive Control RNA (Applied Biosystems®) was added prior to extraction. Negative extraction controls consisted of unpasteurized whole milk added to lysis buffer.

The pan-serotypic rRT-PCR assay was performed using the reagents, parameters and thermal cycling conditions previously reported (23) with primers and probes described by Callahan et al. (24). One microliter per reaction of VetMAX™ Xeno™ Internal Positive Control LIZ™ Assay (Applied Biosystems®) was also included in the reaction mix. All rRT-PCR assays were performed in duplicate using an Applied Biosystems® 7500 Fast Real-time PCR System. Any milk sample with a C_T value of $\leq$50 was considered positive, and was also tested in duplicate on both lineage-specific rRT-PCR assays for A/ASIA/G-VII (25) and O/ME-SA/Ind-2001d (22) using the reagents, parameters and thermal cycling conditions previously reported. Additionally, samples with amplification below the 0.2 fluorescence threshold (which therefore were not considered positive) by the pan-serotypic rRT-PCR assay (termed "inconclusive" for this study), were also tested on the lineage specific rRT-PCR assays, as lower C_T values have previously been observed for the A/ASIA/G-VII rRT-PCR assay when compared with the pan-serotypic rRT-PCR assay (25).

Development of a Model to Predict FMD Virus Concentrations (C_T Values) in Pooled Milk

To assess the limitations of the milk sampling approach, the C_T values of pooled milk samples were predicted using information supplied by the farm, and from the literature. These "predicted" C_T values were then compared with the "observed" C_T values obtained by the pan-serotypic rRT-PCR assays described in the previous section. The values used for each parameter are described below.

a) **Equating C_T Value With the Number of Virus "Units"**

The limit of detection of FMDV RNA in milk using the pan-serotypic rRT-PCR assay was based on a previous experimental cattle infection study (9), as this is the only study in the literature that uses the same rRT-PCR methodology. In the previous study, 10-fold serial dilutions of a whole milk sample from an infected animal gave a limit of detection of 10^{-6} (9). For this study, a viral genome unit value of 1 (subsequently referred to as a "virus unit") was assigned to this last dilution at which FMDV RNA could be detected (i.e., 10^{-6}), and subsequent virus unit values were assigned to each 10-fold dilution on a log scale (**Figure 1**). Linear regression was applied so that a C_T value could be predicted from the fit, when the total virus unit value (V) in the pooled milk was known ($R^2 = 0.9612$, $y = -4.155x + 48.75$).

b) **Estimating the Number of Virus Units Excreted per Cow at Each Stage of Infection (*Ui*)**

Using data from a previous cattle challenge study (9), FMDV RNA could be detected by the pan-serotypic rRT-PCR assay in the milk between 3 and 28 days post infection (DPI), and clinical signs were first observed at 4 DPI. As the day of infection for each cow on the large-scale farm in Saudi Arabia was unknown, the model assumed that the day clinical signs were first recorded was day [D] 0. Consequently, an excretion profile was created using the mean C_T values based on data collected from two in-contact animals from the challenge study (9) between D-1 to D24, subsequently referred to as the "stage of infection" (*i*) in the model (**Figure 2**). Missing values were interpolated, by retrieving values from the fitted line between the two nearest values. From these C_T values, the virus unit value (U) was predicted for each stage of infection (*i*) using the linear regression model fitted in **Figure 1**.

Previous studies have described a reduced level of virus excretion in nasal fluid, saliva, and esophageal–pharyngeal fluid sample types in vaccinated vs. non-vaccinated animals (26–28). As the effect of vaccination on the duration of excretion or quantity of FMD virus in the milk is unknown, additional factors were included to account for this possibility, as milk samples in this study were collected from regularly vaccinated cattle. Data from previous studies were therefore used to inform the model (26–29), where significantly lower levels of viral excretion (by over 10^2 copies/ml) were observed in vaccinated animals compared with unvaccinated animals. Consequently, in the model prediction for this study, three "levels" of viral excretion were adopted: "1" as described above (no vaccination), and then 10-fold reductions of "1/10" and "1/100" (**Figure 2**). In the model prediction, each ("1," "1/10," and "1/100") virus unit value for each stage of infection (*i*) was used separately to determine the effect this change has on the resulting C_T value in the pooled milk sample. Additionally, the reduction was assumed to remain constant throughout the course of infection (D-1 to D24).

c) **Determining the Number of Cattle at Each Stage of Infection (*Ni*) per Sampling Date (*t*)**

Using records of the onset of clinical signs for each cow and the movement data of individual cows between houses available from the farm, the number of cows at each stage of infection (N_i) per sampling date (t) per house was calculated.

d) **Determining the Reduction in Milk Yield for Infected Cattle**

The only milk yield data available from the farm was the average milk yield per house, per sampling date. To enable simplification of the model, it was assumed that in each

house all lactating cows produced equal volumes of milk (M_u) which was considered a reasonable assumption as cattle were placed into houses on the basis of stage of lactation and milk production.

Due to limited studies quantifying the reduction in milk yield during FMDV infection in highly vaccinated cattle, original milk yield data from a large-scale Holstein-Friesian dairy farm in Kenya that reported a FMD outbreak in August 2012 (30, 31), were used to inform this study. For our study, the mean milk yield from 189 cattle was calculated for each 5 day period during infection (D0 to D4, D5 to D9, D10 to D14, D15 to D19, D20 to D24) as a percentage of the mean yield before infection ("normal yield": D-10 to D-1). ANOVA and Welch two sample *T*-tests demonstrated a significant difference between D5 to D9 and normal yield ($p = 0.001$), where the value of D5 to D9 was found to be 87% of the "normal yield." Therefore, a value of 87% of the normal yield (M_i) was employed for each cow at stage D5–D9 of infection when determining the final number of virus units in a pooled milk sample.

e) **Determining the Final Number of Virus Units in a Pooled Milk Sample per Sampling Date [*F(t)*].**

Using the input parameters calculated in *a)* to *d)*, the final number of virus units in a pooled milk sample per sampling date *[F(t)]*, per house, can be calculated using the following equation:

$$F(t) = \frac{\sum_{i=-1}^{24} M_i U_i N_i(t)}{\sum_{i=-1}^{24} M_i N_i(t) + M_U(H - \sum_{i=-1}^{24} N_i(t))}$$

Where:

- N_i is the number of cows at infection stage i
- U_i is the number of virus units excreted per cow at infection stage i
- M_i is the amount of milk produced by a cow in infection stage i
- M_U is the amount of milk produced by a healthy cow
- H is the total number of cows contributing to the milk pool

f) **Predicting C_T Values for Each Sampling Date (t)**

Using the value of *F(t)* for each house the C_T value was predicted from the linear regression model fitted in section Equating C_T value with the number of virus "units."

Statistical Analyses

All data analyses were performed using R (version 3.5.3) (32) within the RStudio IDE (33). In order to compare the "observed" C_T values obtained from pooled milk samples with "predicted" C_T values, values were plotted for visual comparison. For each sampling date (t), "predicted" and "observed" C_T values were assigned a 0 or 1 for a negative (C_T of >50) or positive (C_T of ≤50) result, respectively. Additional diagnostic cut-off C_T values of 45 and 40 were also investigated. Contingency tables were constructed for each house, and for all houses combined using each virus unit value level (i.e., "1," "1/10," and "1/100"), for which sensitivity, specificity, the proportion of observed agreement (A_{obs}) and the Cohen's Kappa statistic (κ) (34) were calculated.

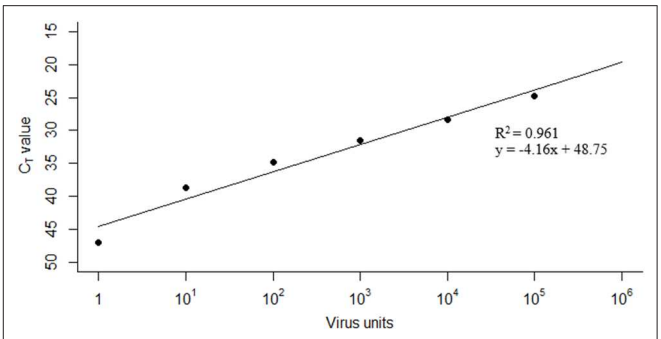

FIGURE 1 | Linear regression used to predict C_T values from total virus unit values. Data taken from limit of detection studies performed by Armson et al. (9).

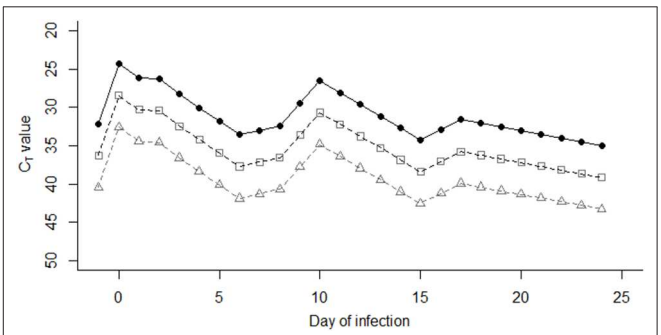

FIGURE 2 | Virus unit values (U) were assigned to each stage of infection (i) between days −1 and day 24 post infection, based on mean C_T values of two animals in studies performed by Armson et al. (9) (closed circles). Open squares and triangles indicate the C_T values represented by "1/10" virus units, and "1/100" virus units, respectively.

TABLE 1 | Summary of outbreak data on the large-scale dairy farm in Saudi Arabia.

Variable		
Total number of lactating cattle during study period (approximate)	4,000	
Number of lactating houses	17	
Number of lactating animals per house[a] (mean, median, range)	227 (237, 44–240)	
Number of lactating houses affected (%)	10 (58.8)[b]	4 (23.5)[c]
Number of clinical cases of FMD[d]	107[b]	33[c]
Overall incidence risk (number of cases/total livestock on farm) (%)	2.8[b]	0.87[c]
Date of index case	02/09/2015[b]	15/02/2016[c]

[a] *Calculated on milk sampling days throughout the study period.*
[b] *A/ASIA/GVII outbreak.*
[c] *O/ME-SA/Ind-2001 outbreak.*
[d] *Case definition used by the farm for FMD was any animal seen salivating with any of the following additional clinical signs: mouth lesions, feet lesions, teat lesions, fever, reduced feed intake, and lameness.*

Potential clustering by management house was accounted for by using a random-effects bivariate model which was used to produce the presented sensitivity and specificity estimates (35).

RESULTS

Epidemiology of the FMD Outbreaks

Throughout the study period, the mean number of lactating cows in each house was 227 (median 237, range 44–240). Details of the farm and clinical incidence for the two FMD outbreaks are shown in **Table 1**. Based on the total number of cattle present on the farm, the overall incidence risk was 2.8% and 0.87% for the two separate outbreaks beginning on 02/09/2015 and 15/02/2016, respectively. The epidemic curves with corresponding sampling periods are shown in **Figure 3A**.

Pooled Milk

During the study period 732 milk samples were collected of which 42 (5.7%) were positive using the pan-serotypic rRT-PCR (**Table 2**, **Figure 3B**). Of these positive samples ($n = 42$), and for those not considered positive but had very low amplification below the fluorescence threshold of 0.2 ("inconclusive," $n = 22$), 32.8% were positive by the A/ASIA/G-VII rRT-PCR assay, and 9.4% were positive by the O/ME-SA/Ind-2001d rRT-PCR assay (**Figure 3C**, **Supplementary Data File 2**). Additionally, 3.1% of the samples tested on the lineage specific assays were positive for both lineages. Of the samples that were positive on the pan-serotypic rRT-PCR assay, 19/42 (45.2%) could not be typed. Of the samples that were inconclusive on the pan-serotypic assay, 3/22 (13.6%) were positive for A/ASIA/G-VII, and 1/22 (4.5%) was positive for O/ME-SA/Ind-2001d.

Correlation Between Epidemiological Data and FMDV RNA in Pooled Milk

Laboratory results from the pooled milk samples were directly compared against clinical data collected during the FMD outbreaks. The first period of clinical disease was seen in lactating cows between the 02/09/2015 and 24/09/2015 ($n = 99$), with two recurrences of clinical disease in a smaller number of cows in mid-October ($n = 1$) and the first half of November 2015 ($n = 7$) (**Figure 3A**). Clinical samples (vesicular epithelium/fluid) were collected from clinically affected animals ($n = 3$) in September and October 2015, and were characterized as belonging to the A/ASIA/G-VII lineage. Further clinical disease was recorded at the beginning of February 2016 ($n = 33$) and a clinical sample identified the strain as from the O/ME-SA/Ind-2001d lineage. Visual comparison of the epidemic curve and temporal representations of rRT-PCR results indicates some clustering of positive pooled milk samples around the occurrence of new clinical cases but with a wider distribution (**Figure 3**). Clustering of lineage A/ASIA/G-VII positive results can also be seen from the commencement of sampling to the end of November, concurrent with reports of this lineage from clinical samples. The clinical incidence in lactating cows over the whole study period was 3.6% (**Table 1**), while FMDV genome was detected in 5.7% of pooled milk samples (**Table 2**). A contingency table was constructed to determine the sensitivity (Se) and specificity (Sp) of the pan-serotypic rRT-PCR, using the number of new clinical cases observed on milk sample collection days for all houses sampled as the gold standard: Se = 49.3% (95% confidence interval (CI): 30.7–68.1%), Sp = 92.5% (95% CI: 90.0–94.4%) (**Supplementary Data File 1**).

FMDV genome was detected in pooled milk in 17 out of the 19 (89.5%) sampled houses compared to 14/19 (73.7%) houses that reported clinical cases. Of the latter, 13 houses were PCR positive at some point during the outbreaks (**Figure 4**, **Supplementary Data Files 3, 4**). Furthermore, four houses were positive by rRT-PCR with no recorded clinical cases at any time during the outbreaks. There were also a total of eight samples taken where the rRT-PCR result was negative but there were new clinical cases observed on that day.

Predicting C_T Values in Pooled Milk

Predicted C_T values were obtained for each house and compared with the observed C_T values from the pan-serotypic rRT-PCR (**Figure 4**, **Supplementary Data Files 3, 4**). The potential effect of reduced virus excretion that may occur due to vaccination was also investigated, where C_T values were predicted for the different levels of virus excretion to accommodate the possible impact of FMDV vaccination ("1," "1/10," and "1/100") (**Figure 4**, **Supplementary Data Files 3, 4**). Predicted C_T values were not calculated for some houses due to a lack of available epidemiological data required for the analysis, or because the house was used as a quarantine pen to isolate new cases of FMD at the start of the outbreak, and therefore regular milk samples were not collected (Houses 17 and 18). Additionally, House 12 is not included in **Figure 4** as both the observed and predicted results were all negative.

Visual comparison of observed vs. predicted C_T values revealed instances where (i) positive results were obtained for both observed and predicted, with C_T values that were generally comparable, (ii) positive results were obtained for the predicted values only, and (iii) positive results were obtained for the observed results only, although this was less frequent than when comparing observed C_T values with new clinical cases (**Figure 4**, **Supplementary Data Files 3, 4**).

The lowest predicted C_T values (i.e., the highest viral RNA concentration) obtained for "1," "1/10," and "1/100" were 30.4, 34.5, and 38.7, respectively, compared with 31.6 for the observed results. A reduction in viral excretion increased the predicted C_T values and, in some instances, decreased the duration for which milk samples from a house would remain positive ($C_T \leq 50$). Additionally, applying a diagnostic cut-off value of 45 or 40 decreased the likelihood and duration of predicted positive C_T values. Contingency tables for all houses combined indicated that a virus excretion level of "1/10" with a diagnostic cut-off C_T value of 40 generated results closest to those of the observed rRT-PCR results (Se = 38.1% [95% CI: 23.2–55.6%], Sp = 95.1% [95% CI: 92.7–96.7%], $A_{obs} = 0.95$, $K = 0.31$) (**Supplementary Data File 5**). A reduction in sensitivity and increase in specificity was observed when these values were compared with estimates of sensitivity and specificity using records of new clinical cases as the "gold standard."

DISCUSSION

This study aimed to expand on previous work to determine the utility of testing pooled milk by rRT-PCR as an alternative approach for FMD surveillance in vaccinated dairy herds. During the 6 month study period, 732 pooled milk samples were collected

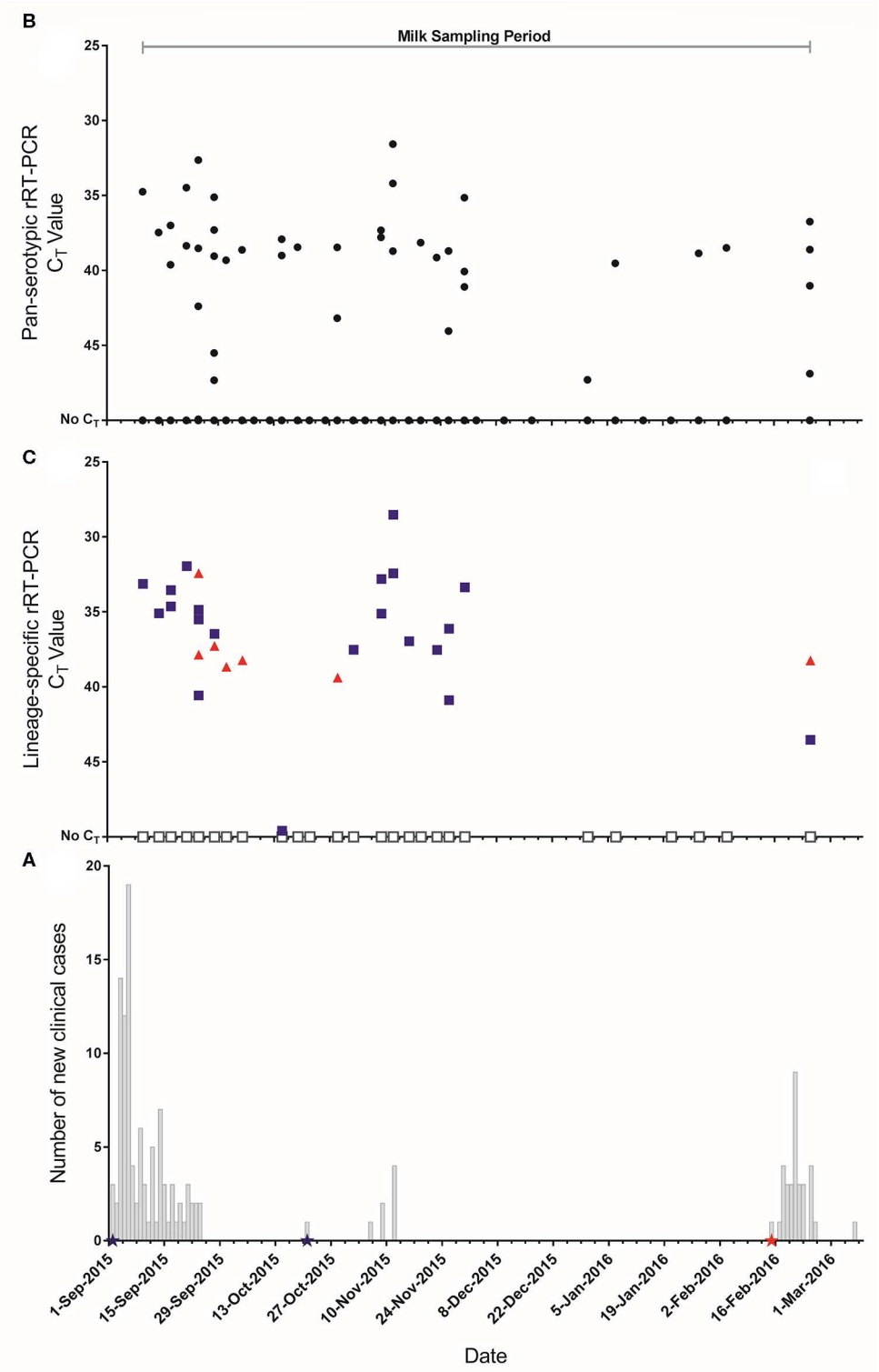

FIGURE 3 | (A) Epidemic curves of FMD outbreaks on the farm. Stars represent dates where clinical samples (vesicular epithelium/fluid) were collected and submitted to the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) and reported as ★: A/ASIA/G-VII, ★: O/ME-SA/Ind-2001d. **(B)** C_T values from the pan-serotypic rRT-PCR assay (●) for pooled milk samples collected from 19 lactating houses in the large scale dairy farm in Saudi Arabia throughout the study period ($n = 732$). **(C)** C_T values for each lineage specific rRT-PCR assay for samples that tested positive ($C_T \leq 50$), or where very low amplification was observed (below the threshold), in the pan-serotypic rRT-PCR assay. ■: A/ASIA/G-VII. ▲: O/ME-SA/Ind-2001d. □: Sample could not be typed.

TABLE 2 | Summary of milk sample results for all rRT-PCR assays for the large-scale dairy farm in Saudi Arabia.

Variable	Farm
Duration of milk sampling (weeks)	25
Number of houses that milk samples were collected from	19
Number of pooled milk samples tested	732
Number positive[a] by pan-serotypic rRT-PCR assay (%)	42 (5.7%)
Number positive[a] by A/ASIA/G-VII rRT-PCR assay (%)	21/64[b] (32.8%)
Number positive[a] by O/ME-SA/Ind-2001d rRT-PCR assay (%)	6/64[b] (9.4%)

[a] *Positive results are those with at least one well giving a C_T of ≤50.*
[b] *22 samples were considered "inconclusive" (amplification was observed below the fluorescence threshold of 0.2) and were therefore also tested by the lineage-specific rRT-PCR assays.*

from a large-scale dairy farm housing ∼4,000 cattle during an FMD outbreak.

The first objective of this study was to determine whether detection and characterization of FMDV by rRT-PCR was possible from pooled milk samples and compare these results with epidemiological data recorded during the outbreaks. This is the first study we are aware of showing that FMDV genome can be detected in milk samples from regularly vaccinated cattle using a proportional in-line milk sampler on a large-scale dairy farm. The mean C_T values obtained in the pan-serotypic rRT-PCR assay were high (>31), most likely due to the dilution of milk from a relatively small number of infected animals in groups of lactating cattle numbering up to 240 and collectively producing in excess of 10,000 liters per day. These results confirm the hypotheses from previous laboratory and modeling studies that suggested FMDV genome could be detected at these dilutions during outbreaks in field settings (13, 16, 36).

Lineage-specific rRT-PCR assays (22, 25) confirmed the presence of the A/ASIA/G-VII and O/ME-SA/Ind-2001d lineages in the pooled milk samples, and this was supported by reports from samples collected from clinical cases that were sent separately for laboratory testing. Reports for these samples demonstrated that the two outbreaks were caused by different FMD viral lineages, the first due to the A/ASIA/G-VII lineage, and the second the O/ME-SA/Ind-2001d lineage, both of which are thought to have emerged recently from South Asia (21, 22). The rRT-PCR results from the pooled milk samples suggest that there was a period of co-circulation or possible even co-infection with FMD viruses from these lineages. Co-infection in clinical samples from individual cattle in Saudi Arabia has been reported previously (37), though it is unknown if this occurred during the study period given that samples were taken and tested from only three clinical cases. Indeed, during this study, the collection of a variety of sample types from numerous individual animals throughout the period of infection and beyond (e.g., vesicular lesion material, blood, nasal/oral swabs and milk) may have allowed for the detection of co-infection, and may have also enabled a more thorough validation of the pooled milk surveillance approach.

Although the farm routinely vaccinated with a high potency, polyvalent FMD vaccine, it has been recently demonstrated that the serotype A components of this vaccine are not antigenically matched, and generate poor cross-protection in a potency test against A/ASIA/G-VII viruses (38). Furthermore, although individual serotype O components (such as O-3039) appear to be antigenically matched, or (O-Manisa) provide experimental protection (39) against O/ME-SA/Ind-2001d viruses, studies under field conditions (20) showed that the polyvalent vaccine used on this farm did not provide adequate heterologous cross-protection to provide full herd immunity against field viruses from the A/ASIA/G-VII and O/ME-SA/Ind-2001d lineages. This may explain why cattle still became clinically affected during the study period, albeit with a low overall incidence risk. Indeed, the A/ASIA/G-VII lineage was detected in more pooled milk samples compared to O/ME-SA/Ind-2001d during the entire study period, consistent with expected vaccine performance from respective *in vitro* vaccine-matching data and experimental studies (38, 39), A. Ludi, personal communication). The detection of a greater number of positive milk samples for the A/ASIA/G-VII lineage could also be due to the relative performance of the typing rRT-PCR assays, as in previous validation studies, lower C_T values for the A/ASIA/G-VII lineage typing assay have been demonstrated compared to the pan-serotypic rRT-PCR assay (indicating an increased sensitivity) (25), whilst C_T values for the O/ME-SA/Ind-2001d typing assay have been demonstrated to be comparable to the pan-serotypic rRT-PCR (22).

To validate the use of pooled milk for the surveillance of FMDV on this large-scale farm, pan-serotypic rRT-PCR results from the pooled milk samples were compared with the clinical incidence of FMD during the study period. At the farm level there were four temporal clusters of clinical cases with gaps of at least 15 days between these clusters. Visual appraisal of the data indicated FMDV rRT-PCR results to be generally correlated with these clusters although they showed a wider distribution around and in between the clusters of clinical cases. Comparison of the onset of individual clinical cases and the assay results on milk sampling days at the house level, revealed only 6 occasions when milk samples were positive and a new clinical case was recorded on the same day. There were also occasions when either (i) positive milk samples were obtained when there were no new clinical cases on that day, or (ii) there were new clinical cases occurring but a positive result was not observed in the milk. This resulted in a low sensitivity and moderate specificity for the pooled milk rRT-PCR assay (49.3 and 92.5%, respectively). However, this approach is limited by only comparing the assay results with the onset of new clinical cases on the sampling day which does not account for FMDV genome shedding in pre-clinical, convalescent, or subclinically infected animals.

To attempt to account for these limitations, "observed" C_T values obtained by the pan-serotypic rRT-PCR assays were compared with "predicted" C_T values for each house based on detailed epidemiological and cattle movement data from the farm, and data from recent literature. Although these results were similar, compared with the onset of clinical cases there was a reduction in sensitivity and an increase in specificity. It is likely that this may be due to the reduced number of sampling points available for the predictive analysis, due to a lack of epidemiological data available from two of the houses. It is possible that this reduced sensitivity (i.e., instances where there

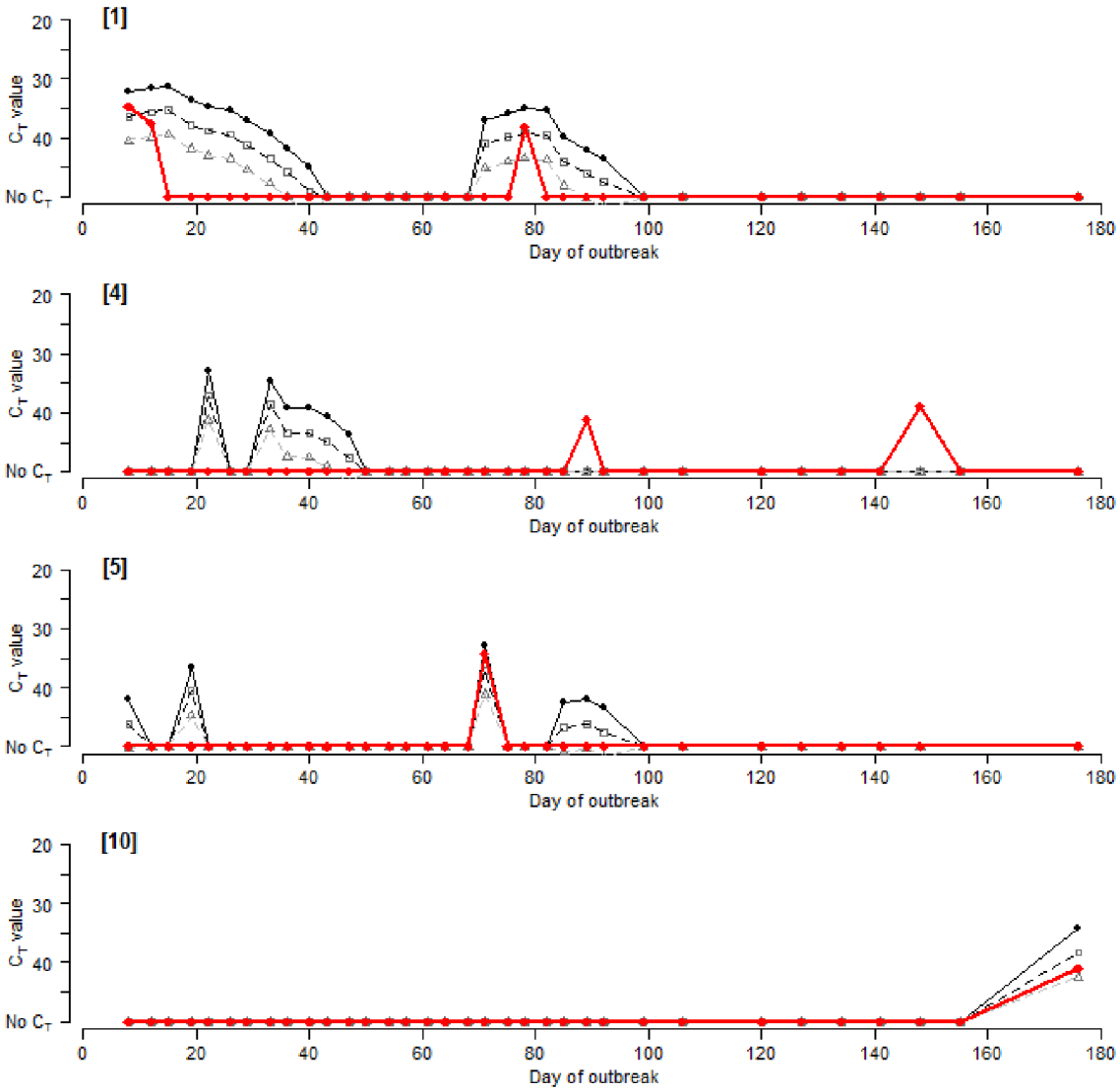

FIGURE 4 | "Observed" C_T values for the rRT-PCR of pooled milk samples (♦) vs. "Predicted" C_T values at "1" viral excretion (●), "1/10" (□) and "1/100" (△), for selected management houses 1, 4, 5, and 10. Results for the remaining houses are included in **Supplementary Data Files 3, 4**.

were positive "predicted" results but negative "observed" rRT-PCR results of the pooled milk), was due to a lower quantity and shorter duration of viral excretion in the milk of these vaccinated infected cattle, than was assumed in the model. This theory supports findings by Leeuw et al. (40) and Orsel et al. (26) who were unable to detect FMD virus in the milk of well-vaccinated cattle after challenge. However, these previous studies used a homologous or efficacious vaccine to the challenge strain and Leeuw et al. (40) only focussed on the detection of infectious live virus instead of FMDV RNA. As there are no other studies known to have considered viral excretion into the milk of vaccinated cattle, data used to inform the model was based on those studies that measured viral excretion from vaccinated and non-vaccinated animals in alternative samples such as nasal fluid, saliva, and esophageal–pharyngeal fluid (26–28). The authors acknowledge the limitation of this approach, particularly since the quantity and duration of viral excretion seemed to have a substantial impact on the likelihood of predicting a positive result in the milk. Consequently, further investigation into the effect of vaccination on viral excretion in milk is required and would enhance the predictive ability of the model.

Management practices on the farm may also have contributed to the low sensitivity of the pooled milk rRT-PCR assay. These include the inconsistent removal of clinical cases and milking practices during the study period in response to the outbreak, with the potential for increased sensitization of farmers to disease as the outbreak progressed, resulting in a decreased chance of milk from an infected cow contributing to the milk pool. Additionally, the proportional in-line sampling method may not be truly representative of all cattle in the group, as reported previously (41). Although the in-line sampler is designed to represent the whole milking, it has been demonstrated that

this method may terminate sampling early (41) and milk from infected cattle may be excluded from the sample tested leading to false negative results. This may explain the low sensitivity obtained for this FMDV detection system compared with what was predicted in the model. Other methods, for example, collecting a sample from the bulk tank after thorough agitation, may be more representative (42), and could be considered for future studies.

During the study period there were also instances when there were positive rRT-PCR results in the milk samples but no new clinical cases observed, or indeed "infected" (D-1 to D24) cows present in the house that would excrete virus into the milk pool. The possibility that these "false positives" are due to laboratory contamination cannot be excluded. However, the laboratory methodology used in this study has been shown to be highly specific (data not shown), and as there were a high number of "negative" samples it is unlikely that these results are due to either laboratory contamination or non-specific amplification. Alternative explanations for this observation include spill-over of virus between houses as they were being milked (i.e., virus from an infected animal in one house may have been carried over to the milk from the subsequent house, generating false-positive results for an otherwise negative house) as there was no milk line disinfection between houses. There is also the possibility of delays in clinical case detection, sub-clinical infections or mild clinical cases that may not have been noticed by farm workers. Subclinical infections in vaccinated animals have been reported previously (43–45) and this is a possible explanation for the prolonged period between cases (up to 27 days) although it is unknown whether the outbreaks on this farm were prolonged circulation or due to new virus introductions.

This is the first study to evaluate the use of pooled milk as a surveillance sample for the detection of FMDV on large-scale dairy farms in endemic regions. This study demonstrates that rRT-PCR testing of pooled milk may be utilized for FMD surveillance and may reveal underlying sub-clinical FMD infection. More representative sampling methods should be investigated that may increase the sensitivity of this approach including investigations into the required frequency of sample collection and an exploration on how the dairy value chain may be exploited for FMD surveillance. Subsequently, this methodology could be integrated into FMD surveillance programs providing significant benefits over conventional surveillance strategies. The similarities in the farming system evaluated in this study and dairy farms in FMD-free countries highlights the potential of this surveillance approach for use in disease-free regions in the event of an incursion of FMDV, to allow rapidly identification of infected herds, tracing the source and spread of infection and to screen infected premises to ensure disease freedom.

DATA AVAILABILITY STATEMENT

All datasets analyzed for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because samples used were collected as part of the farm's usual milking process—no change to animal's behavior/routine from normal. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BA carried out laboratory work, data analysis, and wrote the manuscript. NL and DK led the study design. NL assisted with data interpretation and statistical analysis and helped draft the manuscript. SG assisted with statistical analysis and formulated the model equation. IQ facilitated the collection of samples in Saudi Arabia. VM contributed reference laboratory expertise and assistance to test diagnostic samples. All authors were involved in editing and approving of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00264/full#supplementary-material

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Duration of Contagion of Foot-And-Mouth Disease Virus in Infected Live Pigs and Carcasses

Carolina Stenfeldt[1,2], *Miranda R. Bertram*[1,3], *George R. Smoliga*[1], *Ethan J. Hartwig*[1], *Amy H. Delgado*[4] *and Jonathan Arzt*[1]*

[1] *Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, NY, United States,* [2] *Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, United States,* [3] *PIADC Research Participation Program, Oak Ridge Institute for Science and Education, Oak Ridge, TN, United States,* [4] *Monitoring and Modeling, Center for Epidemiology and Animal Health, Animal and Plant Health Inspection Service, United States Department of Agriculture, Fort Collins, CO, United States*

Data-driven modeling of incursions of high-consequence, transboundary pathogens of animals is a critical component of veterinary preparedness. However, simplifying assumptions and excessive use of proxy measures to compensate for gaps in available data may compromise modeled outcomes. The current investigation was prospectively designed to address two major gaps in current knowledge of foot-and-mouth disease virus (FMDV) pathogenesis in pigs: the end (duration) of the infectious period and the viability of FMDV in decaying carcasses. By serial exposure of sentinel groups of pigs to the same group of donor pigs infected by FMDV A24 Cruzeiro, it was demonstrated that infected pigs transmitted disease at 10 days post infection (dpi), but not at 15 dpi. Assuming a latent period of 1 day, this would result in a conservative estimate of an infectious duration of 9 days, which is considerably longer than suggested by a previous report from an experiment performed in cattle. Airborne contagion was diminished within two days of removal of infected pigs from isolation rooms. FMDV in muscle was inactivated within 7 days in carcasses stored at 4°C. By contrast, FMDV infectivity in vesicle epithelium harvested from intact carcasses stored under similar conditions remained remarkably high until the study termination at 11 weeks post mortem. The output from this study consists of experimentally determined data on contagion associated with FMDV-infected pigs. This information may be utilized to update parameterization of models used for foot-and-mouth disease outbreak simulations involving areas of substantial pig production.

Keywords: foot-and-mouth disease, foot-and-mouth disease virus, FMD, FMDV, pig, transmission, contagion

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***Correspondence:**
Jonathan Arzt
jonathan.arzt@usda.gov

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INTRODUCTION

Foot-and-mouth disease (FMD) is a high-impact viral disease capable of infecting all cloven-hoofed domestic livestock and numerous wildlife species (1). The causative agent, foot-and-mouth disease virus (FMDV; genus: *Aphthovirus*, family: *Picornaviridae*) causes initial infection via the upper respiratory- or gastrointestinal tracts (depending on host species), followed by systemic generalization with lameness, inappentence, and vesicular lesions on the feet and in the mouth as characteristic clinical findings (2, 3).

FMD is endemic in large parts of the world, including most of Africa, the Middle East, and Asia, as well as certain regions of South America (4). By contrast, Europe, North America, Australia, and New Zealand, are kept free of FMD by means of strict regulation of import of animals and animal products as well as by extensive control programs designed to rapidly detect potential incursions. Due to the potentially catastrophic consequences associated with incursions of FMDV into previously free countries (5), substantial efforts are invested into veterinary preparedness and contingency planning. Such endeavors often include mathematical modeling of FMD outbreaks within defined geographic regions for the purpose of estimating disease spread and impact, and to evaluate the effect of applied control measures (6). Outbreak simulations are critically dependent upon appropriate parameterization of models. Essential input parameters need to reflect the detailed structure of the susceptible population, as well as intrinsic factors of host-pathogen interactions that capture the progression of infection and contagiousness within individual animals.

Model parameterization for FMD outbreak simulations is often based upon meta analyses of published experimental studies (7–9). Although there are clear benefits of utilizing available published data for this purpose, simplifying assumptions and extensive use of proxy values are often needed to compensate for gaps in available data. Specifically, the timing of infectiousness in relation to initial exposure and the appearance of clinical signs of disease is of critical value for modeling within-herd transmission of FMD. However, infectiousness, i.e., the ability of an infected animal to transmit infection to in-contact animals, is rarely directly quantified in controlled FMDV experiments. Instead, proxies such as detection of viral genome in secretions, or observed clinical signs of disease are often used to estimate infectiousness for model parameterization (7, 8).

Previous works have demonstrated that the selection and definition of proxies can lead to greatly varying estimates for the onset and duration of infectiousness in FMDV-infected cattle and pigs (10, 11). Such variation in input parameters can lead to substantially different estimates of outbreak dispersal and duration in downstream modeling. Additionally, it was demonstrated that failure to account for preclinical (incubation phase) infectiousness in FMDV-infected pigs substantially underestimated the outcomes of FMD outbreak simulations in areas of dense pig production (10). However, a factor that has received less attention, but that is potentially of similarly critical importance for FMD modeling, is the end of infectiousness in infected hosts, which contributes to definition of the total infectious period. This factor would likely be of greatest impact in scenarios in which disease detection is delayed, or when timely depopulation of infected farms may not be possible. The latter of those circumstances will likely be an issue if an FMD incursion were to affect multiple large livestock holdings, as available resources for depopulation and carcass disposal may be overwhelmed (12–14).

Unless properly disposed of, carcasses of infected animals on depopulated farms may represent a substantial source of contagion (15). It has been reported that FMDV in carcasses will become inactivated due to post mortem acidification (16, 17). Although this has proven to be the case in muscle (18), the greatest viral loads during clinical FMD are in vesicular epithelium at peripheral sites (19). These sites are less likely to be affected by systemic acidification and core temperature changes due to autolysis and are by their peripheral location at the exterior of the carcass also in direct contact with the environment.

This current study reports the descriptive results from an experiment that was prospectively designed to evaluate the end of infectiousness in group-housed FMDV-infected pigs. Additional output includes longitudinal sampling of muscle and vesicle epithelium from carcasses of pigs that had been euthanized during the clinical phase of FMD and air sampling in rooms from which infected pigs had been removed.

METHODS

Virus

The virus used for the current study was a cattle-derived isolate of FMDV A24 Cruzeiro that had been passaged once in pigs. The pathogenesis and transmission characteristics of this specific virus isolate in pigs have been described in detail in previous publications (19–21).

Animals

Animal experiments were carried out within BSL3Ag research facilities at the Plum Island Animal Disease Center, New York. All experimental procedures were approved by the institutional animal care and use committee that functions to ensure ethical and humane treatment of animals (protocol 231-17). The animals used were female Yorkshire pigs weighing ∼25 kg at delivery. The pigs were delivered to the facility as one batch and allowed two weeks of acclimation in the facility prior to start of the study.

Study Design

Sixteen pigs were divided into four groups of four pigs each. Group 1, which were assigned as the "donor pigs", were infected with FMDV by intra-oropharyngeal (IOP) inoculation as previously described (22). In brief; sedated pigs were placed in dorsal recumbency and 2ml of diluted inoculum was deposited onto the surface of the tonsil of the soft palate using a blunt-ended cannula. Each pig received a challenge dose of 100, 50% infectious doses titrated *in vivo* in pig heel bulb epithelium (23), which corresponded to 10^7, 50% tissue culture infectious doses ($TCID_{50}$) when back-titrations were performed using the highly sensitive LFBKαvβ6 cell line (24, 25). At 3 pre-determined time points, corresponding to 5, 10, and 15 days post inoculation (dpi) of the donor pigs, the four donor pigs were relocated to co-habitate with 4 pigs of study groups 2, 3, and 4, respectively, for 24 h each. The contact exposure consisted of co-housing the 4 donor pigs with the 4 susceptible recipients within separate animal isolation rooms with 17.5 m^2 (group 2) or 10.2 m^2 (groups 3 and 4) available floor area. Feed was provided on the floor at the start of the contact exposure period. After each of the 24 h exposure periods, the donor pigs were moved back to their original room and remained in isolation until the subsequent exposure period. After completion of exposure of study group

4 (16 dpi of the donors) the donor pigs were euthanized and removed from the study. The contact-exposed pigs in groups 2–4 were monitored for 14 days post exposure (dpe), or until development of fulminant FMD.

Sample Collection

Antemortem samples consisted of whole blood obtained by jugular venipuncture, and oropharyngeal fluid (OPF) obtained by swabbing the oropharynx using a large cotton swab. Swabs were immediately submerged in 2 ml of minimum essential media with 25 mM HEPES and were subsequently centrifuged to extract absorbed fluid. Blood samples were separated by centrifugation and aliquots of serum and OPF were stored at −70°C until further processing. Samples were collected prior to inoculation or exposure (0 dpi/dpe). The donor pigs were sampled every other day from 0 to 6 dpi, and again at 10 and 15 dpi. Contact-exposed pigs were sampled every other day from 0 to 10 dpe, and again at 14 dpe. Additional OPF swabs were collected so that samples were obtained from all pigs at the beginning and end of the contact exposure periods (i.e., from donor pigs, additional OPF samplings were done at 5, 11, and 16 dpi, and for contact-exposed pigs at 1 dpe).

The onset and progression of the pigs' clinical status (lesion distribution) was quantitated using a previously described scoring system (26). In brief, each of 16 digits having a characteristic FMDV lesion contributed one point toward a cumulative score, with four additional single points added for lesions within the oral cavity, on the snout, on the lower lip, and on carpal/tarsal skin, respectively, resulting in a maximum score of 20.

FMDV Recovery From Muscle and Vesicular Lesions Post Mortem

The viability of FMDV in muscle and vesicle epithelium after death of the animals was evaluated by post-mortem sampling at repeated time points. Initially, the four pigs from study group 2 of the transmission study reported herein were utilized for this purpose. Based on the findings from these initial samples, additional pig carcasses were recruited from unrelated experimental studies in order to extend the post mortem sampling period and to include a second strain of FMDV. These studies comprised pigs that had been infected with the same strain of FMDV A24 as was used in the transmission study, or with a bovine-derived strain of FMDV O/SKR/2010 (27). All pigs utilized for post-mortem sampling were euthanized during acute FMD by intravenous injection of an overdose of sodium pentobarbital "Fatal plus®" (85.8 mg/kg) under deep sedation (Telazol, Ketamine, and Xylazine at 3, 8, and 4 mg/kg, respectively). Carcasses were stored intact in metal cans lined with plastic bags at 4°C throughout the sampling periods.

From the first batch of FMDV A24-infected pigs (study group 2), samples of vesicular epithelium and skeletal muscle (semitendinosus) were obtained at 0, 1, 3, and 5 days post mortem (dpm). The second batch of FMDV A24-infected pigs were subjected to sampling of vesicular epithelium at 5, 10, and 19 dpm. The third batch of pigs were infected with FMDV O/SKR/2010, and samples of vesicle epithelium were harvested at approximately weekly intervals from 4 to 37 dpm. The fourth batch of pigs were infected with FMDV A24; muscle samples were obtained at 0, 7, and 14 dpm, with vesicle epithelium samples harvested at approximately weekly intervals from 0 to 77 dpm. The final sampling at 77 dpm also included harvest of submandibular lymph nodes, neck skin, and bone marrow.

FMDV recovery in tissue samples obtained from pig carcasses was evaluated by qRT-PCR and virus isolation (VI). Additionally, virus titrations were performed on all vesicle epithelium samples from batch 4 carcasses and on additional tissues harvested at 77 dpm that were VI-positive. Titer values expressed as log10 $TCID_{50}$ were used for statistical analysis of virus decay: a simple linear regression with day as the independent variable and titer as the dependent variable was used to estimate the titer half-life and duration of detection.

Air Sampling

Air sampling in the rooms housing experimental groups 2 and 3 was performed using a Model 1,000 air pump developed by the Program Executive Office for Chemical Biological Defense (PEO-CBD), fitted with an original DFU filter assembly holding two separate Lockheed Martin polyester filter disk (1.0 lm filter, diameter 47 mm, Catalog number DFU-P-24; Lockheed Martin, Washington DC, USA) as previously described (21). The airflow through the unit was 15 l/min, and the pump was placed out of reach of the animals. The filter disk were removed and replaced at 24 h intervals. The pump was left running in between daily sample collections, and the filters were removed prior to cleaning of the animal rooms, with the pump turned off as the room was cleaned to avoid sampling of artifactually re-suspended aerosols. Pumps were started the day before the start of contact exposure, and the first filters (0 dpe) were removed before the FMDV-infected donor pigs entered the room. Air sampling in room 2 continued through 8 days after euthanasia of the contact-exposed pigs and washing of the room. Air sampling in room 3 continued for 24 h after removal of the last pigs.

FMDV RNA Detection in Serum, Swabs and Tissues

Tissue samples (ca 20 mg) were thawed and macerated using a TissueLyser bead beater (Qiagen, Valencia, CA) and stainless steel beads (Qiagen cat. no. 69989). Air filters were cut in quarters and disrupted using a similar approach as for tissue samples, but with washed glass beads (combination of bead sizes 425–600 μm and ≤106 μm; Sigma cat. nos. G4949/G8772). The air filter "homogenate" was subsequently centrifuged to extract the fluid that had been absorbed by the filter. Tissue- and air filer macerates, serum, and swab samples were analyzed using quantitative real-time RT-PCR (qRT-PCR), targeting the 3D region of the FMDV genome (28) with forward and reverse primers adapted from Rasmussen et al. (29), and chemistry and cycling conditions as previously described (30). Cycle threshold values were converted into FMDV genome copy numbers (GCN) per ml by use of a standard curve derived from analysis of 10-fold dilutions of *in-vitro* synthesized FMDV RNA. The equation of the curve of GCN vs. Ct values was further adjusted for dilutions used during processing of samples.

Virus Isolation

Aliquots of macerated tissue samples and air filters were cleared from debris and potential bacterial contamination by centrifugation through Spin-X® filter columns (Costar Cat.no 8163). Clarified samples were subsequently analyzed for infectious FMDV through virus isolation (VI) on LFBK αvβ6 cells (24, 31), following a protocol previously described (32). Presence of FMDV was further confirmed by qRT-PCR analysis of VI cell culture supernatants.

OPF samples obtained from donor pigs at the beginning of each of the 3 contact exposure periods, as well as select post mortem tissue samples (see above), were titrated on LFBK αvβ6 cells in micro-titer plates using similar methods as described above, but with end point titers ($TCID_{50}$) determined using standard methods (33).

RESULTS

Donor Pigs

All 4 pigs within the donor group developed clinical FMD of a similar severity and timeline as has previously been described for this virus isolate (19, 22). Vesicular lesions appeared at 2–3 dpi, and all pigs were viremic and shedding high quantities of FMDV RNA (6.15–8.25 log_{10} GCN/ml) by the first sampling time point at 2 dpi (**Figure 1**, **Table 1**). All four donor pigs had reached their maximum cumulative lesion scores by 4 dpi (**Figure 1**).

Contact Transmission of FMD

Group 2; 5 dpi

At the beginning of the first contact exposure at 5 dpi, the clinical conditions of the donor pigs were improving. They were all non-febrile and ambulant, but with moderate lameness and with ruptured vesicular lesions on their feet. The range of FMDV RNA detected in OPF from the donor pigs at 5 dpi was 6.12–7.08 log_{10} GCN/ml (average 6.66 log_{10} GCN/ml). Three of four donor pigs had measurable FMDV titers in OPF at 5 dpi, ranging from $10^{2.25}$ to $10^{3.25}$ $TCID_{50}$ (**Table 1**). At 6 dpi, corresponding to the end of contact exposure of group 2, the level of FMDV RNA detection in OPF of the donors was 6.08–6.89 log_{10} GCN/ml (average 6.41 log_{10} GCN/ml), and none of the OPF samples had a measurable FMDV titer.

The contact-exposed pigs in group 2, which were exposed to the donors from 5 to 6 dpi, all had moderate levels of FMDV RNA in OPF swabs at the end of the contact exposure (1 dpe; range 5.03-6.85 log_{10} GCN/ml, average 5.75 log_{10} GCN/ml). All 4 pigs in group 2 were viremic at 2 dpe, and all had vesicular lesions at 3 dpe (**Figure 1B**). The pigs in group 2 were euthanized at 4 dpe and were subsequently subjected to post mortem sampling of muscle and vesicular lesions.

Group 3; 10 dpi

At 10 dpi, corresponding to the start of contact exposure of group 3, the donor pigs had recovered from apparent clinical FMD. They were all ambulatory and moving freely without marked lameness. Vesicular lesions were healing, although re-epithelializing vesicular erosions were present on their feet. At 10 dpi, FMDV RNA shedding in OPF of the donor pigs ranged from 4.79 to 6.63 log_{10} GCN/ml (average 5.82 log_{10} GCN/ml). An FMDV titer of $10^{2.62}$ $TCID_{50}$ was measured in 1 of 4 OPF samples obtained at that time point, whereas the OPF of the 3 additional pigs was negative for infectious virus. At the end of exposure of group 3, at 11 dpi, the measured FMDV RNA detection in OPF of the donor pigs was 4.05–6.05 log_{10} GCN/ml (average 5.07 log_{10} GCN/ml; **Table 1**).

FMDV RNA was present in OPF swabs of all the contact-exposed pigs of group 3 at the end of exposure (1 dpe; range 5.64–6.85 log_{10} GCN/ml, average 5.91 log_{10} GCN/ml). Two of the pigs were viremic at 2 dpe, with viremia confirmed in the remaining two pigs at the subsequent sampling at 4 dpe. Two of the pigs had vesicular lesions at 3 dpe, and vesicles were detected at 4 and 5 dpe, respectively, in the remaining 2 pigs (**Figure 1C**). The two pigs that developed vesicles at 2 dpe were euthanized on the subsequent day, and the remaining two pigs were both euthanized at 6 dpe.

Group 4; 15 dpi

At 15 dpi, which was the start of contact exposure of group 4, the clinical conditions of the donor pigs had further improved. All 4 pigs still had recognizable healing FMD lesions on their feet and were shedding detectable quantities of FMDV RNA in OPF (range 4.68–5.31 log_{10} GCN/ml, average 5.12 log_{10} GCN/ml). At the end of the final contact exposure, FMDV RNA levels in OPF from the donor pigs were 4.85–6.28 log_{10} GCN/ml (average 6.11 log_{10} GCN/ml). All virus titrations of OPF obtained from the donor pigs at 15 dpi were negative (**Table 1**).

FMDV RNA was detectable in OPF from all contact-exposed pigs of group 4 at the end of exposure (range 4.49–5.09 log_{10} GCN/ml, average 4.76 log_{10} GCN/ml). However, all subsequent samples were FMDV-negative, and none of the 4 pigs developed clinical FMD (**Figure 1D**).

Detection of FMDV in Air

Air sampling was performed in the rooms housing study groups 2 and 3, starting at 1 day prior to contact exposure, and ending at 8 or 1 day(s) after removal of the pigs from the rooms for group 2 and 3, respectively (**Figure 2**). The inoculated donor pigs were housed with the contact groups from 0 to 1 dpe. In both rooms, detected FMDV RNA levels decreased slightly after removal of the donor pigs (2 dpe), to then increase by the following day (3dpe), suggesting increased virus shedding by the newly infected contact-exposed pigs. The 4 pigs in group 2 were euthanized at 4 dpe, after which the room was rinsed with steam - hot water hosing with no detergent or disinfectant. Low levels of FMDV RNA was detected in air samples from this room for another 7 days (up to 11 dpe), but there were no VI-positive samples after removal of the pigs (**Figure 2**). Two pigs from group 3 were euthanized at 5 dpe, and the other 2 at 6 dpe. The room was similarly washed after removal of the pigs, and the final air filter sample was collected the following day. All air filter samples from room 3 were positive by both qRT-PCR and VI (**Figure 2**).

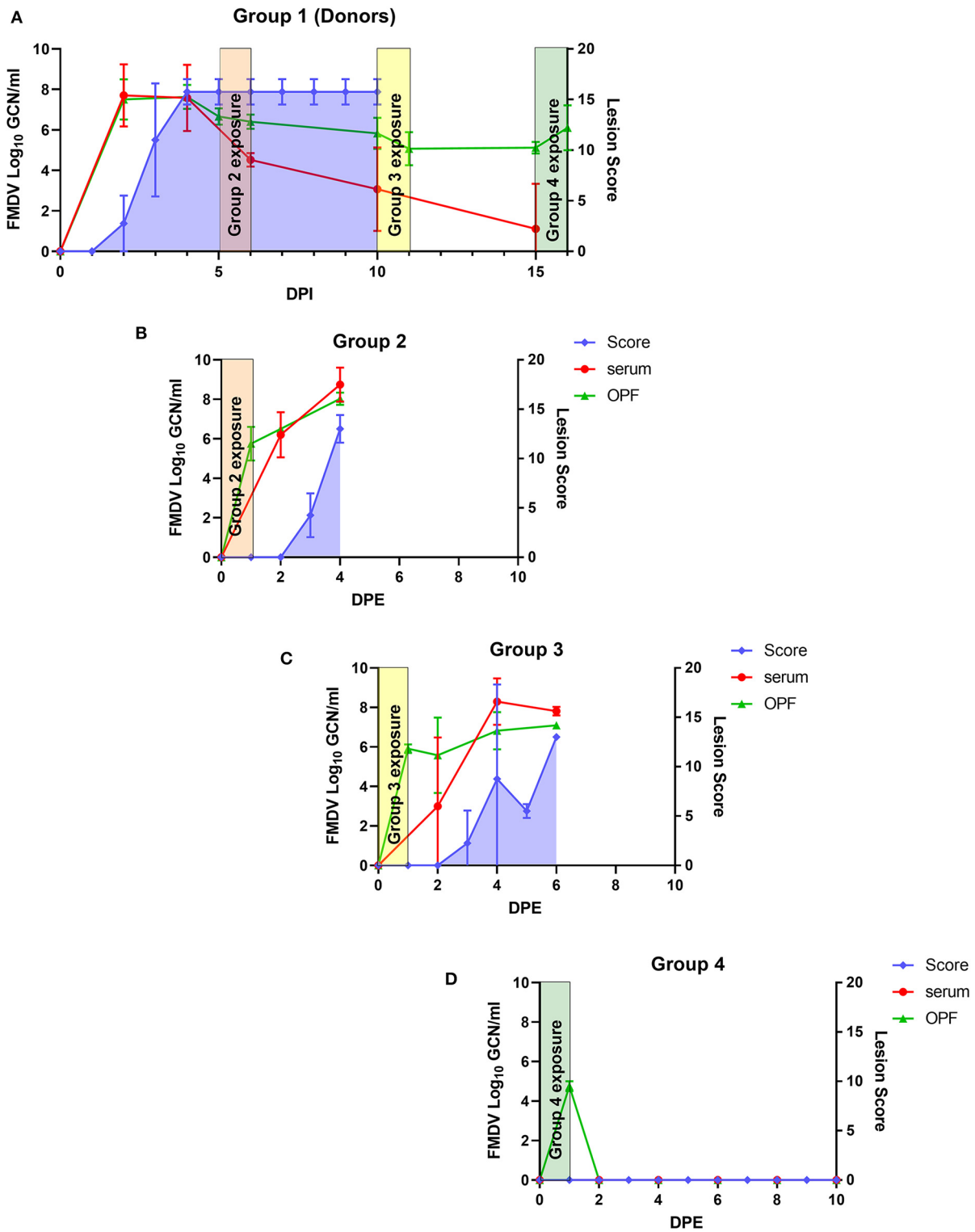

FIGURE 1 | Transmission dynamics of FMDV during late infection. **(A)** Four pigs in group 1 (donors) were infected with FMDV A24 Cruzeiro by intra-oropharyngeal inoculation on day 0. **(B–D)** The 4 donor pigs were subsequently co-mingled with study groups 2, 3, and 4, for 24 h each, on days 5, 10, and 15 post infection, respectively. Graphs show average quantities (geometric means +/- standard deviation) of FMDV RNA (log_{10} GCN/ml) in serum (red) and oropharyngeal fluid (OPF; green), as well as cumulative lesion scores (shaded blue). Orange, yellow, and green rectangles correspond to the time frame during which the donors were housed together with groups 2, 3, and 4, respectively.

TABLE 1 | Donor pig characteristics during contact exposure periods.

Experimental group	Contact exposure time point (dpi of donors at start of exposure)	Mean FMDV RNA in OPF at start of exposure (log_{10} GCN/ml)	Mean FMDV RNA in OPF at end of exposure (log_{10} GCN/ml)	Number of titer-positive OPF samples at start of exposure	Highest FMDV titer in OPF at start of exposure (log_{10} $TCID_{50}$/ml)	Confirmed transmission to contact-exposed pigs
2	5	6.66	6.41	3	3.250	Yes
3	10	5.82	5.07	1	2.625	Yes
4	15	5.12	6.11	0	NA	No

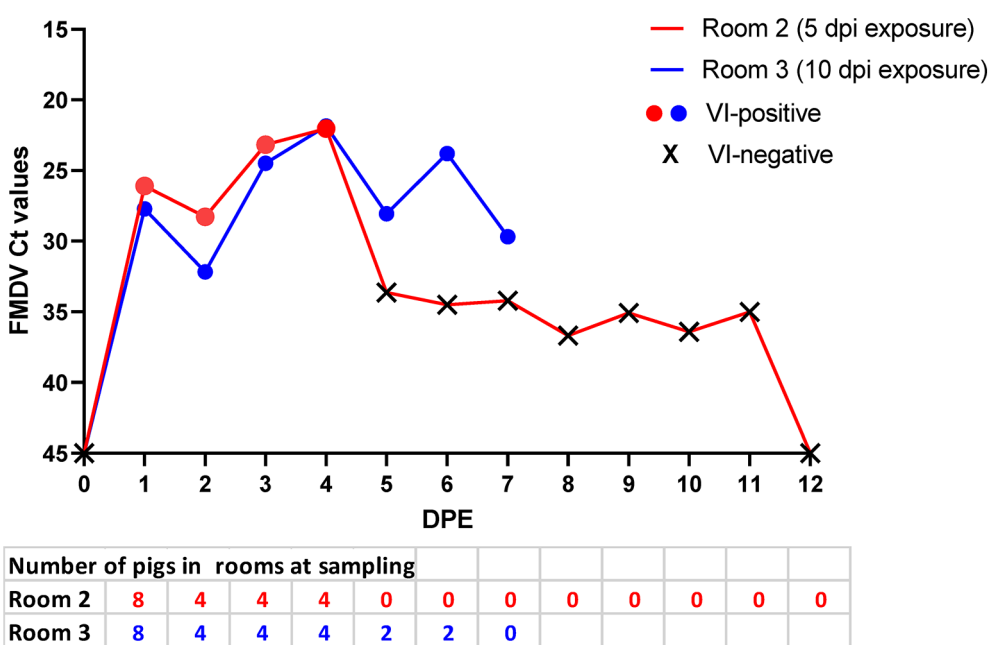

FIGURE 2 | FMDV detection in air samples. FMDV RNA quantities and isolation of infectious FMDV from dry air filters collected from isolation rooms housing pigs that were exposed to FMDV-infected donor pigs for 24 h at 5 (red) or 10 (blue) days post infection of the donors. The Y-axis represents FMDV RNA quantities (Ct values) determined by qRT-PCR. Colored symbols indicate samples from which infectious virus was isolated, while X indicate virus isolation-negative samples. The donor pigs were in the sampled rooms from 0 to 1 days post exposure (dpe). The 4 contact-exposed pigs in group 2 (red) were euthanized and removed at 4 dpe. Two of 4 pigs in room 3 (blue) were euthanized at 4 dpe, and the remaining 2 pigs at 6 dpe. Air sampling continued for 1 or 8 days after removal of the last pig from the rooms.

Post-mortem Viability of FMDV in Skeletal Muscle and Vesicular Epithelium

Post-mortem sampling of vesicle epithelium and skeletal muscle was performed on 4 distinct batches of pig carcasses that had been infected with either FMDV A24 Cruzeiro or FMDV O/SKR/2010, following slightly different sampling schedules.

Samples of semitendinosus muscle were obtained from batches 1 and 4 ($n = 7$), which had both been infected with FMDV A24 Cruzeiro. Low to moderate quantities of FMDV RNA (3.1-6.3 log_{10} GCN/mg) were detected in all muscle samples (**Figure 3**). However, virus isolation was only positive at 0 dpm (directly post euthanasia), 1 dpm (2 of 4 samples), and 5 dpm (1 of 4 samples; **Figure 3**).

By contrast, all vesicle epithelium samples obtained from batches 1 through 4 ($n = 13$) from 0 to 77 dpm were positive both by qRT-PCR and VI. FMDV RNA quantities were substantially higher compared to muscle samples (6.34-8.12 log_{10} GCN/mg; **Figure 4A**). There were no differences in RNA quantities or duration of viability across batches or viruses (the latest samples from FMDV O/SKR/2010-infected carcasses were obtained at 37 dpm).

Virus titrations were performed on all vesicle epithelium samples from batch 4 carcasses (0–77 dpm). Titers ranged from

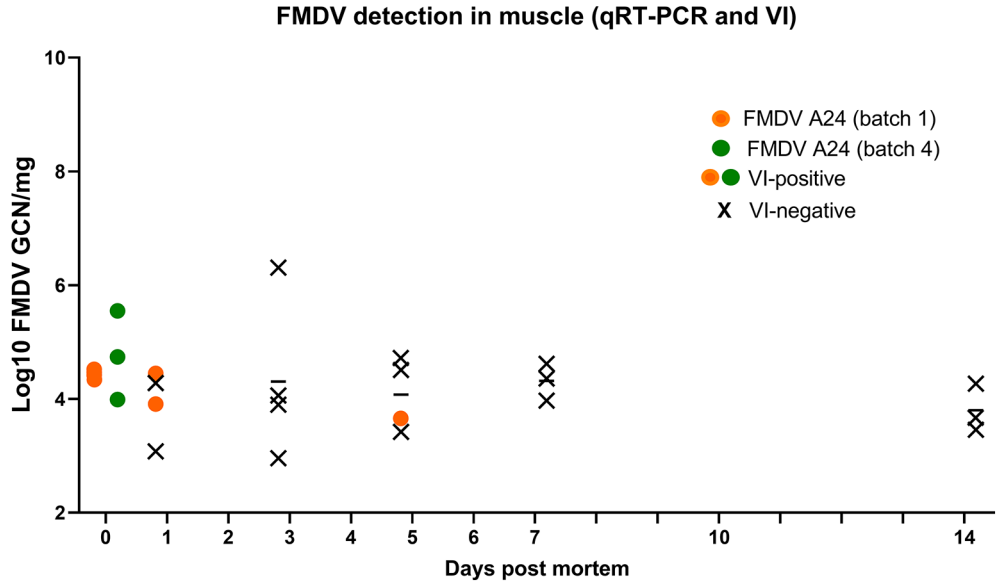

FIGURE 3 | FMDV detection in the semitendinosus muscle of pig carcasses. FMDV RNA quantities (log_{10} GCN/mg) in muscle samples from FMDV-infected pigs obtained from 0 to 14 days post mortem (dpm) from carcasses stored at 4°C. Colored symbols indicate samples from which infectious virus was isolated, while X indicate virus isolation-negative samples. Carcasses from batch 1 ($n = 4$) were samples at 0, 1, 3, and 5 dpm and carcasses from batch 2 ($n = 3$) were sampled at 0, 7, and 14 dpm. All symbols represent individual sample replicates ($n = 7$), horizontal lines are means.

an average of $10^{7.2}$TCID$_{50}$/g at 0 dpm, to an average of $10^{4.4}$ TCID$_{50}$/g at 77 dpm (**Figure 4B**). A simple linear regression fitted to the viral decay data had a negative slope (−0.034). The half-life was estimated to be 128 days and the X-intercept (titer $= 10^0$) was estimated to be 203 days (95% confidence interval = 159 to 295 days).

FMDV RNA was detected in submandibular lymph nodes, neck skin, and bone marrow harvested from FMDV A24-infected carcasses at 77 dpm (3.66–4.89 log_{10} GCN/mg; **Figure 5**). Two bone marrow samples were VI-positive, with titers of $10^{3.25}$ and $10^{3.35}$ TCID$_{50}$/g, respectively (**Figure 5**).

DISCUSSION

Data-driven models for FMD outbreak simulations represent a critical component of FMD preparedness in countries that are normally free of FMD (6). Although a considerable amount of data describing FMD progression in infected animals can be extrapolated from published experimental studies, only a fraction of such studies were originally designed to evaluate disease transmission. Replacement of actual transmission data by more readily available proxy measures can lead to substantially skewed estimates for infectivity (10, 11). Additionally, as disease transmission is highly context-dependent, it is important that estimates used for model parameterization are appropriate for the host species and housing/management conditions represented in the model. Previous studies have shown that individual housing of animals for one-on-one contact transmission trials can substantially reduce disease transmission (34). Thus, even though a one-to-one study design allows for precise measurements of virus shedding from individual animals for transmission proxy evaluation, the set-up may provide skewed estimates as the transmission characteristics of the disease are altered compared to conventional group-housing settings.

In this current investigation, we addressed a critical knowledge gap related to the end (duration) of infectiousness in group-housed FMDV-infected pigs. A single group of donor pigs subjected to simulated-natural FMDV exposure were used to expose sequential groups of contact pigs at pre-determined time points during late infection. The outcome of this trial demonstrated that transmission occurred at 5 and 10 dpi, but not at 15 dpi. The clinical conditions of the donor pigs improved throughout the study. At 5 dpi, although non-febrile and ambulant, there was still visible lameness and some reluctance to walk within the group. At 10 dpi, the donor pigs were more active, and were freely walking/running during the clinical examination and relocation to the contact exposure room. Although moderate quantities of FMDV RNA were measured in OPF of the donor pigs at 10 dpi, the titers of infectious virus were below the limit of detection in all but 1 donor pig at the start of contact exposure. There were, however, healing FMD lesions at the coronary bands of all the donor pigs, and it is highly possible that the contagion of donor pigs at this time point was associated with virus in residual lesions rather than in aerosols or secretions. This is further supported by the fact that FMDV in secretions would be expected to be associated with secretory immunoglobulin during later stages of infection.

Virus shedding, as defined by detection of FMDV RNA in OPF, was still detectable in samples from the donor pigs at the

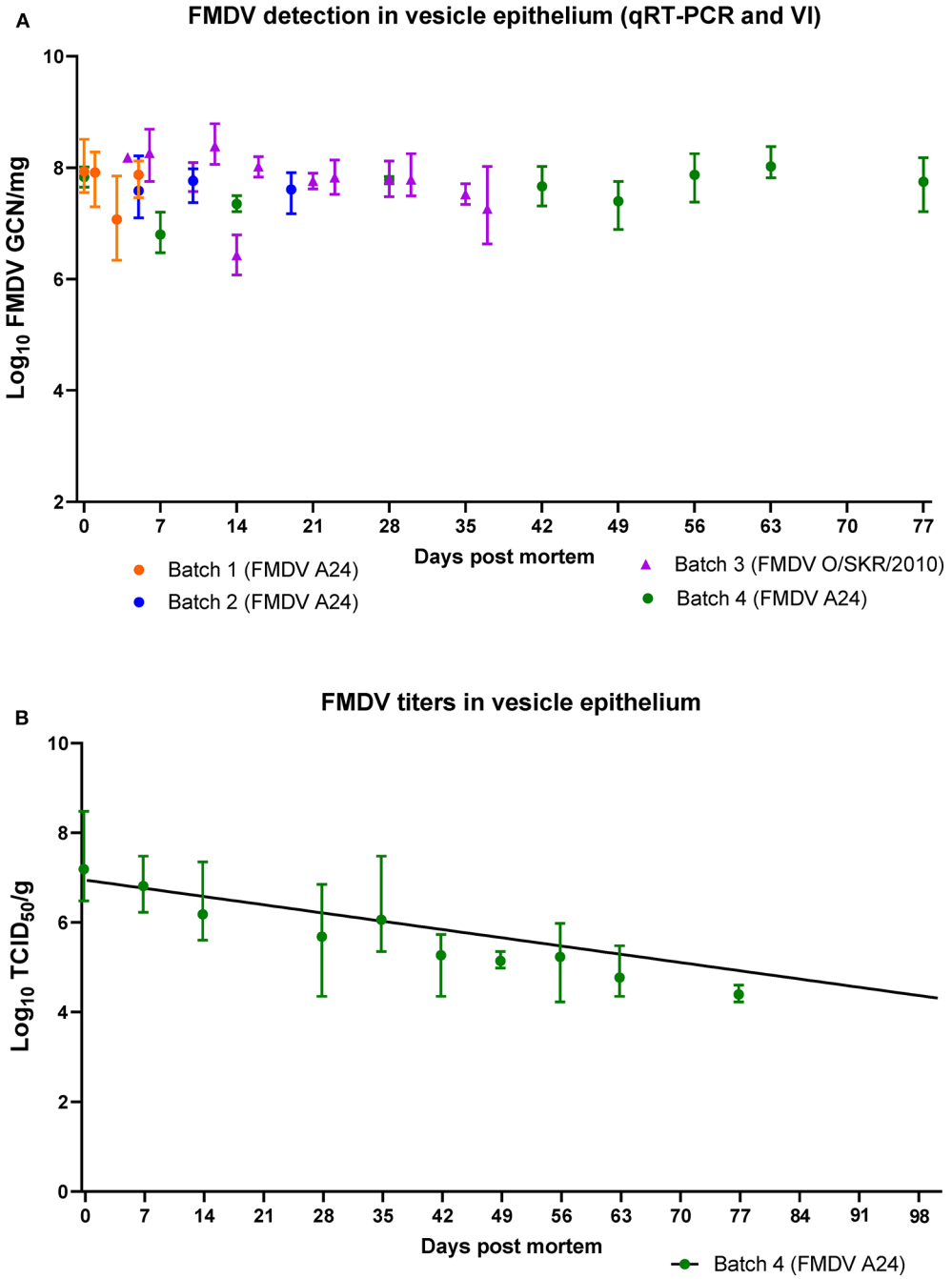

FIGURE 4 | FMDV detection in vesicle epithelium of pig carcasses. **(A)** FMDV quantities (log_{10} GCN/mg) in samples of vesicle epithelium obtained from 4 separate batches of pig carcasses infected with FMDV A24 Cruzeiro or FMDV O/SKR/2010 and stored at 4°C from 0 to 77 days post mortem (dpm). All samples were positive by virus isolation. **(B)** FMDV titers (log10 $TCID_{50}$/g) in vesicle epithelium obtained from 3 carcasses infected with FMDV A24 Cruzeiro from 0 to 77 dpm. All plotted values represent geometric means and range. The fitted line represents a simple linear regression with day as the independent variable and individual sample titers as the dependent variable (slope = −0.034, half-life = 128 days).

beginning and end of the last contact exposure at 15–16 dpi. Furthermore, OPF from all the contact-exposed pigs in group 4 was positive for FMDV RNA at the end of the contact exposure. These findings support the concept of a minimum quantity of FMDV being required to cause infection in exposed pigs (35–37), and that the cumulative amount of virus that the pigs in group 4 were exposed to, was below the threshold of infectivity.

We have previously used a similar experimental design to demonstrate that FMDV-infected pigs are capable of transmitting disease ∼1 day prior to the appearance of clinical signs (10, 20).

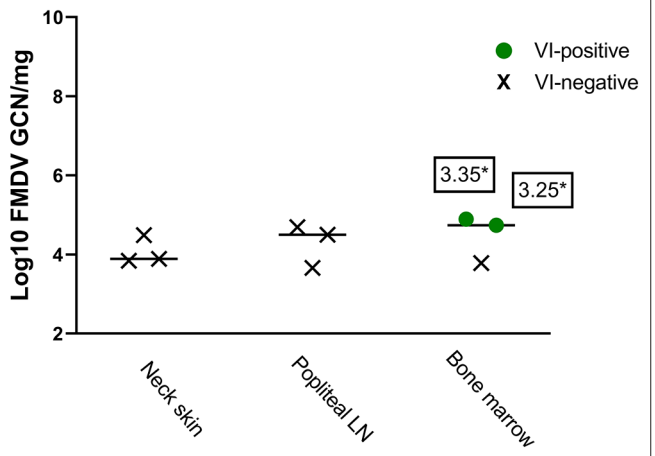

FIGURE 5 | FMDV detection in select tissue samples at 77 days post mortem. FMDV quantities (log_{10} GCN/mg) in samples of submandibular lymph nodes, neck skin, and bone marrow harvested from carcasses of pigs infected with FMDV A24 Cruzeiro after 77 days of storage at 4°C. Colored symbols indicate samples from which infectious virus was isolated, while X indicate virus isolation-negative samples. *FMDV titers in two samples of virus isolation-positive bone marrow were $10^{3.35}$ and $10^{3.25}$ $TCID_{50}$/g, respectively. All symbols represent individual sample replicates ($n = 3$), horizontal lines are means.

During early infection, successful transmission was associated with increasing quantities of FMDV RNA detected in OPF of the donor pigs (20, 37). The findings from this current study suggests that a similar quantitative shedding approach is not appropriate to determine the end of the infectious period. Specifically, the FMDV RNA quantities detected in OPF from the donor pigs were similar between the second exposure at 10-11 dpi and the third exposure at 15–16 dpi despite the transition from contagious to non-contagious status. Based upon this finding, it is likely that the contagion associated with pigs in late phases of FMD is not due to FMDV present in OPF.

The experimental approach of using the same group of donor pigs to expose multiple groups of contact pigs was intended to minimize sources of variation other than the time post infection of the donors. Although this design may have resulted in additional stress of the donor pigs as they were moved between the contact groups, we believe this would not have affected experimental transmission since contagion was not correlated with shedding of FMDV in donors. Rather, the remnant vesicle epithelium on the donor pigs contained high quantities of infectious virus, and this is likely to have been the most substantial determinant of transmission during late stages of infection.

The combined output from the current and previous transmission studies suggests that the infectious period for FMDV-infected pigs lasts for at least 9 days (1 through 10 dpi), which is substantially longer than what has been published for cattle (11). Furthermore, this suggests that the rate of propagation of an FMD outbreak may be substantially impacted by the livestock composition within the affected region.

Nonetheless, it is noteworthy that the cumulative evidence from direct transmission experiments herein and previously published (20, 30) indicate that the actual infectious period in FMDV-infected pigs is shorter than what would have been estimated if using detection of FMDV RNA in OPF as a proxy for infectiousness.

The transmission study reported herein was performed using a single FMDV strain and limited animal numbers. It would thus be relevant to expand the data set by performing similar investigations using additional FMDV strains since previous experimental studies have suggested that there may be strain-specific variability in FMDV transmission parameters (21) and environmental stability.

FMDV virulence and host tropism are known to vary by strain rather than serotype as is demonstrated by the FMDV O Cathay lineage which differs from other serotype O FMDVs by being highly infectious to pigs, but not cattle (38, 39). Therefore, in order to generate robust and representative data, experimental determinations of end-of-infectiousness should ideally be performed for multiple strains within relevant serotypes.

We have previously shown that infectious FMDV could be detected in ambient air for up to 6 days after removal of infected pigs from a room that housed vaccinated (clinically protected) cattle (40), whereas no virus was detected in rooms housing similarly vaccinated cattle that had been infected by intra-nasopharyngeal deposition of the same virus (unpublished). In this current investigation, isolation of virus from air filters was only possible for up to 24 h after removal of infected pigs, while detection of FMDV RNA continued for up to 7 days. It is likely that the presence of cattle in the room of the previous study had a substantial impact on the humidity and temperature in the room, possibly affecting environmental survival of the virus. Additional environmental sampling, such as swabbing of dry surfaces, would be needed to better assess the residual contagion in mechanically cleaned spaces.

FMDV is known to be sensitive to high temperatures and pH values outside a range of 7–7.5 (41–43). It has also been shown that FMDV in muscle tissue gets inactivated within few days post mortem, even when stored at low temperatures (18), which was consistent with the findings from the current study. This is presumed to be associated with the generation of lactic acid from glycogen due to autolysis in muscle. In the current study, the post-mortem viability of FMDV in vesicle epithelium was substantially different, as virus isolation from lesion sites was positive, with measurable virus titers, as far out as 77 days post mortem. Due to biosafety regulations, the carcasses used to evaluate FMDV postmortem viability in the current study were stored in a designated cold storage room at 4°C, and we were not able to evaluate virus viability at higher temperatures. However, this temperature would realistically simulate an FMD outbreak occurring during the colder season of the year in temperate climate zones. As an example of that phenomenon, it was reported that cold weather substantially impeded decontamination efforts and contributed

to dissemination of the November 2010 FMDV serotype O outbreak in South Korea (44).

There is limited published information regarding viability of FMDV in animal products other than meat through prolonged post-mortem durations. One available review paper by Cottral (18) summarizes the data available at that time, and reports that FMDV remained viable in bone marrow stored at 1–4°C for up to 7 months, while virus could be isolated from lesions from guinea pigs for as long as 2 years (18). Conversely, more recent studies of alterations of temperature and pH in animal carcasses in warm climatic conditions suggest that FMDV would likely become inactivated within 24 h when ambient temperatures reach 30–35°C (45). Additional data is needed to evaluate FMDV viability in vesicle epithelium at varying ranges of temperature and humidity. In this current study, carcasses were kept intact in order to assess if autolysis, putrefaction, and associated acidification occurring within tissues and body cavities would affect conditions at peripheral lesion sites. Based upon the findings of long-term viability of FMDV in porcine tissues, further studies should be performed assessing viability of additional viral strains with longer duration and variations in ambient conditions.

CONCLUSIONS

The current investigation demonstrated that FMDV A24-infected pigs were capable of transmitting disease as late as 10 days post infection, which corresponded to 9 days after presumed onset of infectivity (20), and 8 days after appearance of clinical signs. The same pigs were non-contagious at 15 dpi. There is clearly some uncertainty associated with this range as there were no contact trials performed between 10 and 15 dpi. However, one important detail to note is that pigs that were largely clinically recovered, with only minor remnants of FMD lesions, were still capable of transmitting disease. Airborne contagion diminished shortly after removal of infected animals and mechanical cleaning of isolation rooms. However, evidence from previous studies suggest that this may vary substantially, likely depending on relative humidity. Although FMDV in muscle was inactivated within few days post mortem, contagion in vesicle epithelium from intact carcasses stored at 4°C remained high through 11 weeks. This finding should be considered in relation to appropriate handling and disposal of animal carcasses during FMD outbreaks, especially in cold weather conditions. describing the end of infectiousness in pigs and FMDV viability in post-mortem porcine tissues update the available parameters for modeling of FMD in populations of pigs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Plum Island Animal Disease Center Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CS coordinated and executed the animal experiments and drafted the manuscript. MB contributed to study design, execution of the animal experiments, and interpretation of results. GS and EH performed sample analyses and interpretation of data. JA, AD, and CS conceived, coordinated and oversaw the work. All authors have critically reviewed and revised the manuscript and approved the final product.

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Mechanisms of Maintenance of Foot-and-Mouth Disease Virus Persistence Inferred From Genes Differentially Expressed in Nasopharyngeal Epithelia of Virus Carriers and Non-carriers

James J. Zhu[1], Carolina Stenfeldt[1,2], Elizabeth A. Bishop[1], Jessica A. Canter[1,3], Michael Eschbaumer[4], Luis L. Rodriguez[1] and Jonathan Arzt[1]**

[1] USDA-ARS, Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, Orient, NY, United States, [2] Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, United States, [3] Plum Island Animal Disease Center, Oak Ridge Institute for Science and Education (ORISE), Orient, NY, United States, [4] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald, Germany

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Alejandra Victoria Capozzo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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Suresh H. Basagoudanava, ICAR-Indian Veterinary Research Institute, Bengaluru Campus, India

***Correspondence:**
James J. Zhu
james.zhu@usda.gov
Jonathan Arzt
Jonathan.arzt@usda.gov

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Foot-and-mouth disease virus (FMDV) causes persistent infection of nasopharyngeal epithelial cells in ∼50% of infected ruminants. The mechanisms involved are not clear. This study provides a continued investigation of differentially expressed genes (DEG) identified in a previously published transcriptomic study analyzing micro-dissected epithelial samples from FMDV carriers and non-carriers. Pathway analysis of DEG indicated that immune cell trafficking, cell death and hematological system could be affected by the differential gene expression. Further examination of the DEG identified five downregulated (chemerin, CCL23, CXCL15, CXCL16, and CXCL17) and one upregulated (CCL2) chemokines in carriers compared to non-carriers. The differential expression could reduce the recruitment of neutrophils, antigen-experienced T cells and dendritic cells and increase the migration of macrophages and NK cells to the epithelia in carriers, which was supported by DEG expressed in these immune cells. Downregulated chemokine expression could be mainly due to the inhibition of canonical NFκB signaling based on DEG in the signaling pathways and transcription factor binding sites predicted from the proximal promoters. Additionally, upregulated CD69, IL33, and NID1 and downregulated CASP3, IL17RA, NCR3LG1, TP53BP1, TRAF3, and TRAF6 in carriers could inhibit the Th17 response, NK cell cytotoxicity and apoptosis. Based on our findings, we hypothesize that (1) under-expression of chemokines that recruit neutrophils, antigen-experienced T cells and dendritic cells, (2) blocking NK cell binding to target cells and (3) suppression of apoptosis induced by death receptor signaling, viral RNA, and cell-mediated cytotoxicity in the epithelia compromised virus clearance and allowed FMDV to persist. These hypothesized mechanisms provide novel information for further investigation of persistent FMDV infection.

Keywords: foot-and-mouth disease virus, FMDV, microarray analysis, persistent infection, pharyngeal epithelia, chemokine expression, NFκB signaling pathways, the Th17 response

INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most contagious and economically devastating animal viral diseases. FMD virus (FMDV), a positive-sense single-stranded RNA virus of the family *Picornaviridae* (genus *Aphthovirus*), is the etiological agent of the disease. Susceptible hosts include domesticated and wild cloven-hoofed animals. Infection in cattle initiates via the respiratory tract. During primary infection, the virus replicates locally in the nasopharynx or lungs depending on exposure conditions (1–3). The infection subsequently spreads via the bloodstream (viremia) to secondary replication sites causing typical vesicles at specific regions of the oral cavity, feet, and occasionally other sites. Mortality is generally low in adults, but persistent infection can occur for long periods (30 days−5 years) with virus persisting at the primary infection sites (e.g., nasopharynx) in a high percentage (∼50%) of infected cattle, buffalo and sheep (4–8). Specifically, FMDV persistent replication sites in cattle were localized to the epithelial cells of the dorsal soft palate and pharynx (9) and, more precisely, the follicle-associated epithelia of the nasopharyngeal mucosa (7, 10). Although various studies have failed to demonstrate natural transmission from FMDV carrier cattle (8), it has been demonstrated that oropharyngeal fluid from carrier cattle is infectious to naïve cattle (11).

Extensive *in-vivo* studies have been conducted in order to elucidate the mechanisms of persistent FMDV infection in cattle. Zhang and Alexandersen (12) and Zhang et al. (13) showed that declining rate of FMDV RNA levels in oropharyngeal fluid samples during early infection differed between carriers and non-carriers and proposed that differences in the host's abilities to either clear the virus or to support virus replication may determine the establishment of FMDV persistent infection. There was significantly higher anti-FMDV IgA production in carriers than in non-carriers (7, 14, 15), indicating antibodies are not effective in complete clearance of FMDV infection. In addition, the lymphocyte proliferative response of peripheral blood mononuclear cells to FMDV antigens was higher in non-carriers than in carriers (16).

Expression levels of a small number of candidate genes such as cytokines (7, 10, 17, 18) and microRNA (19) have been quantitated in FMDV carriers and non-carriers by qRT-PCR. However, these results do not provide detailed mechanisms involved in persistent infection. Broader transcriptomic studies using microarrays have been conducted to obtain genome-wide expression profiling of tissues targeted for persistent FMDV infection. A transcriptomic analysis showed that the lungs, susceptible to early infection but not persistent infection, expressed significantly higher levels of TNF cytokines and the associated receptors than the pharyngeal tissues that are susceptible to both primary and persistent FMDV infection (20). However, it is unknown if these same differences between the tissues exist between FMDV carriers and non-carriers. Another transcriptomic study of pharyngeal tissues from carriers and non-carriers indicated that inducible regulatory T cells (Treg) especially type 1 regulatory T cells (Tr1) could play a role in persistent infection based on cytokine and Tr1-expressed genes being differentially expressed between carriers and non-carriers (21).

Further transcriptomic investigation using RNA prepared from micro-dissected nasopharyngeal epithelia suggested that persistent FMDV infection is associated with compromised apoptosis and a reduced cellular immune response based on some most-differently expressed genes (22). These results could further explain the differences between carriers and non-carriers. Immunohistochemistry analysis using anti-CD3, anti-CD8, and anti-γδTCR antibodies showed no differences in the numbers of detected cell populations between carriers and non-carriers (22). The current study is a continued analysis of all differentially expressed genes (DEG) from previously published expression data (22) derived from micro-dissected nasopharyngeal epithelium samples of FMDV carriers and non-carriers during the persistent phase of FMDV infection in order to identify additional mechanisms involved. Pathway analyses using the list of all detected DEG show that genes involved in immune cell trafficking were over-represented by DEG including four chemokines known to play key roles in mucosal immunity. Other immune-related DEG support the downregulated chemokine expression in carriers and suggest that reduced recruitment of neutrophils, antigen-experienced T cells and dendritic cells in carriers could lead to compromised virus clearance and allow FMDV to persist.

METHODS AND MATERIALS

Gene Expression Data

The microarray data used in this study and the details of the animal experiments have been reported (21, 22). The data were produced using a custom bovine gene expression 60-mer oligonucleotide microarray designed based on gene expression information displayed on the bovine genome in the UCSC Genome Browser (https://genome.ucsc.edu/index.html). Microarrays and reagents were manufactured by Agilent Technologies (San Jose, CA) and the lab procedures were conducted based on the protocols and equipment recommended by the manufacturer. For comparison of the gene expression levels between carriers and non-carriers, microarray expression data from the micro-dissected pharyngeal epithelia of three carriers (persistently infected by FMDV A24 for >28 days) were compared to those from the corresponding micro-dissected tissues of four non-carriers that had cleared FMDV as reported by Stenfeldt et al. (22). For comparison of gene expression between the micro-dissected pharyngeal epithelia samples and whole tissue macerates of nasopharyngeal samples from the corresponding anatomical site, normalized mean expression data from the micro-dissected pharyngeal epithelia of sixteen animals (22) were compared to the data from the whole tissue macerates of nineteen cattle as reported by Eschbaumer et al. (21).

Statistical Analysis

R scripts implemented with the LIMMA package (23) were used to normalize and analyze the microarray data as previously described (21). All signal intensities (averaged photons per pixel) used in the statistical analysis were Log_2 transformed.

Genes differentially expressed between carriers and non-carriers with a false discovery rate (FDR) of 0.10 or smaller and an expression difference of at least 50% were considered as statistically significant genes in the transcriptomic study. This FDR significance threshold increases the detection power (fewer false negatives/type II errors) with a false positive (type I error) rate of 0.10 in declared DEG, or one false positive in 10 DEG, compared to FDR at 0.05 (one in twenty) to balance type I and type II errors. The means of normalized signal intensities (photons per pixel) of ACTA1, ACTA2, and ACTB were used as the internal controls to normalize the expression data to account for differences in the methods of data acquisition between micro-dissected epithelia (22) and whole tissue macerates (21).

Pathway Analysis

All bovine genes included in the microarray design were mapped to human reference genes using computer analysis via NCBI BLAST and/or manual annotation by aligning the microarray probe sequences on bovine genome sequences displayed on the UCSC Genome Browser using BLAT program (https://genome.ucsc.edu/cgi-bin/hgGateway). The list of upregulated and downregulated genes associated with the human Entrez Gene ID was analyzed with Ingenuity Pathway Analysis (IPA) (Qiagen, Maryland) and a NCBI Functional Annotation Bioinformatics Microarray Analysis program (DAVID Bioinformatics Resources version 6.8) to identify the biological pathways significantly over-represented by DEG. The biological functions of DEG were based on scientific publications obtained from the PubMed website (https://www.ncbi.nlm.nih.gov/pubmed/) listed as cited references or the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene/) listed as NCBI.

Biological Inferences

Biological inferences were based on (i) reported biological functions of DEG, (ii) gene expression levels based on microarray averaged signal intensity and (iii) magnitudes (fold difference) of upregulated or downregulated expression, assuming that (1) genes with a higher signal intensity and larger differential expression play a bigger biological role in their gene group and (2) upregulated expression enhances gene activities and vice versa. Differential expression of genes with cell-specific expression was also used to infer the differences in the number of the cells. Genes with no significant differential expression (FDR $>$ 0.10) but known to play important roles in the relevant biological pathways/processes associated with DEG were also used as references or supporting results for DEG and/or DEG related mechanisms. Probabilities of differential expression at gene levels are listed as *P*-values along with FDR. Genes downregulated or upregulated in carriers compared to non-carriers were expressed as negative and positive values (fold changes), respectively. Multiple DEG involved in a known immune mechanism were used in the formulation of hypothesis. All immune mechanisms known to play roles in virus clearance based on our literature review were considered for candidate mechanisms.

Proximal Promoter Analysis

The nucleotide sequences up- and down-stream of the gene transcription start sites of four mucosal chemokines (RARRES2/chemerin, CXCL15, CXCL16, and CXCL17) were downloaded from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/). The core promoters were predicted using Neural Network Promoter Prediction (NNPP version 2.2 software) (24). The proximal promoters, 500-bp up-stream and 250-bp down-stream nucleotide sequences of the TATA box or CpG island sequences associated with the transcription starting sites, were used in the prediction of transcription factor binding sites (TFBS) using the TESS 2.0 program (25) and JASPAR database (http://jaspar.genereg.net/downloads/) with a log-odd score of 7.0 or higher (default value at 6.0). Overlapping TFBS for the same transcription factors were counted if the sites differed by at least five nucleotides. AP-1, CREBs, NFκB, IRF3, and STAT1/STAT2 were selected to represent the transcription factors activated by MAPK, NFκB, IRF3 and interferon signaling pathways.

RESULTS

Pathway Analysis

There were 1,505 probes with significantly downregulated expression in carriers compared to non-carriers and 1,097 probes with upregulated expression. Among the genes associated with these probes, there were 1,281 downregulated and 951 upregulated genes that could be mapped to human or mouse genes. This gene set including both up- and down-regulated genes was used in the NCBI DAVID and IPA pathway analyses. The KEGG and REACTOME pathway analyses using the NCBI DAVID program showed no significant pathways associated with these DEG. In contrast, pathway analysis with the IPA program detected several significant associations. The top five canonical pathways significantly over-represented by DEG were (1) integrin signaling, (2) Wnt/β-catenin signaling, (3) PI3K/AKT signaling, (4) colorectal cancer metastasis signaling, and (5) chronic myeloid leukemia signaling (**Figure 1A**). The top five upstream regulators were TP53, ESR1, HNF4A, TP63, and beta-estradiol (ordered based on probability) (**Figure 1A**). None of the top upstream regulator genes were differentially expressed between carriers and non-carriers.

The top five molecular and cellular functions that were significantly associated with the differential expression were (1) cell death and survival, (2) gene expression, (3) cellular assembly and organization, (4) cellular function and maintenance, and (5) cellular development (**Figure 1B**). The top five inferred physiological system development and function were (1) organism survival, (2) tissue morphology, (3) organism development, (4) hematological system development and function, and (5) immune cell trafficking (**Figure 1B**). Because cell death/apoptosis and immune cell development, function and trafficking play important roles in immunity against virus infections, DEG involved in these functions were examined in detail.

Among 24 functions associated with immune cell trafficking (**Table 1**), the top five functions with the lowest *p*-values ranging

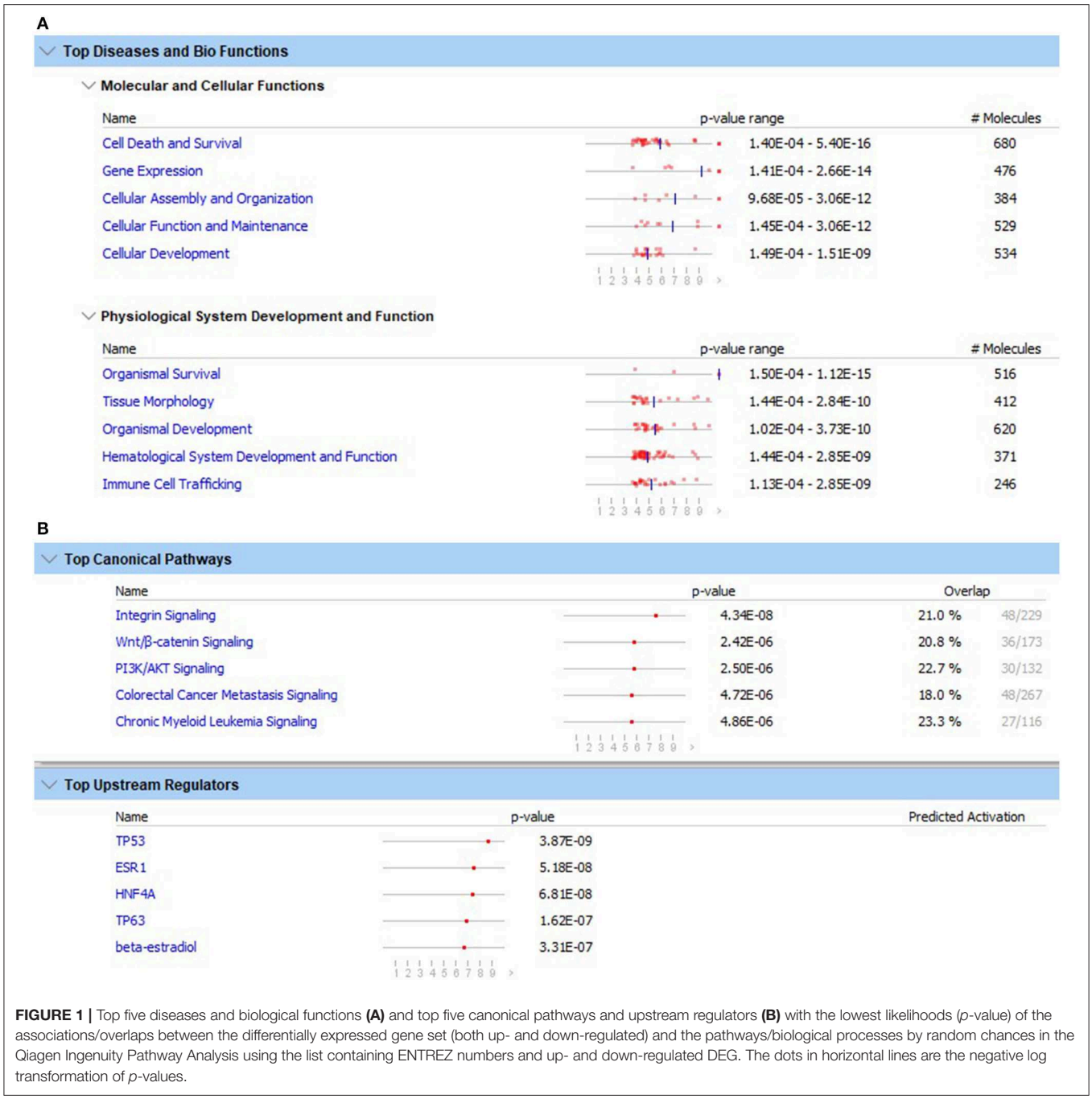

FIGURE 1 | Top five diseases and biological functions **(A)** and top five canonical pathways and upstream regulators **(B)** with the lowest likelihoods (*p*-value) of the associations/overlaps between the differentially expressed gene set (both up- and down-regulated) and the pathways/biological processes by random chances in the Qiagen Ingenuity Pathway Analysis using the list containing ENTREZ numbers and up- and down-regulated DEG. The dots in horizontal lines are the negative log transformation of *p*-values.

from 3.05E-07 to 2.85E-09 were associated with leukocyte movement/infiltration and neutrophil movement. Based on these findings, the differential expression of chemokines was further examined as listed in **Table 2**, followed by differential expression of the genes supporting or regulating the differential chemokine expression in **Table 3**. For DEG involved in immune cell development and function, differential expression of cytokines, cytokine signaling pathways and other immune regulatory genes together with their function was analyzed (**Tables 4**–**6**). The DEG involved in apoptosis were evaluated for their potential roles in affecting apoptosis induced by viral RNA, death receptor signaling and cell-mediated cytotoxicity as in **Table 7**. These DEG were used to formulate the candidate immune mechanisms involved in the maintenance of FMDV persistent infection as shown in **Figure 3**. The TFBS in proximal promoter regions (**Table 8**) were predicted as the supporting results of the hypothesis.

Chemokines and the Receptors

Six chemokine genes were differentially expressed between carriers and non-carriers. Only one of these chemokines (CCL2) was significantly upregulated by 6.8-fold in carriers compared

TABLE 1 | The *p*-values of the number (#) of differentially expressed genes (DEG) in 24 functions (top five with lowest *p*-value in bold fonts) of immune cell trafficking by random chances in the analyses of diseases and functions using Qiagen Ingenuity Pathway Analysis program.

Function	Diseases or functions annotation	*p*-value	# of DEG
Accumulation	Accumulation of neutrophils	1.13E-04	20
Activation	Activation of leukocytes	1.33E-05	129
	Activation of lymphocytes	1.67E-05	89
	Activation of mononuclear leukocytes	2.37E-05	92
Adhesion	Adhesion of immune cells	5.11E-05	70
	Adhesion of mononuclear leukocytes	5.85E-05	33
Cell movement	Cell movement of leukocytes	**2.85E-09**	169
	Cell movement of phagocytes	1.60E-07	121
	Cell movement of neutrophils	**3.05E-07**	71
	Cell movement of granulocytes	2.03E-06	81
	Cell movement of mononuclear leukocytes	5.64E-05	93
	Cell movement of antigen presenting cells	9.20E-05	73
Cell rolling	Cell rolling of leukocytes	3.90E-05	20
	Cell rolling of phagocytes	1.09E-04	11
Cellular infiltration	Cellular infiltration by leukocytes	**7.11E-07**	89
	Cellular infiltration by phagocytes	4.18E-06	63
	Infiltration by neutrophils	7.32E-05	39
	Cellular infiltration by macrophages	1.08E-04	39
Chemotaxis	Chemotaxis of leukocytes	1.87E-05	73
	Chemotaxis of phagocytes	6.74E-05	60
	Chemotaxis of neutrophils	1.11E-04	35
Homing	Homing of leukocytes	**6.63E-07**	82
	Homing of mononuclear leukocytes	8.38E-05	44
Migration	Leukocyte migration	**1.23E-08**	196

to non-carriers (**Table 2**). CCL2 has chemotactic activity for inflammatory monocytes and NK cells (26–28). Additionally, higher expression of C5AR1, CD16, CD300A, CD300LD, GZMA, GZMM, KLRB1, MMD, and NCR2 including an unannotated KLR in carriers compared to non-carriers (**Table 3**) supports increased migration of monocytes and NK cells into the persistently infected epithelia. Monocyte to macrophage differentiation-associated (MMD) is highly expressed in mature differentiated macrophages after migration to tissues but is absent in monocytes (106). CD16, specifically expressed on NK cells and some monocytes/macrophages, can activate antibody directed cell-mediated cytotoxicity (54, 107), whereas KLR receptors and NCR2 are receptors highly expressed on NK cells (56). Granzymes are cytotoxicity effectors expressed in cytotoxic T cells and NK cells (108) and Treg cells (109). C5AR1 is expressed in myeloid cells including macrophages and granulocytes (37). CD300 receptors which are mainly expressed on myeloid cells play a fundamental role in immune regulation (61).

The expression of five chemokines (CCL23, CXCL15, CXCL16, CXCL17, and RARRES2, also named chemerin) was significantly downregulated in carriers compared to non-carriers by 2.5- to 17.9-fold (**Table 2**). These chemokines all have chemotactic activities for antigen presenting cells, antigen-experienced T cells, monocytes, NKT cells and/or neutrophils (29–36). Chemerin, CXCL15, CXCL16, and CXCL17 were expressed at higher levels by 2.2-, 5.0-, 6.1-, and 1.7-fold, respectively, in the micro-dissected epithelia than in the whole tissues (**Figure 2**), indicating epithelium-specific expression. The differential expression of chemokines could potentially result in reduced recruitment of dendritic cells, antigen-experienced T cells and neutrophils to the nasopharyngeal epithelium of the carriers compared to the non-carriers. This hypothesis was further supported by analysis of DEG associated with expression of the chemokines of interest (**Table 3**).

Two chemokine receptors, CXCR1 and CXCR2, known to be predominately expressed on neutrophils (27, 40), were expressed at significantly lower levels in carriers than in non-carriers (**Table 3**). Cathepsins are components of neutrophil granules, and the expression of three cathepsins (CTSC, CTSF, and CTSV) was significantly lower in carriers than in non-carriers. CD38 is important for neutrophil migration (38, 39), whereas RGS5 inhibits neutrophil chemotaxis and trafficking (41). CD38 expression was lower and RGS5 was higher in carriers than in non-carriers. The expression of C3, an important component for activation of the classical and alternative complement activation pathways that lead to production of C5a to recruit neutrophils (110), was downregulated by 15- fold in carriers compared to non-carriers (**Table 3**). All ELR+ CXCLs have chemotactic activity for neutrophils (27, 32). Although only CXCL15 showed significant differential expression, the total normalized signal intensity of six bovine ELR+ CXCLs including CXCL1, CXCL2, CXCL3, CXCL5, CXCL8 and CXCL15 was 2.4 times lower in carriers than non-carriers (**Table 2**), further supporting lower expression of neutrophil-recruiting chemokines in carriers.

Other DEG supported reduced recruitment of antigen-experienced T cells and dendritic cells in nasopharyngeal epithelium of FMDV carriers (**Table 3**). CD44 is a cell marker for antigen-experienced T cells including T effector, central memory T, T effector memory and T resident memory cells but not naïve T cells (43). CD44 expression was downregulated by nearly threefold in carriers compared to non-carriers. There were three effector T-cell-expressed genes (CD27, CD73 and CD244 (42, 44–48) with $\geq$ 4.4-fold decreased expression in carriers. LAT, IKT and two TRBC2 (T cell receptor beta constant 2) involved in TCR signaling (49–51) displayed >3-fold downregulated expression in carriers. By contrast, genes highly expressed on naïve T and natural intraepithelial lymphocytes such as CD45R, CD62L, and CD161 (52, 53, 55) were expressed significantly higher in carriers than in non-carriers. The expression of T cell marker genes, CD2, CD3D, CD4, CD8A, and CD8B was not significantly different. Dendritic cells express CLEC9A/DNGR1, CD276, VISTA and VTCN1 (57–60). These four genes were expressed at significantly lower levels in carriers than in non-carriers. These results suggest a reduced recruitment of dendritic

TABLE 2 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR), fold differences (+ and – values as up- and down-regulated in carriers compared to non-carriers, respectively) and chemotactic activities of chemokine genes differentially expressed between the nasopharynx epithelia of carriers and non-carriers.

Gene	MSI	FDR	Fold	Chemotactic activity[a]	References
CCL2	841	0.03	6.8	Inflammatory monocytes and NK cells	(26–28)
CCL23	422	0.10	−17.9	Resting T cells, monocytes & neutrophils	(29)
Chemerin	1188	0.02	−5.7	Immature DC and macrophages (Mϕ)	(30, 31)
CXCL15	525	0.08	−7.6	Neutrophils	(32)
CXCL16	13128	0.04	−2.5	Activated CD8+/CD4+ T, IEL and NKT cells	(33–35)
CXCL17	3028	0.01	−3.5	Immature DC, Mϕ, CD8+ Tem and Trm cells	(36)
ELR+CXCLs[b]	2530	n/a	−2.4	Neutrophil > monocyte, NK, CD8+ T cells	(27)

[a] *DC, dendritic cells; Mϕ, macrophages; IEL, intraepithelial lymphocytes; NKT, natural killer T cells; Tem, memory T effector; Trm, resident memory T cells.*
[b] *ELR+CXCLs: CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, and CXCL15, which total signal intensity is the sum of the signal intensity of each ELR+CXCL chemokine in carriers and non-carriers.*

cells and antigen-experienced T cells within the nasopharyngeal epithelium of FMDV carriers.

Cytokines and the Receptors

Ten cytokines belonging to interferon (IFNA and IFNL), IL-1 (IL1A, IL33, and IL36A), IL-2- (IL7 and IL15) or TNF (TNF, TNFSF10, and TNFSF15) families were expressed at significantly higher levels in carriers than in non-carriers (**Table 4**). All significantly upregulated cytokines were proinflammatory or immune-stimulatory except IL33. IL33 activates Th1 and Th2 cells, group 2 innate lymphoid cells, and CD8+ T cells, and it also plays a key role in suppressing Th17 and promoting Treg (95). Among the significant cytokines, TNF and IL33 were expressed at the highest levels and were the most upregulated (approximately 15-fold). Other cytokines significantly upregulated by ∼6- to 10-fold were IL1A, IL7, IL36A, and TNFSF15, whereas IFNA, IFNL, IL15, and TNFSF10 were expressed at levels 1.5- to 2.5-fold higher in carriers than in non-carriers (FDR $\leq$ 0.1). The signaling of IL-1 cytokines can be inhibited by soluble IL1RAP (sIL1RAP, an alternative 3' end transcript) (111), and the sIL1RAP expression level was significantly higher in carriers than in non-carriers (the only differently expressed receptor of the cytokine DEG) was not differentially expressed between carriers and non-carriers. The expression of a receptor (IL10RA) of IL10, an immunosuppressive cytokine (112), was nearly 3-fold higher in carriers than in non-carriers. These results suggest that 15-fold upregulation of IL33 could significantly suppress the Th17 response.

There are also several cytokine genes differentially expressed at gene levels ($P \leq 0.05$). IL1RN is an IL-1 antagonist. IL6 and IL23 are important cytokines stimulating Th17 cell differentiation (113). IL22 plays a key role in mucosal immunity by stimulating inflammatory responses and inducing S100s and defensin expression (94). EBI3 or IL35B is a part of an immune inhibitory cytokine, IL35. The expression of IL1RN, IL6, IL22, IL23A, and EBI3/IL35B was upregulated in carriers at $p \leq 0.05$. Among the receptors of the differentially expressed cytokines, IL6R, IL15R, and IL1RL1 (ST2, IL33 receptor) were expressed at higher levels ($p \leq 0.02$) in carriers than in non-carriers, whereas IL12RB1 (a part of IL23 receptors) was expressed at a lower level ($p = 0.02$; FDR = 0.19) in carriers than in non-carriers. The expression of IL10 was different between carriers and non-carriers.

Signal Transducing Genes

There were several DEG in interferon, IRF3, MAPK and NFκB signaling pathways that could negatively impact cytokine signaling in carriers (**Table 5**). Toll-like receptor 6 (TLR6) was expressed 7.7-fold lower in carriers compared to non-carriers. The expression of ATK1 and AKT3, which are involved in interferon signaling (63, 114), were significantly lower in carriers than in non-carriers. There were eight signal transducers (MAP3K9, MAPK3, MAPK8IP1, MAPKAPK3, MAPKAPK5, TAB1, TRAF3, and TRAF6) and two transcription factors (CREB5 and NFKB2) in the IRF3, MAPK and NFκB signaling pathways (64–66), which expression levels were significantly downregulated in carriers compared to non-carriers (**Table 5**). However, one kinase, CHUK/IKKα essential for non-canonical NFκB signaling pathway but dispensable for canonical NFκB signaling pathway (67) was expressed at a significantly higher level (3.1-fold) in carriers than in non-carriers, whereas the expression of two other kinases (IKKB and IKKG) crucial for the canonical pathway was downregulated by >3-fold at close to a significant level (FDR = 0.12 and 0.15), suggesting the differential expression was in favor of activation of non-canonical NFκB signaling pathway.

Interestingly, four signaling enhancers of the IRF3 and/or canonical NFκB pathways, OTUB1 (68), RNF128 (69), TGFB2-OT1 (70) and TRIM52 (71) were expressed at significantly lower levels in carriers than in non-carriers. By contrast, four inhibitors, HIVEP2 (72), NKIRAS1 (73), NLK (74) and NKRF (75) of the canonical NFKB signaling pathway were expressed 2.8 to 11.8-fold higher in carriers than in non-carriers. Additionally, two immune inhibitory microRNAs, MIR221 (77, 78) and MIR503HG (79) as well as one proinflammatory one, MIR155, which also has both anti-apoptotic activity (115, 116) and an inhibitory effect on interferon signaling (76), were upregulated

TABLE 3 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR) and fold differences (+ and – values as up- and down-regulated in carriers compared to non-carriers, respectively) of differentially expressed genes supporting decreased numbers of neutrophils, antigen-experienced T cells and dendritic cells and increased number of naïve and natural intraepithelial lymphocytes.

Group	Gene	MSI	FDR	Fold	Expressing cells/functions[a]	References
Neutrophil	C3	16129	0.03	−15	Neutrophil chemoattractant C3a	(37)
	CD38	241	0.02	−8.1	Neutrophils/migration	(30, 38, 39)
	CTSC	23238	0.04	−2.6	Neutrophils/granule component	NCBI
	CTSF	1084	0.01	−11.7		
	CTSV	5645	0.04	−3.4		
	CXCR1	102	0.09	−2.3	Neutrophils/ELR+ CXCL receptor	(27, 40)
	CXCR2	259	0.06	−3.8		
	RGS5	426	0.06	5.4	Neutrophils/migration inhibitor	(41)
Antigen-specificT cell	CD27	556	0.00	−4.4	CD8+ effector T cells	(42)
	CD44	13684	0.07	−2.9	Antigen-experienced T cells	(43)
	CD73	1244	0.05	−17.0	CD8+ effector T cells and Treg	(44–46)
	CD244/2B4	450	0.03	−4.9	NK cells and CD8+ effector T cells	(47, 48)
	LAT	248	0.02	−6.9	T cells/TCR signaling	(49–51)
	ITK	1469	0.09	−3.5	T cells/TCR signaling	(51)
	TRBC2	1074	0.09	−5.1	T cells/TCR beta chain	(49–51)
	TRBC2	1113	0.05	−3.4		
	CD45R	360	0.01	14.3	Naïve T cells	(52, 53)
	SELL/CD62L	1747	0.01	4.3	Naïve T and central Tm cells	(52, 53)
	CD2	716	0.31	−5.3	T cells	NCBI
	CD3D	1700	0.59	2.0	T cells	NCBI
	CD4	420	0.72	−1.4	CD4+ T cells	NCBI
	CD8A	1601	0.82	−1.5	CD8+ T and IEL	NCBI
	CD8B	120	0.65	1.8	CD8+ T cells	NCBI
Antigen-non-specific T cells	CD16	89	0.02	2.1	NK cells and monocytes	(54)
	GZMA	91	0.05	4.6	NK cells, cytotoxic T cells, Treg	NCBI
	GZMM	66	0.08	2.5		
	KLRB1/CD161	111	0.09	4.0	NK cells and natural IEL	(55)
	KLR (unknown)	164	0.00	3.8		
	NCR2	625	0.00	10.4	NK cell receptors	(56)
	NCR3	243	0.27	4.4		
Dendritic cells	CD276/B7-H3	228	0.04	−2.7	Antigen presenting cells	(57)
	CLEC9A/DNGR1	93	0.07	−2.2	DC/cross-presentation	(58)
	VISTA/B7-H5	1182	0.05	−5.0	Antigen presenting cells	(59)
	VTCN1/B7-H4	2468	0.04	−5.0	Antigen presenting cells	(60)
Macro-phages	C5AR1	62	0.01	2.0	Myeloid cells, Mϕ M1 activation	(37)
	CD300A	66	0.02	1.6	Macrophages	(61)
	CD300LD	154	0.06	2.5	Macrophages	(61)
	MMD	914	0.03	9.3	Macrophages	(62)

[a] *DC, dendritic cells; IELs, intraepithelial lymphocytes; Mϕ, macrophage; TCR, T cell receptor.*

in carriers. These findings suggest that the interferon, IRF3, MAPK and especially canonical NFκB signaling pathways could be negatively impacted by the differential gene expression during persistent FMDV infection.

Immune Cell-Associated Genes

MFSD6 recognizes certain MHC-I molecules and mediates MHC-I restricted killing by macrophages (117). The expression levels of MFSD6and a non-classical MHC-I were 3.9 and 7.7 times lower in carriers compared to non-carriers (**Table 6**). Similarly, a ligand (NCR3LG1) of NK cell receptor 3 (NCR3) (80) was expressed at a significantly lower level in carriers than in non-carriers, whereas a NK cell cytotoxicity inhibitory soluble ligand (NID1) of NCR2 (81) was significantly upregulated by 12.1-fold in carriers vs. non-carriers. This differential expression could inhibit macrophage- and NK cell-mediated cytotoxicity in carriers.

Intraepithelial lymphocytes (IEL) are heterogenous T cells reside within the epithelial layer of mucosal and barrier tissues. CD69 is a receptor expressed by several subsets of tissue

TABLE 4 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR) and fold differences (+ and –values as up- and down-regulated in carriers compared to non-carriers, respectively) of cytokines and the receptors differentially expressed between the nasopharynx epithelia of carriers and non-carriers.

Gene group	Gene	MSI	*p*-value	FDR	FD	Function
IFN, Type 1	IFNA	70		0.10	1.5	Anti-viral cytokines
IL-1 family	IL1A	102		0.04	6.0	Proinflammatory
	IL36A	104		0.00	9.9	
	IL1RN	5405	0.02	0.18	1.8	Anti-inflammatory or stimulate Treg cells
	IL33/IL1F11	578		0.02	14.7	
	IL1RL1/ST2	55	0.02	0.18	2.2	
	sIL1RAP	115		0.07	4.2	
IL-2 family	IL7	139		0.04	8.2	T & B cell development
	IL7R	3099	0.07	0.33	2.1	IL7 receptor
	IL15	100		0.06	1.7	↑NK & CD8+ T cells
	IL15R	146	0.01	0.12	3.3	IL-15 receptor
IL-10 family	IFNL	184		0.04	2.5	Anti-viral cytokines
	IL10	112	0.92	0.98	−1.1	Immune inhibition
	IL10RA	3866		0.06	2.9	
	IL22	42	0.04	0.25	1.6	↑antimicrobial proteins
TNF family	TNF	494		0.00	15.0	Inflammation/apoptosis
	TNFSF10/TRAIL	305		0.04	2.3	Apoptosis
	TNFSF15/TL1A	401		0.10	7.1	Inflammation/apoptosis
Th17-related	IL6	139	0.02	0.19	1.3	↑Th17 differentiation
	IL6R	2696	0.01	0.13	4.5	IL-6 receptor
	IL23A	540	0.01	0.11	4.2	↑Th17 differentiation
	IL12RB1	387	0.02	0.19	−7.0	IL-23 receptor
	Il17A	161	0.12	0.34	3.4	↑Th17 response
	IL17F	227	0.15	0.49	−1.9	
	IL17RA	951		0.06	−2.4	IL17A and IL17F receptor
Anti-inflammatory	EBI3/IL35B	83	0.02	0.21	3.6	Immune suppression

resident immune cells such as IEL. It plays a key role in regulating T cell differentiation and activities depending on the cells [86]. Three probes of CD69 consistently showed ∼5-fold upregulated expression (FDR = 0.00) in carriers compared to non-carriers (**Table 6**). On the other hand, two genes (CD27 and CD244), playing important roles in activating T cells especially CD8+ cytotoxic T cells and NK cells (42, 47, 48), were expressed at significantly lower levels in carriers. Additionally, TNFRSF19 Inhibits TGFβ Signaling (84) and TGFβ signaling suppressed T cell cytotoxicity and promote immune tolerance (85), whose expression was significantly downregulated in carriers. The differential expression of these genes could inhibit IEL cytotoxicity.

T-bet is required for the development of CD8aa+ IEL (88, 89). T-bet expression increases in non-CD4+ T cell-helped resident memory T cells, which suppresses CD103 expression (87). The expression of T-bet was significantly upregulated in carriers compared to non-carriers, whereas CD103 expression was downregulated ($P = 0.03$). A probe of bovine immunoglobulin delta heavy chain constant region mRNA (NCBI accession #: AF411240) displayed significant 2.3-fold increased signal intensity in carriers than in non-carriers, indicating more of γδ T cells in the epithelium of carriers than in non-carrier. γδ T cells are known to have regulatory functions in mucosal immunity (86). These results indicate that T cells in the epithelium (also called as IEL), especially γδ T cells, might play a role in FMDV persistent infection.

RORC is a Th17-specific transcription factor. RORC and STAT3 promotes Th17 differentiation (90). TIAM1 forms a complex with RORC in the nuclear compartment of Th17 cells and together they bind and activate the IL17 promoter (91). TIAM1 expression was downregulated by 20.8-fold in carriers compared to non-carriers, whereas the expression levels of RORC and STAT3 were lower in carriers than in non-carriers ($P \leq 0.03$). On the other hand, CD69, ETS1, IL33 and STAT5 are negative regulators of Th17 differentiation and activity (90, 92, 118, 119). The expression levels of these four genes were 4.4- to 14.7-fold higher in carriers than in non-carriers. Th17 cells increase the expression of IL33 receptor (IL1RL1) upon inflammation in mucosa and IL33 induces acquire immunosuppressive properties in Th17 cells (93). IL1RL1 expression was higher in carriers than in non-carriers ($P = 0.02$). These results suggest that the Th17 response could be suppressed in carriers.

Tr1 is an antigen-specific FOXP3^{-} regulatory T cell and CD49B and PRDM1 are Tr1 markers (96, 97). CD49B and PRDM1 expression was significantly higher in carriers than in non-carriers (**Table 6**). Two genes associated with Tr1 differentiation (EBI3 and MAF) (96, 120) were also expressed

TABLE 5 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR) and fold differences (+ and –values as up- and down-regulated in carriers compared to non-carriers, respectively) of differentially expressed genes in interferon, IRF3, MAP, and NFκB signaling pathways.

Gene group	Gene	MSI	FDR	Fold	Function	References
TLR	TLR6	147	0.05	−7.7	Activate NFκB, IRFs and MAPKs	NCBI
Signal transducers	AKT1	2378	0.05	−2.5	Enhance interferon signaling & NFκB activation	(63, 64)
	AKT3	1000	0.01	−6.3		
	CHUK/IKKα	326	0.06	3.1	Non-canonical NFκB signaling	(65)
	IKBKB/IKKβ	6116	0.12	−4.3	Canonical NFκB signaling	
	IKBKG/IKKγ	1693	0.15	−3.3	Canonical NFκB signaling	
	MAP3K9	767	0.01	−5	MAP kinases in MAP signaling pathways	NCBI (66)
	MAPK3	1810	0.06	−10.6		
	MAPK8IP1	168	0.03	−1.8		
	MAPKAPK3	20022	0.09	−1.8		
	MAPKAPK5	537	0.08	−5.1		
	TAB1	1366	0.03	−2.9	Signaling transducers in IRF3, MAP and NFκB signaling pathways	(64–67)
	TRAF3	1980	0.02	−3.4		
	TRAF6	267	0.00	−12.4		
NFκB signaling enhancers	OTUB1	2939	0.01	−4.9	Specific ubiquitin iso-peptidase	(68)
	RNF128	1467	0.00	−4.3	E3 ubiquitin ligase	(69)
	TGFB2-OT1	226	0.05	−3.6	Activate NFκB RELA	(70)
	TRIM52	128	0.03	−3.1	Enhance NFκB signaling	(71)
NFκB signaling inhibitors	HIVEP2	1033	0.05	11.8	Inhibit NFκB in DNA binding	(72)
	NKIRAS1	318	0.03	4.6	Inhibit IKKβ activity	(73)
	NLK	375	0.03	9.7	Inhibit co-activators of NFκB	(74)
	NKRF	147	0.07	2.8	Nuclear inhibitor of NFκB	(75)
Transcription factors	CREB5	264	0.00	−22.5	Co-activated with NFκB by TLR	(65, 66)
	NFKB2	2376	0.03	−4.1	Non-canonical NFκB signaling	(65)
	RELA	4183	0.08	3.8	Canonical NFκB signaling	(65)
miRNAs	MIR155HG	219	0.00	12.7	Suppress interferon signaling	(76)
	MIR221	540	0.05	2.9	↓ IFNβ expression & LPS signaling	(77, 78)
	MIR503HG	165	0.05	2.4	Inhibit NFκB signaling	(79)

higher in carriers ($p = 0.02$ and 0.1, respectively). FOXP3 and ThPOK are a CD4+ T cell-specific and Treg-specific transcription factors, respectively. FOXP3+ Treg cells lose expression of ThPOK and FOXP3 after migration to the mucosal epithelium and convert to CD4+ intraepithelial lymphocytes (IEL) (88, 98). The expression levels of FOXP3 and ThPOK but not CD4 were significantly lower in carriers compared to non-carriers (**Table 6**), indicating increased recruitment of FOXP3+ Treg cells to the epithelium during the establishment of FMDV persistent infection.

Apoptosis and Inflammatory Mediators

IPA pathway analysis indicated cell death and survival could be affected by the differential gene expression. Apoptosis is a well-known cell death mechanism that plays a role in immunity against viral infection. The expression of four pro-apoptotic genes (CASP3, BNIPL, BCL2L14, and BCL2L1) was significantly downregulated by 3.3- to 8.3-fold and one anti-apoptotic gene (BNIP2) upregulated by 9.2-fold in carriers compared to non-carriers (**Table 7**). CASP3 is a critical caspase in the down-stream of apoptosis pathways that is activated by eternal and external signals such as virus infection, death receptor ligands (TNF, TRAIL, etc.) and cell-mediated cytotoxicity. TP53 is a tumor suppressor gene and is the top upstream regulator detected in this study. Two TP53-interacting genes, TP53BP1 (TP53 binding protein 1) and TP53RK (TP53 regulating kinase), were expressed at significantly lower levels by > 7-fold in carriers than in non-carriers (**Table 7**). TRAF3 and TRAF6 downregulated in carriers (**Table 5**) could also inhibit viral RNA-induced apoptosis activated by RIG-I-like receptor-induced IRF3 mediated pathway (64). These findings suggest that apoptosis triggered by virus infection might be inhibited in the nasopharyngeal epithelium of FMDV carriers as detected with IPA analysis.

Four downregulated (PTGR1, PTGES, PTGES2, and PTGS1) and one upregulated (PLA2G2A) genes involved in leukotriene B4 (LTB4) and prostaglandin production (103–105) were expressed at higher levels in carriers than in non-carriers (**Table 7**). The differential expression suggests there is imbalance between LTB4 (↑) and prostaglandin (↓) production. Higher expression of LTB4R, Leukotriene B4 receptor 1 (5.7-fold at $P = 0.02$, **Table 7**), supports increased production of LTB4 in carriers. Interestingly, a probe of bovine immunoglobulin epsilon heavy chain constant region mRNA (NCBI accession #: AY221098) displayed 3.9-fold increased signal intensity in carriers than

TABLE 6 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR) and fold differences (+ and –values as up- and down-regulated in carriers compared to non-carriers, respectively) of genes regulating or expressed on immune cells.

Group	Gene	MSI	*P*	FDR	Fold	Functions	References
Macrophage	MFSD6	2626		0.03	−3.9	MHC-I restricted killing by Mϕ	(80)
	MHCIb	1019		0.09	−7.7	Non-classical MHC Class I	NCBI
NK cell	NCR3LG1	616		0.07	−4.8	Membrane NCR3 ligand	(81)
	NID1	346		0.01	12.1	Extracellular NCR2 ligand	(82)
T cells or intraepithelial lymphocytes (IEL)	CD69	121		0.00	5.7	Inhibit Th17 and CD8+ Teff cells, stimulate Treg cells and increase tryptophan uptake	(82, 83)
[-3mm]	CD69	268		0.00	4.9		
	CD69	277		0.00	4.9		
	CD98	3005	0.20	0.55	−1.6	Transport of tryptophan	(83)
	LAT1	7268		0.05	−2.8		
	CD27	556		0.00	−4.4	CD8+ T cell cytotoxicity	(42)
	CD244/2B4	450		0.03	−4.9	NK and CD8+ T cell cytotoxicity	(47, 48)
	TNFRSF19	1468		0.00	−9.4	↓TGFβ effect on CD8+ cells	(84, 85)
	IGHD	587		0.01	2.3	IgD on γδ T cells	(86)
	CD103	3984	0.03	0.21	−2.5	Resident memory T cells	(87)
	T-bet	564		0.05	4.5	↑ in non-helped Trm	(88, 89)
Th17 cell	RORC	748	0.03	0.24	−5.9	Th17 transcription factor	NCBI
	STAT3	17482	0.01	0.13	−1.5	Promote Th17 differentiation	(90)
	TIAM1	3826		0.00	−20.8	Activate IL-17 promoter	(91)
	ETS1	881		0.01	4.7	Suppress Th17 differentiation	(92)
	IL1RL1/ST2	55	0.02	0.18	2.2	↑ on stimulated Th17	(93)
	IL22	42	0.04	0.25	1.6	Inhibit Th17 response	(94)
	IL33	578		0.02	14.7	↓ Th17	(95)
	STAT5B	2118		0.08	4.4	Inhibit Th17 cell differentiation	(90)
Tr1 cell	CD49B	216		0.05	4.0	Tr1 cell marker	(96)
	EBI3/IL27B	83	0.02	0.21	3.6	Stimulate Tr1 differentiation	(96)
	MAF	1729	0.01	0.13	2.1	Tr1 transcription factor	(96)
	PRDM1	2302		0.08	2.9	Tr1 cell marker	(97)
Treg cell	FOXP3	116		0.07	−2.1	Treg transcription factor	(98)
	ThPOK	3191		0.09	−2.8	CD4+ transcription factor	(98)

in non-carriers (P = 0.02), indicating higher levels of IgE in carriers and indirectly supporting the increased production of LTB4 based on the role of LTB4 in allergy (102). The LTB4 and prostaglandin imbalance has been reported to play a role in *Mycobacterium tuberculosis* persistent infection (121).

Transcription Factor Binding Sites

TATA box sequences were detected in the proximal promoter regions of CXCL15, CXCL16 and CXCL17 but not in chemerin. There is a CpG island sequence overlapping transcription start sites of chemerin and CXCL16 genes. There was at least one transcription factor binding site (TFBS) for RELA (A transcription factor activated by the canonical NFκB signaling pathway) detected in the proximal promoters of all four of these chemokine genes (**Table 7**). There were at least two TFBS for IRF3 and three for STAT1::STAT2 in these proximal promoters except for the chemerin promoter. No TFBS for FOS::JUN, IRF3 or STAT1::STAT2 were found in the chemerin proximal promoter, but five NFKB1 (another transcription factor activated by the canonical NFκB signaling pathway) TFBS were detected in the region. Several TFBS for IRF3, NFκB, and STAT1::STAT2 overlap, suggesting that multiple transcription factors can bind to the same sequences to regulate the transcription at these sites. These predicted TFBS suggest that the transcription of these four chemokine genes could be regulated by the interferon, IRF3, MAPK and/or canonical NFκB signaling pathways.

DISCUSSION

Persistent infection is not unique to FMDV as most, if not all, picornaviruses can persistently infect cells both *in-vitro* and *in-vivo* (122). In some virus families, virus-specific factors promoting persistent infection have been described (e.g., in lymphocytic choriomeningitis virus Clone 13) (123), but it has been demonstrated that virus mutations or specific viral genomic characteristics are not the decisive factors in the establishment of persistent FMDV infection *in-vitro* or *in-vivo* (124, 125). To date, it is not clear which specific mechanisms allow FMDV to persist in some hosts and why available vaccines can protect animals from clinical disease while not preventing or curing subclinical (primary) or persistent infection of the upper respiratory tract. In the current study, we used Ingenuity Pathway Analysis (IPA) to analyze all DEG and found that immune cell trafficking could be significantly impacted by genes differentially

TABLE 7 | Mean expression levels (microarray signal intensity, MSI), false discovery rates (FDR) and fold differences (+ and – values as up- and down-regulated in carriers compared to non-carriers, respectively) of genes involved in apoptosis or inflammatory mediator production.

Group	Gene	MSI	*P*	FDR	Fold	Functions	References
Apoptosis	CASP3	5887		0.07	−6.9	Apoptosis activating caspase	(99)
	BNIP2	708		0.01	9.2	Anti-apoptosis	NCBI
	BNIPL	30865		0.04	−3.2	Pro-apoptosis	NCBI
	BCL2L14	832		0.02	−8.3	Pro-apoptosis	NCBI
	BCL2L1	1371		0.02	−8.2	Pro-apoptosis	NCBI
	TP53BP1	1386		0.06	−7.2	Pro-apoptosis via NFκB	(100)
	TP53RK	1042		0.07	−7.3	Pro-apoptosis via TP53	(101)
Inflammatory mediators	IGHE	66	0.02	0.16	3.9	IgE heavy chain	NCBI
	LTB4R	170	0.02	0.18	5.7	Leukotriene B4 receptor 1	(102)
	PTGR1_s	1554		0.00	−10.3	Inactivation of leukotriene B4	NCBI
	PTGR1_l	259	0.41	0.74	−1.8		
	PLA2G2A	161		0.00	18.5	Production of leukotriene B4 and prostaglandins	(103–105)
	PLA2G2A	211		0.00	15.7		
	PTGES	240	0.01	0.11	−2.6	Prostaglandin E synthesis	
	PTGES2	715		0.00	−7.1		
	PTGS1	1499	0.04	0.27	−3.3	Prostaglandin G/H synthesis	

TABLE 8 | The numbers and locations of predicted transcription factor (TF) binding sites (TFBS) in the proximal promoters (CpG island or 500 bp upstream and 250 bp downstream of TATA box) of chemerin, CXCL15, CXCL16, and CXCL17 genes.

Gene	Type	TF[a]	TFBS	TFBS Location in the proximal promoters[b]
Chemerin	CpG	NFkB1	5	80–92, 229–241, 264–276, 296–308, 328–340
		RELA	2	81–91, 523–532
CXCL15	TATA	IRF3	6	220–240, 277–297, 305–325, 664–684, 690–710, 730–750
		RELA	1	281–290
		STAT1::STAT2	4	304–318, 372–386, 665–679, 731–746
CXCL16	TATA and CpG	CREB5	1	124–135
		FOS::JUN	1	124–135
		IRF3	5	409–429, 414–434, 420–440, 426–446, 698–706
		NFKB1	1	519–531
		RELA	2	519–528, 789–798
		STAT1::STST2	3	419–433, 525–539, 693–707
CXCL17	TATA	FOS::JUN	1	70–82
		IRF3	2	363–383, 381–401
		RELA	1	388–397
		STAT1::STAT2	3	364–378, 388–402, 524–537

[a] *Underlines indicate that TFBS are present in all proximal promoters.*

[b] *Underlines indicate overlapping of TFBS for different transcription factors in the proximal promoter.*

expressed between the nasopharyngeal epithelium of FMDV carriers and non-carriers.

It is well-known that immune cell trafficking is controlled by chemokines, and these immune cells express various receptors to respond to chemokine gradients. In the mucosa, epithelial cells express several chemokines to recruit immune cells to control mucosal infections and maintain homeostasis (126). Among five chemokine genes that were downregulated in the nasopharyngeal mucosa of FMDV carriers, chemerin, CXCL15, CXCL16, and CXCL17 have been reported to be expressed by epithelial cells (32, 34, 127, 128). Our results (**Figure 2**) also show that the expression of these four genes was higher in the micro-dissected epithelium than in whole tissue macerates of samples from the corresponding anatomic sites.

The mucosal immune system is separated into inductive and effector sites based upon anatomical and functional properties (129). Antigen presenting cells (APC) such as dendritic cells bind antigens in effector sites and migrate to mucosa-associated lymphoid tissues (inductive sites) where they present antigen epitopes to T cells and induce a specific set of chemokine receptors on the T cells to specifically migrate back to the mucosa (the effector sites). Chemerin recruits plasmacytoid dendritic cells, immature myeloid dendritic cells, macrophages and natural killer cells by binding to three different receptors; ChemR23, GPR1, and CCRL2 (30, 130). Downregulated expression of

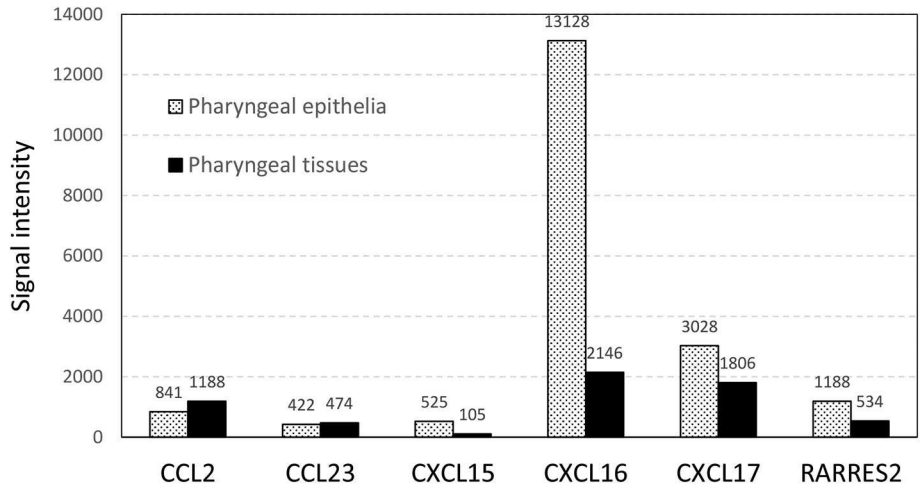

FIGURE 2 | The normalized microarray signal intensity (photons per pixel) of six differentially expressed chemokines in total RNA prepared from micro-dissected pharyngeal epithelia and whole pharyngeal tissues.

chemerin in carriers could reduce recruitment of dendritic cells to the epithelium, which could hinder the induction and reactivation of an adapted immunity. A potentially reduced recruitment of dendritic cells in FMDV carriers was further supported by downregulated expression of four APC-expressed genes (CD276/B7-H3, CLEC9A/DNGR1, VISTA/B7-H5, and VTCN1/B7-H4 (**Table 2**).

CXCL16 and CXCL17 are ELR- CXCL chemokines. CXCL16 has a strong chemotactic activity for activated CD8+ T cells, CD4+ T cells, NKT cells and intraepithelial lymphocytes (IEL) but weak or no activity for unstimulated T cells (33, 35). The expression of CXCL16 receptor (CXCR6) on T cells in the lung is correlated with local protective immunity against *Mycobacterium tuberculosis* (131). Like CXCL16, CXCL17 is chemotactic for antigen-experienced memory CD8+ T cells such as memory T effector (Tem) and resident memory (Trm) cells (132). CXCR8 is the receptor of CXCL17. CXCL17 null mice developed fewer CXCR8+ CD8+ Tem and Trm cells and exhibited greater herpes virus replication and susceptibility to latent herpes infection in the mucosa compared to wild-type mice (132). Like chemerin, CXCL17 also recruits antigen presenting cells such as immature dendritic cells (36, 133). Significant downregulation of CXCL16 and CXCL17 could reduce recruitment of antigen-experienced T cells to the infected epithelium, which then reduced the killing of infected cells.

In contrast, the recruitment of NK cells into persistently infected epithelium might increase based on upregulated expression of CCL2, CD16, and two KLR receptors (**Table 3**). NK cells can kill FMDV infected cells and the cytotoxicity can be enhanced by cytokines such as IL-2, IL-15, IL-18, and IFN-α (134). IFNA, IFNL, and IL15 were expressed in significantly higher levels in carriers than in non-carriers; however, downregulated NCR3 ligand (NCR3LG1/B7-H6) and upregulated extracellular NCR2 ligand (NID1), a NK cell cytotoxicity inhibitor (81) could significantly reduce interaction between infected epithelial cells and NK cells and compromise the effectiveness of NK cells in killing of FMDV infected cells. Therefore, the killing of FMDV infected epithelial cells by both antigen-specific cytotoxic T cells and NK cells could be compromised as discussed earlier and later based on upregulated CD69.

Although killing of infected cells reduces virus replication, additional mechanisms such as extracellular traps and phagocytosis by neutrophils or macrophages are needed to limit virus spread and clear infection from the host. All ELR+ chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8, have a chemotactic activity for neutrophils via binding to CXCR2 (27). CXCL6 and CXCL8 can also bind to CXCR1 (135). CXCR1 and CXCR2 are known to be predominately expressed on neutrophils (27, 40). Downregulated expression of ELR+ chemokines (**Table 2**), CXCR1 and CXCR2 and other neutrophil-expressed genes in carriers (**Table 3**) strongly supports reduced recruitment of neutrophils in carriers. Because neutrophils produce CCL23 (136), lower CCL23 expression in carriers also indirectly supports our hypothesis.

CXCL15 is another ELR+ CXCL chemokine reported in mice and has a strong chemotactic activity for neutrophils (32). CXCL15-null mice were more susceptible to *Klebsiella pneumoniae* infection than wild-type mice (137). We have recently identified a novel bovine CXCL15 in cattle (138). The expression of CXCL15 was significantly downregulated in carriers compared to non-carriers and total expression of all seven bovine ELR+ CXCLs was also 2.4-fold lower in carriers (**Table 2**). CXCL15 gene appears to be not functional in water buffalo (*Bubalus bubalis*) due to early stop codon mutation (138). Interestingly, there is limited evidence that Asian buffalo are more susceptible to persistent FMDV infection based on higher percentage of FMDV persistence in buffalo than in cattle in one study (139), which indirectly supports the role of CXCL15 in FMDV persistence.

It is well-known that neutrophils are the most numerous circulating immune cells and play a critical role in the first line of defense against infections by engulfing and destroying pathogens as well as secreting anti-microbials, cytokines and chemokines to recruit other immune cells (140, 141). Neutrophils can also release neutrophil extracellular traps (NET) to immobilize and inactivate viruses (142). NET are extracellular fibril matrices composed of granule proteins and chromatin released by activated neutrophils (143), which has been reported to play a role in eliminating virus infections (144, 145).

To understand why the expression of chemerin, CXCL15, CXCL16, and CXCL17 chemokines was downregulated in carriers, we first examined the expression of cytokines. Unexpectedly, all significantly differentially expressed cytokine genes were upregulated in carriers and all are proinflammatory (**Table 4**). Upregulated expression of the cytokines probably was due to increased recruitment of monocytes into the epithelium of FMDV carriers based on upregulated CCL2, C5AR1, CD16, CD300, CD300LD, MIR155HG, and MMD expression (**Tables 2**, **3**, **5**). MMD overexpression has been shown to increase TNF production in a macrophage cell line (62). Co-culture of IL-15-stimulated NK cells with blood mononuclear cells induced TNF production in macrophages, which in turn induced CD69 expression on lymphocytes (146). CD69 and IL15 were also significantly upregulated in carriers in the current study (**Table 4**).

TNF was the most upregulated cytokine in carriers. The expression of another TNF cytokine (TNFSF10 or TRAIL) also known to be able to kill virus-infected cells via death receptor signaling to induce apoptosis (147) was also upregulated in carriers. The expression levels of TNF and TRAIL were higher in the lung (the tissue susceptible to acute FMDV infection but resistant to persistent infection) than in pharyngeal tissues (20). It may be concluded that TNF and TRAIL probably are not very effective in killing FMDV infected cells in carriers because apoptosis induced by these two cytokines could be suppressed by the significantly down-regulated (6.9-fold) expression of a key apoptosis activator, CASP3 (99) and other apoptosis-related genes listed in **Table 7**. Interestingly, TP53 (a tumor suppresser gene) was the top regulator detected in this study. TP53BP1 interacts with TP53 and NFκB and can sensitize breast cancer cells to apoptosis induced by TNF treatment (100). TP53RK is a TP53 kinase that can phosphorylate and activate TP53 (101). Therefore, significantly downregulated CASP3, TB53RK and TP53BP1 together with TRAF3 and TRAF6, the signal transducers in RIG-I-induced IRF3 mediated pathway of apoptosis (64), strongly support the suppression of apoptosis induced by death receptor signaling, virus RNA and cell-mediated cytotoxicity in carriers.

IL33 was the second most upregulated (14.7-fold) cytokine in FMDV carriers. The receptor of IL33 is constitutively expressed on mast cells, group 2 innate lymphoid cells and Tregs. IL33 plays a key regulatory role in mucosal immunity (95). This cytokine promotes the accumulation and function of myeloid-derived suppressor cells (148) and regulatory T-cells in the intestine (149), induces alternative activation of macrophages (150) and has a potent suppressive effect on innate antiviral immunity (151). Epithelial cells are the major source of IL33 production in the intestine during inflammation (93). Stimulation of IL33 changed the Th17 expression profile in favor of an immunosuppressive phenotype (93). The expression of IL33 in macrophages can be induced by aryl hydrocarbon receptor (AHR) (152). It is well-known that AHR plays a critical role in mucosal immunity (153).

Interestingly, CD69 can increase uptake of L-tryptophan through LAT1-CD98 for converting tryptophan into AHR ligands to activate AHR signaling (83) and CD69 was one of the most consistently upregulated genes in carriers (**Table 6**). Significantly downregulated CD98/LAT1 in carriers (**Table 6**) could reduce the effect of upregulated CD69 on AHR ligand production. However, CD69 also has a broad immune suppressive effect via receptor signaling (82). The expression of CD69 and miR-155 (also upregulated in carriers in this study) are coregulated in a positive-feedback loop to promote Treg cell differentiation (154). Increased CD69 expression enhances immunosuppressive function of regulatory T-cells (155, 156), suppressed Th17 cell differentiation (82, 118, 157) and T cell cytotoxicity (158).

The expression of CD69 on lymphocytes in the gut appears to depend on the microflora because germ-free mice and the ablation of microflora decreased CD69 expression (159, 160). Sustained expression of CD69 on activated T lymphocytes depends on non-canonical NFκB signaling (161). Interestingly, our results indicate that the activation of upregulated IL1s and TNFs in NFκB signaling pathways were routed to the non-canonical pathway due to significantly upregulated IKKα and downregulated IKKβ and IKKγ (**Table 5**). The activation of the non-canonical pathway is known to play a role in peripheral immune tolerance and secondary lymphoid tissue development (71). FMDV persistent infection was observed in the follicle-associated epithelia of the nasopharyngeal mucosa (7, 10).

Next we examined the differential expression of genes involved in cytokine signaling pathways. The results listed in **Table 5** could significantly suppress the expression of downregulated chemokines in carriers. IL17A and IL17F activate NFκB signaling pathways and play an important role in mucosal immunity via inducing chemokine expression to recruit neutrophils (162, 163). It induces neutrophil recruiting ELR+ CXCL chemokines in epithelial cells (164) and increases the stability of CXCL1 and CXCL5 mRNA (165, 166). Interestingly, the expression of IL17RA (the IL17A and IL17F receptor) was significantly downregulated in carriers, which could inhibit the response of epithelial cells to IL17 stimulation. Additionally, downregulated NFkB signaling enhancers and CHUK/IKKα and upregulated signaling inhibiters, IKKβ and IKKγ (**Table 5**) could play a significant role in the downregulated chemokine expression.

Third, we examined more DEG that could suppress the immune response. DEG listed in **Table 6** indicate that the cytotoxicity of macrophages, NK cells and CD8+ cytotoxic T cells and especially the Th17 response could be suppressed in carriers. As stated earlier for IL33 and CD69, there were two more DEG upregulated in carriers (ETS1 and STAT5B) known to suppress Th17 differentiation (90, 92, 119), whereas

TIAM1 needed for IL17 expression (91) was downregulated by 20-fold in carriers (**Table 6**). These results could explain why upregulated Th17-stimulatory cytokines did not result in higher IL17 expression in carriers (**Table 4**). Therefore, the suppression on the Th17 response could also play a role in FMDV persistent infection.

Finally, we predicted the TFBS in the proximal promoters of the four epithelium-expressed chemokines; chemerin CXCL15, CXCL16, and CXCL17, to infer if inhibition of the signaling pathways could reduce the expression of these four chemokine genes. The results of the promoter analysis indicate that the expression of all four chemokines relies on the activation of canonical NFκB (NFKB1 and RELA) transcription factors, whereas CXCL15, CXCL16, and CXCL17 may also depend on IRF3, interferon and MAPK signaling pathways. CXCL15 expression has been shown to increase under inflammatory conditions such as antigen and LPS stimulation and infection (32, 167). IFNγ and TNF stimulates CXCL16 and CXCL17 RNA expression in cultured cells (126, 168). IFNγ and TNF appear to have a synergetic effect on CXCL16 expression, indicating that multiple transcription factors act together to regulate expression of this chemokine. Interestingly, the differential expression of DEG listed in **Table 5** could inhibit these signaling pathways especially canonical NFκB signaling pathway, which is known to be activated by IL-17, IL-33, and TNF as stated earlier.

Our previous study using whole pharyngeal tissues showed cytokine and Tr1-expressed genes being differentially expressed between carriers and non-carriers (21). In current study, only two Tr1 marker genes (CD49B and PRDM1) were significantly upregulated in carriers, and one transcription factor (MAF) and one cytokine (IL27B) critical for Tr1 differentiation (96) were upregulated ($p \leq 0.02$) at non-significant levels. The expression

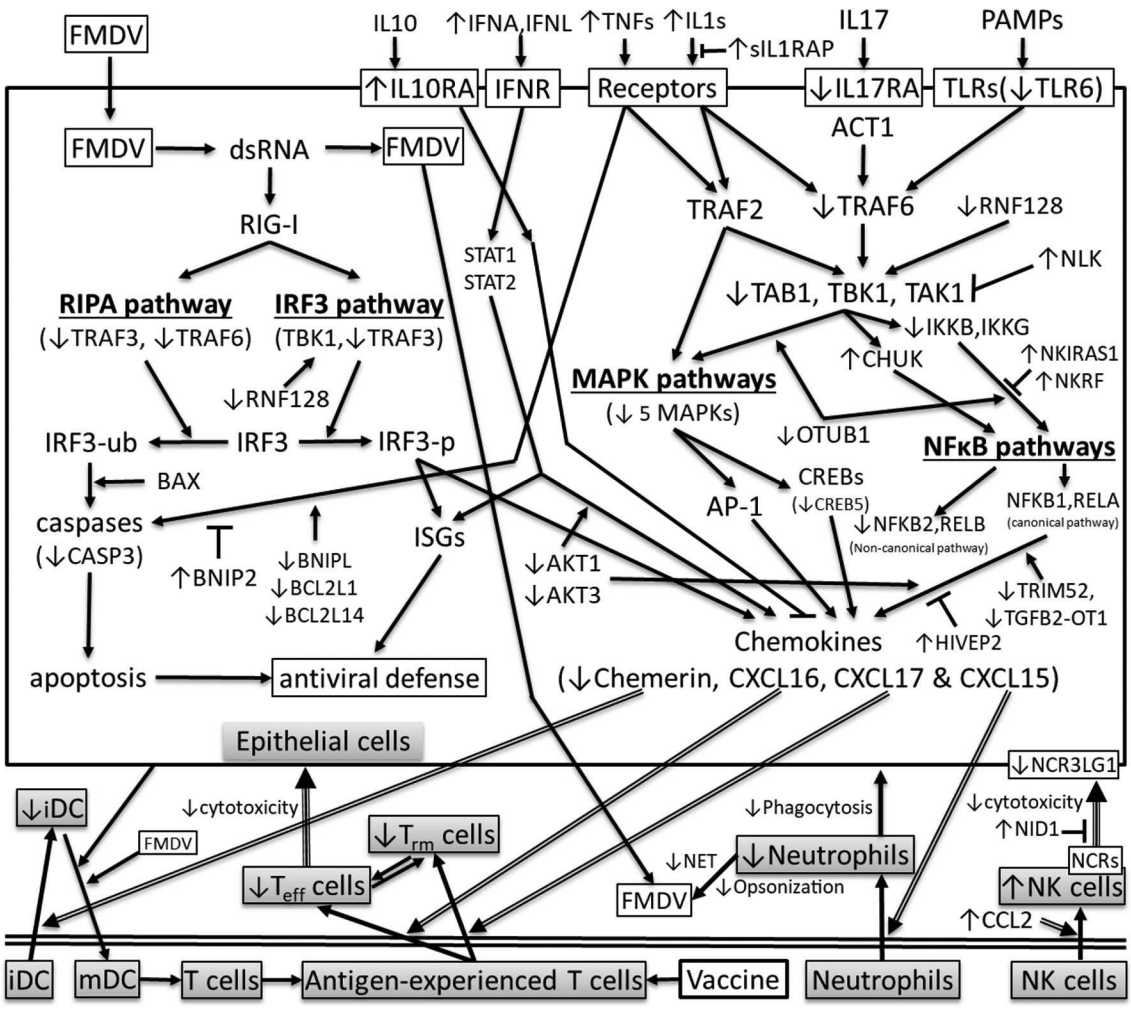

FIGURE 3 | Differentially expressed genes in cytokine signaling pathways that could affect the transcriptions of chemerin, CXCL15, CXCL16, and CXCL17 in the pharyngeal epithelial cells of FMDV carriers (↑ or ↓: significantly up- or downregulated in carriers compared to non-carriers, respectively; arrow: stimulation; ⊤ inhibition; iDC and mDC: immature and mature DC; IRF3-ub and IRF3-p: ubiquitinated and phosphorylated IRF3, respectively; ISGs: interferon-stimulated genes; MAPKs: mitogen-activated protein kinases; NCRs: NK cell receptors; NET: neutrophil extracellular traps; RIPA: RIG-I-like receptor-induced IRF3 mediated pathway of apoptosis; T_{eff}: effector T cells; T_{rm}: memory T cells; double line arrow: cell recruitment; double line: separation between epithelium and other tissues).

of IL10, a typical cytokine produced by Tr1 cells, was not differentially expressed. EBI3, one of an immunosuppressive cytokine IL35 dimer, was upregulated ($p = 0.02$) at a non-significant level. The differences could be due different tissue sampling in the study. Most regulatory T cells reside in the lamina propria instead of the epithelia (129).

A higher significant threshold (FDR ≤ 0.05) was used in the previous study of this set of microarray data used in current study (22). Most of the DEG listed in current study was not evaluated in the previous study. Immunohistochemistry analysis using anti-CD3D, anti-CD8A and anti-γδTCR antibodies showed no differences in the numbers of the detected cell populations between carriers and non-carriers (22). These antibodies could not distinguish between naïve and antigen-experienced effector T cells. The results generally agree with the results found in current study of no differences in overall T cell population sizes, though the results of the current study suggest increased naïve T cell/natural IEL- and decreased effector T cell-recruitment.

In conclusion, the IPA pathway analysis suggests that the detected differential gene expression could affect cell death and survival, immune cell trafficking and hematological system development and function. Four chemokines (chemerin, CXCL15, CXCL16, and CXCL17) recruiting neutrophils, antigen-experienced T cells and/or dendritic cells were downregulated in FMDV carriers, whereas macrophage and NK cell recruiting CCL2 was upregulated. Other DEG support the differential expression of the chemokines, as shown throughout these analyses. Although all differentially expressed cytokines were upregulated and proinflammatory, the DEG in signaling pathways suggested that the interferon, IRF3, MAPK, and NFκB signaling pathways especially the canonical NFκB pathway could be inhibited in carriers. The TFBS predicted from the proximal promoters indicated that the expression of these downregulated chemokines depends on the activation of these signaling pathways. DEG such as CASP3, CD69, IL17RA, IL33, NCR3LG1, NID1, TP53BP1, TRAF3, and TRAF6 indicated that the Th17 response, NK cell cytotoxicity and apoptosis could be suppressed in carriers. Therefore, based on our results and published gene functions, we hypothesize that (1) under-expression of chemokines that recruit neutrophils, antigen-experienced T cells and dendritic cells, (2) blocking NK cell binding to infected cells and (3) suppression of cell-mediated cytotoxicity-, death receptor signaling- and viral RNA-induced apoptosis compromised virus clearance and allowed FMDV to persist as shown in **Figure 3**. These hypothesized mechanisms indicate that vaccines may not be effective in curing FMDV persistent infection. This study provides novel insights for further investigation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Michael Eschbaumer, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104058.

ETHICS STATEMENT

The animal study was reviewed and approved by Plum Island Institutional Animal Care and Use Committee (Protocol numbers 209-12-R, 209-15-R).

AUTHOR CONTRIBUTIONS

JZ designed the bovine microarray and the microarray experiment, conducted gene annotation, bioinformatic analysis, and wrote the manuscript. JA and CS conceived and executed the primary animal studies and laboratory analysis. CS collected and analyzed the animal samples and performed tissue microdissection. EB performed gene annotation. ME performed microarray analyses and statistical analysis of the data. All authors reviewed and edited in the manuscript.

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Experimental Infection of Foot and Mouth Disease in Indian Sheep and Goats

Madhanmohan Muthukrishnan†, Nagendrakumar Singanallur Balasubramanian† and Srinivasan Villuppanoor Alwar†*

Foot and Mouth Disease Virus Laboratory, Research and Development Centre, Indian Immunologicals Limited, Hyderabad, India

Foot-and-mouth disease (FMD) is an economically important contagious disease of livestock mainly cattle, buffalo, sheep, goats, and pig. There is limited data available on pathogenesis of foot and mouth disease in goats. In the study, the sheep and goats were infected experimentally with a serotype O foot-and-mouth disease virus by different challenge routes. The sheep and goats challenged by coronary band route and coronary band and intra-dermo-lingual route exhibited FMD clinical signs at 2–5 days post challenge. Whereas intra-dermo-lingual challenged sheep and goats did not exhibit FMD clinical signs. Live virus could be isolated from blood of infected sheep and goats at 2–5 days post challenge. Viral RNA could be detected from blood of infected sheep and goats at 1–10 days post challenge. The neutralizing antibody titre was detected at 10 days post challenge and maintained up to 35 days post challenge in all infected sheep and goats. Non structural protein (NSP) antibodies were detected as early as 5–10 days post challenge and remain positive up to 35 days post challenge in the infected sheep and goats. In conclusion, the pathogenesis of sheep and goats with serotype O foot and mouth disease virus by different challenge routes could be demonstrated.

Keywords: foot-and-mouth disease, serotype O, experimental infection, sheep, goats

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Francois Frederick Maree, Agricultural Research Council of South Africa (ARC-SA), South Africa

***Correspondence:**
Madhanmohan Muthukrishnan
muthukrishnan.madhanmohan@gmail.com

†Present address:
Nagendrakumar Singanallur Balasubramanian, FMD Risk Management Project, CSIRO Health & Bio-Security, Australian Centre for Disease Preparedness, Geelong, VIC, Australia
Srinivasan Villuppanoor Alwar, National Dairy Development Board, Hyderabad, India
Madhanmohan Muthukrishnan, Veterinary University Training and Diagnostic Centre, Tamil Nadu Veterinary and Animal Sciences University, Madurai, India

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INTRODUCTION

Foot-and-Mouth Disease (FMD) is an infectious disease which causes severe economic loss to the livestock sector (1). FMD is caused by FMD virus (FMDV), a member of family *Picornaviridae* and genus *Aphthovirus* affecting all the cloven footed animals. FMDV exists as seven distinct serotypes viz., O, A, C, Asia 1, Southern African territory 1(SAT1), SAT2, and SAT3. In India, incidence of FMD is reported throughout the country with the prevalence of FMDV serotypes O, A and Asia 1 (2). Cattle and buffalo are vaccinated biannually with inactivated FMD trivalent vaccine to control FMD in India. However, sheep and goats are not included in FMD control program (3, 4). Sheep and goats play an important role in the livelihood of a large percentage of small and marginal farmers and landless laborers in India. India constitutes around 148.88 million heads of goat population and 74.26 million heads of sheep population in the world. Moreover, cattle, buffalo, sheep and goats are grazed together in India (5). FMD outbreaks in sheep and goats are reported in India (6, 7).

There is paucity of information on the role of goats in FMD epidemiology and transmission. FMD infected sheep and goats transmitted the sub-clinical infection to cattle, buffalo, sheep and goats and FMD vaccination in sheep and goats could prevent the transmission of FMD to

cattle, buffalo, sheep and goats (8). Usually, sheep and goats showed mild or unapparent FMD clinical signs (9, 10). Moreover, FMD infected goats showed typical oral and foot lesions in India (11). However, there is no detailed account of the pathogenesis of the disease in these small ruminants, especially in goats. This preliminary report describes pathogenesis of sheep and goats experimentally infected with type O foot and mouth disease virus using different challenge routes.

MATERIALS AND METHODS

Cell Line and Viruses

Baby Hamster kidney (BHK) and primary bovine thyroid (BTY) cells were provided by the tissue culture laboratory at Research and Development Centre, Indian Immunologicals Limited (IIL), Hyderabad. BTY cells were grown using Hely cell growth medium supplemented with 10% adult bovine serum and antibiotics cocktail (penicillin, neomycin and polymyxin). O/IND/R2/75 virus was received from the virus seed laboratory, IIL, Hyderabad.

Experimental Animals

Eight Nellore sheep and eight Osmanabadi goats of either sex (6–12 months of age) were obtained from the holding farm of IIL, Hyderabad. These animals were reared in the farm from one month of age and were screened by three rounds of testing for FMDV-non-structural protein (NSP) antibodies using PrioCHECK® FMDV NS kit (Prionics Lelystad B.V., The Netherlands). All the animals were NSP seronegative in all the three tests. Additionally, the animals were tested for the absence of virus in the oesophagopharyngeal fluids (Probang samples) thrice by virus isolation on primary bovine thyroid cells (12) followed by antigen ELISA (13) and RT-PCR (14).

Challenge Virus Preparation

Challenge virus O/IND/R2/75 was prepared and titrated by standard methods as described previously (15).

Experimental Design

One sheep and goat each were inoculated with O/IND/R2/75 cattle challenge virus by intra-dermo-lingual, coronary band and by both sites in 0.1 ml quantity in each site. The animals were monitored for 24–72 h for signs of FMD (passage 1). For a second passage, epithelial tissue collected from vesicles was triturated in 0.04 M phosphate buffer followed by centrifugation at 3000 xg. The clear supernatant was used to inoculate one sheep and goat each by intra-dermo-lingual, coronary band and by both sites in 0.1 ml quantity in each site respectively. The animals were monitored for 24–72 h for signs of FMD. Two sheep and two goats was included as unchallenged control and maintained throughout the study period. Experiments were conducted in a bio-secure animal isolation unit at IIL, Hyderabad. The studies involving animals were reviewed and approved by Institutional Animal Ethics Committee, Indian Immunologicals Limited, Hyderabad and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Department of Animal Husbandry and Dairying, Government of India.

Clinical Scoring

The sheep and goats were observed for clinical signs of disease and temperatures recorded daily. A subjective scoring system (16) was used to evaluate the progression of disease in these animals with slight modification (8).

Sample Collection and Processing

Clotted blood for serology and NSP antibody was collected at days 0, 5, 10, 15, 21, 28, and 35 post-challenge. Heparinized blood was collected daily up to 10 dpc. Heparinized blood (200 µl) was mixed with 300 µl of lysis buffer (Roche Diagnostics, Germany) for analysis by real-time RT-PCR (qRT-PCR) and stored at −70°C. Heparinized blood (1 ml) was used for virus isolation (VI) (15).

Virus Isolation

Heparinized blood samples were examined for the presence of live virus by primary bovine thyroid (BTY) cell culture inoculation (9). BTY tubes were inoculated with 250 µl sample (5 tubes per sample) and incubated in a stationary position for 30 min at 37°C. The tubes were then gently washed with 0.04 M phosphate buffer containing antibiotics and 2 ml of virus maintenance medium was added prior to incubation at 37°C on roller drums. At 24, 48, and 72 h post inoculation, cell monolayer was examined for cytopathic effect (CPE). The presence of FMDV in cultures showing CPE was confirmed using an antigen ELISA (10). BTY cell culture supernatants from samples showing no sign of CPE after 72 h were pooled and re-passaged once and the absence of FMDV was confirmed by the antigen ELISA as mentioned above (15).

Virus Neutralizing Antibody Test (VNT)

Virus neutralization tests were performed for the sera in flatbottomed tissue culture grade micro titre plates (Nunclon™, Denmark) as described previously (17). Antibody titres were expressed as the reciprocal of the final dilution of serum in the serum/virus mixture which neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point (18).

Non Structural Protein Antibody Test

Antibodies to FMDV NSP 3ABC were tested using PrioCHECK®FMDV NS kit (Prionics Lelystad B.V., The Netherlands) (19).

Quantitative Real-Time RT-PCR Assay for Detection of Viral RNA

The amount of viral RNA in blood was quantified by qRT-PCR (20). The total nucleic acid was extracted from liquid samples with MagNApure LC total nucleic acid isolation kit (Roche Diagnostics GmbH, Germany) using an automated nucleic acid robotic workstation (MagNApure LC, Roche Diagnostics GmbH, Germany). For the generation of standard curves, a FMDV RNA standard was synthesized *in vitro* from a plasmid containing a 79 base pair insert of the internal ribosomal entry site (IRES) of a type O FMDV (kindly provided by Dr. Donald P. King, Institute for Animal Health, UK) using a MEGAscript® T7 kit (Ambion, USA) as described previously (21) in an IQ®5

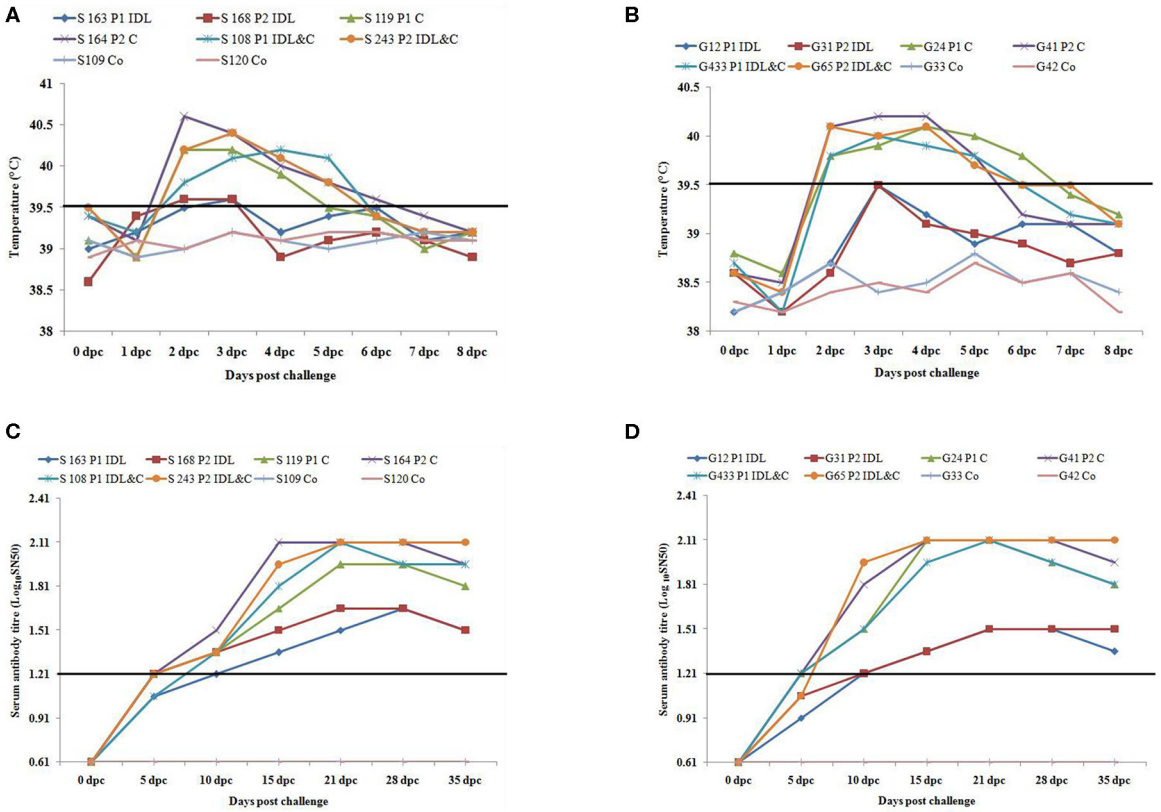

FIGURE 1 | (A) Rectal temperature (°C) of challenged (intra-dermo-lingual (IDL), coronary band (C) and by both route) and control sheep. P1, passage 1; P2, Passage 2. Solid line indicates normal temperature of sheep. **(B)**. Rectal temperature (°C) of challenged (intra-dermo-lingual (IDL), coronary band (C) and by both route) and control goats. P1, passage 1; P2, Passage 2. Solid line indicates normal temperature of goats. **(C)** Virus neutralization titres of challenged and control sheep (Expressed as the log10 reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infectious units). Solid line indicates neutralizing antibody titre >1.2 $\log_{10}$SN50 is considered positive. **(D)** Virus neutralization titres of challenged and control goats (Expressed as the log10 reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infectious units). Solid line indicates neutralizing antibody titre >1.2 $\log_{10}$SN50 is considered positive.

Multicolor Real-time PCR detection system (BioRad, USA). The results from all samples were analyzed using Bio-Rad iQ®5 optical system software and CT values were assigned to each reaction (18). Viral RNA was quantified using a standard curve derived from the standard RNA preparation at different concentrations (10^8-10^1) (22).

RESULTS

Development of Clinical FMD

Sheep inoculated by intra-dermo-lingual route did not exhibit clinical signs of FMD in both passages. Sheep inoculated by coronary band route showed clinical signs of FMD such as inappetance, panting, pyrexia (≥40 °C) (**Figure 1A**), lameness and vesicles in foot and mouth at 2–5 dpc. Sheep inoculated by both intra-dermo-lingual/coronary band routes showed FMD clinical signs at 5 dpc. Goats inoculated by intra-dermo-lingual route did not exhibit clinical signs of FMD in both passages. Goats inoculated by coronary band route showed clinical signs of FMD such as inappetance, panting, pyrexia (≥40 °C) (**Figure 1B**), lameness and vesicles in foot and mouth at 2–5 dpc. Goats inoculated by both intra-dermo-lingual/coronary band routes also showed FMD clinical signs at 3 dpc. The unchallenged control sheep and goats did not show any FMD clinical signs (**Table 1**).

Detection of Virus/ Virus Nucleic Acid in Blood

In passage 1, virus could not be isolated form challenged sheep and goats (S163, S119, S108, G12, G24, and G433) irrespective of challenge routes. In passage 2, infectious virus was isolated form intra-dermal-lingual route challenged sheep (S168) and goat (G31) at 3 dpc and coronary band challenged sheep (S164) and goat (G41) at 2–5 dpc. Whereas in intra-dermal-lingual and coronary band route challenged sheep (S243) was positive for virus isolation at 4–5 dpc. In the case of intra-dermal-lingual and coronary band route challenged goat (G65) virus was isolated on 2 and 5 dpc.

Viral RNA ($10^{8.44}$–$10^{9.45}$ viral RNA copy numbers/ml of blood) was detected as early as 1 dpc from the sheep (S119P1 and S164P2) and goat (G41P2) inoculated by coronary route.

TABLE 1 | Lesion score of challenged and control sheep and goats.

Groups	Animal No	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc
Intra-dermo-lingual	S163 P1	0	0	0	0	0	0	0	0	0
	S168 P2	0	0	0	0	0	0	0	0	0
	G12 P1	0	0	0	0	0	0	0	0	0
	G31 P2	0	0	0	0	0	0	0	0	0
Coronary band	S119 P1	0	0	1	3	3	3	2	0	0
	S164 P2	0	0	2	5	6	6	5	4	4
	G24 P1	0	0	1	2	2	2	0	0	0
	G41 P2	0	0	1	4	6	6	5	3	0
Intra-dermo-lingual and coronary band	S108 P1	0	0	0	0	0	0	0	0	0
	S243 P2	0	0	1	2	2	3	2	2	0
	G433 P1	0	0	1	1	2	0	0	0	0
	G65 P2	0	0	1	3	2	1	0	0	0
Control	S109	0	0	0	0	0	0	0	0	0
	S120	0	0	0	0	0	0	0	0	0
	G 33	0	0	0	0	0	0	0	0	0
	G42	0	0	0	0	0	0	0	0	0

S, Sheep; G, Goats; P1, Passage 1; P2, Passage 2; dpc, days post challenge.
Lesion in inoculated feet−1; Mouth lesions−2; Lesion in one foot other than the inoculated foot−2; lesion in two feet other than the inoculated feet−3; lesion in three feet other than the inoculated feet−4. The scores were then added.

TABLE 2 | Virus isolation and quantification of FMD viral RNA copy numbers (Log_{10} RNA copy numbers/ ml of blood) from blood of challenged and control sheep and goats.

Groups	Sheep/ goats	0 dpc		1 dpc		2 dpc		3 dpc		4 dpc		5 dpc		6 dpc		7 dpc		8 dpc		9 dpc		10 dpc	
		VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR
Intra-dermo-lingual	S 163 P1	-	0	-	0	-	9.46	-	9.47	-	9.43	-	0	-	0	-	9.4	-	0	-	0	-	0
	S 168 P2	-	0	-	0	-	9.45	+	9.42	-	10.5	-	0	-	0	-	0	-	0	-	0	-	9.43
	G12 P1	-	0	-	0	-	0	-	0	-	9.43	-	9.48	-	0	-	0	-	0	-	0	-	0
	G31 P2	-	0	-	0	-	0	+	9.42	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Coronary band	S 119 P1	-	0	-	9.45	-	9.44	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	S 164 P2	-	0	-	9.44	+	9.44	+	9.42	+	9.51	+	9.57	-	0	-	9.51	-	0	-	0	-	9.42
	G24 P1	-	0	-	0	-	9.44	-	8.45	-	0	-	9.45	-	0	-	0	-	0	-	0	-	0
	G41 P2	-	0	-	8.44	+	8.66	+	9.82	+	9.61	+	9.37	-	0	-	8.42	-	0	-	0	-	7.42
Intra-dermo-lingual And coronary band	S 108 P1	-	0	-	0	-	9.49	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	S 243 P2	-	0	-	0	-	9.57	-	9.52	+	9.42	+	9.48	-	0	-	0	-	0	-	0	-	0
	G433 P1	-	0	-	0	-	8.49	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	G65 P2	-	0	-	0	+	8.57	-	8.52	-	8.42	+	8.48	-	0	-	0	-	0	-	0	-	0
Control	S109	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	S120	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	G 33	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
	G42	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0

S, Sheep; G-Goats; P1, Passage 1; P2, Passage; + Positive for virus isolation; Negative for virus isolation; dpc, days post challenge.

Viral RNA ($10^{9.42}$-$10^{10.47}$) was detected in all the inoculated sheep between 1 and 10 dpc irrespective of challenge routes. Viral RNA ($10^{7.42}$-$10^{9.82}$) was detected in all the inoculated goats between 1 and 10 dpc. All the unchallenged control sheep and goats were negative for virus isolation and viral RNA from blood samples (**Table 2**).

FMDV NSP Antibody Response

Four inoculated sheep (S163, S119, S108 and S243) were positive for NSP antibody on 10 dpc while other sheep NSP antibodies were observed on 15–35 dpc. Three inoculated goats (G12, G31 and G433) were positive for NSP antibody on 5 dpc while in other goats NSP antibodies were observed on 15–35 dpc. Both

TABLE 3 | FMDV NSP antibody responses of challenged and control sheep and goats.

Groups	Animal No	0 dpc	5 dpc	10 dpc	15 dpc	21 dpc	28 dpc	35 dpc
Intra-dermo-lingual	S163 P1	N	N	P	P	P	P	P
	S168 P2	N	N	N	P	P	P	P
	G12 P1	N	P	P	P	P	P	P
	G31 P2	N	P	P	P	P	P	P
Coronary band	S119 P1	N	N	P	P	P	P	P
	S164 P2	N	N	N	P	P	P	P
	G24 P1	N	N	P	P	P	P	P
	G41 P2	N	N	N	P	P	P	P
Intra-dermo-lingual andcoronary band	S108 P1	N	N	P	P	P	P	P
	S243 P2	N	N	P	P	P	P	P
	G433 P1	N	P	P	P	P	P	P
	G65 P2	N	N	P	P	P	P	P
Control	S109	N	N	N	N	N	N	N
	S120	N	N	N	N	N	N	N
	G 33	N	N	N	N	N	N	N
	G42	N	N	N	N	N	N	N

S, Sheep; G-Goats; P1, Passage 1; P2, Passage 2; P, Positive for NSP antibody; N, Negative for NSP antibody; dpc, days post challenge.

the unchallenged control sheep and goats were negative for NSP antibody up to 35 dpc (**Table 3**).

Virus Neutralizing Antibody Response

The neutralizing antibody titer was detected in all inoculated sheep and goats at 10 dpc ($>$ 1.2 $\log_{10}SN_{50}$). However, the highest neutralizing antibody titer was detected between 10 and 35 dpc (2.1 $\log_{10}SN_{50}$) in sheep and goats inoculated by coronary and both by coronary and intra-dermo- lingual route. Both the unchallenged control sheep and goats had no serum neutralizing antibody titre up to 35 dpc (**Figures 1C,D**).

DISCUSSION

The experiment described the preliminary results on pathogenesis and development of FMD in sheep and goats by inoculating the type O FMD virus in three different challenged routes. The development of clinical signs was observed. The viral RNA levels in blood were quantified.

The incubation period of natural FMDV infection is normally between 3 and 8 days in sheep (23), but can be as short as 24 h following experimental infection (23, 24). In the current study, lesions were evident in three sheep and four goats on the 2nd day of challenge.

Sheep and goats inoculated by coronary band route showed clinical signs of FMD such as inappetance, panting, pyrexia ($\geq$40 °C), lameness and vesicles in foot and mouth at 2–5 dpc. This finding was in accordance with the earlier experiments in sheep (25–27) and goats (28). Hughes et al. (29) reported intra nasal inoculation of FMDV also resulted in generalized infection in sheep. Sheep and goats inoculated by both intra-dermo-lingual/coronary band routes also showed FMD clinical signs whereas sheep and goats inoculated by intra-dermo-lingual route did not produce clinical signs of FMD. It should be noted that although sheep and goats inoculated by the intra-dermo-lingual route showed no signs of generalized infection, virus could be isolated from blood samples and viral RNA was detected in blood samples up to 10 dpc. Furthermore, Lazarus, et al. (30) presented that indigenous South African goats manifested FMD clinical signs by challenging intra-dermo-lingual route with SAT1 virus pool. However, the clinical signs of FMD may be influenced by the virus strain and the breed of sheep and goats (31). In the present study, type O virus and Indian breed of sheep and goats were used. This may be the reason for intra-dermo-lingual challenged sheep and goats did not show the clinical signs of FMD.

Intra-dermo-lingual route of inoculation in cattle, dental pad/gum route of inoculation in buffalo (32) and intra dermal inoculation in the heel bulb in pigs (33) of FMDV resulted in generalized disease.

Ryan et al. (27) reported that all inoculated ewes developed viraemia at 1 dpi and viral RNA levels then peaked at 2 dpi. In the current study, viral RNA was detected as early as 1dpc, viral RNA level then peaked at 2–5 dpc from the inoculated sheep and goats. Virus was isolated from blood of inoculated sheep and goats up to 2–5 dpc as reported by Parida et al. (34).

Infection with live foot and mouth disease virus induces non structural antibody response in animals (35). Earlier studies (15, 34) revealed antibodies against 3ABC in sheep at 10 dpc. In the current experiment, all the sheep were positive for NSP antibodies on 10 dpc and continued up to the end of the experiment (35 dpc). In case of goats NSP antibodies were detected as early as 5 dpc and continued up to the end of the experiment (35 dpc). This finding was in accordance with the earlier experiment in sheep and goats (8, 36).

In the present study, neutralizing antibody titre was detected in all the inoculated sheep and goats at 10 dpc and the peak antibody titre was detected between 10 and 35 dpc. Dellers et al.

(25) reported neutralizing antibodies were first detected 60 h post inoculation and initial peak titers occurred by 10th day in inoculated sheep.

In this study statistical analysis could not be carried out due to the small number of animals in each group ($n = 2$). In coronary band and Intra-dermo-lingual challenge group of animals received double the dose of challenge virus (0.2 ml) against the Intra-dermo-lingual challenge group (0.1 ml) and coronary band challenge group (0.1 ml), respectively. These are the limitations of this study. So, further study with increased number of animals and statistical analysis is warranted to confirm this result.

CONCLUSION

The sheep and goats were infected experimentally with a serotype O foot-and-mouth disease virus by different challenge routes. The sheep and goats challenged by coronary band route and coronary band and intra-dermo-lingual route exhibited FMD clinical signs at 2–5 days post challenge. Whereas intra-dermo-lingual challenged sheep and goats did not exhibit FMD clinical signs. Live virus could be isolated from blood of infected sheep and goats at 2–5 days post challenge. Viral RNA could be detected from blood of infected sheep and goats at 1–10 days post challenge. The neutralizing antibody titre was detected at 10 days post challenge and maintained up to 35 days post challenge in all infected sheep and goats. NSP antibodies were detected as early as 5–10 days post challenge and remain positive up to 35 days post challenge in the infected sheep and goats. In conclusion, the pathogenesis of sheep and goats with serotype O foot and mouth disease virus by different challenge routes could be demonstrated.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ supplementary material.

ETHICS STATEMENT

The studies involving animals were reviewed and approved by Institutional Animal Ethics Committee, Indian Immunologicals Limited, Hyderabad and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Department of Animal Husbandry and Dairying, Government of India.

AUTHOR CONTRIBUTIONS

SV designed, coordinated, reviewed and corrected the manuscript. MM and NS performed the experiments, completed analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: MM, NS, and SV were employed by the company Indian Immunologicals Limited, Hyderabad, India.

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Evaluation of Cell Lines for the Isolation of Foot-and-Mouth Disease Virus and Other Viruses Causing Vesicular Disease

Ashley R. Gray, Britta A. Wood, Elisabeth Henry, Mehreen Azhar, Donald P. King and Valérie Mioulet*

Vesicular Disease Reference Laboratory, The Pirbright Institute, Surrey, United Kingdom

The most sensitive cell culture system for the isolation of foot-and-mouth disease virus (FMDV) is primary bovine thyroid (BTY) cells. However, BTY cells are seldom used because of the challenges associated with sourcing thyroids from FMDV-negative calves (particularly in FMD endemic countries), and the costs and time required to regularly prepare batches of cells. Two continuous cell lines, a fetal goat tongue cell line (ZZ-R 127) and a fetal porcine kidney cell line (LFBK-$\alpha_V\beta_6$), have been shown to be highly sensitive to FMDV. Here, we assessed the sensitivity of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells relative to primary BTY cells by titrating a range of FMDV original samples and isolates. Both the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells were susceptible to FMDV for >100 passages, and there were no significant differences in sensitivity relative to primary BTY cells. Notably, the LFBK-$\alpha_V\beta_6$ cell line was highly sensitive to the O/CATHAY porcine-adapted FMDV strain. These results support the use of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ as sensitive alternatives to BTY cells for the isolation of FMDV, and highlight the use of LFBK-$\alpha_V\beta_6$ cells as an additional tool for the isolation of porcinophilic viruses.

Keywords: foot-and-mouth disease virus, virus isolation, cell line, diagnosis, vesicular viruses

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****Correspondence:***
Ashley R. Gray
ashley.gray@pirbright.ac.uk

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, which results in widespread economic burden (1). The major cause of global spread is the transboundary movement of animals, and as such, animal trade is restricted in countries where the disease is present (2). Foot-and-mouth disease virus (FMDV; family *Picornaviridae*, genus *Aphthovirus*) is the causative agent, and there are seven different serotypes [O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2, and SAT 3; (3)], with many different topotypes within each serotype (4).

Control of FMD is underpinned by rapid and accurate diagnosis. Virus isolation using susceptible cell cultures is beneficial for the amplification of virus for downstream diagnostic tests, including FMD serotyping by antigen enzyme linked immunosorbent assay (ELISA) (5) and sequencing of the VP1 region of the genome (6). Cell cultures are also required to produce FMDV vaccines, which are currently based on inactivated whole virus preparations (7). Control of FMD through vaccination is complicated by limited cross serotype/topotype immunity and therefore, vaccine matching field isolates using susceptible cell lines is an essential tool for appropriate vaccine selection (7).

Primary bovine thyroid (BTY) cell cultures are the most sensitive system for the isolation of FMDV (8), but their use is not widespread because of the difficulties obtaining tissue, the time and expense required to prepare the cells, and the fact that the cells have a relatively short life span. Immortalized cell lines, such as baby hamster kidney fibroblasts (BHK-21) and pig kidney (IB-RS-2) cells, provide a stable source of susceptible cultures, but are generally less sensitive to FMDV (8). Nonetheless, porcine cells (e.g., IB-RS-2) are commonly required for the isolation of FMDV strains that have naturally adapted to infect pigs (9), such as the serotype O/CATHAY topotype, which do not replicate in BTY cells. For diagnostic laboratories, it is also important that cell culture systems are able to support the propagation of viruses that cause clinical disease that are indistinguishable from FMDV, such as swine vesicular disease virus (SVDV), vesicular exanthema of swine (VESV), vesicular stomatitis virus (VSV), and Seneca Valley virus (SVV).

Fetal porcine kidney (LFBK-$\alpha_V\beta_6$) cells, which have been engineered to express bovine $\alpha_V\beta_6$ integrin, a principal cellular receptor of FMDV, and fetal goat tongue cells (ZZ-R 127) are two continuous cell lines that are highly sensitive to FMDV (10–12). A number of studies have utilized the LFBK-$\alpha_V\beta_6$ and ZZ-R 127 cell lines for the isolation of FMDV from different clinical samples (13–20). In previous studies, the ZZ-R 127 cell line provided similar sensitivity to FMDV as primary BTY cells (10) and LFBK-$\alpha_V\beta_6$ cells (21), however to our knowledge the LFBK-$\alpha_V\beta_6$ cell line has not been compared to BTY cells. The World Reference Laboratory for FMD (WRLFMD; The Pirbright Institute, UK) currently utilizes BTY and IB-RS-2 cells for the diagnosis of FMDV. In this study, the diagnostic capabilities of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells lines were evaluated using epithelium suspensions from a range of FMDV serotypes/subtypes, as well as the effects of different sample matrices commonly used for the isolation of FMDV. Through comparative titrations, we assessed the longevity of sensitivity of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells lines to FMDV isolates alongside BTY and IB-RS-2 cells. Finally, the ability of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells lines to propagate representative isolates of VESV, VSV, and SVV was also determined.

MATERIALS AND METHODS

All experiments were conducted at The Pirbright Institute in high-containment laboratories that meet the *Minimum Biorisk Management Standards for Laboratories Working with Foot-and-Mouth Disease Virus* of the European Commission for the Control of Foot-and-Mouth Disease (22).

Cells

BTY cells were prepared weekly incorporating variations from the method previously described in Snowdon (23). Briefly, bovine calf thyroids were obtained from an abattoir, dissociated using dispase II (Gibco), and cultured using Eagle's Glasgow minimal essential medium (GMEM; Sigma) supplemented with 12 mL/L field antibiotics (0.002 mg/mL amphotericin B, 10^{-4} MU/mL penicillin, 49 μg/mL neomycin, 98 U/mL polymyxin B, sterile water), 10 mL/L L-glutamine (Sigma), and 10% adult bovine serum (ABS; Sigma). The BTY cells were counted using a Fuchs-Rosenthal counting chamber and the concentration normalized to a seeding density of 6×10^5 cells/mL. The BTY cells were cultured in Nunc™ flat-sided cell culture tubes (5.5 cm^2; Thermo Fisher Scientific) using 2 mL of cell suspension and incubated stationary at 37°C. After 96 h, the media was discarded from each tube and replaced with GMEM (Sigma) supplemented field antibiotics and L-glutamine as above and between 2 and 10% ABS (Sigma). The percentage of ABS used was dependent on the average level of confluency observed in 10 tubes after 96 h (e.g., <40% confluence – 10% ABS, 40–60% confluence – 7% ABS, 60–90% confluence – 5% ABS, >90% confluence – 2% ABS). After the media change, the cell culture tubes were incubated with rotation at 37°C until use.

IB-RS-2 cells were maintained in T-175 cell culture flasks using GMEM (Gibco) supplemented with 10% adult bovine serum (Sigma). The seed stocks were passaged to reach 90–100% confluency in 72 to 96 h. The IB-RS-2 cells were prepared in Nunc™ cell culture tubes using 2 mL of cell suspension at a concentration between 0.5 and 6×10^5 cells/mL to reach 90–100% confluency between 24 and 96 h. Seed flasks and cell culture tubes were incubated stationary at 37°C until use.

ZZ-R 127 cells, supplied by the Friedrich-Loeffler-Institute (Greifswald-Insel Riems, Germany), were maintained in T-175 cell culture flasks using Dulbecco's modified Eagle medium: F12 (DMEM; Lonza) supplemented with 10% fetal bovine serum (Gibco). The seed stocks were passaged to reach 90–100% confluency in 96 h. The ZZ-R 127 cells were cultured in Nunc™ cell culture tubes using 2 mL of cell suspension at a concentration of 0.65×10^5 cells/mL to reach 90–100% confluency in 96 h. Seed flasks and cell culture tubes were incubated stationary at 37°C until use.

LFBK-$\alpha_V\beta_6$ cells (11, 12), supplied by the Animal and Plant Health Inspection Service, Diagnostic Service Section at the Plum Island Animal Disease Center (Long Island, NY, USA), were maintained in T-175 cell culture flasks using DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco). The seed stocks were passaged to reach 90–100% confluency in 72 h. The LFBK-$\alpha_V\beta_6$ cells were cultured in Nunc™ cell culture tubes using 2 mL of cell suspension at a concentration of 2×10^5 cells/mL to reach 90–100% confluency in 72 h. Seed flasks and cell culture tubes were incubated stationary at 37°C until use.

Preparation of primary cell cultures and passaging of continuous cell lines were performed inside a class 2 microbiological safety cabinet. Biocontainment procedures were required for the maintenance of IB-RS-2 cells and LFBK-$\alpha_V\beta_6$ cells, which are persistently infected with classical swine fever (CSF) virus (24) and a non-cytopathic bovine viral diarrhea virus (BVDV; Rodriguez LL, personal communication, 2019), respectively. All virus isolations and titrations were performed using monolayers of 90–100% confluency cultured in Nunc™ cell culture tubes. All cell culture tubes received minimal essential media (MEM; Gibco) supplemented with 6 mL/L field antibiotics and 2% fetal bovine serum (Gibco) to sustain cell cultures after the addition of virus and negative matrices.

Virus Stocks

In line with the OIE manual (25), FMDV and SVDV original suspensions were prepared by homogenizing vesicular epithelium as a 10% solution in M25 buffer (35 mM disodium hydrogen phosphate, 5.7 mM potassium dihydrogen phosphate, sterile water). The tissue was homogenized with sterile sand (Sigma) using a sterilized pestle and mortar. The suspension was clarified by centrifugation at 3,000 g for 10 min at 4°C.

Epithelial suspensions tested in the diagnostic sensitivity experiments were either used immediately after preparation or aliquoted and stored at −80°C. The suspensions of FMDV/A/IRN/24/2012, FMDV/O/KUW/4/2016 and SVDV/UKG/77/80 prepared for the longevity of sensitivity experiments were mixed 1:1 with glycerol (VWR chemicals) for long term storage at −20°C. SVV, VESV, and VSV New Jersey isolates of known high viral titers were selected from the WRLFMD virus collection.

Virus Titrations

Virus titrations were performed in parallel to compare the relative sensitivity of the cell lines to FMDV and SVDV. Virus stocks were serially diluted 10-fold in M25 buffer. Cells ($n = 4$ or 5 tubes per cell line) were washed with 2 mL sterile phosphate buffer saline (PBS; Severn Biotech) before adding 2 mL of MEM (Gibco). The cell tubes were then inoculated with 0.2 mL of the appropriate virus dilution and incubated with rotation at 37°C for 72 h, after which the cells were visually examined under a microscope for cytopathic effect (CPE). For each cell line, viral titers were calculated using the Spearman-Karber method and expressed as Log_{10} $TCID_{50}$/mL, where a higher viral titer in a cell line correlated to a lower limit of detection and greater analytical sensitivity.

A/IRN/24/2012 and O/KUW/4/2016 glycerinated epithelium suspensions were initially titrated with BTY cells to establish baseline titers (6.6 and 7.8 Log_{10} $TCID_{50}$/mL, respectively). The continued sensitivity of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ to FMDV was assessed by titrating A/IRN/24/2012 or O/KUW/4/2016 during continued passaging of the cell lines; all titrations were performed in parallel with BTY and IB-RS-2 cells. A/IRN/24/2012 was used for 9 months (16th May 2017 to 6th February 2018) until viral titers began to decrease across all cell lines, possibly due to sample degradation, and was replaced with O/KUW/4/2016 that was used for 10 months (12th February 2018 to 18th December 2018).

SVDV/UKG/77/80 glycerinated epithelium suspension was initially titrated with IB-RS-2 cells to establish a baseline titer (3.8 Log_{10} $TCID_{50}$/mL). The sensitivity of the LFBK-$\alpha_V\beta_6$ cell line to SVDV was assessed over time by titrating UKG/77/80 during continued passaging of the cell line; all titrations were performed in parallel with IB-RS-2 cells. The ZZ-R 127 cell line was not included in these experiments because SVDV does not propagate in this cell line (10).

FMDV Diagnostic Sensitivity

Forty epithelium suspensions (**Table 1**), representing five serotypes and thirteen topotypes of FMDV (O $n = 20$, A $n = 8$, SAT 1 $n = 4$, SAT 2 $n = 3$, and Asia 1 $n = 5$), were either retrieved from −80°C storage or prepared from epithelial tissue. Titrations were performed with BTY, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells for all samples, except for the O/CATHAY topotype. O/CATHAY is a porcine adapted strain and does not replicate in BTY cells. The O/CATHAY samples were titrated using IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells.

TABLE 1 | Number of epithelium suspensions tested by serotype and lineage.

Serotype	Topotype	Lineage	Sub-lineage	No of isolates
O	CATHAY	–	–	8
	SOUTH EAST ASIA	Mya-98	–	1
	MIDDLE EAST SOUTH ASIA	Ind-2001	d	2
			e	1
		PanAsia	–	1
		PanAsia2	ANT-10	1
			BAL-09	2
			QOM-15	1
	WEST AFRICA	–	–	1
	EAST AFRICA 2	–	–	1
	EAST AFRICA 3	–	–	1
A	ASIA	Iran-05	FAR-11	2
			SIS-13	2
			SIS-10	1
		G-VII	–	2
	AFRICA	G-IV	–	1
SAT 1	III	–	–	1
	III (WZ)	–	–	2
	X	–	–	1
SAT 2	VII	Alx-12	–	2
		Lib-12	–	1
ASIA 1	ASIA	Sindh-08	–	3
		–	–	2
	Total			40

Twenty-six diagnostic porcine epithelium suspensions originating from Hong Kong were inoculated onto BTY, IB-RS-2, and LFBK-$\alpha_V\beta_6$ cells. Each cell tube ($n = 4$ or 5 tubes per cell line) was washed with 2 mL sterile PBS and then inoculated with 0.2 mL of sample. The tubes were incubated stationary at 37°C for 30 min and after incubation, each tube received 2 mL MEM. Cell culture tubes were incubated at 37°C with rotation and examined microscopically for CPE every 24 h up to a maximum of 96 h. All isolated samples were then characterized by antigen ELISA (5) and VP1 sequencing.

Matrix Cytotoxicity

To determine whether sample matrices have an effect on cell monolayers, undiluted bovine serum, milk, probang, and whole blood were inoculated onto BTY, IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells, and a 10% fecal suspension (SVDV sample type) was inoculated on IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells. Each cell tube ($n = 4$ per cell line) was washed with 2 mL sterile PBS and then inoculated with 0.2 mL of the matrix. The tubes were incubated

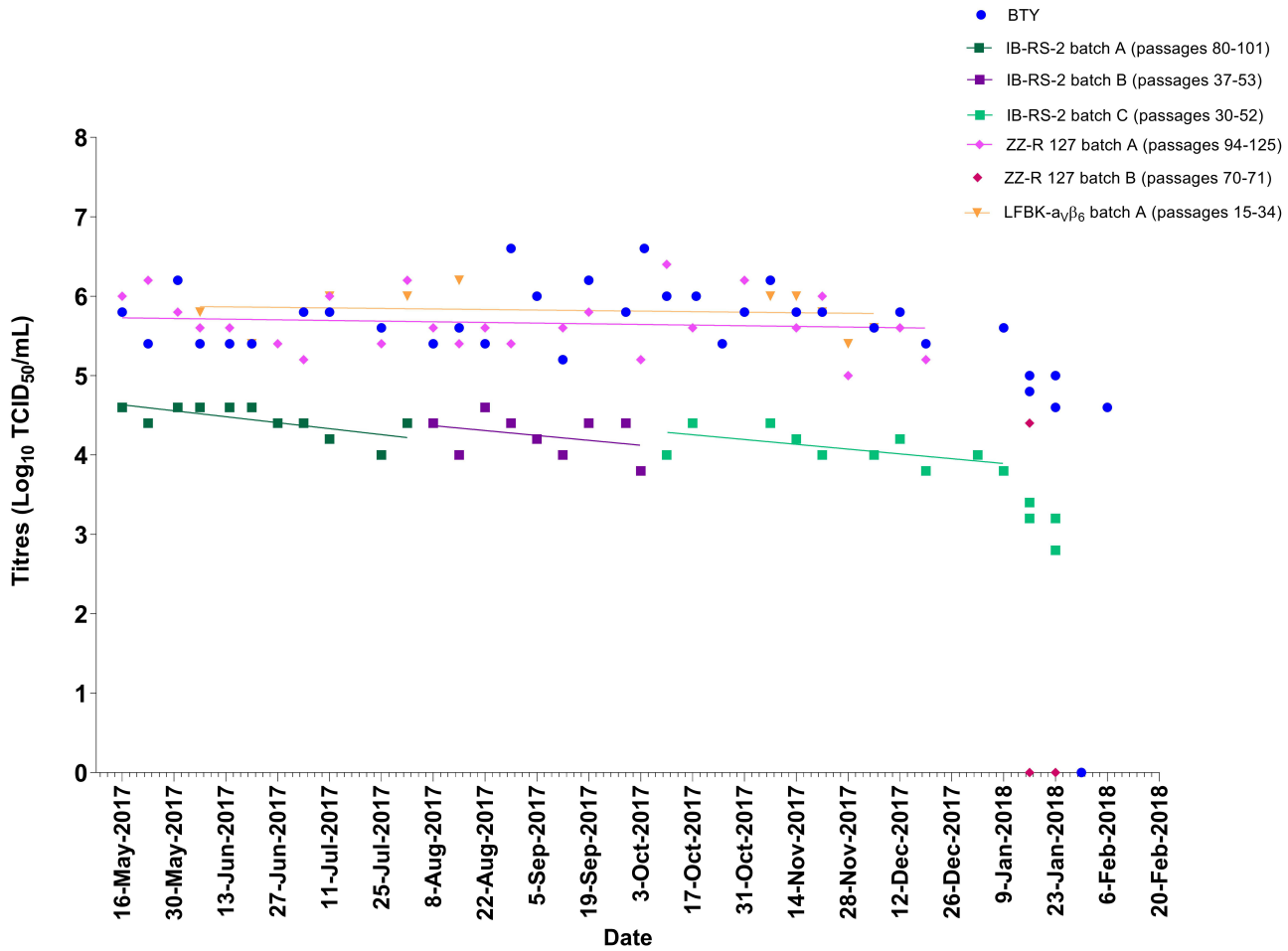

FIGURE 1 | Titers of FMDV A/IRN/24/2012 epithelium suspension tested on BTY, IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells. The lines represent the trend of titers over time. No trendline is present for BTY cells as these are independent, weekly batches.

stationary at 37°C for 30 min. After incubation, the monolayers were washed at least 3 times with 2 mL sterile PBS before adding 2 mL MEM to each tube. Cell culture tubes were incubated at 37°C with rotation for 72 h, and then examined microscopically for cytotoxicity.

SVV, VESV, and VSV

BTY, IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells ($n = 3$ tubes per cell line) were assessed for their ability to propagate SVV, VESV, and VSV. Cell culture tubes were washed with 2 mL sterile PBS and each tube received 2 mL MEM. Tubes were inoculated with 0.2 mL of SVV, VESV, or VSV, and then incubated at 37°C with rotation for 72 h. After 72 h, the cell monolayers were examined microscopically for CPE.

Statistical Analysis

Average viral titers for FMDV/A/IRN/24/2012 and FMDV/O/KUW/4/2016 amongst BTY, IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells were compared using Kruskal-Wallis and *post-hoc* Dunn's multiple comparisons tests. Average viral titers for SVDV/UKG/77/80 between IB-RS-2 and LFBK-$\alpha_V\beta_6$ were compared using the Mann-Whitney test. Where epithelial suspensions were tested amongst cell lines and provided a single data point, the differences in sensitivity to FMDV for ZZ-R 127 and LFBK-$\alpha_V\beta_6$ were compared to BTY cells independently, using paired *t*-tests. Statistical analysis was not performed on the O/CATHAY sensitivity data due to the low number of isolates detected. Statistical analyses were performed on log transformed titer values using Graphpad Prism 8.1.2. $P < 0.05$ were considered significant.

RESULTS

Longevity of Sensitivity to FMDV and SVDV

Over a 19-month period, weekly titrations were performed on BTY, IB-RS-2, ZZ-R 127, and/or LFBK-$\alpha_V\beta_6$ cells using FMDV A/IRN/24/2012 (**Figure 1**) or O/KUW/4/2016 (**Figure 2**) epithelium suspensions; not all cell types were available each

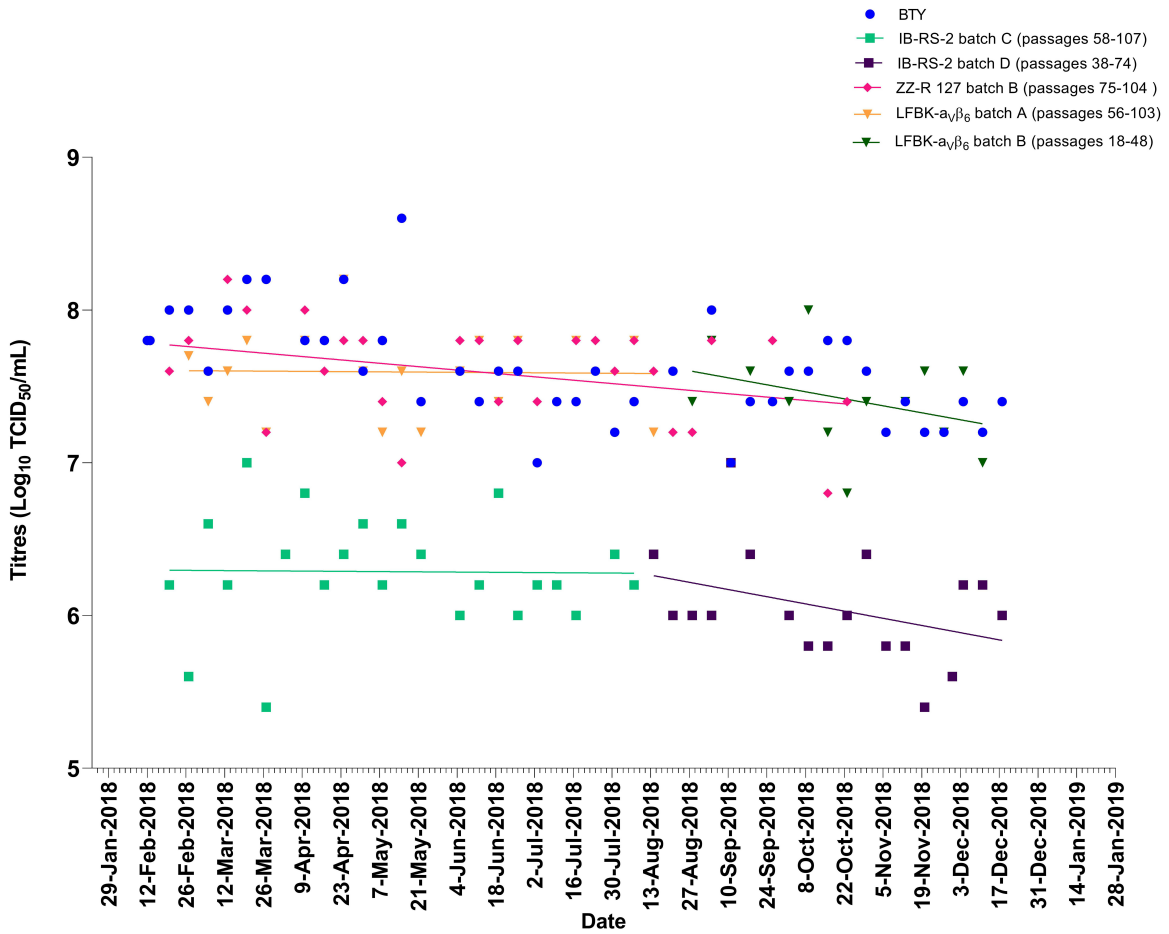

FIGURE 2 | Titers of FMDV O/KUW/4/2016 epithelium suspension tested on BTY, IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$. The lines represent the trend of titers over time. No trendline is present for BTY cells as these are independent, weekly batches.

week, resulting in minor gaps in testing. The viral titers obtained from the weekly batches of BTY cells were within ± 1 $\log_{10}$. The longevity of sensitivity for IB-RS-2 cells was inconsistent between batches (range 9–35 weeks), and in each case, the cells gradually lost their sensitivity over time, as evident by the decreasing titers (**Figure 1**). Once a batch of IB-RS-2 cells lost sensitivity, a new batch was revived for testing. The LFBK-$\alpha_V\beta_6$ and ZZ-R 127 cell lines remained sensitive to FMDV for >100 passages, although the LFBK-$\alpha_V\beta_6$ cells underwent senescence at passage 105 and the batch of cells were replaced. The two batches of ZZ-R 127 cells were replaced (after 33 and 43 weeks) before a noticeable decline in sensitivity to FMDV could be observed.

The average viral titers of epithelium suspensions FMDV/A/IRN/24/2012 (mean ± standard deviation; BTY; 5.9 ± 0.3, ZZ-R 127; 5.8 ± 0.4, LFBK-$\alpha_V\beta_6$; 5.9 ± 0.3 and IB-RS-2; 4.3 ± 0.3 Log_{10} TCID_{50}/mL) and FMDV/O/KUW/4/2016 (BTY; 7.9 ± 0.3, ZZ-R 127; 7.7 ± 0.3, LFBK-$\alpha_V\beta_6$; 7.7 ± 0.3 and IB-RS-2; 6.4 ± 0.4 Log_{10} TCID_{50}/mL) were significantly different by cell type ($p < 0.001$). For both FMDV A/IRN/24/2012 and O/KUW/4/2016, the sensitivity of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells were comparable to BTY cells; however, the sensitivity of the IB-RS-2 cells was significantly lower than BTY, ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells ($p < 0.0001$).

Over an 8-month period, weekly titrations were performed on IB-RS-2 and/or LFBK-$\alpha_V\beta_6$ cells using SVD/UKG/77/80 (**Figure 3**). IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells were not available each week, hence the minor gaps (maximum of 4 weeks) in testing. IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells remained sensitive to SVDV for >100 passages. The LFBK-$\alpha_V\beta_6$ cells lost sensitivity to SVDV at passage 104, as indicated by the lack of viral titer (**Figure 3**). The LFBK-$\alpha_V\beta_6$ trend lines indicate that titers decreased overtime similar to the IB-RS-2 cell line. The average titer for the SVDV/UKG/77/80 epithelium suspension was significantly higher in the LFBK-$\alpha_V\beta_6$ than IB-RS-2 cells ($p < 0.001$; 5.2 ± 1.2 and 4.5 ± 0.5 Log_{10} TCID_{50}/mL, respectively),

Detection of FMDV in Diagnostic Epithelium Suspensions

Thirty-two epithelium suspensions were titrated using BTY, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells and the limit of detection

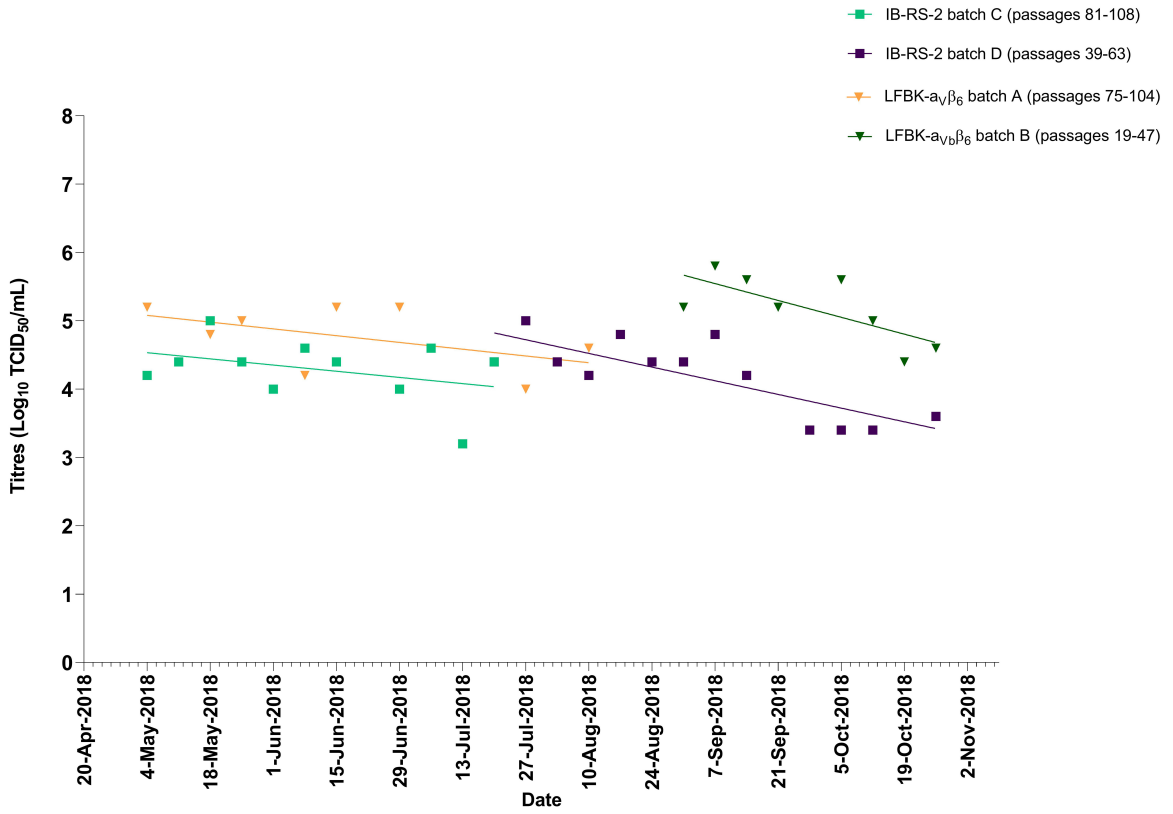

FIGURE 3 | Titers of SVDV/UKG/77/80 epithelium suspension tested on IB-RS-2 and LFBK-$\alpha_V\beta_6$. The lines represent the trend of titers over time.

compared by calculating the relative viral titer generated in these different cell systems. In the majority of samples tested (29 of 32), the analytical limit of detection for the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ was comparable to that of the primary BTY cells (**Figures 4**, **5**). Overall, there was no significant difference in analytical sensitivity between BTY and either ZZ-R 127 or LFBK-$\alpha_V\beta_6$ ($p > 0.05$). Nonetheless, the LFBK-$\alpha_V\beta_6$ cells showed a high degree of diagnostic capability by successfully propagating virus from all epithelium suspensions tested, whereas three viral suspensions were unable to replicate in either the BTY or ZZ-R 127 cells. A/PAK/25/2016 was undetected in BTY cells, despite originally being isolated in this cell type, and A/TUR/8/2015 and O/SRL/3/2017 were undetected in ZZ-R 127 cells.

Eight O/CATHAY epithelium suspensions were titrated using IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells. Despite the eight suspensions being originally isolated in IB-RS-2 cells during diagnostic testing at the time of submission, only one epithelium suspension (HKN/5/2016) was able to generate a titer in IB-RS-2 cells during repeat testing in this study. Although the epithelium suspensions had been stored at −80°C since use, it is likely that the viral titer of the samples had decreased during storage and through freeze-thawing. The majority of these samples (6 of 8) did not cause CPE in the ZZ-R 127 cells; only two epithelium suspensions, HKN/11/2017 and HKN/5/2016, generated viral titers. In contrast, all eight samples replicated in LFBK-$\alpha_V\beta_6$ cells. The LFBK-$\alpha_V\beta_6$ cells had increased sensitivity to the O/CATHAY topotype in comparison to IB-RS-2, with higher titers observed for all eight epithelium suspensions correlating to a lower limit of detection.

Initially, six porcine epithelium suspensions, negative for virus isolation using BTY and IB-RS-2 cells but positive for FMDV genome, were inoculated onto LFBK-$\alpha_V\beta_6$ cells. From these six suspensions, two viruses were isolated in LFBK-$\alpha_V\beta_6$ cells (**Table 2**). A further 20 porcine epithelium suspensions originating from Hong Kong SAR were inoculated onto BTY, IB-RS-2, and LFBK-$\alpha_V\beta_6$ cells in parallel at the time of submission. Out of these 20 suspensions, 12 viruses were isolated in LFBK-$\alpha_V\beta_6$ cells only. In total, viruses were isolated in 14/26 samples using LFBK-$\alpha_V\beta_6$ cells, which otherwise would not have been undetected, and were subsequently characterized by antigen ELISA and VP1 sequencing as O/CATHAY topotype.

Effects of Sample Matrices

No cytotoxicity was observed in the BTY cultures for any of the matrices tested. Cytotoxicity was not observed in IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$ cells for serum, probang fluid and milk; however, whole blood caused cytotoxicity in all four replicates of each of the continuous cell lines

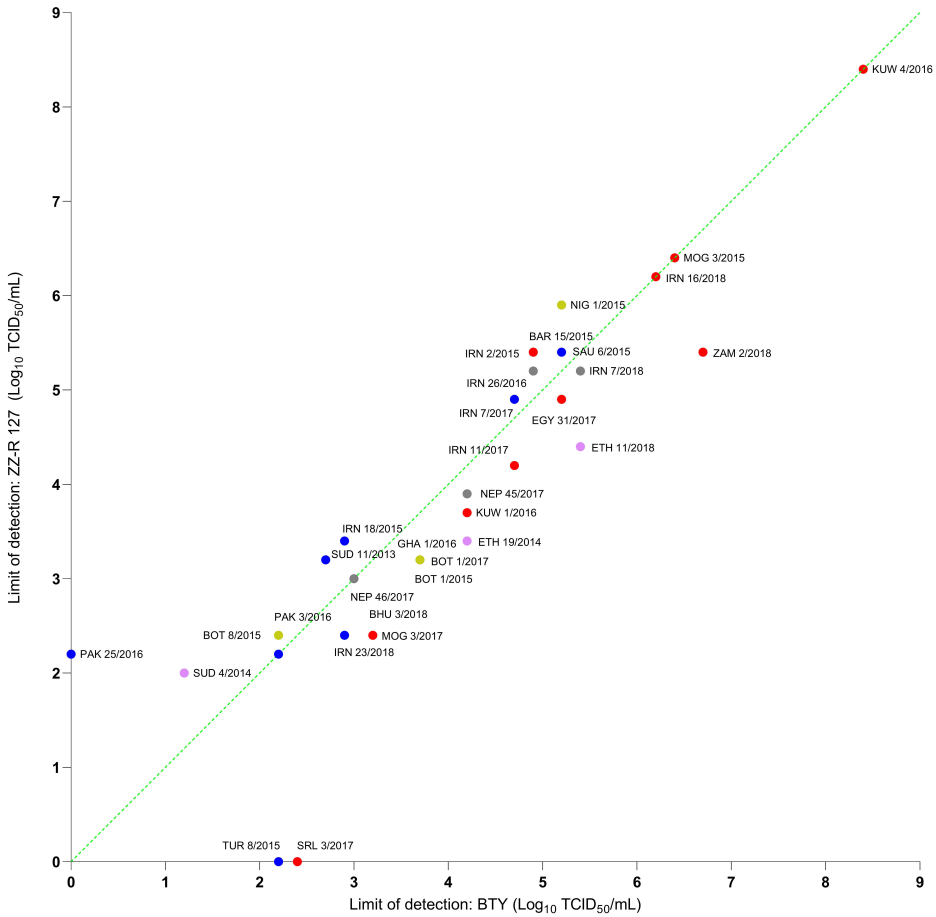

FIGURE 4 | Titers of epithelium suspensions tested on primary BTY cells and ZZ-R 127 cells. Samples are color coded based on FMDV serotype as follows: **O**, **A**, **SAT 1**, **SAT 2**, and **Asia 1**. The dotted green line indicates where the limit of detection was identical between the cells; samples with data points above the line indicate a lower limit of detection in ZZ-R 127 and samples with data points below the line indicate a lower limit of detection in BTY cells.

where patches of adherent cells were stripped from the tube surfaces. No cytotoxicity was caused by the 10% pig fecal suspension, which was inoculated onto IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells.

Susceptibility to Other Vesicular Viruses

No CPE was observed in BTY cells 72 h after inoculation with SVV, VESV, and VSV, indicating BTY cells cannot propagate these viruses (**Table 3**). The ZZ-R 127 cells were able to propagate VESV and VSV, producing CPE in each of the replicates, whereas SVV was unable to propagate as indicated by the lack of CPE. The IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells were able to support the replication of all three vesicular viruses tested.

DISCUSSION

In this study, we have shown that the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines were susceptible to FMDV for >100 passages (**Table 4**), and the analytical limit of detection of these cell lines was comparable to primary BTY cell cultures. In comparison, the sensitivity of the IB-RS-2 cell line was significantly lower than ZZ-R 127, LFBK-$\alpha_V\beta_6$, and BTY cells. Our results highlight the known decreased sensitivity of these cells to FMDV (8). The IB-RS-2 cells lost sensitivity over time, but the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells remained consistently sensitive during progressive sub-culturing (**Figures 1**, **2**). These data confirmed previous studies that reported the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines were highly sensitive to FMDV (10, 11, 21).

In comparison to IB-RS-2, our data highlight the increased longetivity of the ZZ-R 127 and LFBK-avb6 cell lines to support FMDV replication. We anticipate that these findings will be broadly transferable to other laboratories, but specific cell batches and culture conditions may influence these results. Therefore, prior to use for routine diagnostics, we recommend that cell sensitivity should be monitored using dilutions of a well-characterized reference FMD virus.

When the diagnostic sensitivities of ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells were assessed using a range of FMDV field strains, the LFBK-$\alpha_V\beta_6$ cells detected all 32 samples (**Table 4**) whereas, the ZZ-R 127 and BTY cells detected 30 and 31 samples, respectively. The

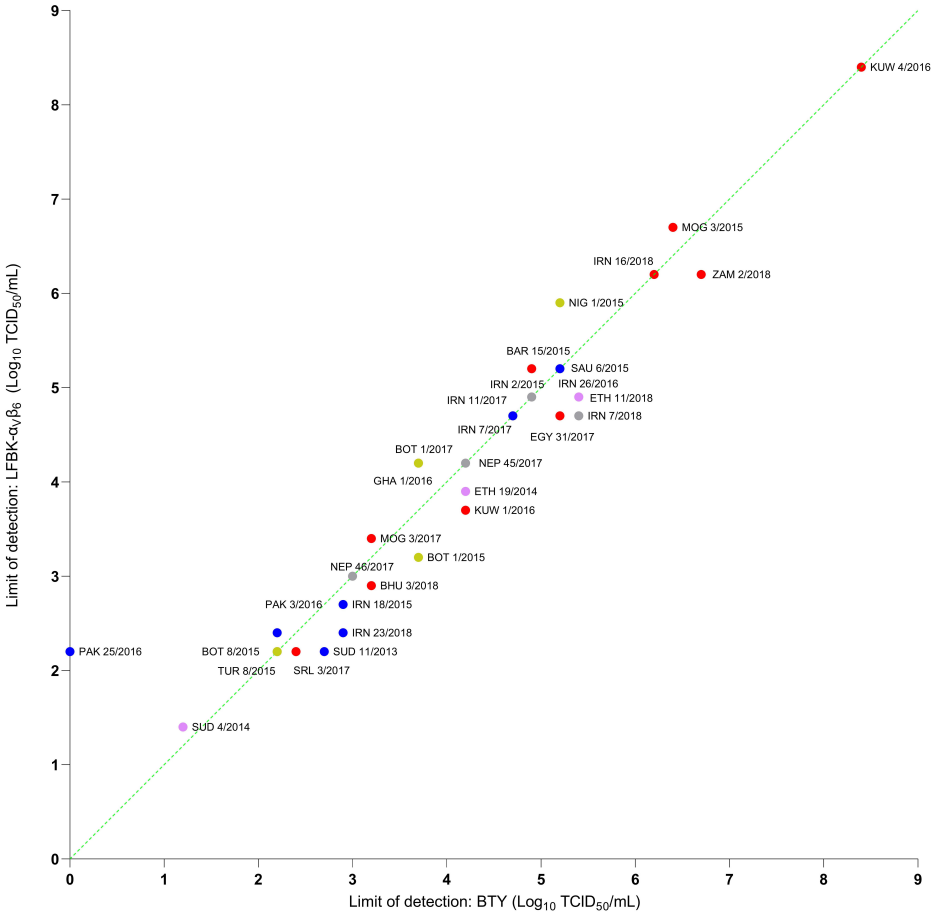

FIGURE 5 | Titers of epithelium suspensions tested on primary BTY cells and LFBK-$\alpha_v\beta_6$ cells. Samples are color coded based on FMDV serotype as follows: **O**, **A**, **SAT 1**, **SAT 2**, and **Asia 1**. The dotted green line indicates where the limit of detection was identical between the cells; samples with data points above the line indicate a lower limit of detection in LFBK-$\alpha_v\beta_6$ and samples with data points below the line indicate a lower limit of detection in BTY cells.

32 samples were selected to encompass multiple FMDV serotypes and topotypes (serotype O $n = 20$, A $n = 8$, SAT 1 $n = 4$, SAT 2 $n = 3$, and Asia 1 $n = 5$). Serotype C was not included in this study because it is not known to be circulating; it was last detected in Kenya and Brazil in 2004 (26). No SAT 3 epithelium suspensions were tested due to limited availability of material.

Cell lines of porcine origin are utilized for the detection of pig-adapted FMDV topotypes (e.g., O/CATHAY) and other porcinophilic vesicular viruses. Here, we demonstrated that LFBK-$\alpha_V\beta_6$ cells were sensitive to SVDV for >100 passages, and provided a significantly higher limit of detection than the IB-RS-2 cell line (**Figure 3**). The LFBK-$\alpha_V\beta_6$ cells were also highly susceptible to infection with isolates from the pig-adapted O/CATHAY FMDV topotype. Overall, our data demonstrated that the LFBK-$\alpha_V\beta_6$ cell line is more sensitive to FMDV and SVDV than IB-RS-2, possibly because of the constitutive expression of the bovine $\alpha_V\beta_6$ integrin receptor. The only potential disadvantage of the LFBK-$\alpha_V\beta_6$ cell line is that they are contaminated with a non-cytopathic BVDV (Rodriguez LL, personal communication, 2019).

The most common sample type submitted to the WRLFMD for the diagnosis of vesicular diseases is epithelium from vesicular lesions. FMD virus can be isolated from other samples types, including whole blood, serum, milk, probang fluid, and feces; however, these matrices can cause detrimental effects to cells, and thus compromise virus isolation. Of the matrices tested, primary BTY cells were the most robust, in that no cytotoxicity was observed. No cytotoxicity was observed in ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells after inoculation with serum and probang fluid, supporting the findings that these cell types can be used to isolate FMDV from serum and probang of experimentally infected animals (21). The only matrix that caused cytotoxicity was the undiluted bovine whole blood, which stripped patches of cells from the monolayers of IB-RS-2, ZZ-R 127, and LFBK-$\alpha_V\beta_6$.

While virus isolation is a sensitive diagnostic test, the observation of CPE is not virus specific. There are several notifiable diseases that are clinically indistinguishable from FMDV, such as VSV, VESV, and SVV, which cause similar CPE in cell culture. Of the four cell types tested, IB-RS-2 and LFBK-$\alpha_V\beta_6$ cells were the most versatile, in that VSV, VESV, and SVV were all

TABLE 2 | Number of CPE positive replicates for BTY, IB-RS-2, and LFBK-$\alpha_V\beta_6$ post-inoculation with porcine samples received from Hong Kong ($n = 26$).

Sample reference	BTY	IB-RS-2	LFBK-$\alpha_V\beta_6$	FMDV 3D C_T values
HKN 5/2017*	0/5	0/5	1/4	28.68
HKN 2/2018*	0/5	0/5	0/5	25.57
HKN 3/2018*	0/5	0/5	0/5	20.22
HKN 4/2018*	0/5	0/5	2/5	25.72
HKN 7/2018*	0/5	0/5	0/5	32.91
HKN 9/2018*	0/5	0/5	0/5	28.30
HKN 10/2018	0/5	0/5	0/5	27.91
HKN 11/2018	0/5	0/5	5/5	21.53
HKN 12/2018	0/5	0/5	3/5	38.37
HKN 13/2018	0/5	5/5	5/5	24.94
HKN 14/2018	0/5	0/5	5/5	No C_T
HKN 15/2018	0/5	0/5	5/5	33.94
HKN 16/2018	0/5	0/5	5/5	34.51
HKN 17/2018	0/5	0/5	4/5	39.08
HKN 18/2018	0/5	0/5	3/5	32.92
HKN 19/2018	0/5	0/5	0/5	35.89
HKN 20/2018	0/5	5/5	5/5	18.83
HKN 21/2018	0/5	1/5	5/5	34.43
HKN 22/2018	0/5	0/5	0/5	36.35†
HKN 23/2018	0/5	5/5	5/5	22.71
HKN 1/2019	0/5	2/5	4/4	33.05
HKN 2/2019	0/5	0/5	4/4	35.99
HKN 4/2019	0/5	0/5	4/4	33.02
HKN 5/2019	0/5	0/5	4/4	No C_T
HKN 6/2019	0/5	0/5	3/4	34.76
HKN 7/2019	0/5	0/5	4/4	36.36†

Samples were received to the WRLFMD between 2017 and 2019. All isolated viruses were confirmed as O/CATHAY topotype by VP1 sequencing. FMDV 3D qRT-PCR C_T values are included for comparison and are an average of two replicates.
**LFBK-$\alpha_V\beta_6$ inoculated independently from BTY and IB-RS-2 cells.*
†Sample provided a C_T value in only one replicate.

TABLE 3 | Number of replicates per cell line with CPE after inoculation with SVV, VESV, and VSV.

Cell line	BTY	ZZ-R 127	LFBK-$\alpha_V\beta_6$	IB-RS-2
SVV-MN-88-36695	0/3	0/3	3/3	3/3
VESV-K54	0/3	3/3	3/3	3/3
VSV-New Jersey	0/3	3/3	3/3	3/3

able to replicate, confirming previous results that the LFBK-$\alpha_V\beta_6$ cells are capable of propagating VESV and VSV (11). Cell lines currently available for the isolation of SVV include swine testis cells, porcine kidney, IB-RS-2 and BHK (27). To our knowledge, this is the first time LFBK-$\alpha_V\beta_6$ cells have been identified as a resource for the isolation of SVV.

As mentioned, primary BTY cells are accepted as the most sensitive cell culture for the isolation of FMDV, but their preparation is expensive and labor intensive (23). Hence, diagnostic laboratories would benefit from a continuous cell line with the same sensitivity as primary BTY cells. Although sensitivity comparisons have been performed between BTY and ZZ-R 127 (10) and between ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cells (21), this is the first study to compare LFBK-$\alpha_V\beta_6$ and primary BTY cells. The results indicate that both ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines are suitable alternatives to BTY cells for the isolation of FMDV. Furthermore, the LFBK-$\alpha_V\beta_6$ cells have multiple advantages in that the cells grow quickly in cell culture, remained stable for >100 passages, and were able to support growth of all the other vesicular viruses tested. This contrasts with the ZZ-R 127 cells which grow slowly and were not able to support growth of SVV.

Rapid and accurate diagnosis underpins the control of FMDV. Although virus isolation is not a rapid diagnostic test (i.e., it can take 1–6 days to isolate a virus), it is necessary for downstream testing, such as vaccine matching. At WRLFMD, FMD serotype is most commonly determined using a polyclonal antigen ELISA (5), or a monoclonal antigen ELISA (28). Epithelium suspensions prepared from clinical samples can be tested directly on an ELISA, but only approximately a third of samples submitted to the WRLFMD contain the concentration of viral antigen needed for detection [e.g., minimum concentration of 1–2 ng/mL of virus antigen for detection with the polyclonal antigen ELISA (29)]. In addition, samples such as blood, serum, probang fluid, milk, and feces cannot be tested directly on ELISA. Consequently, clinical samples such as these must be isolated in cell culture before testing with a serotyping antigen ELISA.

Recently, lineage-specific real-time RT-PCR assays have been developed to circumvent the need for virus isolation and the handling of "live" virus (30–33). However, due to the diversity of FMDV topotypes and the rapid mutation rate of the RNA genome (34), these assays need to be tailored to geographic regions and require ongoing monitoring of sensitivity. Additionally, these assays are "dead end tests," as the material produced cannot be used for downstream testing, such as vaccine matching. Although serotyping real-time RT-PCRs have advantages, these assays cannot yet replace virus isolation.

Currently, the use of sensitive cell cultures are required for testing vaccine efficacy to a particular field strain (25). The virus neutralization test requires the serial passage of an FMDV isolate to generate a high viral titer and is dependent on the use of continuous cell lines, such as BHK and IB-RS-2, to determine the ability of antibodies to neutralize "live" virus. While both ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines represent suitable alternatives, it is expected that the LFBK-$\alpha_V\beta_6$ cell line will undergo validation for virus neutralization tests in the WRLFMD due to their susceptibility to a wider range of FMDV strains, including the O/CATHAY topotype.

CONCLUSIONS

In this study, the FMDV sensitivity of the ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines were comparable to primary BTY cells, and significantly higher than the IB-RS-2 cell line (**Table 4**). In

TABLE 4 | Summary comparing the results among BTY, ZZ-R 127, LFBK-$\alpha_V\beta_6$, and IB-RS-2.

		Cell type			
		BTY	**ZZ-R 127**	**LFBK-$\alpha_V\beta_6$**	**IB-RS-2**
Duration of FMDV sensitivity		3–4 weeks per batch	>100 passages	>100 passages	>100 passages
Duration of SVDV sensitivity		ND	ND	>100 passages	>100 passages
Sensitivity of cell lines	FMD/A/IRN/24/2012	5.8	5.7	5.8	4.3
	FMD/O/KUW/4/2016	7.9	7.7	7.7	6.4
(Avg. Log_{10} $TCID_{50}$/mL)	SVD/UKG/77/80	ND	ND	5.2	4.5
Detected FMDV epithelium suspensions		31/32	30/32	32/32	ND
Detected O/CATHAY epithelium suspensions		ND	2/8	8/8	1/8
Detected O/CATHAY diagnostic submissions		0/26	ND	19/26	5/26
Susceptibility to other vesicular viruses		None	VESV, VSV	VESV, VSV, SVV	VESV, VSV, SVV
Matrices causing cytotoxicity		None	Whole blood	Whole blood	Whole blood

ND, not done.

addition, the LFBK-$\alpha_V\beta_6$ cells were significantly more sensitive to SVDV than the IB-RS-2 cells and exhibited a high diagnostic capability for detecting the O/CATHAY pig-adapted FMDV strain. Overall, ZZ-R 127 and LFBK-$\alpha_V\beta_6$ cell lines have been confirmed as sensitive tools for FMDV diagnostic testing. The LFBK-$\alpha_V\beta_6$ cells outperformed the IB-RS-2 throughout testing and therefore, have been identified as a highly sensitive porcine cell line for the routine detection of FMDV strains and porcinophilic vesicular viruses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AG, VM, BW, and DK conceived the study. DK obtained funding. AG, VM, BW, MA, and EH performed virus titrations and inoculations. AG performed data interpretation and wrote the manuscript. All authors read and approved the manuscript content.

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A New Cage-Like Particle Adjuvant Enhances Protection of Foot-and-Mouth Disease Vaccine

*Juan Bidart[1,2], Claudia Kornuta[1,2], Mariela Gammella[1], Victoria Gnazzo[3], Ivana Soria[1], Cecilia Langellotti[1,2], Claudia Mongini[1,2], Roxana Galarza[4], Luis Calvinho[5], Giuliana Lupi[2,6], Valeria Quattrocchi[1], Ivan Marcipar[2,6] and Patricia Zamorano[1,2,7]**

[1] Instituto de Virología e Innovaciones Tecnológicas-IVIT, CICVyA, INTA-CONICET, Hurlingham, Argentina, [2] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [3] Instituto Nacional de Medicina Tropical, Puerto Iguazú, Argentina, [4] Agencia de Extensión Rural Chascomus, INTA, Chascomus, Argentina, [5] Estación Experimental Agropecuaria Rafaela, INTA, Rafaela, Argentina, [6] Facultad de Bioquímica y Ciencias Biológicas – Universidad Nacional del Litoral, Santa Fe, Argentina, [7] Universidad del Salvador, Buenos Aires, Argentina

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***Correspondence:**
Patricia Zamorano
zamorano.patricia@inta.gob.ar

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Foot-and-Mouth Disease (FMD) is an acute viral disease that causes important economy losses. Vaccines with new low-cost adjuvants that stimulate protective immune responses are needed and can be assayed in a mouse model to predict their effectiveness in cattle. Immunostimulant Particle Adjuvant (ISPA), also known as cage-like particle adjuvant, consisting of lipid boxes of dipalmitoyl-phosphatidylcholine, cholesterol, sterylamine, alpha-tocopherol, and QuilA saponin, was shown to enhance protection of a recombinant vaccine against *Trypanosoma cruzi* in a mouse model. Thus, in the present work, we studied the effects on the magnitude and type of immunity elicited in mice and cattle in response to a vaccine based on inactivated FMD virus (iFMDV) formulated with ISPA. It was demonstrated that iFMDV–ISPA induced protection in mice against challenge and elicited a specific antibody response in sera, characterized by a balanced Th1/Th2 profile. In cattle, the antibody titers reached corresponded to an expected percentage of protection (EPP) higher than 80%. EPP calculates the probability that livestock would be protected against a 10,000 bovine infectious doses challenge after vaccination. Moreover, in comparison with the non-adjuvanted iFMDV vaccine, iFMDV–ISPA elicited an increased specific T-cell response against the virus, including higher interferon gamma (IFNγ)+/CD8+ lymphocyte production in cattle. In this work, we report for first time that an inactivated FMDV serotype A vaccine adjuvanted with ISPA is capable of inducing protection against challenge in a murine model and of improving the specific immune responses against the virus in cattle.

Keywords: FMDV, ISPA, vaccine, adjuvant, protection, immune responce

INTRODUCTION

Foot-and-Mouth Disease (FMD) is an acute, highly contagious viral vesicle disease, which infects cloven-hoofed animals including livestock—cattle, pigs, sheep, goats, and buffaloes—as well as wild species—deer, antelopes, wild pigs, elephants, giraffes, and camelids (1).

The economic losses produced by Foot-and-Mouth Disease Virus (FMDV) infection in bovines and pigs are due to physical and productive deterioration rather than mortality. Indeed, mortality

rates are low in adult animals, although they are often high in young ones due to myocarditis. However, for countries that export animals and their products, the most relevant economic impact is connected with restrictions on international trade (1). Routine vaccination with inactivated FMDV (iFMDV) can significantly reduce the economic impact of this disease.

FMDV has seven serotypes, known as A, C, O, Asia, SAT 1, SAT 2, and SAT 3. Different strains are used in different countries for vaccine formulation. Serotype A/Argentina/2001 (A2001), isolated in an outbreak of FMD in Argentina in 2000, was used in the present study as proof of concept (2).

In previous work, we developed an experimental murine model using FMDV O1 Campos that proved useful to evaluate the potency of FMDV vaccines. Although mice are not naturally infected by FMDV, experimental infections can be performed by intraperitoneal (ip) inoculation. In the murine model, the humoral and protective responses against FMDV in mice are correlated with cattle (3–6).

Commercial vaccines contain inactivated virus and adjuvants to boost the immune response. Adjuvants improve the immune response elicited against inactivated antigens, direct the immune response to a particular profile, increase the number of responding individuals, reduce the amount of vaccine doses, and/or allow attainment of homogeneous immune responses (7). It is of great importance to find new adjuvants that allow reducing the amount of virus in vaccines and that induce Th1/Th2 responses. Other desirable characteristics include stability and low cost. Immune stimulating complexes (ISCOMs) are capable of developing a Th1/Th2 balanced immune response, in addition to increasing cytotoxic responses (8–11). ISCOMs are spherical particles of ∼40 nm in diameter, composed of phospholipids, cholesterol, and saponin, which can retain the antigen through hydrophobic interactions (8, 12). They have been applied to the development of several registered vaccines for veterinary applications (10). Recently, an empty cage-like particle formulation similar to one of this type of adjuvant, ISCOMATRIX®, was described. It was named Immunostimulating Particle Adjuvant (ISPA) and contains dipalmitoyl-phosphatidylcholine (DPPC), cholesterol (CHO), stearylamine (STEA), alpha-tocopherol (TOCO), and Quil A saponin (11, 13). This adjuvant was shown to surpass conventional adjuvants by improving humoral and cellular CD4/CD8 responses (11). Notably, it was demonstrated that vaccination with the transialidase protein of *Trypanosoma cruzi* (mTS) formulated with ISPA induced increased humoral and cellular immune responses that protected mice against challenge with these parasites (11, 13). Importantly, ISPA preparation can be easily scaled up.

In this work, we report the effect of ISPA as adjuvant for an inactivated FMDV vaccine both in a murine model and in cattle.

MATERIALS AND METHODS

Animals

All experiments involving the use of animals were carried out according to National Agricultural Technology Institute (INTA) Ethics Manual "Guide for the Use and Care of Experimental Animals," under protocol number 24/2016.

Male BALB/c mice, 8–12 weeks old from La Plata University, Argentina, were used.

Calves seronegative for FMDV by enzyme-linked immunosorbent assay (ELISA), ∼8–10 months old, were used in the experiment.

Virus

Binary ethylenimine (BEI)-iFMDV A/Argentina/2001 serotype (provided by Biogenesis Bago, Buenos Aires, Argentina) was used in ELISA assays and in the experimental vaccine formulation. Infectious A/Argentina/2001 serotype, provided by Argentine National Service of Animal Health (SENASA), was used for viral challenge. All experiments involving infectious virus were performed in the BSL-4 OIE (World Organization for Animal Health) facilities at the Institute of Virology, INTA.

Infective Dose of FMDV for Viral Challenge

To select the infective dose of FMDV, serotype A, groups of 4 mice each were intraperitoneally (ip) inoculated with 500 μL of $10^{1.5}$ $TCID_{50}$/mL, $10^{2.5}$ $TCID_{50}$/mL, or $10^{3.5}$ $TCID_{50}$/mL and monitored for viremia at 24, 48, and 72 h postinfection (hpi) as described in Quattrocchi et al. (5). Briefly, heparinized blood withdrawn at different hpi was spread onto BHK-21 cell monolayers grown in 48-well plates and incubated at 37°C in a 5% CO_2 atmosphere. Then, cell monolayers were washed twice with sterile phosphate-buffered saline (PBS). Fresh D-MEM supplemented with 2% fetal calf serum (FCS) was added and the cells were incubated for 48 h at 37°C in 5% CO_2. It was considered that animals were infected if the cell monolayer presented cytopathic effects after a blind passage. Clinical signs, including apathy, ruffled fur, respiratory distress, watery eye discharge, and loss of weight, were daily monitored from 0 to 96 hpi. An infective dose of $10^{2.5}$ $TCID_{50}$ was selected out of the results of these experiments.

Inactivated FMDV Dose to Vaccine Formulation

To select the iFMDV vaccine dose, dilutions of inactivated FMDV in PBS containing 1, 0.5, 0.3, or 0.1 μg in a final volume of 0.2 mL were prepared. Groups of mice ($n = 8$) were subcutaneously (sc) inoculated with these formulations and challenged with an ip injection of $10^{2.5}$ $TCID_{50}$/mL of infectious FMDV, A2001 serotype, after 21 days postvaccination (dpv). Twenty-four hours later, viremia was evaluated as described earlier. Animals were considered protected if viremia was absent at this time point, as established in previous studies (4–6, 14–16). Percentages of protection were calculated as 100× (protected/challenged mice). A dose of 0.3 μg of iFMDV was selected from the results obtained because induction of 50% of protection and the adjuvant effect can be detected.

ISPA Production

ISPA adjuvant is composed of alpha-tocopherol (TOCOP), phosphatidylcholine (DPPC), sterylamine (STEA), cholesterol (CHOL), and QuilA saponin. The ISPA particles have a cage-like structure of 73.0 ± 1.5 nm size as assessed by dynamic light scattering. First, liposomes were prepared with the final proportions of TOCOP: 0.00074% (0.017 mM), DPPC: 0.320%

(4.35 mM), STEA: 0.0216% (0.8 mM), and CHOL: 0.143% (3.70 mM). The suspension was then extruded through a 50-nm-pore membrane and a QuilA saponin solution in acetate buffer was added to liposomes (6.5 mg/300 μL per mL of liposomes) and extruded through a 50-nm-pore membrane (11, 13).

Vaccine Formulations and Vaccination Experiments

The vaccines to be applied in mice were formulated with (1) 0.3 μg of iFMDV in PBS (iFMDV) or (2) 0.3 μg of iFMDV in PBS mixed with 6 μL of ISPA (iFMDV–ISPA), in a final volume of 0.2 mL/dose. BALB/c mice were immunized with (1) iFMDV ($n = 5$), (2) iFMDV–ISPA ($n = 5$), (3) commercial vaccine ($n = 5$), (4) 6 μL of ISPA ($n = 2$), or (5) PBS ($n = 2$) by the sc route. Mice were challenged at 21 dpv as described earlier.

The vaccines used in cattle were formulated with (1) 12 μg of iFMDV in PBS, according to Mattion et al. (2), or (2) the same formulation with 1 mL of ISPA, in a final volume of 2 mL/dose. Cattle ($n = 4$, per group) were vaccinated sc at days 0 and 48 as follows: (1) iFMDV, (2) ISPA–iFMDV, or (3) commercial vaccine. The commercial vaccine consisted of a water-in-oil single emulsion containing O1/Campos, A24/Cruzeiro, A/Arg/2000, and A/Arg/2001 iFMDV and was provided by Biogénesis Bagó.

Measurement of Total IgG and Isotypes Against FMDV by Sandwich ELISA

Total antibodies (Ab) against FMDV were assessed by ELISA as described previously (3–5) Briefly, Greiner Microlon® plates were coated ON at 4°C with anti-FMDV rabbit serum in carbonate–bicarbonate buffer, pH 9.6. After three washing steps, plates were blocked for 30 min at 37°C with polyvinylpyrrolidone blocking solution in the case of mouse sera (0.5 M NaCl/0.01 M phosphate buffer/0.05% Tween-20/1 mM EDTA/1% polyvinylpyrrolidone 30–40 K, pH 7.2) or with PBS/10% FCS in the case of bovines sera. An optimal dilution of inactivated FMDV was added in blocking solution. Plates were incubated at 37°C for 30 min. Then, serially diluted mouse sera (1:4) or bovine sera (1:5) in blocking solution were added. After 1 h 20 min incubation at room temperature, plates were washed and an optimal dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (H+L) (KPL®), HRP-conjugated anti-mouse isotypes (Southern Biotech, Birmingham, AL, USA), HRP-labeled goat anti-bovine IgG antibody (KPL®), or HRP-labeled goat anti-bovine IgG1 or IgG2 antibody (KPL®) was added. Plates were incubated for 1 h at room temperature and then washed. Ortho-phenylenediamine (1,2-benzenediamine) dihydrochloride (Sigma Aldrich, St. Louis, MO, USA) (OPD)/H_2O_2 was used as the peroxidase substrate. Reactions were stopped by use of 1.25 M H_2SO_4 and A_{492} was measured in an absorbance microplate reader. Positive and negative control sera were included in every plate. The cut-off was established as the mean of the values of negative sera ($n = 10$) plus two standard deviations.

Measurement of Total FMDV-Specific Antibodies by Liquid-Phase ELISA

A liquid-phase ELISA test was used according to Hamblin et al. (17), with modifications (1). Briefly, Greiner Microlon® plates were coated overnight at 4°C with rabbit anti-FMDV serum diluted to the optimal concentration in carbonate–bicarbonate buffer, pH 9.6. After washing with 0.05% Tween-20/phosphate buffered saline (PBST), plates were blocked with PBST/1% ovalbumin (blocking buffer) for 30 min at 37°C. Mice or bovine sera were serially diluted (1:10) in blocking buffer in separate tubes and a fixed amount of inactivated FMDV was added. After 1 h of incubation at 37°C with shaking, the virus–antibody mixtures were transferred to the blocked plates, and incubated for 1 h at 37°C. An optimal dilution of guinea pig anti-FMDV serum in PBS/2% normal bovine serum/2% normal rabbit serum was added for detection, followed by 1 h of incubation at 37°C. Plates were washed and peroxidase-conjugated anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA, USA) serum diluted in the same buffer was added, followed by 1 h of incubation at 37°C. OPD/H_2O_2 was used as peroxidase substrate as described earlier and A_{492} was measured in a microplate reader. Strong positive, weak positive, and negative bovine reference sera were included in each test for validation. Antibody titers were expressed as the negative logarithm of the highest dilution of serum that causes an inhibition of color development higher than 50% in the average values of the control samples.

Neutralizing Antibody Titers

Sera samples were examined for anti-FMDV neutralizing antibodies as described before (16). Briefly, serial dilutions of complement inactivated sera were incubated for 1 h at 37°C with 100 $TCID_{50}$ of infective FMDV. Then virus–serum mixtures were seeded on BHK-21 monolayers. After 40 min at 37°C, fresh DMEM/2% FCS was added to the monolayers, which were incubated at 37°C, under 5% CO_2. Cytopathic effects were observed after 48 h.

Lymphoproliferation Assay

Murine splenocytes were obtained 21 days after immunization. Animals were anesthetized and euthanized by cervical dislocation and spleens were removed.

Cattle Peripheral Blood Mononuclear Cells (PBMCs) were obtained as described previously (18) by centrifugation of bovine blood in a Ficoll-Paque™ plus gradient (GE Healthcare, Chicago, IL, USA).

Murine splenocytes or PBMCs were labeled with 3 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS for 30 min at 37°C. Labeled cells were added to 96-well plates (5×10^5 cell/well) in complete RPMI 1640 media supplemented with 10% FCS and 50 mM 2-mercaptoethanol and were stimulated with (1) mock, (2) 2.5 μg/mL of iFMDV, or (3) 5 μg/mL of concanavalin A (Sigma Aldrich) as positive control. Cells were incubated at 37°C in 5% CO_2 atmosphere for 4 days, and then 0.2% paraformaldehyde was added and cell proliferation was analyzed by flow cytometry using FACSCalibur® (Becton Dickinson, San Jose, CA, USA) and Flowing Software (Turku

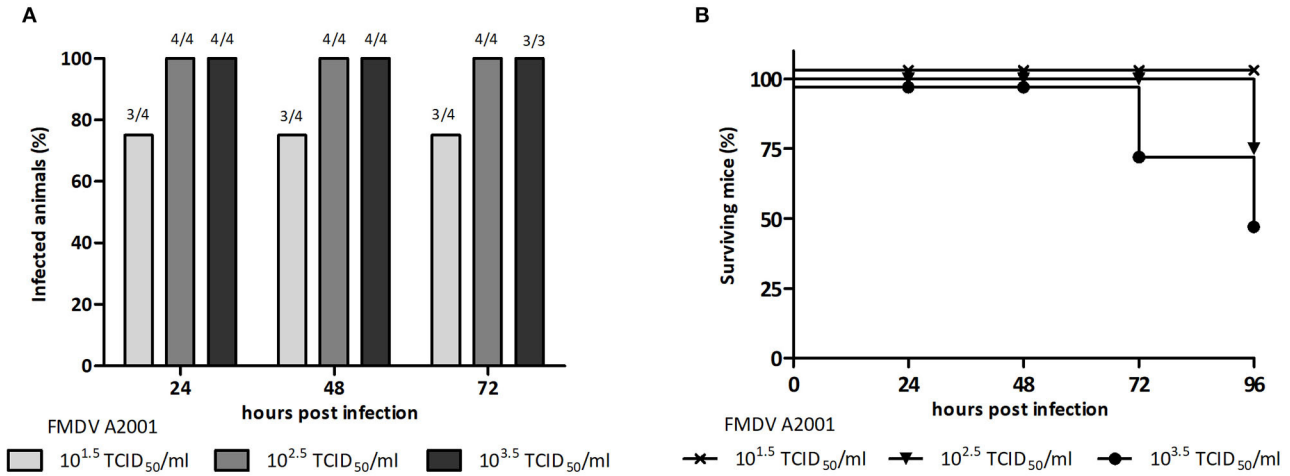

FIGURE 1 | Selection of the infective dose of FMDV, serotype A. Groups of BALB/c mice ($n = 4$) were ip inoculated with 500 μL of $10^{1.5}$TCID$_{50}$, $10^{2.5}$TCID$_{50}$, or $10^{3.5}$TCID$_{50}$/mL of infectious FMDV A/Argentina/2001, and viremia was analyzed at 24, 48, and 72 hpi. **(A)** Percentage of infected animals at 24, 48, and 72 hpi and **(B)** percentage of surviving mice at 24, 48, 72, and 96 h after inoculation with infective FMDV.

Center for Biotechnology, Finland). Results were expressed as delta proliferation and were calculated as the difference between the percentage of proliferating cells stimulated with inactivated virus and the percentage of proliferating cells without stimuli. An example of flow cytometry gating strategy adopted in this article is depicted in **Supplementary Figure 1**.

Surface and Intracytoplasmatic Staining for IFN-γ-Producing Cells Detection

PBMC were incubated in complete RPMI 1640 media supplemented with 10% FCS and 50 mM 2-mercaptoethanol and were stimulated with (1) mock, (2) 2.5 μg/mL of iFMDV, or (3) 5 μg/mL of concanavalin A (Sigma Aldrich, St. Louis, MO, USA) as positive control. Cells were incubated for 18 h in the presence of brefeldin A (BD GolgiPlug™) (according to manufacturer recommendations). After washing, cells were fixed in 0.5% paraformaldehyde and permeated with saponin (0.1% in PBS). Permeated cells were incubated for 20 min at RT with Alexa Flour 647 anti-bovine interferon gamma (INF-γ; clone CC302, AbD Serotec, Oxford, UK) or isotype-matched control antibody. After 20 min, cells were washed twice and stained for 30 min at 4°C with anti-bovine CD4, clone CC8 (AbD Serotec) plus FITC anti-bovine IgG (polyclonal, Jackson ImmunoResearch); PE anti bovine CD 8 (clone CC63, Bio-Rad) or FITC anti-bovine WC1 (clone CC15, AbD Serotec). Cells were then washed and fixed with 0.2% paraformaldehyde. Flow cytometry was performed in a BD FacsCalibur and analyzed with Flowing Software (Turku Center for Biotechnology, Finland). An example of flow cytometry gating strategy adopted in this article is depicted in **Supplementary Figure 2**.

Statistical Analysis

The GraphPad InStat® program (GraphPad, San Diego, USA) was used. Differences between groups were analyzed by applying the non-parametric Kruskal–Wallis test, followed by Mann–Whitney *U*-test for comparisons between two groups. A $p < 0.05$ was considered as an indicator of significant differences.

RESULTS

Selection of the Infective Dose for Viral Challenge in Mice

A previously developed murine model for FMDV serotype O vaccine testing was adjusted in this study to serotype A (3–5, 19). With the aim of selecting the viral challenge dose, unvaccinated mice were inoculated with different viral infective doses of FMDV ($10^{1.5}$, $10^{2.5}$, or $10^{3.5}$ TCID$_{50}$ infectious FMDV/mL) and viremia was assessed at 24, 48, and 72 hpi. Mice were also examined for clinical signs until 96 hpi. All mice inoculated with $10^{2.5}$ or $10^{3.5}$ TCID$_{50}$ infectious FMDV/mL, but only 80% of those inoculated with $10^{1.5}$ TCID$_{50}$ infectious FMDV/mL, presented positive viremia at all studied time points (**Figure 1A**). Survival was 100% at 24 and 48 hpi with all doses used. At 72 hpi, one mouse of the group inoculated with $10^{3.5}$ TCID$_{50}$/mL died, and at 96 hpi, one mouse each from the $10^{2.5}$ TICD$_{50}$/mL and the $10^{3.5}$ TICD$_{50}$/mL groups died (**Figure 1B**). As shown in **Table 1**, clinical signs started to appear at 24 hpi in mice inoculated with $10^{2.5}$ TCID$_{50}$/mL and $10^{3.5}$ TCID$_{50}$/mL and at 48 hpi, all animals in these groups showed signs, including apathy, ruffled fur, and others. Conversely, no mice infected with $10^{1.5}$ TCID$_{50}$/mL showed observable clinical signs at any time of the experiment.

Taking into account these results, the dose of $10^{2.5}$ TICD$_{50}$/mL infectious FMDV serotype A and the time point of 24 hpi were chosen to, respectively, perform and assess viral challenge assays.

Selection of the iFMDV Dose for Vaccine Formulation With ISPA as Adjuvant

To analyze the modulatory effect of ISPA adjuvant on the immune response, a dose of inactivated FMDV capable of inducing 50% protection was first selected. To this end, mice were

TABLE 1 | Clinical signs in mice inoculated with different FMDV A/Argentina/2001 serotype doses.

	24 h			48 h			72 h			96 h		
	$10^{1.5}$	$10^{2.5}$	$10^{3.5}$	$10^{1.5}$	$10^{2.5}$	$10^{3.5}$	$10^{1.5}$	$10^{2.5}$	$10^{3.5}$	$10^{1.5}$	$10^{2.5}$	$10^{3.5}$
Apathy	0/4	0/4	0/4	0/4	4/4	4/4	0/4	4/4	3/3[a]	0/4	3/3[a]	2/2[b]
Ruffled fur	0/4	1/4	3/4	0/4	4/4	4/4	0/4	4/4	3/3[a]	0/4	3/3[a]	2/2[b]
Respiratory distress	0/4	0/4	1/4	0/4	4/4	4/4	0/4	4/4	3/3[a]	0/4	2/3[a]	2/2[b]
Watery eye discharge	0/4	0/4	0/4	0/4	0/4	2/4	0/4	1/4	2/3[a]	0/4	1/3[a]	2/2[b]
Loss of weight	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	1/3[a]	0/4	2/3[a]	2/2[b]

Results are expressed as number of mice with signs/total number of infected mice. Groups of BALB/c mice (n = 4) were ip inoculated with $10^{1.5}$, $10^{2.5}$, or $10^{3.5}$ $TCID_{50}$/mL of infectious FMDV, A/Argentina/2001 serotype, and observed for disease indications at 24, 48, 72, and 96 hpi.

[a] *One animal in this group died.*

[b] *Two animals in this group died.*

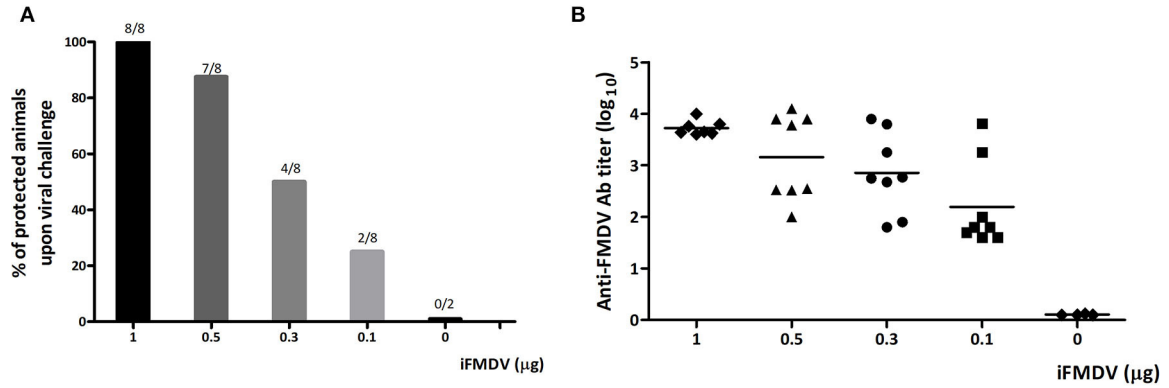

FIGURE 2 | Selection of iFMDV dose for vaccination of BALB/c mice. Groups of mice ($n = 8$) were vaccinated with 1, 0.5, 0.3, or 0.1 μg of iFMDV in PBS and challenged with infective virus after 21 dpv. **(A)** Percentages of protected animals on viral challenge. Group 0 corresponds to animals inoculated with PBS. Animals were considered protected if viremia was absent at 24 h post challenge. Protection percentages were calculated as 100× (number of vaccinated animals without viremia/number of vaccinated animals). **(B)** Antibodies against FMDV elicited by vaccination with different amounts of iFMDV measured by ELISA at 21 dpv.

vaccinated with 1, 0.5, 0.3, or 0.1 μg of iFMDV in PBS and at 21 dpv challenged with infectious FMDV, serotype A. A dose-dependent protective effect was observed (**Figure 2A**), as well as a concomitant decrease in antibody titers with decreasing amounts of virus (**Figure 2B**). Fifty percent of mice vaccinated with 0.3 μg of iFMDV were protected upon viral challenged, so this dose was chosen for vaccine formulations.

iFMDV–ISPA Vaccine Confers Total Protection Against FMDV in Mice With a Single-Dose Immunization

The protective efficacy of the inclusion of ISPA as adjuvant in an iFMDV vaccine (iFMDV–ISPA) was tested in mice. Groups of mice were vaccinated with iFMDV, iFMDV–ISPA, a commercial vaccine (Biogénesis Bagó), ISPA, or PBS (negative control) and challenged with infective FMDV at 21 dpv (**Figure 3**). Notably, while protection with iFMDV alone was achieved in 40% of mice, inclusion of ISPA in the formulation increased protection levels to 100% as well as the commercial vaccine. Animals in mock vaccinated groups inoculated with ISPA or PBS were not protected, indicating that the viral challenge was conducted properly.

Murine-Specific FMDV Antibodies and Neutralizing Antibodies Are Increased When ISPA Is Used as Adjuvant

Antibody (Ab) responses elicited by iFMDV, iFMDV–ISPA, the commercial vaccine, ISPA, and PBS were evaluated at 14 and 21 dpv. Total specific FMDV Abs titers were significantly higher ($p < 0.001$) as measured by liquid-phase ELISA in the iFMDV–ISPA group as compared to the iFMDV group (**Figure 4A**). Importantly, when the virus neutralization test (VNT) was applied, neutralizing antibody titers at 21 dpv were significantly higher in the iFMDV–ISPA group as compared to the iFMDV group (1.6 ± 0.1 vs. 0.95 ± 0.05, $p < 0.001$). Neutralizing Ab titers in the iFMDV–ISPA group were similar to those in the commercial vaccine group (**Table 2**). Ab levels in the iFMDV–ISPA group were similar to those in the commercial vaccine group.

Analysis of isotype profiles at 21 dpv showed that the iFMDV–ISPA group achieved higher IgG1 and IgG2a titers ($p < 0.05$ and $p < 0.001$, respectively) than the iFMDV group, and the profile was similar to that of the commercial vaccine group (**Figure 4B**). IgG2b titers were also higher in the iFMDV–ISPA group than in the iFMDV group ($p < 0.001$). Finally, there were significantly

higher IgG3 titers ($p < 0.001$) in the iFMDV–ISPA group than in the iFMDV and the commercial vaccine groups.

Immunization With iFMDV–ISPA Induces a Specific Cellular Immune Response Against FMDV in Mice

At 21 dpv, FMDV-specific T-cell stimulation levels were significantly higher in splenocytes derived from mice immunized with iFMDV–ISPA ($p < 0.01$) or with commercial vaccine ($p < 0.05$) than in those derived from iFMDV, ISPA, or PBS-inoculated mice (**Figure 5**).

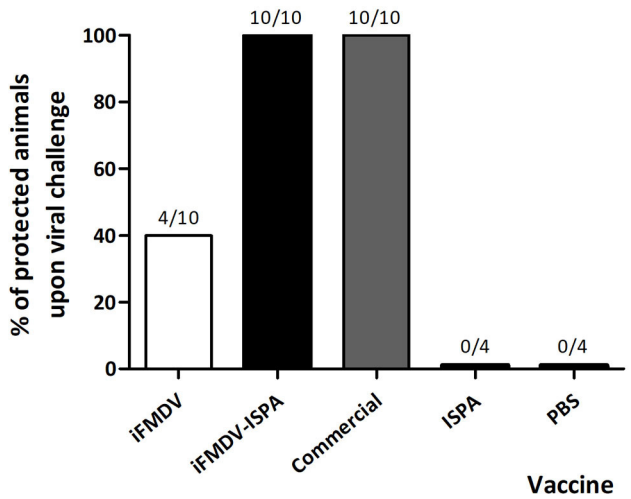

FIGURE 3 | Protection on viral challenge elicited by different vaccines. Groups of mice ($n = 10$) were vaccinated with iFMDV, ISPA-iFMDV, or a commercial FMD vaccine, and groups of mice ($n = 4$) were vaccinated with ISPA or PBS alone, and challenged with infective FMDV at 21 dpv. Protection was calculated as described for **Figure 2**. Results are representative of two independent experiments.

iFMDV–ISPA Vaccine Induces an Increase of FMDV Abs in Cattle

After promising results obtained in the murine model, the immune efficacy of the iFMDV–ISPA vaccine was studied in cattle, a natural host of the virus.

FMDV serologically negative calves ($n = 4$ per group) were inoculated (at days 0 and 48) with iFMDV (12 μg) or iFMDV (12 μg)-ISPA, a commercial vaccine (at day 0) or PBS (negative control).

At 30 dpv, calves vaccinated with iFMDV–ISPA displayed an increment in the elicited specific humoral response as compared to individuals vaccinated with iFMDV alone ($p < 0.05$), when measured by liquid-phase ELISA (**Figure 6A**).

As shown in **Figure 6B**, at 30 dpv, the iFMDV–ISPA vaccine induced significantly higher levels of IgG1 isotype antibodies against FMDV than the iFMDV vaccine ($p < 0.05$). Moreover, IgG2 titers also presented significant differences ($p < 0.05$) among groups. There were no statistically significant differences in isotype profiles in the iFMDV–ISPA and the commercial vaccine group.

VNT results at 30 dpv also showed a significant increase ($p < 0.05$) in Ab titers in the iFMDV–ISPA group as compared to the iFMDV group (**Table 3**). However, at 48 dpv, decreases in total and neutralizing Ab titers were observed in the

TABLE 2 | Virus neutralizing antibody titers (VNT) in mice at 21 dpv with different vaccines.

Vaccine	VNT (mean Ab titers ± standard deviation)
iFMDV	0.95 ± 0.05
iFMDV–ISPA	1.7 ± 0.1***
Commercial	1.8 ± 0.1***
ISPA	<1.0
PBS	<1.0

*Titers are expressed as log_{10} of the reciprocal of the serum dilution that neutralizes 50% of 100 $TCID_{50}$ of infective FMDV, using the fixed virus–variable serum method. ***Significant differences with respect to the iFMDV group ($p < 0.001$).*

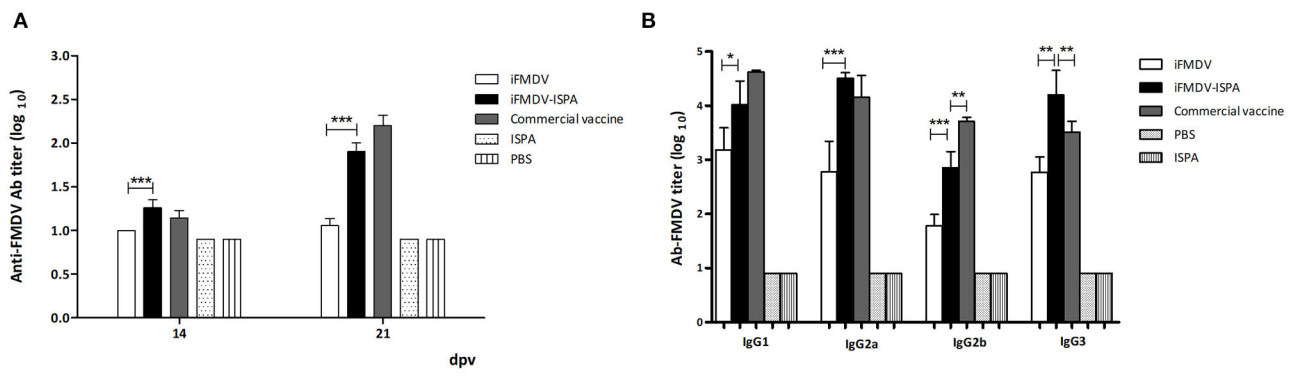

FIGURE 4 | Antibodies against FMDV elicited by different vaccines in mice. FMDV specific antibody titers were measured by **(A)** liquid-phase ELISA at 14 and 21 dpv. Each bar represents the mean ($n = 5$) Ab titer ± SD in each group. **(B)** Isotype profile of vaccinated animals at 21 dpv. Data are expressed as the mean Ab titer ± SD. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

iFMDV–ISPA group. Due to a decreases in VNT, a second dose was administered to cattle, which resulted in an increase ($p < 0.05$) at 76 dpv in the seroneutralizing Abs titers in the iFMDV–ISPA group as compared to the iFMDV group. Remarkably, these VNT values were similar to the VNT induced by the commercial vaccine group. These values are associated with an 80% Expected Percentage os Protection (20). EPP calculates the probability that livestock would be protected against a 10 000 bovine infectious doses challenge after vaccination (1).

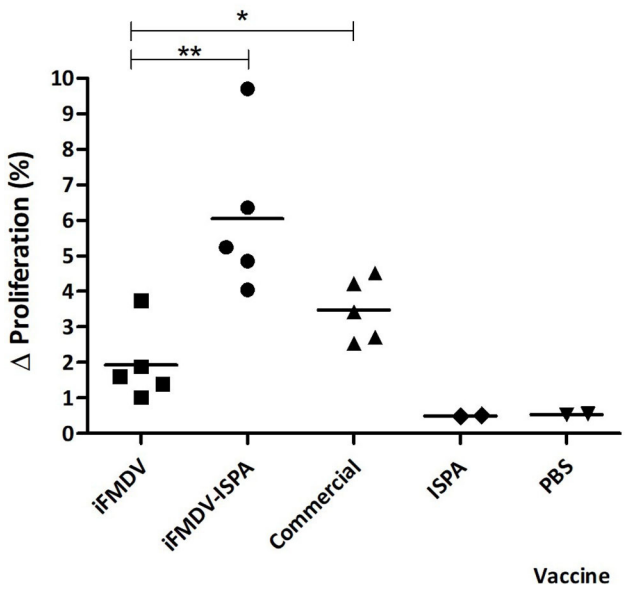

FIGURE 5 | Cellular immune response in mice splenocytes at 21 dpv. Animals were vaccinated with iFMDV, ISPA-iFMDV, ISPA, or PBS. Splenocyte proliferative response after stimulation with iFMDV was measured by CFSE loss. Results are expressed as the difference (Δ%) between the percentage of proliferating splenocytes stimulated with inactivated virus and the percentage of proliferating splenocytes without stimuli. $^{**}p < 0.01$; $^{*}p < 0.05$.

Immunization With iFMDV–ISPA Induces a Specific Cellular Immune Response Against FMDV in Cattle

When PBMCs from vaccinated calves were stimulated with iFMDV, a significantly increased lymphoproliferative response ($p < 0.001$) was evident in iFMDV–ISPA compared to iFMDV (**Figure 7A**). No significant differences were detected between the iFMDV–ISPA and the commercial vaccine group ($p = 0.075$).

On the other hand, when lymphocytes stained with anti-bovine CD4, anti-bovine CD8, and anti-bovine INF-γ and then studied by flow cytometry, the percentages of IFNγ+/CD8+ lymphocytes from iFMDV–ISPA vaccinated calves were higher than in animals vaccinated with iFMDV alone ($p < 0.05$) (**Figure 7B**). Concerning CD4+ lymphocytes, a tendency of an increased production of IFNy was also observed in the

TABLE 3 | Virus neutralizing antibody titers at 30, 48, and 76 days after inoculating cattle with different vaccines.

	VNT (mean of Ab titers ± standard deviation)		
Vaccine	**30 dpv**	**48 dpv**	**76 dpv**
iFMDV	1.02 ± 0.02	0.85 ± 0.03	1.4 ± 0.1
iFMDV–ISPA	1.8 ± 0.2*	1.2 ± 0.1*	2.2 ± 0.4*
Commercial	2.1 ± 0.2**	2.0 ± 0.4**	2.6 ± 0.2**
PBS	<1.0	<1.0	<1.0

Titers are expressed as log_{10} of the reciprocal of the serum dilution that neutralizes 50% of 100 $TCID_{50}$ infective FMDV, using the fixed virus–variable serum method. Significant differences against iFMDV group: $^{*}p < 0.05$; $^{**}p < 0.01$.

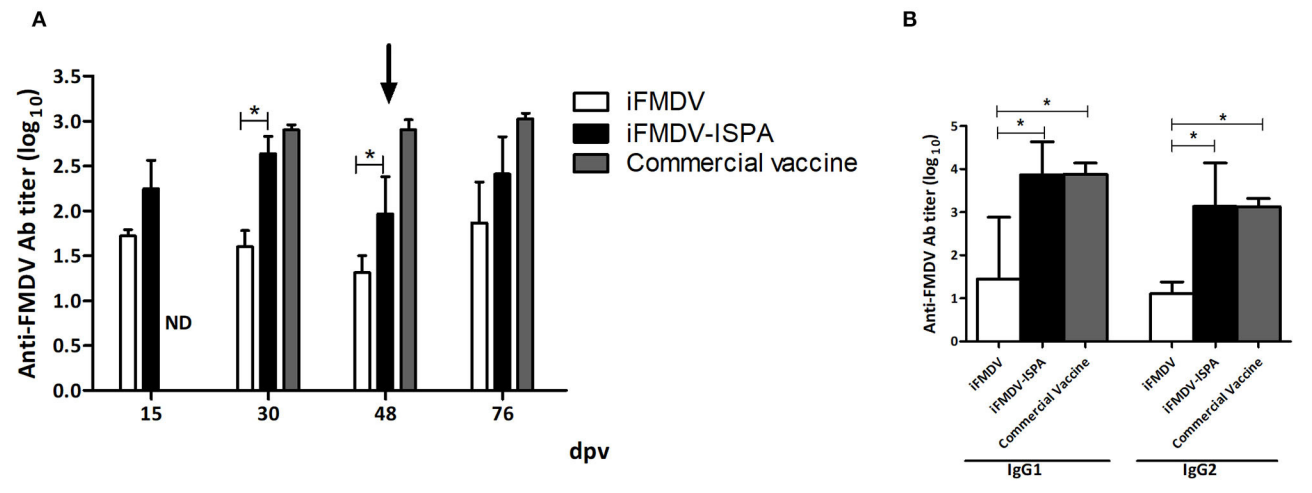

FIGURE 6 | Humoral response elicited in cattle by different vaccines. FMDV-specific antibody titers were measured by liquid-phase ELISA. **(A)** Each bar represents the mean Ab titer ± SEM ($n = 4$) at 15, 30, 48, and 76 dpv. **(B)** Isotype profiles at 30 dpv, expressed as mean Ab titers ± SD. $^{*}p < 0.05$.

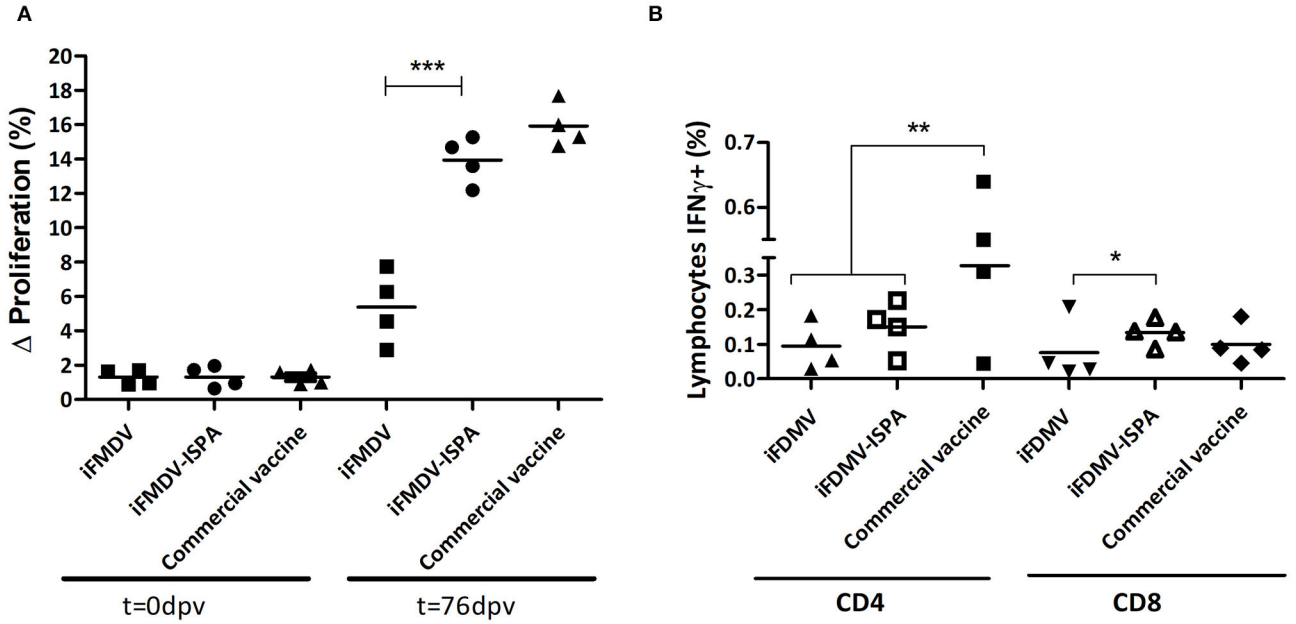

FIGURE 7 | Cellular immune response in cattle. **(A)** Lymphocyte proliferative response after stimulation with iFMDV, A/Arg/2001 serotype, measured by CFSE loss. Differences (Δ%) were calculated as (% proliferating PBMCs stimulated with inactivated virus − % proliferating PBMCs without stimuli). **(B)** Percentages of CD8+/IFNγ$^+$ or CD4+/IFNγ$^+$ T cells in PBMCs of cattle immunized with iFMDV or iFMDV–ISPA, at 76 dpv. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

iFMDV–ISPA group as compared to the iFMDV group, although the difference was not statistically significant ($p = 0.72$).

On the other hand, at 76 dpv, there were no statistically significant differences in the amounts of γδ T cells or IFN+/γδ T cells in the iFMDV–ISPA-immunized with respect to the iFMDV-immunized calves (data not shown).

DISCUSSION

In this work, we used a mouse model to examine the capacity of an iFMDV formulation containing new cage-like particles (ISPA), as a new generation adjuvant, to elicit a protective and specific immune response to FMDV. The results of the immunological immune response profile obtained in the murine model were confirmed in calves.

In the murine model, all animals vaccinated with iFMDV–ISPA were protected against homologous viral challenge while the protection percentages induced by a non-adjuvanted iFMDV vaccine were inferior. Individuals vaccinated with ISPA alone were not protected against viral challenge, showing that the protective response corresponded to an adaptive response against the virus and was not due to innate immune mechanisms induced by the adjuvant.

Total and seroneutralizing Abs against FMDV were significantly elevated in mice that received iFMDV–ISPA as compared to the group vaccinated with iFMDV alone. These results correlate with the protection induced on challenge. It is noteworthy that neutralizing antibody titers showed a good correlation with protection levels, substantiating the notion that they are an *in vitro* reflection of the immune response that occurs *in vivo* (1, 21, 22).

In addition, all isotypes of specific IgG were increased in iFMDV–ISPA group as compared to the group vaccinated with iFMDV alone, being IgG2a-b/IgG1 ratio also higher. It has been reported that murine macrophages could have a virus clarifying action by complement-fixing isotypes IgG2a, IgG2b, and IgG3 (5, 21, 23). The FcγI receptor (FcγRI), expressed in dendritic cells, monocytes and macrophages binds to these isotypes (24, 25). According to Klaus et al. (26) and Kipps et al. (27), IgG2a and IgG2b are the most effective isotypes in complement activation as well as in antibody-mediated cellular immune responses. Using the murine model to evaluate the quality of FMDV vaccines, Gnazzo et al. (6) reported that vaccine protection is associated not only with total FMDV antibody levels but also with the IgG2b/IgG1 ratio and the avidity of sera. Moreover, it has been reported that mice inoculated with iFMDV plus some adjuvants generate a complement-fixing IgG profile that correlates with protection on FMDV challenge (3, 28).

When the specific cellular response to the virus was studied, an increased lymphoproliferative response was evident in mice immunized with iFMDV–ISPA. These results suggest that the ISPA adjuvant improves the adaptive immune response against FMDV, reaching results similar to those obtained with the commercial vaccine. Ostrowski et al. (29) and Langellotti et al. (30) reported that vaccination of mice with inactivated FMDV induces T-cell responses and has been shown to increase CD8+ numbers in the spleen. Moreover, ISPA-iFMDV formulation triggers proliferation and IFNγ production in FMDV-specific CD4+ and CD8+ T lymphocytes (data not shown). It is well-described that IFN-γ is involved in the isotype switch of immunoglobulins, leading to an increase in the IgG2a and IgG2b types (31). This result is in agreement with the high levels of

IgG2a and IgG2b obtained and the protection levels observed in the iFMDV–ISPA group. Previous work describes that ISCOMs improve the dendritic cross-presentation (9, 32–34). These data indicate that iFMDV adjuvanted with ISPA generates a strong cellular response, in accord with previous reports of studies that used cage-like particles.

Similar to what was observed in mice, the iFMDV–ISPA formulation generated an increase in anti-FMDV antibody titers in calves as compared to the iFMDV vaccine alone. In addition, animals immunized with iFMDV–ISPA displayed similar VNT titers as those immunized with a commercial vaccine approved by SENASA for vaccination in Argentina. Noteworthy, the commercial vaccine contains FMDV serotype A24/Cruzeiro, A/A2001, O1 Campos, and A/Arg2000, all of which bear epitopes that participate in the immune response against FMDV.

In cattle, numerous studies show a correlation between antibody titers against FMDV elicited by vaccination and *in vitro* and *in vivo* protection on experimental viral challenge. These correlations have allowed estimation of the Expected Percentage Protection to the homologous infection using titers of systemic α-FMDV Ab measured by liquid-phase ELISA or viral seroneutralization (19; 21; 1). Total and neutralizing anti-FMDV Ab titers reached in the iFMDV–ISPA group correspond to an EPP above 80% (35, 36). Importantly, an acceptable inactivated vaccine should induce 75% protection in cattle (1). Moreover, in cattle, IgG1 and IgG2 isotype titers were higher when ISPA was included as adjuvant in iFMDV vaccines. Bovine macrophages and neutrophils possess an immunoglobulin receptor to which IgG2 can bind (37). However, there are reports in which high IgG1 titers were related to high protection against FMDV challenge (38, 39). IgG1 is involved in both pathogen opsonization and seroneutralization in bovines. The particular role of each bovine IgG isotype in the response against FMDV has not been deeply characterized yet. In addition, IgG1/IgG2 ratio > 1 is related to FMDV protection and it is used as a protection parameter when there are low VNTs (39, 40).

Regarding cellular responses, *in vitro* T-cell stimulation was significantly higher in cattle PBMCs of the iFMDV–ISPA group than of the iFMDV group. In addition, IFNγ production was increased in CD8+ PBMCs derived from iFMDV–ISPA-immunized cattle. Thus, we here demonstrate that the ISPA-FMDV vaccine induces a cellular immune response in these bovines by inducing IFNγ secretion and raising viral-specific PBMC proliferation. Moreover, IgG1 is usually taken as a parameter of cellular immune response activation (41, 42).

The role of FMDV cellular immunity responses in a target species, such as the bovine, is still unclear, although many reports indicate its relevance to fight the infection. In this way, specific T-cell-mediated antiviral responses have been observed in cattle after infection or vaccination (43–45). Also, FMDV vaccination induces rapid T-cell responses, and FMDV-specific CD4+ T-cell proliferation has been detected as early as 7 dpv (46). T-helper cells are necessary for the induction of isotype switching to generate high-affinity antibodies and to reach a protective neutralizing response to vaccination with iFMDV (47). On the other hand, CD8+ T-cell–mediated immune responses to FMDV have been reported in pigs (45, 48, 49) and cattle (43, 50, 51). Vaccination with the conventional iFMDV vaccine induces circulating memory CD8+ T cells which, upon an appropriate stimulus, can be expanded and are cytotoxic (51). Stenfeld et al. (52) demonstrated the role of a CTL response in preventing the FMDV carrier state in vaccinated cattle. Besides, the percentage of CD4+ lymphocytes and the CD4/CD8 ratio after vaccination may serve as a parameter to select young sires with a high immune response against FMDV (53). Moreover, IFN-γ displays activity against FMDV (54), by controlling viral replication and spreading within the host through natural killer cell and macrophage activation (55). Thus, a positive correlation between IFN-γ response and vaccine-induced protection as well as reduction of long-term persistence of FMDV has been observed in cattle (56).

Cattles numbers included in this pilot study was equal to those used in other preliminary studies on vaccine candidates (48, 57–59), although it is not enough for statistical analysis (60). However, the results obtained serve as a proof of concept of the usefulness of ISPA as adjuvant for FMDV vaccines.

Future work will be devoted to examining whether vaccine formulations containing ISPA promote the virus presentation to the immune effectors, and in this way enhance the immune response generated and the protection obtained. Some authors have reported that ISCOMs induce local recruitment, activation, and maturation of immune cells, such as dendritic cells; granulocytes; F4/80 int cells; and T, B, and NK cells (10, 61, 62), increasing in this way the chances of the antigen to come into contact with immune cells. In addition, Brok et al. (34) proved that saponin-based adjuvants enhance antigen cross-presentation by dendritic cells and T-cell activation. Moreover, Prochetto et al. have proved that a vaccine for *Trypanosoma cruzi* formulated with ISPA and a recombinant trans-sialidase fraction favorably modulates the regulatory arm of the immune system to reach immune protection against the parasite (13).

In conclusion, ISPA displays an important adjuvant activity for FMDV vaccines, increasing and modulating the humoral and cellular responses in vaccinated mice and cattle and yielding enhanced protection against challenge.

DATA AVAILABILITY STATEMENT

The data are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional para el Cuidado y Uso de Animales de Experimentación (CICUAE) - Centro de Investigación en Ciencias Veterinarias y Agronómicas del INTA.

AUTHOR CONTRIBUTIONS

JB: collaboration in work designing, acquisition, analysis, interpretation of field and laboratory data, drafting, final approval of the version to be published, and ensuring that questions related to the accuracy or integrity of the work were appropriately

investigated and resolved. CK, MG, VG, IS, CL, CM, RG, LC, GL, VQ, and IM: acquisition and analysis of laboratory data for the work, critical revision for intellectual content, final approval of the version to be published, ensuring that questions related to the accuracy, and integrity of laboratory work were appropriately investigated and resolved. PZ: conception and design of the work, critical revision of the work for important intellectual content, final approval of the version to be published, ensuring that questions related to the accuracy, and integrity of the work were appropriately investigated and resolved. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00396/full#supplementary-material

Supplementary Figure 1 | The figure shows representative dot plots used for selecting the lymphocyte region based on side sideward scatter (SSC) on the *y*-axis and forward side scatter (FSC) on the *x*-axis. Lymphocyte proliferative response after stimulation with iFMDV is shown. **(A)** Representative dot plots from mice splenocytes at 0 dpv and CFSE loss (H-3 gate). **(B)** Representative dot plots from mice splenocytes at 21 dpv and CFSE loss (H-3 gate). **(C)** Representative dot plots from bovine PBMCs at 0 dpv and CFSE loss (H-3 gate). **(D)** Representative dot plots from bovine PBMCs at 76 dpv and CFSE loss (H-3 gate).

Supplementary Figure 2 | The figure shows representative dot plots, from bovine PBMCs, used for selecting the lymphocyte region based on side sideward scatter (SSC) on the *y*-axis and forward side scatter (FSC) on the *x*-axis. Then, we selected the CD8 region based on fluorescence anti-CD8 stain on the *y*-axis and CD4 region based on fluorescence anti-CD4 stain on the *x*-axis. PBMCs incubated for 18 h with iFMDV are shown.

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A Wide-Ranging Antiviral Response in Wild Boar Cells Is Triggered by Non-coding Synthetic RNAs From the Foot-and-Mouth Disease Virus Genome

*Miguel Rodríguez Pulido, Ranjitha H. B.† and Margarita Sáiz**

Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid, Spain

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Mariano Pérez-Filgueira, National Agricultural Technology Institute, Argentina

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Fayna Diaz San Segundo, Plum Island Animal Disease Center, United States

***Correspondence:**
Margarita Sáiz
msaiz@cbm.csic.es

†Present address:
Ranjitha H. B., ICAR-Indian Veterinary Research Institute, Bengaluru, India

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Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious viral disease that affects multiple cloven-hooved hosts including important livestock (pigs, cattle, sheep and goats) as well as several wild animal species. Crossover of FMDV between domestic and wildlife populations may prolong virus circulation during outbreaks. The wild boar (*Sus scrofa*) is considered a reservoir of various pathogens that can infect other wildlife, domestic animals, and humans. As wild boar and domestic pigs are susceptible to the same pathogens and can infect each other, infected wild boar populations may represent a threat to the pig industry and to international trade. The ncRNAs are synthetic non-coding RNA transcripts, mimicking structural domains in the FMDV genome, known to exert a broad-spectrum antiviral and immunomodulatory effect in swine, bovine and mice cells. Here, we show the type I interferon-dependent, robust and broad range antiviral activity induced by the ncRNAs in a cell line derived from wild boar lung cells (WSL). Transfection of WSL cells with the ncRNAs exerted a protective effect against infection with FMDV, vesicular stomatitis virus (VSV), swine vesicular disease virus (SVDV) and African swine fever virus (ASFV). Our results prove the biological activity of the ncRNAs in cells of an FMDV wild animal host species against a variety of viruses affecting pigs, including relevant viral pathogens of epizootic risk.

Keywords: foot-and-mouth-disease virus, antivirals, wild boar, non-coding RNA, wildlife

INTRODUCTION

Foot-and-mouth disease (FMD) is a severe, highly contagious and transboundary viral disease that has a significant economic impact affecting the production of livestock and disrupting regional and international trade in animals and animal products. The causative agent of FMD is foot-and-mouth disease virus (FMDV), a member of the family *Picornaviridae*. FMDV isolates are classified into seven different serotypes and all of them have been found in wildlife (1). The capacity of the wild boar (*Sus scrofa*) for FMDV transmission has been reported and the prolonged viral secretion along with mild clinical disease raised the concern that wild boars may spread FMD (2, 3). However, our knowledge on the clinical manifestations of FMD in wild boars and their actual contribution to transmission during field outbreaks is very limited (4–6).

Wild boars are extremely adaptable, presenting a current geographic range that comprises territories from three continents. The Eurasian wild boar is widely distributed in Europe and hunting bags reveal a massive increase in the population in recent decades. This population growth may lead to increased contact with the domestic pig, consequently increasing the risk of transmission of pathogens (7, 8). As a result, infected wild boar populations may represent a threat to the pig industry and to international trade. How this affects the risk of FMD in Europe is a relevant aspect to be considered (5).

Here, we have assayed the antiviral activity in wild boar cells of three synthetic non-coding RNA molecules derived from the FMDV genome (ncRNAs) against FMDV and other relevant viral pathogens of domestic swine. The ncRNAs mimic in sequence and structure the 5′-terminal S fragment (S), the internal ribosome entry site (IRES) and the 3′ non-coding region (3′NCR), respectively (9, 10). These small and non-infectious RNA transcripts are known to elicit a robust antiviral effect based on type I interferon (IFN) induction through both Toll-like and retinoic acid-inducible gene-I (RIG-I)-like receptors (TLR and RLR, respectively) signaling pathways (11–14). The IRF3-dependent activation of the antiviral responses triggered by the 3′NCR transcripts in swine and bovine cells has been described (15). The FMDV S fragment has also been involved in modulation of innate responses in host cells (16). In previous work, we showed the enhancing effect of the IRES transcripts on the specific B- and T-cell mediated immune responses elicited by a conventional inactivated FMD vaccine in pigs, increasing the rate of protection against FMDV challenge (17). With the aim of testing the biological activity and potential application of these immunomodulatory RNA molecules in FMDV wild host species, the immune response and antiviral spectrum of the ncRNAs has been analyzed in a wild boar cell line (WSL). Our results show that transfection of wild boar cells with the ncRNAs triggered a solid and broad range innate immune response. The antiviral activity induced in transfected WSL cells effectively inhibited infection by FMDV and also by three other relevant viruses: vesicular stomatitis virus (VSV) and swine vesicular disease virus (SVDV)—two RNA viruses causing vesicular disease in pigs—and moreover, by African swine fever virus (ASFV), a complex DNA viral pathogen causing a highly virulent disease of domestic swine with devastating consequences for swine industries and food security globally.

MATERIALS AND METHODS

Cells and Viruses

WSL cell line was developed in Günther Keil laboratory (Friedrich-Loeffler-Institut, Greifswald, Germany) from wild boar lung cells (18). WSL cells were shown to have a macrophage lineage origin with the loss of some specific myeloid markers (19). Vero cells were obtained from ATCC. Swine kidney epithelial IBRS2 cells were obtained from CISA-INIA. WSL, Vero, and IBRS2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin-streptomycin and 2 mM L-glutamine (Gibco). FMDV O1BFS isolate, vesicular stomatitis virus (VSV) Indiana, swine vesicular disease virus (SVDV) SPA 93 and African swine fever virus BA71 V9 (adapted to Vero cells) were used for infection experiments. Information of titers of FMDV, VSV, SVDV and ASFV viral stocks used in this study, as well as those in WSL cells is shown in **Table 1**.

RNA Synthesis, Transfection, and RT-PCR

RNA *in vitro* transcripts corresponding to the 3′NCR (186 nt including a 58-nt polyA tail) or S fragment (5′-terminal 403 nt) of the FMDV O1K genome were synthetized using T3 RNA polymerase (NEB) and previously described plasmids as templates that were linearized with *Not*I prior to *in vitro* transcription (10). RNA corresponding to the IRES of FMDV CS8 (470 nt) was generated by *in vitro* transcription with T7 RNA polymerase (NEB) from a pGEM-derived clone (20) linearized with *Xho*I. Next, DNA was removed from the RNAs preparations by treatment with RQ1 DNase (1 U/μg; Promega). Then, RNAs were extracted with phenol-chloroform, ethanol-precipitated, and finally resuspended in water. The RNA was quantified by spectrometry and its size and integrity were analyzed by electrophoresis. RNAs were denatured/renatured by heating at 92°C for 5 min, incubation for 10 min at room temperature, and then kept on ice until transfection. In some experiments, *E. coli* MRE600 tRNA (Roche) and pI:C (Invivogen) were used as negative and positive controls, respectively. Transfection was performed using Lipofectamine 2000 (Invitrogen). Approximately 1×10^6 WSL cells were transfected with 40 μg/ml 3′NCR, S, IRES transcripts or tRNA. For RT-PCR analysis, cells were harvested at different times following transfection. Then, total RNA was extracted, quantified by spectrometry and treated for DNA removal with Turbo DNA-free kit (Ambion). RNA aliquots were subsequently analyzed by RT-PCR for amplification of swine IFN-β and Myxovirus resistance gene 1 (Mx1) or GAPDH as described (14, 21). Amplification products were detected and analyzed by electrophoresis on agarose gels (2–2.5%).

Antiviral Activity Assays

The paracrine antiviral activity of the supernatants from ncRNA-transfected WSL cells against VSV or FMDV was assayed on WSL cells, while the activity against SVDV was assayed on IBRS2 cells. The assays were performed basically as described (10). Briefly, WSL cells were transfected for 24 h with 40 μg/ml

TABLE 1 | Viral titers of the viruses used in the study in WSL cells compared to those in the cell lines where viral stocks were grown.

Virus	Titer in WSL	Titer of viral stock (cell line)
FMDV	3×10^6 pfu/ml	1×10^7 pfu/ml (SK6)
VSV	4.9×10^8 pfu/ml	2.9×10^9 pfu/ml (BHK21)
SVDV	2×10^7 pfu/ml[(a)]	3.7×10^7 pfu/ml (IBRS2)
ASFV	2.4×10^6 $TCID_{50}$/ml[(b)]	1.4×10^6 pfu/ml (Cos)

[(a)]*supernatants from SVDV-infected WSL cells were titered in IBRS2 cells.*
[(b)]*supernatants from ASFV-infected WSL cells were titered in Vero cells.*

of the ncRNAs transcripts, pI:C (Invivogen), tRNA or mock-transfected with PBS. Fresh monolayers of WSL (or IBRS2 for SVDV) cells were incubated for 24 h with the transfection supernatants (serial dilutions), washed, and infected with 50–100 PFU/10^6 cells (MOI of 0.5–1 $\times$ 10^{-4}) of VSV, FMDV or SVDV. Next, the plaques were counted 24 h after infection with VSV and FMDV or 48 h after infection with SVDV, respectively. Where indicated, the blockade of the antiviral activity in the transfection supernatants was assessed by previous incubation of the supernatants with 2 μg of specific neutralizing monoclonal antibodies against swine IFN-α (K9; PBL InterferonSource) for 1 h at 37°C. Antiviral activity was expressed as the reciprocal of the highest dilution of the corresponding supernatant reducing the number of plaques by 50%.

A VSV infection inhibition assay was performed to assess the autocrine antiviral activity induced in WSL cells by transfection with the FMDV ncRNAs. For that, WSL cells were mock-transfected or transfected with 40 μg/ml of tRNA, S, IRES or 3′NCR transcripts and infected 24 h after transfection with 50–100 PFU/10^6 cells (MOI of 0.5–1 $\times$ 10^{-4}) of VSV. Cytophatic effect (CPE) was monitored by plaque assay on semi-solid medium 24 h after infection.

To test the autocrine antiviral effect of ncRNA transfection in WSL cells against ASFV, WSL cells were mock-transfected or transfected with IRES RNA as above, and 24 h later, infected with ASFV at an MOI of 2. Cell extracts were collected at 4, 8, or 16 h after infection. The viral titers were determined by plaque assay in Vero cells at 5 days post-infection and expressed as TCID50/ml.

Immunoblot Analysis

Detection of total IRF3, phospho-IRF3 and Mx1 was performed by SDS-PAGE. IRES-transfected WSL cells were washed twice in ice-cold PBS and harvested in PBS supplemented with 1% NP-40, 1 mM DTT and 1X Complete protease inhibitor cocktail (Roche) at the indicated times after transfection. Cell extracts (20 μg) were run on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane and probed with the specific primary antibody. Then, incubation of the blots with the corresponding secondary antibody HRP conjugate (Thermo Scientific Pierce) was performed. Proteins were detected by chemiluminescent detection (NZY standard ECL, NZYTech) followed by exposure to X-ray film. The following primary antibodies were used in this study: rabbit monoclonal anti-Phospho-IRF3 (Ser 396) (4D4G, Cell Signaling), rabbit polyclonal anti-IRF3 (FL-425, Santa Cruz Biotech), mouse monoclonal anti-Mx1 (AM39, Acris Antibodies) and rabbit polyclonal anti-βII tubulin (22).

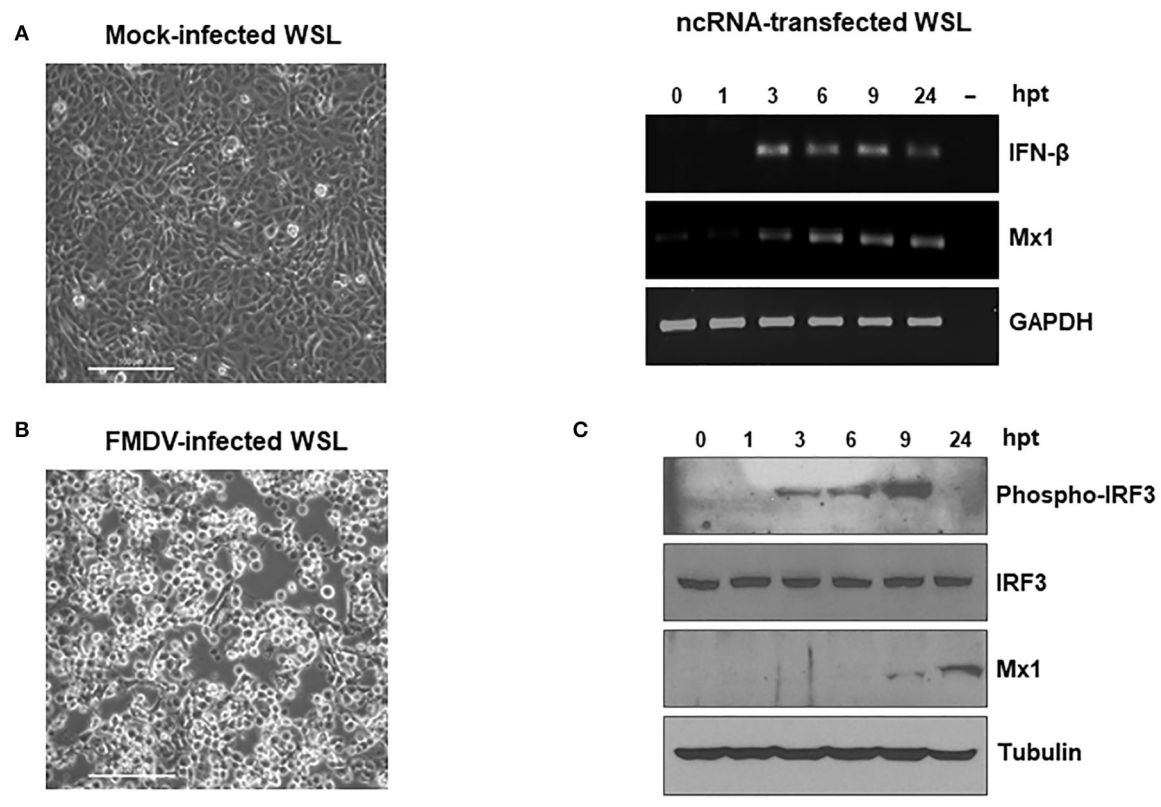

FIGURE 1 | WSL cells are susceptible to FMDV infection and induce innate immune responses upon transfection with the ncRNAs. **(A)** WSL cells were infected with FMDV O1BFS at an MOI of 0.01 or mock-infected. Images were captured 24 h after infection. Scale bars, 100 μm. **(B,C)** WSL cells were transfected with IRES RNA and cell lysates were collected at the indicated times after transfection for RT-PCR analysis of IFN-β, Mx1 and GAPDH mRNAs **(B)** or for protein detection of phospho-IRF3, total IRF3, Mx1, and tubulin by immunoblot **(C)**.

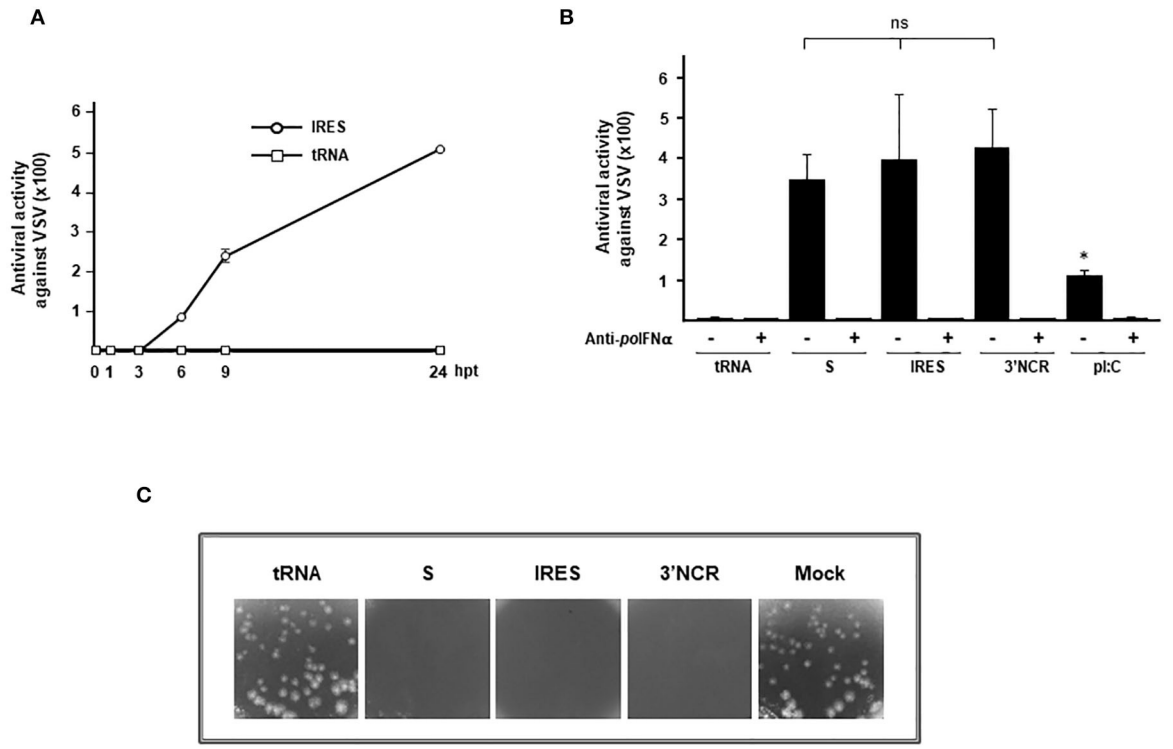

FIGURE 2 | Antiviral activity against VSV induced in WSL cells by transfection with the ncRNAs. **(A)** Antiviral activity of supernatants from IRES-transfected WSL cells, collected at different times after transfection and corresponding to lysates analyzed in **Figures 1B,C**. Supernatants from WSL cells transfected with tRNA were also analyzed as a control. The antiviral activity was assayed on fresh WSL monolayers against VSV infection. Data are average of triplicates ± SD. **(B)** The antiviral activity in supernatants from WSL cells transfected with S, IRES, 3′NCR transcripts, pI:C or tRNA for 24 h was assayed on fresh WSL monolayers against VSV. Where indicated, supernatants were incubated previously with antibodies against swine IFN-α. Data are average of triplicates from two independent experiments ± SD (*$p <$ 0.05; ns, not significant). Antiviral activity was expressed as the reciprocal of the highest supernatant dilution needed to reduce the number of VSV plaques by 50%. **(C)** Autocrine antiviral activity in WSL cells transfected with the ncRNAs. WSL cells were transfected with each ncRNA, tRNA or mock-transfected and 24 h later infected with VSV. A comparison of the CPE induced after 24 h of infection is shown.

Statistical Analysis

For comparison of data, the unpaired Student's *t*-test for independent samples was used with the IBM SPSS Statistical (v.24) software; statistically significance was considered for a $p < 0.05$. As mentioned in the corresponding figure legends, ns indicates not significant ($p > 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

RESULTS AND DISCUSSION

The FMDV IRES Triggers a Type I IFN-Dependent Innate Immune Response in Wild Boar Cells

To assess the potential protective effect of the ncRNAs in wild boar cells against FMDV infection, we first tested the susceptibility of WSL cells to the virus. When WSL were infected with FMDV, a clear CPE could be observed, being the extent of it dependent on the MOI used. **Figure 1A** shows the comparison between WSL monolayers 24 h after either infection with FMDV at an MOI of 0.01 or mock infection. Next, the effect of transfection with the IRES transcripts on IFN-β mRNA induction in WSL cells was analyzed. The 470 nt long RNA transcripts corresponding to the IRES in the 5′ NCR of the FMDV genome conferred the highest levels of protection against FMDV in mice (11) and were also able to enhance the immune response of an FMD vaccine in mice and pigs (17, 23). The RT-PCR analysis of WSL cells transfected with the IRES showed the induction of IFN-β mRNA, being detectable from 3 to 24 h after transfection (**Figure 1B**). The mRNA levels of Mx1 were also analyzed. *Mx1* is an IFN-stimulated gene (ISG) involved in anti-FMDV response in swine and bovine cells (24–28). Mx1 mRNA induction was also observed between 3 and 24 h after transfection with IRES transcripts in WSL cells (**Figure 1B**). We were also able to detect the expression of Mx1 protein at 9 and 24 h post-transfection, indicating that the gene induction observed led to productive translation of the protein (**Figure 1C**). As a result of viral infection and subsequent activation of the signaling routes (including TLR and RLR pathways) leading to promote an antiviral state, the cytoplasmic inactive form of IRF3 undergoes phosphorylation of a series of serine residues,

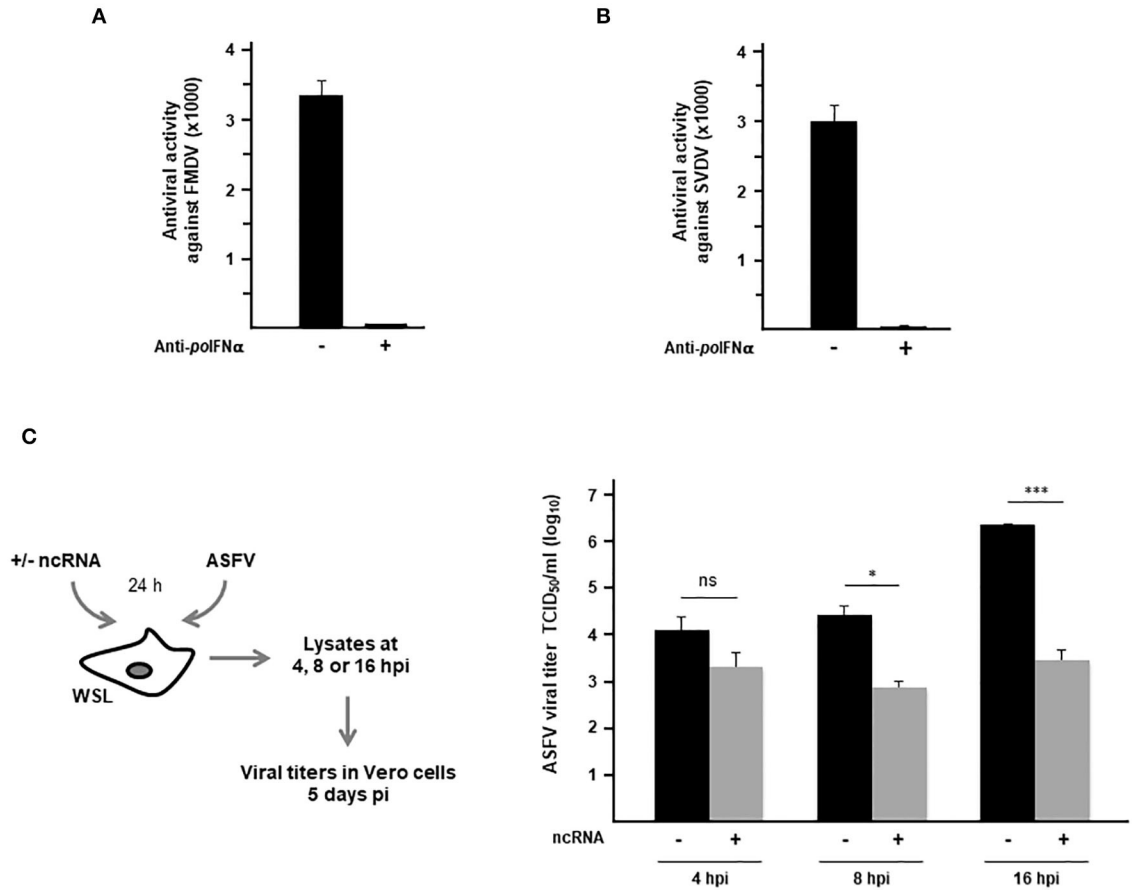

FIGURE 3 | Antiviral activity against FMDV, SVDV and ASFV induced in WSL cells by transfection with the ncRNAs. **(A,B)** Supernatants from WSL cells transfected with IRES RNA for 24 h were assayed on fresh WSL monolayers against infection with FMDV **(A)** or on IBRS2 cells against infection with SVDV **(B)**. Where indicated, supernatants were incubated previously with antibodies against swine IFN-α. Antiviral activity was expressed as the reciprocal of the highest supernatant dilution needed to reduce the number of FMDV plaques by 50%. Data are mean ± SD of triplicates. **(C)** WSL cells were transfected with IRES transcripts or mock-transfected and 24 h later infected with ASFV at an MOI of 2. Lysates were collected at 4, 8 or 16 h after infection and viral titers were determined in Vero cells after 5 days of infection. Data are average of triplicates ± SD (*$p < 0.05$; ***$p < 0.001$; ns, not significant).

dimerization and translocation to the nucleus where a protein complex is formed for activation of the type I IFN and ISG genes (29). To address whether IRF3 was being activated in ncRNA-transfected WSL cells, the levels of phosphorylated and total IRF3 in the lysates were analyzed by immunoblot (**Figure 1C**). While total IRF3 levels remained stable over time, phospho-IRF3 was initially detected at 3 h after transfection, reaching maximal levels at 9 h post-transfection, the later time coinciding with the initial detection of Mx1 protein (**Figure 1C**). Our results show that the FMDV ncRNAs can trigger type I IFN-dependent innate immune responses in wild boar cells.

Transfection With the ncRNAs Confers Protection Against VSV, FMDV, SVDV, and ASFV Infection in WSL Cells

Having shown the upregulation of IFN-β and ISGs in IRES-transfected WSL cells (**Figures 1B,C**) we sought to analyze whether the innate immune response elicited was associated with measurable antiviral activity. For that, we first tested the supernatants corresponding to IRES-transfected WSL for paracrine antiviral activity against VSV. As shown in **Figure 2A**, the antiviral activity increased over time being first detected at 6 h after transfection and reaching maximal levels 24 h post-transfection (around 500). No antiviral activity could be measured in supernatants from WSL cells transfected with tRNA (**Figure 2A**). Next, the paracrine antiviral activity in supernatants from WSL cells 24 h after transfection with each ncRNA or with pI:C (a double stranded RNA analog) was tested against VSV (**Figure 2B**). High levels of antiviral activity were found in all supernatants from ncRNA-transfected cells, with no statistically significant differences between S, IRES or 3′NCR RNAs. Transfection with pI:C induced 3–3.8-fold lower levels of antiviral activity than the FMDV ncRNAs. No sign of cytotoxicity or negative effect on cell viability was observed after transfection with any of the RNAs analyzed. In all cases, incubation with an anti-swine IFN-α antibody abrogated the antiviral activity in the supernatants (**Figure 2B**). This is in agreement with previous

work showing that IFN-α mainly accounts for the antiviral activity in swine transfected or FMDV infected cells (10, 30) despite the early induction of IFN-β mRNA observed. A possible explanation for this may be that IFN-β is translated at lower levels or that its turnover rate mRNA/protein is very rapid (30). With the aim of testing the autocrine antiviral activity induced by the ncRNAs in wild boar cells, WSL monolayers were transfected with tRNA, S, IRES, 3′NCR RNAs, or mock-transfected and 24 h later, cells were infected with VSV. While the CPE observed in tRNA- and mock-transfected cells was equivalent, no sign of infection was detected in WSL cells transfected with each of the three ncRNAs, suggesting that transfected cells were protected against VSV infection (**Figure 2C**). Altogether, these results show that the FMDV ncRNAs are able to induce a fast and potent autocrine and paracrine antiviral response in wild boar cells against VSV.

With the purpose of exploring the activity of the FMDV ncRNAs in wild boar cells against relevant viral pathogens affecting domestic pigs, antiviral activity assays were carried out against FMDV, SVDV and ASFV. When the paracrine antiviral activity in supernatants from WSL cells transfected with IRES transcripts was assayed against FMDV infection, very high levels of protection were observed with an average titer over 3,000 which was completely abrogated by previous treatment with anti-swine IFN-α antibodies (**Figure 3A**). Similarly, very high levels of antiviral activity against SVDV infection were observed (**Figure 3B**). In this case, supernatants of transfected WSL cells were assayed in swine kidney IBRS2 cells, as infection with SVDV induced a diffuse cell detachment but not a clear CPE in WSL cells (see **Table 1**). The role of wild boar in SVDV transmission is still controversial. While it is considered likely to be susceptible to SVDV infection, serological surveys suggest that wild boars do not serve as reservoir hosts in Europe (31). Next, we wanted to test whether ncRNA transfection in wild boar cells might have an inhibitory effect against infection with ASFV. Persistence of ASFV in wild boar in Eastern Europe remains a significant threat to domestic pig populations globally (32). Unlike VSV, FMDV, or SVDV, ASFV is a genetically complex double stranded DNA virus. As shown in **Figure 3C**, the differences between viral titers recovered from WSL cells that had been previously transfected with the IRES transcripts, compared with those in mock-transfected cells, increased over time as infection proceeded, and reaching statistical significance at 8 h post-infection. Remarkably, an 800-fold reduction in viral titers in IRES-transfected wild boar cells was observed at 16 h after infection with ASFV (**Figure 3C**).

WSL cells used in this study have a macrophage lineage origin. Though some of the viruses tested in WSL cells replicate mainly in epithelial cells, porcine cell lines developed from alveolar macrophages have been shown to be a valuable tool for viral pathogenesis and immune function studies, being susceptible to a wide variety of viruses including VSV and SVDV (33).

To conclude, the current study presents new data on the antiviral effect of the ncRNAs in wild boar cells, a wild animal host species for FMDV and many other pathogens affecting domestic pigs with a potential relevance in FMD epidemiology, especially considering the increasing population of the wild boar in Europe. Our results show a robust and broad range of antiviral activity against FMDV, other viruses causing vesicular disease in swine (VSV and SVDV) and ASFV, being the later, together with FMDV, a major concern in animal health worldwide. The possibility of implementing antiviral strategies in wild animals in contact with farm species during outbreaks is an interesting point for debate and further studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MR and MS designed the study. MS obtained funding and wrote the manuscript. MR and RH performed the experiments. All authors analyzed the data, revised, and approved the manuscript.

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Immunogenicity of a Dendrimer B_2T Peptide Harboring a T-Cell Epitope From FMDV Non-structural Protein 3D

Rodrigo Cañas-Arranz[1†], Patricia de León[1†], Mar Forner[2†], Sira Defaus[2], María J. Bustos[1], Elisa Torres[1], David Andreu[2], Esther Blanco[3*] and Francisco Sobrino[1*]*

[1] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Madrid, Spain, [2] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain, [3] Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain

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***Correspondence:**
David Andreu
david.andreu@upf.edu
Esther Blanco
blanco@inia.es
Francisco Sobrino
fsobrino@cbm.csic.es

[†]These authors have contributed equally to this work

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Synthetic dendrimer peptides are a promising strategy to develop new FMD vaccines. A dendrimer peptide, termed B_2T-3A, which harbors two copies of the major FMDV antigenic B-cell site [VP1 (140–158)], covalently linked to a heterotypic T-cell from the non-structural protein 3A [3A (21–35)], has been shown to protect pigs against viral challenge. Interestingly, the modular design of this dendrimer peptide allows modifications aimed at improving its immunogenicity, such as the replacement of the T-cell epitope moiety. Here, we report that a dendrimer peptide, B_2T-3D, harboring a T-cell epitope from FMDV 3D protein [3D (56–70)], when inoculated in pigs, elicited consistent levels of neutralizing antibodies and high frequencies of IFN-γ-producing cells upon *in vitro* recall with the homologous dendrimers, both responses being similar to those evoked by B_2T-3A. Lymphocytes from B_2T-3A-immunized pigs were *in vitro*-stimulated by T-3A peptide and to a lesser extent by B-peptide, while those from B_2T-3D- immunized animals preferentially recognized the T-3D peptide, suggesting that this epitope is a potent inducer of IFN-γ producing-cells. These results extend the repertoire of T-cell epitopes efficiently recognized by swine lymphocytes and open the possibility of using T-3D to enhance the immunogenicity and the protection conferred by B_2T-dendrimers.

Keywords: FMDV, vaccines, dendrimer peptides, T-cell epitopes, swine

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals that is caused by a virus belonging to the *Picornaviridae* family: FMD virus (FMDV). Although the mortality rate is low, FMD is feared in farm industry and animal health because, in an outbreak, massive culling of infected or suspected animals is mandatory, with devastating economic impact. In addition, FMD control is costly in endemic countries in which current vaccines based on inactivated viruses are being used for disease control (1). Nevertheless, several drawbacks associated with these vaccines have led FMD-free countries to follow non-vaccination policies, increasing the risk of disease reintroduction and severe outbreaks (2). Therefore, the development of safer and effective vaccines is a major priority for FMD control including those based on viral subunits (3–5).

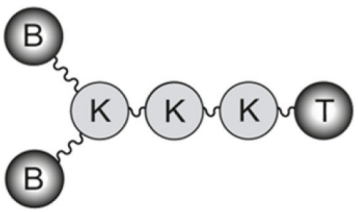
Targeting capsid protein VP1 for the induction of neutralizing anti-FMDV antibodies was one of the first attempts to produce peptide-based subunit vaccines. Among the advantages of peptide vaccines are: (i) safety, as non-infectious material is used, and no reversion to virulence is possible, (ii) DIVA condition (efficient serological distinction between infected and vaccinated animals), (iii) easy to handle and store (no cold chain is required), (iv) chemical stability, and (v) affordable large scale production. The first attempts to produce vaccines based on synthetic VP1 capsid protein were reported in the early 80s (6), but later reports evidenced the low immunogenicity of VP1, probably due to non-native folding when expressed in a non-capsid protein context. Since then, peptides corresponding to the G-H loop in VP1 have been used as the main component of FMD peptide vaccines (7–9). An important advantage of this B-cell epitope is that it is structurally continuous and easy to mimic as a peptide.

Despite the vaccine potential of FMDV peptides, the main limitation faced during decades was their weak immunogenicity when compared with conventional vaccines that use inactivated virus as immunogen (10), a limitation that may lead to selection of antigenic variants in partially immunized animals (11). Optimization of the B-cell sites and inclusion in peptide vaccines of specific T-cell epitopes recognized by different MHC molecules capable of evoking adequate T-cell responses, are requirements for optimal production of FMDV neutralizing antibodies (nAbs) and have therefore been included in the composition of linear vaccine peptides (12–15). Nevertheless, since classical linear peptides barely achieved levels of protection in livestock as those required for their use as commercial vaccines (11, 16), multimerization strategies have been developed to overcome this low-immunogenicity. One of these approaches relies on so-called multiple antigenic peptides (MAPs), in which the B-cell epitope branches out from a lysine core scaffold giving rise to a dendrimer display (17). Interestingly, two doses (2 mg each) of a dendrimer peptide displaying four copies of the G-H loop from a type C FMDV linked to a heterotypic and highly conserved T-cell epitope from FMDV 3A protein [3A (21–35)], were able to protect pigs against homologous FMDV challenge (18). More remarkably, downsized versions bearing two copies of the B-cell epitope afforded full protection in swine against an epidemiologically relevant type O FMDV even upon a single peptide dose (19, 20).

The protective responses elicited by B_2T-3A and other related dendrimeric constructs (hereafter B_2T-dendrimers), associate with the induction of high titers of nAb and the activation of specific lymphocytes that would provide T-cell help for effective production of nAbs (18, 19). Besides, such T-cell epitopes can also stimulate T-cell subsets leading to the expression of IFN-γ, a cytokine with a relevant role in the antiviral response (21). Thus, a further characterization of the functional role of the T-cell epitope(s) recognized by swine lymphocytes in the B_2T-dendrimers is relevant to understand how they work and to design vaccine improvements. Moreover, the MHC restriction phenomenon can limit the recognition by T-cells of B_2T-3A and related peptide dendrimers among different pig individuals as well as between FMDV host species (22, 23). This makes the functional characterization of B_2T-dendrimers encompassing T-cell epitopes other than T-3A an interesting goal.

Here, we show that a B_2T-dendrimer (termed B_2T-3D) including the porcine T-cell epitope identified in the 3D FMDV protein [3D (56–70)] previously shown to be promiscuous and heterotypic T-cell epitope (24) can elicit in pigs nAbs titers and IFN-γ-producing cells at levels similar to those induced by the dendrimer peptide B_2T-3A.

TABLE 1 | B_2T bivalent dendrimeric constructions.

	B_2T dendrimers	
General structure[a]		
B epitope	acetyl-PVTNVRGDLQVLAQKAARTC-amide	
T-3A	AAIEFFEGMVHDSIK-amide	
T-3D	IFSKHRGDTKMSAED-amide	
Peptides	**B_2T-3A**	**B_2T-3D**
MW[b]	6742.8 Da	6770.8 Da
HPLC[c]	6.9 min (98%)	5.1 min (98%)

[a] *B_2T(mal) construct with the B epitope linked to a 3-maleimidopropionic acid unit attached in the Lys core and linked to the T cell epitope (T3-A or T-3D).*
[b] *Experimental peptide mass obtained by LC/MS.*
[c] *Retention time on a C18 column (Luna, 4.6 × 50 mm, 3 mm; Phenomenex) eluted with a 20–60% linear gradient of solvent B (0.036% TFA in MeCN) into solvent A (0.045% TFA in H_2O) over 15 min. In parenthesis, homogeneity of purified material.*

MATERIALS AND METHODS

Peptides

The B-cell epitope from FMDV (O/UK/11/2001), VP1 (residues 140–158), and the T-cell epitopes 3A (residues 21–35) and 3D (residues 56–70) were synthesized by Fmoc-solid phase synthesis (SPPS), purified by reverse-phase liquid chromatography (RP-HPLC) and characterized by mass spectrometry (MS). B_2T-dendrimers were prepared by conjugation in solution of two B-cell peptides containing and additional C-terminal Cys (free thiol form) with one T-cell epitope N-terminally elongated with two Lys residues followed by an extra Lys branching point further derivatized into two maleimide groups (**Table 1**). The B_2T-3A and B_2T-3D constructs were obtained via thiol–maleimide ligation at pH 6.0, purified by RP-HPLC and characterized by MS (18, 19, 25).

Viruses

The FMDV stocks (O/UK/11/2001), O/SKR, O_1Manisa, O_1BFS (The Pirbright Institute, UK) and O_1Campos (OPS-PanAftosa) were amplified in IBRS-2 cells and type C CS8-c1 virus (26) was amplified in BHK-21 cells.

Animals

Mice

Groups of five 5-to-6-week-old outbred female mice were (Swiss ICR-CD1, Envigo) were maintained under standard housing conditions at CBMSO animal facility. Mice were immunized subcutaneously at days 0 and 21 with 100 μg of each B_2T-dendrimer peptide emulsified in Montanide ISA 50V2 (Seppic-France) and euthanized at day 40. Blood samples were collected at days 0, 21, and 40 post-immunization (pi). Experimental procedures were conducted in accordance with protocols approved by the CSIC Committees on Ethical and Animal Welfare and by the National Committee on Ethics and Animal Welfare (PROEX 034/15).

Pigs

White cross-bred Landrace female pigs, 9–12 weeks-old (20 Kg), were maintained in a conventional farm facility at the Departamento de Reproducción Animal, INIA, Madrid. Groups of four pigs were immunized with B_2T-3A or B_2T-3D at day 0 with 2 ml of Montanide ISA 50V2 emulsion containing 2 mg of the corresponding peptide and boosted at day 21 pi. Two additional pigs were PBS-inoculated and maintained as controls. Blood samples were collected at days 0, 7, 14, 21, 28, 35, and 70 pi to obtain serum and peripheral blood mononuclear cells (PBMCs). The study was approved (CBS2014/015 and CEEA2014/018) by the INIA Committees on Ethics of Animal Experiments and Biosafety, and by the National Committee on Ethics and Animal Welfare (PROEX 218/14).

Virus Neutralization Test (VNT)

Neutralization assays were performed in 96-well culture plates. Serial 2-fold dilutions of each serum sample (in DMEM containing 2% fetal bovine serum) were incubated with 100 infection units−50% tissue culture infective doses ($TCID_{50}$)– of FMDV (O/UK/11/2001) for 1 h at 37°C. Then, a cell suspension of IBRS-2 cells in DMEM was added and plates were incubated for 72 h. Monolayers were controlled for development of cytopathic effect (cpe), fixed, and stained. End-point titers were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of homologous FMDV in 50% of the wells (19). For cross neutralization assays, incubation of sera with the panel of FMD viruses (O/SKR, O_1Manisa, O_1BFS, and O_1Campos) that belonged to different type O topotypes was performed in parallel to that of the homologous isolate O/UK/11/2001 and the negative control type C CS8-c1 virus. The antigenic relationship of viruses was calculated by the ratio r_1 = nAb titers against the heterologous virus/nAb titer against homologous virus, as reported (27).

Detection of Anti-FMDV Antibodies by ELISA

Specific antibodies were assayed by ELISA as described (19) using plates coated with peptide B (1 μg) that were incubated with 3-fold dilutions of serum and detected using HRP-conjugated protein A. Plates were read at 450 nm and titers expressed as the reciprocal of the last serum dilution given an absorbance range of two standard deviations above the background (serum at day 0) plus 2 SD.

PBMC Isolation and IFN-γ Detection by ELISPOT

Porcine PBMCs were isolated from blood samples collected in Vacutainer tubes EDTA-K2, diluted 1:1 in PBS and then used to obtain PBMC by density-gradient centrifugation with Histopaque 1077 (Sigma) and Leucosep tubes (Greiner Bio-One) as described (28). For the IFN-γ ELISPOT assay 2.5×10^5 PBMCs were shed in triplicate wells of Immobilon-P plates (Merck Millipore) coated as reported (19) and *in vitro* stimulated with 50 μg/ml of their respective immunogenic peptides. As positive or negative controls, cells were incubated with 10 μg/ml of phytohaemagglutinin (Sigma) or only with medium, respectively. After 48 h at 37°C and 5% CO_2, plates were washed and incubated with a biotinylated mouse anti-pig IFN-γ (clone P2C11, BD) followed by streptavidin:HRP (BD). The frequency of peptide-specific T-cells was expressed as the mean number of spot-forming cells/10^6 PBMCs, with background values (number of spots in negative control wells) subtracted from the respective counts of stimulated cells. These experiments were performed using outbred domestic pigs with different individual genetic backgrounds. In any case, the levels of animal-to-animal variation did not exceed those observed in other related studies (11).

Statistical Analyses

Differences among peptide-immunized groups in FMDV-antibody titers and number of IFN-γ producing cells were analyzed using the Student's *t*-test. Values are cited in the text as mean ± SD. All *p*-values are two sided, and $p < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism Software 5.0.

RESULTS

Analysis of the Humoral Immune Response Elicited by B_2T-3D FMDV Dendrimer in a Mouse Model

The mouse strain Swiss ICR (CD1®) offers the possibility of conducting immunogenic studies in outbred populations that mimic the heterogeneous genetic background of natural FMDV hosts (29). As previous results showed that peptide B_2T-3A was able to induce significant levels of nAbs in outbred Swiss ICR mice, this strain was used to evaluate the immunogenicity of the dendrimer B_2T-3D. To this end, groups of five mice were immunized with each B_2T-dendrimer construction.

Total IgG antibodies against B-cell peptide were measured by ELISA after one dose (day 21 pi) or two doses of peptide (day 40 pi). After the first dose, B_2T-3A induced antibody titers (3.4 ± 0.9 $\log_{10}$), whereas no antibodies were detected in any animal from the B_2T-3D group (**Figure 1A**). After the peptide boost, antibody titers increased significantly in the B_2T-3A group

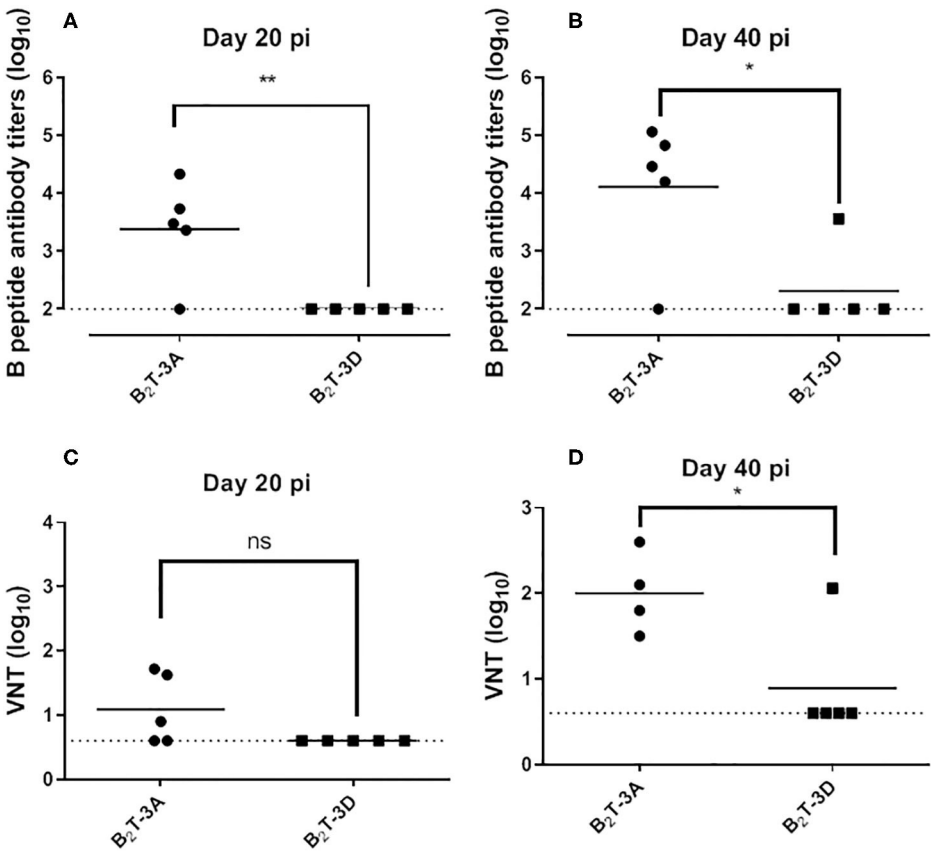


FIGURE 1 | FMDV antibodies elicited in mice by B_2T-3A and B_2T-3D dendrimers. Total IgG antibodies against peptide B detected by ELISA in sera from immunized mice after the first (day 21 pi) **(A)** and the second peptide dose (day 40 pi) **(B)**. Virus neutralization titers, expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of homologous FMDV, after the first **(C)** and second peptide dose **(D)**. Each point represents the mean of a triplicate value of a single animal. Horizontal bars indicate the mean of each group. Statistically significant differences are indicated by asterisks (*) for $p < 0.05$ and (**) for $p < 0.005$; (ns) statistically non-significant difference. A representative experiment out of five is presented.

(4.1 ± 1.2 $\log_{10}$) while only one animal from B_2T-3D group showed detectable antibodies (**Figure 1B**).

Next, neutralizing activity against homologous FMDV was analyzed in sera from immunized mice. At day 21 pi, nAbs were detected in animals from B_2T-3A (1.1 ± 0.6 $\log_{10}$). In contrast, none of the mice immunized with B_2T-3D displayed detectable nAbs (**Figure 1C**). After the boost, the titers increased in the B_2T-3A group (2 ± 0.5 $\log_{10}$), while only one of the B_2T-3D immunized mice showed detectable levels of nAbs (**Figure 1D**).

These results suggest that the T-3D epitope incorporated in the B_2T-dendrimeric construction is not efficiently recognized as a T-helper epitope in Swiss ICR mice.

Immunogenicity in Swine of a B_2T Construction Harboring a T-Cell Epitope From FMDV Non-structural Protein 3D

The above results indicate that the 3D epitope previously identified as a T-cell epitope in swine was not efficiently recognized by murine lymphocytes (**Figure 1**). To confirm its potential to immunomodulate the response to B_2T-dendrimers comprising the antigenic B-cell site on the VP1 GH loop in pigs, we decided to test in parallel the immune response elicited by B_2T-3D and B_2T-3A in this species, including the longevity of the response. To this end, groups of four pigs were immunized with 2 mg of B_2T-3A (pigs 80, 81, 82, and 83), B_2T-3D (pigs 84, 85, 86, and 87) or non-immunized (88 and 89). At day 21 the animals were boosted with the same amount of peptide and sera and PBMCs samples were collected at the indicated times. One animal from B_2T-3A group (pig 83) showed a deteriorated health status during the second week of the experiment, being excluded from the analysis.

Dendrimers B_2T-3D and B_2T-3A Elicit Similar Antibody Responses

The total IgG antibodies elicited by the peptides were measured by ELISA. Specific antibodies were detected in both groups at day 14 pi from which a gradual increment was observed. No remarkable boost effect was observed neither in B_2T-3A nor in B_2T-3D immunized pigs and high levels of IgG antibodies were maintained until day 70 (2 months pi) without significant differences between the two groups. As expected, no specific

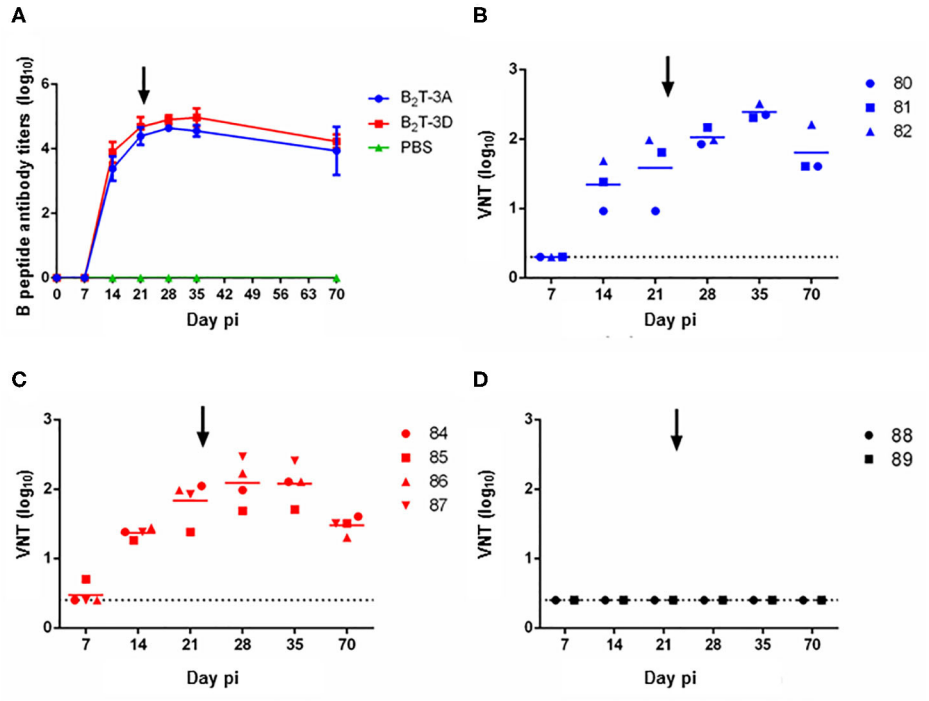


FIGURE 2 | Peptides B_2T-3D and B_2T-3A induce similar antibody responses. **(A)** Total IgG specific antibody titers measured by ELISA in sera collected at different days pi. Points depict mean antibody titers for each group of pigs. VNT in sera from animals immunized with **(B)** B_2T-3A, **(C)** B_2T-3D, and **(D)** non-immunized. Titers are expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of homologous FMDV. Each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each animal group ($n = 4$) and dotted lines the detection limit. The arrows show the day of the boost.

antibodies were detected in the sera from control PBS-inoculated pigs (**Figure 2A**).

Next, the ability of these antibodies to *in vitro* neutralize homologous virus was tested. B_2T-3A-immunized pigs elicited nAbs by day 14 pi (1.3 ± 0.4 $\log_{10}$) that increased by day 21 pi (1.6 ± 0.6 $\log_{10}$). After the second peptide dose, the titers increased reaching an average value of 2 ± 0.1 $\log_{10}$ at day 28 pi, and a peak at day 35 pi (2.4 ± 0.1 $\log_{10}$), following a gradual smooth decrease until day 70 pi (1.8 ± 0.4 $\log_{10}$) (**Figure 2B**).

The nAbs from B_2T-3D vaccinated group followed a similar time course and no significant differences were found when compared with B_2T-3A. At day 14 pi, nAbs titers were first observed (1.4 ± 0.1 log10) and increased at day 21 pi (1.8 ± 0.3 $\log_{10}$). After the boost, the average titers reached the peak at day 28 pi (2.1 ± 0.3 $\log_{10}$) and were maintained until day 35 pi (2.1 ± 0.3 $\log_{10}$). A slight decrease, similar to that observed in the B_2T-3A group, was detected at day 70 pi (1.5 ± 0.1 $\log_{10}$) (**Figure 2C**). No neutralizing activity was found in sera from PBS-inoculated animals at any time point (**Figure 2D**).

Thus, B_2T-3D elicited an antibody response in pigs that paralleled that of B_2T-3A.

T-Cell Responses Elicited by B_2T-3D and B_2T-3A

The ability of peptide B_2T-3D to induce specific T-cell responses was assessed in PBMCs isolated from immunized pigs by ELISPOT analysis of the IFN-γ-secreting cells. In this experiment, the B-cell peptide was included as stimulus for the *in vitro* recall, to address the possibility of its recognition by T-cells. As in previous experiments, intragroup variability was observed in the responses, which was reflected in the presence in each group of high responders (B_2T-3A: pigs 81 and 82; B_2T-3D: pigs 86 and 87) and low responders (B_2T-3A: pig 80; B_2T-3D: pigs 84 and 85). A remarkable primary response of IFN-γ secreting cells was noticed at day 14 pi (**Figure 3**). Interestingly, the two high responder pigs in the B_2T-3D group showed more IFN-γ spots than the higher responders in the B_2T-3A group when their PBMCs were stimulated with the whole homologous dendrimer (1.743 ± 364 for B_2T-3D group vs. 1.206 ± 244 for B_2T-3A group) and the specific T-cell epitope (1.679 ± 453 vs. 959 ± 587). The magnitude of the responses was lower when cells were stimulated with the B-cell peptide, with the higher values being in pigs immunized with B_2T-3A (130 ± 97 vs. 507 ± 183) (**Figure 3**). At day 21 pi the response weaned in both groups reaching similar levels of IFN-γ spots when cells were stimulated with the dendrimer (847 ± 105 vs. 800 ± 224) and the T-cell epitope (785 ± 5 vs. 646 ± 382). At this time, responses against B-cell peptide were clearly lower (42 ± 16 vs. 286 ± 186) (**Figure 3**). After the boost, a non-immediate secondary response was observed at day 35 pi in the two major responders in each group when stimulated with the corresponding dendrimer ($1,073 \pm 132$ vs. $1,161 \pm 87$, respectively). However, when stimulated with the T-cell peptide, the IFN-γ production was

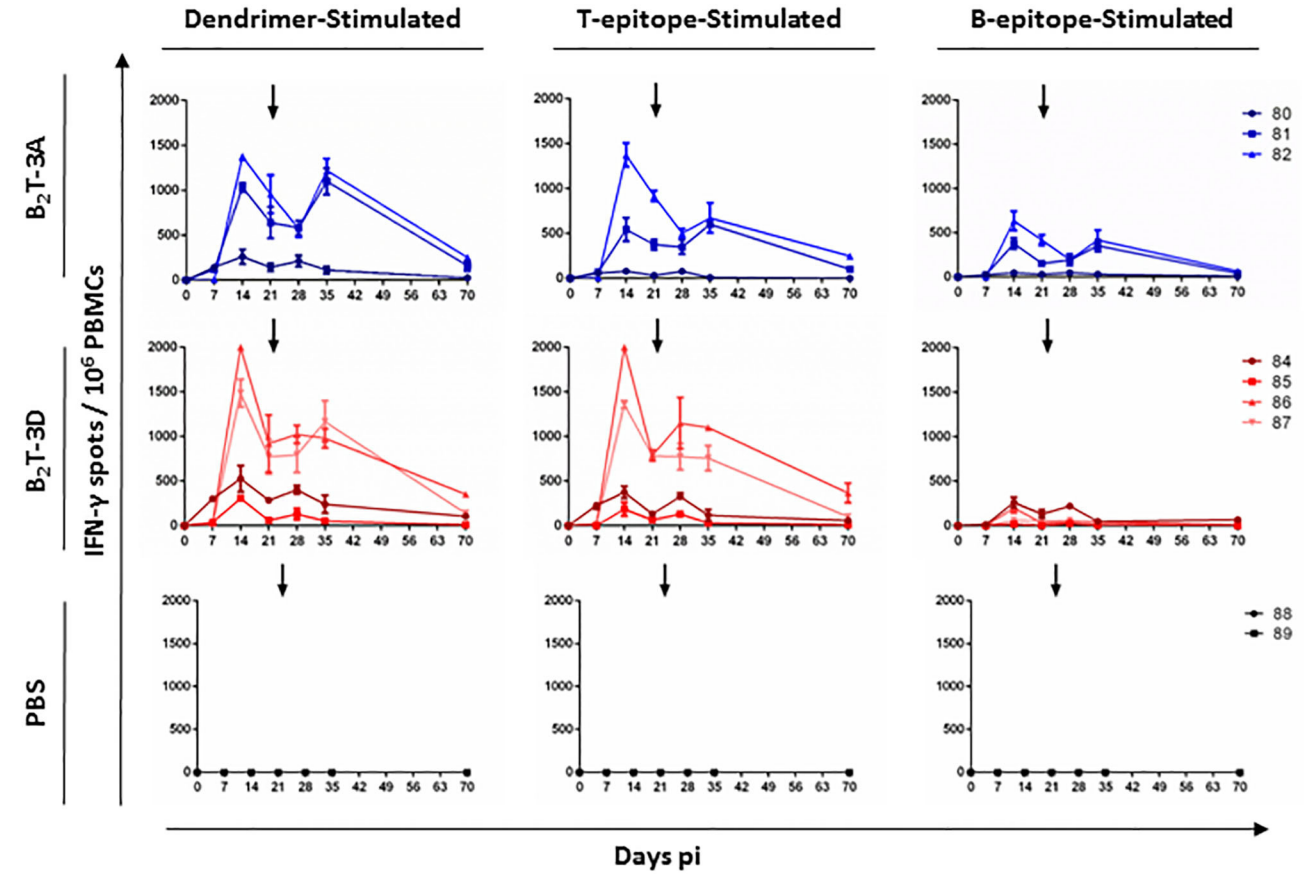


FIGURE 3 | T-cell responses in pigs immunized with B_2T-3A and B_2T-3D constructions. PBMCs isolated from individual animals of each group were collected at different days pi. Cells were stimulated *in vitro* for 48 h with homologous dendrimer, T-cell epitope or B-cell epitope, and the number of cells expressing IFN-γ was measured by ELISPOT. PBMCs stimulated with medium (not shown) were included as a negative control of the assay and subtracted. Each point represents the mean of a triplicate of an individual animal. Arrows show the day of the boost.

higher in the B_2T-3D group (929 ± 242 vs. 637 ± 52). The response dramatically weaned in both groups being scarcely detected at day 70 pi. As expected, non-immunized animals did not induce IFN-γ secreting cells upon stimulation with any of the specific peptides (**Figure 3**).

Interestingly, the frequencies of IFN-γ spots in response to the B-cell peptide in pigs from the B_2T-3D group were considerably lower than those of B_2T-3A immunized animals. These results suggest that the B-cell epitope plays a minor role in cytokine production in B_2T-3D immunized pigs making T-3D epitope a more potent inducer of IFN-γ-producing cells compared to T-3A epitope.

Dendrimers B_2T-3D and B_2T-3A Elicit nAbs Against a Broad Spectrum of Type O FMDVs

The high antigenic diversity of FMDV makes the development of vaccines a challenging issue. Since type O FMDVs are responsible of many of the current FMD outbreaks in endemic countries, a broad-spectrum response is necessary for optimal vaccines against this serotype (30). Therefore, we were interested in assessing the neutralization range afforded in pigs by the B_2T-dendrimers studied. To this end, sera recovered from the pigs vaccinated in this study with peptides B_2T-3A and B_2T3-D, as well as those of pigs previously immunized with B_2T-3A (20, 31) were tested for their ability to neutralize a panel of type O FMDVs (**Figure 4**). The FMDV isolates selected belonged to different type O topotypes, i.e., viruses from different spatiotemporal locations. A non-related serotype C FMDV isolate (CS8-c1) was included as a serotype-specific control.

At day 21 pi, animals immunized with B_2T-3A and B_2T-3D showed nAbs titers against the panel of FMDVs without significant differences among the viruses compared (**Figure 4A**). A similar neutralizing profile was observed at day 28 pi, after the second immunization with the same dose of each of the peptides (**Figure 4B**). The level of neutralization afforded at day 21 pi by B_2T-3A and B_2T-3D against the panel of viruses relative to the homologous isolate O/UK/11/2001 (r1 value; VNT ratio: virus problem/O/UK/11/2001) is shown in **Figures 4C,D**. While for B_2T-3D the data compared belong to the four pigs immunized in this study (**Figure 4D**), for

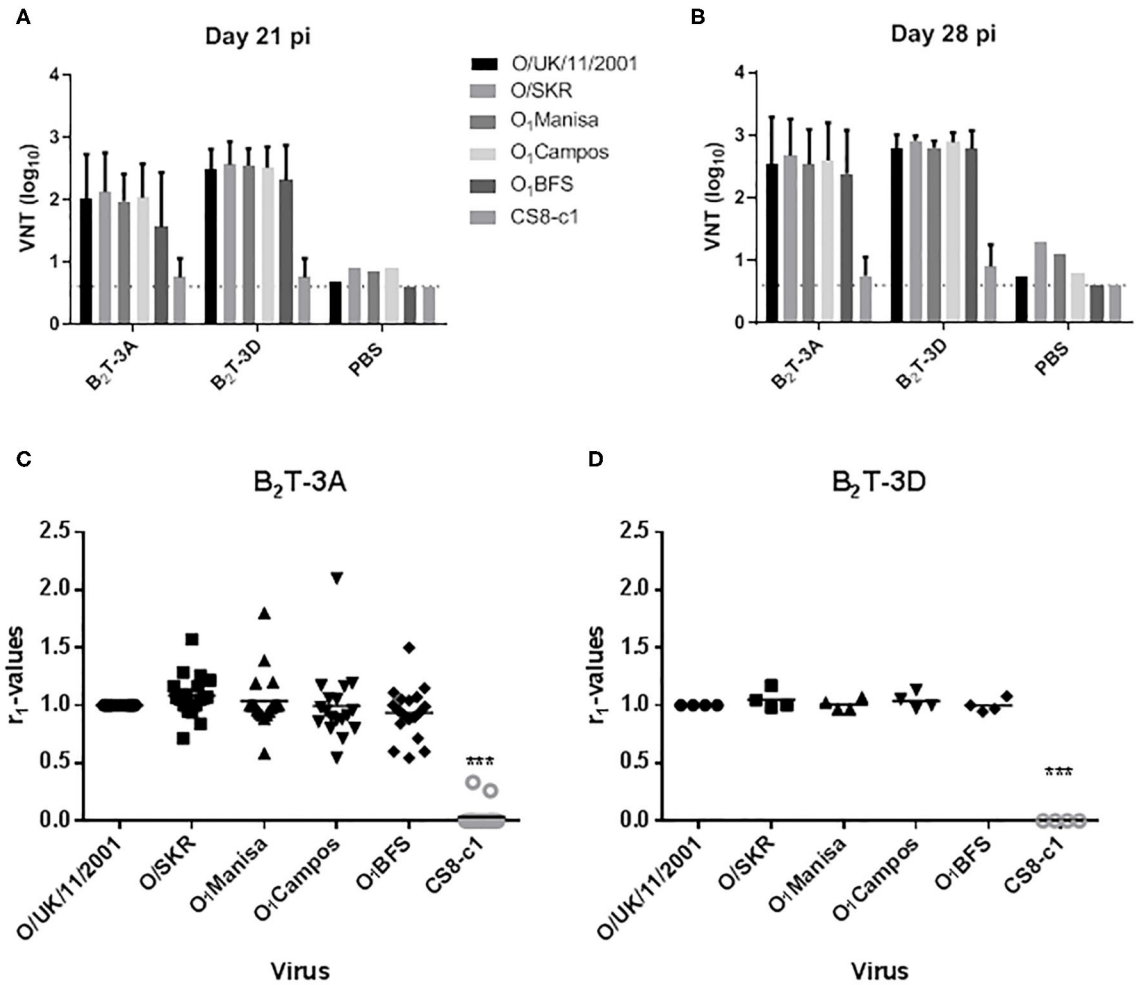


FIGURE 4 | Sera from pigs immunized with B_2T-dendrimers can neutralize a wide panel of different FMDVs type O topotypes. Sera recovered from animals immunized with B_2T-3A and B_2T-3D at days **(A)** 21 and **(B)** 28 pi were tested for its capability to neutralize a panel of different type O FMDVs. Individual columns represent the mean of each group ($n = 4$) ± SD. Values are expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of each FMDV. **(C,D)** Antigenic relationship (r_1) values of the six viruses. The serological match (r_1-values; calculated as described in Materials and Methods) of sera from pigs immunized with **(C)** B_2T-3A including those from previous experiments (20, 31) and **(D)** B_2T-3D is shown and each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each animal group against each virus ($n = 18$ for B_2T-3A and $n = 4$ for B_2T-3D). Statistically significant differences are indicated by asterisks (***) for $p < 0.0005$.

B_2T-3A, a total of 18 animals, including those immunized with this dendrimer in previous works (20, 31) were included (**Figure 4C**). All the viruses tested showed r1 values similar or for some of the animals immunized with B_2T-3A even higher than that of O/UK/11/2001. As expected, the nAbs were serotype-specific and none of the sera from any immunized animal was able to neutralize type C CS8-c1 virus (**Figure 4**).

These results support that B_2T-dendrimers induce a broad anti-FMDV immunity within a serotype, which can be considered as an important valuable asset for their potential use in endemic countries where a wide spectrum of antigenic variable pools of FMDVs can circulate.

DISCUSSION

For a rational FMDV peptide vaccine design, the incorporation of B-cell antigenic sites that fully mimic their native viral conformation and are efficiently recognized by B-cells, as well as species embodying T-cell epitopes that provide an adequate T-cell help and recognition from lymphocytes, are key issues. In this context, the characterization of FMDV-specific epitopes functionally analogous to T-3A is a relevant work to extend the repertoire of T-cell epitopes to be included in dendrimeric vaccines that particularly face the MHC (SLA in swine) polymorphisms of different pig breeding's, thus becoming a potential manner to increase the quality of the immune responses elicited by peptide-based vaccines.

We previously reported that alternative subunit vaccines consisting of multiple FMDV antigenic peptides, including B_2T-3A, induced similar nAb titers in outbred Swiss mice as those elicited in pigs (31, 32). Therefore, in this work we first addressed the immunogenicity of B_2T-3D dendrimer peptide, using this mouse model as a screening system to confirm that this construction, harboring a T-cell epitope identified in swine, retained the ability to elicit FMDV nAbs. Unexpectedly, our results indicate that peptide B_2T-3D did not induce Ab in mice unlike the previous analog B_2T-3A. These results suggest that the T-3D epitope comprised in the B_2T-dendrimeric platform is not efficiently recognized as a T-helper epitope in Swiss mice, probably due to its low affinity for mouse MHC class II haplotypes. Nevertheless, we cannot rule out that an inefficient processing of the epitope and/or conformation alterations affecting to the correct cross-linking of the B-epitope can also be contributing to the lack of antibody induction observed. Thus, further work is required to confirm the lack of recognition of T-3D by murine T-cells. In any case, our results evidence the limitations of mouse models for the analysis of the role of FMDV-specific T-cell epitopes (33–35).

Replacement of T-3A or its combination with other T-cell peptides are possibilities to explore the effect of altering the recognition B_2T constructions by T-cells. As commented above, different T-cell epitopes previously identified in swine were not efficiently recognized by murine lymphocytes. Thus, despite the limited amount of nAbs elicited by B_2T-3D in mice, we selected T-3D to study the effect of its inclusion on the immunogenicity of B_2T-dendrimer in swine, showing that B_2T-3D and B_2T-3A elicited similar antibody responses, with titers being consistent by day 70 pi, as previously reported for B_2T-3A (31).

Animal-to-animal variation observed is a common feature in previous studies with peptide and other subunit vaccines (9, 11, 18, 31, 36, 37). As in all FMDV natural hosts, the genetic background of individual pigs may differ. Thus, a pool of SLA alleles exists in the population and, as mentioned above, this polymorphism can contribute to the individual variability observed (38).

Both B_2T-3A and B_2T-3D elicited consistent levels of neutralizing antibodies. As previously reported, B_2T-3A also induced IFN-γ expressing T-cells that were *in vitro* recalled by T-3A peptide and, interestingly to a lower extent, by B-cell peptide with similar time courses, supporting that both sequences were recognized as T-cell epitopes. Conversely, the IFN-γ expressing cells elicited by B_2T-3D preferentially recognized the T-3D peptide, suggesting that this epitope is a potent inducer of IFN-γ. Further experiments are in progress to confirm the immunostimulatory differences between T-3A and T-3D.

Implementation of efficient vaccination campaigns against FMD requires the use of inactivated viruses capable of eliciting protective responses against circulating and emerging FMDVs, including serotype- specific vaccine isolates into vaccine formulations (39). Thus, because of the wide antigenic range presented by FMDV, an optimal vaccine needs to protect against a wide FMDV spectrum. This is particularly the case for vaccines against type O viruses, which are responsible for major outbreaks in epidemic countries (40).

Initial experiments with linear peptides indicated that the elicited nAbs were able to neutralize not only the homologous virus, whose sequence contains the VP1 GH-loop, but also heterologous FMDV isolates (41). Our results show that dendrimer peptide B_2T-3D elicited, in most cases, high titers of cross-neutralizing antibodies, which, for some isolates, were higher than those against the homologous virus in a similar manner than when using B_2T-3A to immunize pigs. Multiple factors inherent to the assay such as the differences in thermal stability among the viral isolates analyzed, can contribute to explain these observations, which have also been reported for type A FMDV conventionally vaccinated animals (27) and for an adenovirus-vectored type O FMDV vaccine (42). On the other hand, the modular approach used also allows extension to other FMDV serotypes.

In summary, a B_2T-dendrimer incorporating FMDV T-cell epitope T-3D elicits high levels of neutralizing antibodies and a potent response of IFN-γ producing-cells. These results extend the repertoire of T-cell epitopes efficiently recognized by swine lymphocytes and open the possibility of using T-3D to enhance the immunogenicity and the protection conferred by B_2T-dendrimers.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by CSIC Committees on Ethical and Animal Welfare and by the National Committee on Ethics and Animal Welfare (PROEX 034/15) (CBS2014/015 and CEEA2014/018) by the INIA Committees on Ethics of Animal Experiments and Biosafety, and by the National Committee on Ethics and Animal Welfare (PROEX 218/14).

AUTHOR CONTRIBUTIONS

FS, EB, DA, RC-A, PL, and MF conceived and designed the experiments. RC-A, PL, ET, MF, SD, and MB performed the experiments. RC-A, PL, MF, EB, DA, and FS analyzed the data and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Diagnostic and Epitope Mapping Potential of Single-Chain Antibody Fragments Against Foot-and-Mouth Disease Virus Serotypes A, SAT1, and SAT3

Melanie Chitray[1,2*], *Pamela Anne Opperman*[1,3], *Lia Rotherham*[1], *Jeanni Fehrsen*[1,2], *Wouter van Wyngaardt*[1], *Janine Frischmuth*[4], *Elizabeth Rieder*[5] and *Francois Frederick Maree*[1,6]

[1] Agricultural Research Council, Onderstepoort Veterinary Research, Vaccines and Diagnostic Development, Onderstepoort, Pretoria, South Africa, [2] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa, [3] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa, [4] Biotechnology Division, National Bioproducts Institute, Pinetown, South Africa, [5] Plum Island Animal Disease Centre, U.S. Department of Agriculture, Agricultural Research Service, Greenport, NY, United States, [6] Department of Biochemistry, Genetics and Microbiology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa

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Mariano Pérez-Filgueira, National Agricultural Technology Institute, Argentina

Reviewed by:
Charles K. Nfon, National Centre for Foreign Animal Disease (NCFAD), Canada
Sabrina Galdo Novo, University of Buenos Aires, Argentina

***Correspondence:**
Melanie Chitray
chitraym@arc.agric.za

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Foot-and-mouth disease (FMD) affects cloven-hoofed domestic and wildlife animals and an outbreak can cause severe losses in milk production, reduction in meat production and death amongst young animals. Several parts of Asia, most of Africa, and the Middle East remain endemic, thus emphasis on improved FMD vaccines, diagnostic assays, and control measures are key research areas. FMD virus (FMDV) populations are quasispecies, which pose serious implications in vaccine design and efficacy where an effective vaccine should include multiple independent neutralizing epitopes to elicit an adequate immune response. Further investigation of the residues that comprise the antigenic determinants of the virus will allow the identification of mutations in outbreak strains that potentially lessen the efficacy of a vaccine. Additionally, of utmost importance in endemic regions, is the accurate diagnosis of FMDV infection for the control and eradication of the disease. To this end, a phage display library was explored to identify FMDV epitopes for recombinant vaccines and for the generation of reagents for improved diagnostic FMD enzyme-linked immunosorbent assays (ELISAs). A naïve semi-synthetic chicken single chain variable fragment (scFv) phage display library i.e., the *Nkuku*® library was used for bio-panning against FMD Southern-African Territories (SAT) 1, SAT3, and serotype A viruses. Biopanning yielded one unique scFv against SAT1, two for SAT3, and nine for A22. SAT1 and SAT3 specific scFvs were exploited as capturing and detecting reagents to develop an improved diagnostic ELISA for FMDV. The SAT1 soluble scFv showed potential as a detecting reagent in the liquid phase blocking ELISA (LPBE) as it reacted specifically with a panel of SAT1 viruses, albeit with different ELISA absorbance signals. The SAT1svFv1 had little or no change on its paratope when coated on polystyrene plates whilst the SAT3scFv's paratope may have changed. SAT1 and SAT3 soluble scFvs did not neutralize the SAT1 and SAT3 viruses; however, three

of the nine A22 binders i.e., A22scFv1, A22scFv2, and A22scFv8 were able to neutralize A22 virus. Following the generation of virus escape mutants through successive virus passage under scFv pressure, FMDV epitopes were postulated i.e., RGD+3 and +4 positions respectively, proving the epitope mapping potential of scFvs.

Keywords: foot-and-mouth disease, SAT1, SAT3, serotype A, phage display, single-chain variable fragment, epitope, ELISA

INTRODUCTION

Diseases caused by RNA viruses are often difficult to control because of the high mutation rate and the continual emergence of novel genetic and antigenic variants that allow escape from immunity (1). Antigenic differences between viruses play a role in whether the immunity induced by one virus is effective against another. Foot-and-mouth disease (FMD) virus (FMDV), a single-stranded, positive-sense RNA virus, and the prototype member of the *Aphthovirus* genus in the family *Picornaviridae* (2), is an example of an antigenically variable pathogen with the ability to evade the immune system (3–5). Of the seven clinically indistinguishable FMDV serotypes, viruses belonging to the three Southern African Territories (SAT) serotypes display appreciably greater genomic and antigenic variation (6).

Two key research focus areas for enhanced FMD control are improved vaccines that offer a broad immunogenic response and improved specific diagnostic assays (7). However, the high antigenic diversity that exists within the FMDV serotypes hinders FMD control by vaccination, as vaccination against one serotype does not confer protection against another and may only be partially effective against some subtypes within the same serotype (8). This poses serious implications in vaccine design and efficacy where an effective vaccine should include multiple independent epitopes to elicit an immune response (9). The humoral immune response has generally been accepted as the most important factor in conferring vaccine-induced protection against FMD, as a strong correlation has been reported between the levels of virus-neutralizing antibody produced after vaccination and subsequent protection of cattle, one of the main target species for vaccination (10–13). To develop more effective vaccines or peptide vaccines, numerous FMDV studies have been undertaken to identify these neutralizing antigenic sites in more detail (14). Neutralizing antigenic sites have been identified for serotype A (15–17), O (18–21), C (22), Asia-1 (23), and SAT2 (19, 24, 25). However, information regarding the antigenic determinants of SAT serotypes, which are confined geographically to Africa, is scarce (26). Mapped SAT2 epitopes include: (i) βG–βH loop of VP1; (ii) residue 210 in the C-terminus of VP1; (iii) VP1 84–86, 109–111, VP2 71, 72, 133, 134; and (iv) VP1 159, VP2 71–72, 133–134, 148–150 (19, 24, 25, 27, 28). Four independent antigenic determinants were identified for SAT1 viruses i.e., (i) two occurring in the βG–βH loop of VP1; (ii) two simultaneous residues one in VP3 (position 135 or 71 or 76) and one in VP1 (position 179 or 181); (iii) a conformation dependant site within VP1 position 181 and VP2 72; and (iv) VP1 position 111 (24). To date, no neutralizing sites have been determined for viruses of the SAT3 serotype. It has been shown that the majority of FMDV-neutralizing antibodies are directed against conformational epitopes located on the β-barrel connecting loops, especially the highly mobile βG–βH loop in VP1 (15, 18, 26, 29, 30). Therefore, knowledge of the amino acid residues that comprise the antigenic determinants of FMDV, and those that function as protective epitopes in particular, will greatly improve our understanding of virus neutralization *in vivo* (12, 26, 31).

Diagnostic assays hampered by the lack of specificity caused by polyclonal capture and detection antibodies highlighted the need for more specific tests. Monoclonal antibodies are highly specific reagents and are being used for a variety of research and diagnostic purposes within the FMD field and their pivotal role in all aspects of FMD research is now clear. However, traditional monoclonal antibodies, produced using hybridoma technology, and used in diagnostics have several limitations such as its high cost, time-consuming production, and the expertise required (32–34).

The development of large combinatorial antibody libraries based on antibody genes expressed and displayed on phages have revolutionized the selection and isolation of unique antibodies to an antigen and aided in the development of recombinant reagents for ELISA (35). A key advantage of phage display of antibody fragments is that the generation of specific single-chain variable fragment (scFv) or antigen binding fragment (Fab) to a particular antigen can be completed within a few weeks compared to hybridomas taking months. Antibody libraries can be either immune (from immunized donors) or naïve (from non-immunized donors). An immune library will have an antibody range that is highly enriched for antibodies generated in response to a particular immunogen, whereas, the naïve library can advantageously be used for an unlimited array of immunogens (36).

Phage display libraries have been used with success to map epitopes for FMDV for serotype O (37, 38) and the SAT2 serotype (25). The *Nkuku*® phage-display library, which is a large semi-synthetic library of recombinant filamentous bacteriophages displaying scFv's derived from combinatorial pairings of chicken variable heavy and light chains, was used for this study (36). This naïve library has been utilized to generate a variety of antibodies against antigens such as the bluetongue virus, African horse sickness virus, echovirus 1, coxsackievirus B3, FMDV of the SAT2 serotype as well as a mycobacterial 16 kDa antigen (25, 36, 39–42). These studies prove that this library is sufficiently diverse for the recognition of a variety of different haptens, proteins, bacteria, and viruses.

Abbreviations: FMD, foot-and-mouth disease; FMDV, FMD virus; scFv, single-chain variable fragment; MAb, monoclonal antibody.

In this study, phage display technology was used to obtain specific scFvs from panning with FMDV serotype A, SAT1, and SAT3 viruses. This is a novel study as recombinant monoclonal antibodies (scFvs) have not been isolated for FMDV serotype A, SAT1, and SAT3. The scFvs resulting from the biopanning were investigated in virus neutralization assays in the pursuit of epitope identification and for their prospective use as FMDV diagnostic reagents in an ELISA.

MATERIALS AND METHODS

Cell Cultures, Virus Propagation, and Purification

Baby hamster kidney (BHK) strain 21 clone 13 cells (ATCC CCL-10), used for virus propagation and SAT1 and SAT3 serotype neutralization assays, were maintained in Glasgow minimum essential medium (GMEM, Sigma-Aldrich), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 1 × antibiotic-antimycotic solution (Invitrogen), 1 mM L-glutamine (Invitrogen), and 10% (v/v) tryptose phosphate broth (TPB, Sigma-Aldrich. Instituto Biologico Renal Suino-2 (IBRS-2) cells used for A22 virus propagation and virus neutralization tests, were maintained in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone) and 1 × antibiotic-antimycotic solution (Invitrogen). The Mycl-9E10 hybridoma (ECACC 85102202) was cultured in protein-free hybridoma medium (Invitrogen).

The SAT1/KNP/196/91 and SAT3/KNP/10/90 FMDVs used for the biopanning, originated from buffalo in the Kruger National Park (KNP) in South Africa, isolated during 1991 and 1990, respectively (**Table 1**). Also used for biopanning was the A22 virus (**Table 1**), which was obtained from the Pirbright Institute, UK. The SAT1 and SAT3 viruses were propagated on BHK-21 cells whilst A22 was propagated on IB-RS-2 cells prior to sucrose density gradient (SDG) purification of 146S particles. Virus particles were concentrated with 8% (w/v) polyethylene glycol (PEG)-8000 (Sigma-Aldrich) and purified on 10–50% (w/v) sucrose density gradients, prepared in TNE buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM EDTA), as described by Knipe et al. (43). Peak fractions corresponding to 146S virion particles (extinction coefficient E259 nm [1%] = 78.8) were pooled and the amount of antigen (μg) was calculated as described previously (44). In a similar way, viruses utilized for the ELISA assays (**Table 1**) were PEG concentrated, where virus particles were concentrated with 8% (w/v) PEG-8000 (Sigma-Aldrich) and the resulting precipitated pellet was re-suspended in TNE buffer.

TABLE 1 | Detailed list of FMDV SAT1, SAT2, SAT3, and A viruses used in this study.

FMDV Serotype	Virus strain	Passage history*	Genbank accession number
SAT1	KNP/196/91	PK1RS5	DQ009716
	KNP/3/03	PK1RS1	KJ999914
	SAR/33/00	PK1RS2	KJ999908
	BOT/1/06	PK1RS1	KJ999919
	SAR/9/03	PK1RS1	KJ999911
	ZIM/14/98	BTY2RS2	KJ999925
	SAR/2/10	PK1RS2	KJ999913
	ZAM/2/93	PK1RS3	DQ009719
	KNP/10/03	PK1RS2	KJ999916
	SAR/9/81	B1BHK4B1RS2	DQ009715
	NAM/272/98	PK2RS1	KJ999921
SAT3	KNP/10/90	PK2RS2	KF647849
	KNP/14/96	PK1RS1	MK415741
	SAR/1/06	BHK5 BTY1	MK415736
	KNP/8/02	PK2	MK415739
	BOT/6/98	BTY1RS2	MK415742
	KNP/2/03	PK1RS1	MK415738
	KNP/1/03	PK1RS1	MK415737
	SAR/14/01	PK1RS2	MK415740
	ZAM/5/93	PK1RS4BHK6	MK415744
	ZIM/5/91	BTY1RS4	MK415745
	KNP/6/08	PK1RS1	MK415735
	ZIM/11/94	BTY2RS5	MK415743
SAT2	ZIM/7/83	B1BHK5B2RS2	DQ009726
A	A22/IRAQ	B2/TBTY2BHK2RS2	AY593764
	A24/CRUZEIRO	B6BHK2RS3BHK3	AJ251476

**PK, pig kidney cells; RS, Instituto Biologico Renal Suino-2 (IB-RS-2) cells; BTY, bovine thyroid cells; B, Bovine; BHK, baby hamster kidney cells.*

Selection of scFvs Against SAT1/KNP/196/91, SAT3/KNP/10/90, and A22

Selection of virus-specific scFvs from the *Nkuku*® phage display library was performed as described by van Wyngaardt et al. (36) and Opperman et al. (25). Briefly, 2-ml immunotubes (Nunc® Maxisorp), after being coated overnight with purified virus (30 μg/ml) were blocked with 1 × PBS containing 2% (w/v) milk powder (Elite) and incubated with the *Nkuku*® library phage particles (10^{12}-10^{13} transducing particles). Exponentially growing *Escherichia coli* TG1 cells (Stratagene, USA) were infected with eluted phage-displayed scFvs that had bound to the specific viruses before plating on TYE plates (15 g/l agar, 8 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin. Subsequent to overnight incubation, the bacteria were collected and the phagemids rescued by the addition of M13KO7 helper phage. Infected bacterial cells were incubated overnight in 2 × TY medium (16 g/l tryptone, 10 g/l yeast extract, 5 g NaCl/l) containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Phages displaying scFvs were precipitated from the cell-free culture supernatant with one-fifth of the original culture volume of 20% (w/v) PEG-8000 in NaCl and were then suspended in 1 × PBS for use in the next selection round. A total of three such selection rounds were performed. The input and output phages from each selection round was titered to monitor enrichment. The outputs of each consecutive selection round was tested in a polyclonal ELISA. Single clones from the third selection round was tested

as soluble scFvs for specific binding to SAT1/KNP/196/91, SAT3/KNP/10/90, or A22.

Polyclonal Phage ELISA

van Wyngaardt et al. (36) and Opperman et al. (25) described the polyclonal phage ELISA. In short, SDG purified virus (30 μg/ml) of either SAT1/KNP/196/91, SAT3/KNP/10/90, or A22 was used to coat 96-well Maxisorp immunoplates (Nunc®) overnight at 4°C. To confirm the specificity of the phage-displayed scFvs to the respective viruses, 1 × PBS containing 2% milk powder (Elite) was used as a blocking reagent and negative control. Bound PEG-precipitated phage-displayed scFvs, produced at each selection round, were detected with the MAb B62-FE2 (100 ng/ml, Progen Biotechnik) and horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgG (PO260, Dako). After a final wash step, substrate/chromogen solution consisting of 4 mM 3,3′,5,5′-Tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1 M citric acid monohydrate, 0.1 M tri-potassium citrate, pH 4.5) and 0.015% H_2O_2 was added for the colormetric reaction. Following 10 min incubation at room temperature, the color reaction was stopped with 1 M H_2SO_4 and the absorbance values were recorded at an absorbance of 450 nm ($A_{450\ nm}$).

Monoclonal Phage ELISA

Following the third round of panning, monoclonal phage antibodies were screened by randomly selecting individual clones from the titration plates and inoculating it into a 96-well tissue-culture plate (Nunc®) containing 2 x TY medium supplemented with 100 μg/ml ampicillin and 2% (w/v) glucose. The bacteria were grown overnight with shaking at 30°C. Using a 96-well inoculation device (Sigma-Aldrich: Cat. No. R-2508), bacterial cells were transferred from the overnight plate to a second plate containing 150 μl of fresh medium per well-followed by incubation for 2.5 h at 37°C with shaking. Subsequently, 50 μl of medium that contained 2×10^9 of the M13K07 helper phage was added to each well and the plate incubated for 30 min at 37°C without shaking. Thereafter, plates were centrifuged at 600 × g for 10 min, the supernatant fractions were removed and replaced with 150 μl of 2 × TY medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin prior to incubation overnight at 30°C with shaking. Following centrifugation at 600 × g for 10 min to pellet bacterial cells, the supernatant fractions, which contained the phage-displayed scFvs were removed and mixed 1:1 with 1 × PBS containing 4% (w/v) casein and 0.2% (v/v) Tween-20 prior to undergoing ELISA testing as described for the polyclonal phage ELISA above.

Monoclonal Soluble scFv ELISA

The monoclonal soluble scFv ELISA has been described by van Wyngaardt et al. (36) and Opperman et al. (25). The method is similar to the monoclonal phage ELISA described above, except, instead of rescuing phages with the M13K07 helper phage, soluble scFvs were induced by adding 2 × TY containing 100 μg/ml ampicillin and 3 mM IPTG. The anti-c-Myc MAb 9E10, expressed from the murine hybridoma Mycl-9E10 (Sigma-Aldrich) and the polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (P0260; Dako) detected the secreted soluble scFvs. The ELISA colormetric reaction was performed as described above.

DNA Sequencing and Sequence Analysis of Phage-Displayed scFvs

Phagemid DNA for monoclonal scFv ELISA positive clones were sequenced [ABI PRISM™ Big Dye™ Terminator Cycling Ready Reaction Kit v.3.0 (Applied Biosystems)]. The clones were inoculated into 2 x TY medium containing 100 μg/ml ampicillin and 20% (w/v) glucose and phagemid DNA was isolated with a QIAprep® Spin Miniprep Kit (Qiagen) as per the manufacturer's instructions. The OP52 forward primer (5′-CCCTCATAGTTAGCGTAACG-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′), as well as the ABI PRISM™ Big Dye™ Terminator Cycling Ready Reaction Kit v.3.0 (Applied Biosystems) was used to sequence the single clones (36). An ABI 3100 automated sequencer resolved the extension products and all sequences were edited, assembled and translated using BioEdit v.7.0.9 (45) and Sequencher v5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) software.

Large Scale Expression and Purification of Soluble scFvs

Glycerol stocks (150 μl) of selected phage clones were inoculated in 90 ml of 2 × TY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 μg/ml ampicillin and 2% (w/v) glucose and incubated overnight at 30 °C with shaking. A 1:10 dilution of the overnight culture was prepared in 800 ml of fresh 2 × TY medium containing 100 μg/ml ampicillin and 2% (w/v) glucose and incubation, with shaking, continued for a further 8 h after which the bacterial cells were pelleted at 4,000 × g for 30 min. All traces of glucose-containing 2 × TY media was removed and the bacterial pellet resuspended in 1 L of 2 × TY media containing 100 μg/ml ampicillin and 1 mM IPTG and incubated overnight at 30°C with shaking. The expressed soluble scFv was harvested by pelleting the bacterial cells at 4,000 × g for 30 min.

The bacterial pellets were resuspended in 50 ml of TSA buffer (0.05 M Tris, 0.1 M NaCl, 0.02% NaN_3, 0.02% sodium azide; pH 8.0) and treated with 0.01% (v/v) of 100 mg/ml lysozyme for 30 min at 30°C. Freshly prepared 200 mM phenylmethylsulfonyl fluoride [0.01% (v/v)] in isopropanol was added to the bacterial suspension and the suspension mixed by inverting. The bacterial suspension was sonicated for 3 min (30 s pulses with 30 s pauses), where after, the bacterial pellet was collected by centrifugation at 15,000 × g for 30 min and the clear lysate filtered through 0.8 and 0.45 μm filters, respectively. The anti-*myc*-Sepharose column coupled with 143 mg of 9E10 Mab (prepared by Janine Frischmuth, the National Bioproducts Institute, Biotechnology division, Pinetown, South Africa) was washed with TSA buffer before the clear lysate (containing soluble scFvs) was loaded through the column via a peristaltic pump. The column was washed with TSA buffer until the spectrophotometer reading at absorbance A_{280nm} fell below 0.3. Soluble scFvs were eluted from the column with elution buffer (0.1 M glycine, 0.14 M NaCl, pH 2.2) and fractions collected. Peak fractions were pooled

and the scFvs dialyzed (Sigma-Aldrich, dialysis tubing cellulose membrane, 10 mm flat width), for 48 h at 4°C, in 2 L PBS pH 7.4.

Binding Specificity of Soluble scFvs

The specificity of the soluble scFvs was tested with an ELISA essentially performed as described for the monoclonal phage ELISA. ELISA plates were coated in duplicate with 30 μg/ml of purified SAT1/KNP/196/91, SAT2/ZIM/7/83, SAT3/KNP/10/90, A22, or A24 viruses as well as with BHK-21 cell extract, 2% (w/v) sucrose and 1 × PBS containing 2% (w/v) milk powder as negative controls.

Neutralization Assays and Generation of Virus Escape Mutants

IB-RS-2 cells were used to determine the 50% tissue culture infective dose ($TCID_{50}$) of A22 (OIE Terrestrial Manual, 2018). Similarly, BHK-21 cells were used to determine the virus titers for SAT1/KNP/196/91 and SAT3/KNP/10/90. The resulting virus titers were used to calculate the dilutions subsequently used in the virus neutralization test (VNT).

Virus dilutions containing ∼500, 50, and 5 infectious particles were prepared in the appropriate cell medium (RPMI for IB-RS-2 cells and GMEM for BHK-21 cells) and were applied in triplicate wells across a microtiter plate and diluted two-fold down the plate. Virus was incubated for 1 h at 37 °C in an atmosphere of 5% CO_2 after purified scFvs at a concentration between 0.03 and 0.23 mg/ml were added neat to appropriate wells. A control plate without soluble scFvs was included. BHK-21 and IB-RS-2 cells supplemented with 1% (v/v) FCS and antibiotics (virus growth medium, VGM), with a cell count of 0.3×10^6 cells/ml for both cell lines, were subsequently added to the respective microtiter plates. Incubation of microtiter plates then occurred for 72 h at 37°C and fixation and staining with a methylene blue-formaldehyde stain to allow for inspection of the cytopathic effect, which was scored as a measure of neutralization.

To generate virus neutralization escape mutants, the viruses (SAT1/KNP/196/91, SAT3/KNP/10/90, and A22) were passaged under scFv pressure as described by Crowther et al. (19) and Opperman et al. (25). Equal volumes of ca. 25 infectious virus particles were diluted two-fold in GMEM or RPMI medium on a microtiter plate before being mixed with an equal volume of the respective purified scFv (neat), followed by 30 min incubation at 37°C. The virus-scFv complexes were added to either BHK-21 or IB-RS-2 monolayer cells and incubated for 1 h at 37°C. Following incubation, the virus-scFv complexes were removed. Monolayers were washed twice with GMEM or RPMI (Sigma-Aldrich) medium before VGM containing a 1:50 dilution of purified scFvs at a concentration between 0.6 and 4.6 μg/ml were added. All scFvs were tested. Each virus was subjected to four consecutive passages under scFv pressure.

Characterization of Virus Escape Mutants

Virus escape mutants were then characterized by sequencing the Leader- P1-2A coding region (ca. 3 kb) of the virus genome. RNA extraction was performed using the QIAmp viral RNA kit (Qiagen). cDNA was synthesized with SuperScript III first strand synthesis kit (Invitrogen), using the genome specific primer WDA 5′-GAAGGGCCCAGGGTTGGACTC-3′.

The Leader-P1-2A coding region of the escape viruses were amplified using the Expand Long Template PCR System (Roche) and forward primer NCR1 5′-TACCAAGCGACACTCGGGATCT-3′ and reverse primer WDA 5′-GAAGGGCCCAGGGTTGGACTC-3′. Briefly, each 50 μl PCR reaction mixture consisted of 3 μl of the first strand cDNA reaction mixture, 0.3 μM of each oligonucleotide, 2.5 U of Expand Long Template DNA polymerase, 1 × Expand buffer, 0.75 mM $MgCl_2$ and 2 μM of each dNTP. Using a thermocycler (GeneAmp 9700, Applied Biosystems) after initial denaturation at 94°C of 2 min, the reactions were subjected to 35 cycles of 94°C for 20 s, 55°C for 20 s and 68°C for 4 min with a final cycle of 68°C for 7 min to complete the synthesis of all strands.

To determine the nucleotide sequence of the gel-purified amplicons, 0.16 μM of the appropriate oligonucleotide (**Table 2**) and the ABI PRISM Big Dye Terminator Cycling Ready Reaction kit v3.0 (Applied Biosystems) was utilized. The extension products were resolved on an ABI PRISM™ 3100 automated sequencer (Applied Biosystems) and sequences analyzed using the BioEdit v.7.0.9 (45) and Sequencher v5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) software of the ca. 2.2-kb P1-coding region.

Investigation of the SAT1 and SAT3 Soluble scFvs as Capturing Antibodies in an Indirect ELISA

The SAT1 and SAT3 soluble scFvs were tested in an indirect ELISA against a panel of viruses (**Table 1**) to determine whether it can be used as capturing antibodies in routine testing of suspected FMDV cases. The PEG concentrated viruses were titrated in a liquid phase blocking ELISA [LPBE; (46)] to determine the optimal dilution where an absorbance value at 450 nm (A_{450nm}) of ca. one was obtained. The virus dilution of 1:8 was chosen for the scFv ELISA as this was the highest dilution where an A_{450nm} ∼1 (with standard deviation of 0.25, observed after the ELISA colorimetric reaction) was obtained for the viruses tested. The purified, neat scFvs (SAT1scFv1 0.03 mg/ml, SAT3scFv1 0.039 mg/ml, and SAT3scFv2 0.09 mg/ml) were used to coat 96-well Maxisorp immunoplates (Nunc®) overnight at 4 °C, following which, ELISA plates were washed

TABLE 2 | Oligonucleotides used for sequencing the virus escape mutants.

Oligonucleotide	*Sequence
L internal	5′-GWTACGTCGATGARCC-3′
NCR1	5′-TACCAAGCGACACTCGGGATCT-3′
WDA	5′-GAAGGGCCCAGGGTTGGACTC-3′
SEQ 16	5′-GTGGAACAAGCAGAGAGG T-3′
SEQ 18	5′-CAACTGCAACGTCCTTCTC-3′

In selected oligonucleotides, the abbreviation represents ambiguities i.e., W=A or T, R=A or G.

four times with wash buffer [1 × PBS containing 0.05% (v/v) Tween 20]. As a blocking reagent and negative control, 2% milk powder in 1 × PBS was used. Diluted PEG concentrated virus (1:8) was added to the scFv coated plates and incubated for 1 h at 37°C. Following incubation and washing, serotype specific guinea pig antiserum (typing/detecting antibody, ARC-OVR-VDD) was added (working dilution for SAT1 and SAT2 was 1:100 and SAT3 whilst for A it was 1:50) and plates were again incubated for 1 h at 37°C and then washed. The conjugate (rabbit anti-guinea pig IgG conjugated to horseradish peroxidase, Sigma-Aldrich) diluted at 1:80, was added to respective microtiter plate wells, followed by 1 h at 37°C incubation and washing. The ELISA colorimetric reaction followed using substrate-chromogen solution, consisting of 4 mM 3,3′,5,5′-Tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1 M citric acid monohydrate, 0.1M, disodium hydrogen phosphate; pH 4.5) 0.015% (v/v) H_2O_2. Following 10 min incubation at room temperature, the colorimetric reaction was stopped with 1.25M H_2SO_4. The A_{450nm} was determined using a Labsystems Multiskan Plus photometer (Thermo Fisher Scientific). The samples were tested in duplicate wells and the absorbance calculated as an average of the two values for each sample. A positive ELISA result was calculated as two-fold the A_{450nm} value of the average negative control.

Investigation of the SAT1 and SAT3 Soluble scFvs as Detecting Antibodies in an Indirect ELISA

The SAT1- and SAT3-specific soluble scFvs from this study were further investigated for their suitability as a detecting antibody for the FMDV antigen in an ELISA format. 96-well ELISA plates (Nunc®) were coated with either SAT1, SAT2, SAT3, or A specific rabbit antiserum. A 1:8 dilution of PEG concentrated viruses (**Table 1**) were added to the coated 96-well Maxisorp immunoplates (Nunc®) and incubated for 1 h at 37°C. Following a wash step with wash buffer i.e., 1 × PBS containing 0.05% (v/v) Tween 20, undiluted scFvs (SAT1scFv1 0.03 mg/ml, SAT3scFv1 0.039 mg/ml, and SAT3scFv2 0.09 mg/ml) were added and ELISA plates incubated for 1 h at 37°C. Microtiter plates were washed and the soluble scFvs that bound to the FMD antigen were detected with the anti-c-Myc antibody clone 9E10 (Sigma-Aldrich) and 1: 1,000 dilution of horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse IgG (PO260; Dako). The negative control contained 2% milk powder (Elite) in 1 × PBS instead of the scFvs. The substrate/chromogen solution and A_{450nm} determination was performed as described above and a positive A_{450nm} value was considered greater-than or equal to two-fold the average negative control value. The test samples were tested in duplicate and the absorbance calculated as an average of the two values for each test sample. The LPBE was adapted and essentially carried out in conjunction as a comparison of performance of the scFv detecting ELISA and was undertaken as described in the Office International des Epizooties (46).

RESULTS

Selection and Identification of Phage-Displayed scFvs Against FMDV SAT1/KNP/196/91, SAT3/KNP/10/90, and A22

The large semi-synthetic naïve *Nkuku*® phage display library based on chicken immunoglobulin genes, was panned by exposing the recombinant antibody repertoire to SDG purified virions of the FMD SAT1/KNP/196/91, SAT3/KNP/10/90, and A22 viruses. After three consecutive biopanning rounds, polyclonal phage displayed scFvs were tested in a polyclonal ELISA to evaluate enrichment (**Figure 1**) for each of the three biopannings to the specific viruses. The phage outputs from the three consecutive selection rounds were tested and an aliquot of the library prior to panning was included as a non-enriched control (**Figure 1**). Output phages from selection round three resulted in A_{450nm} of 1.05, 1.35, and 2.48 for SAT1/KNP/196/91, SAT3/KNP/10/90, and A22, respectively (**Figures 1A–C**). The *Nkuku*® non-enriched control (selection round 0) produced A_{450nm} results of 0.13, 0.10, and 0.06 for SAT1/KNP/196/91, SAT3/KNP/10/90, and A22, respectively. An increase of at least eight-fold in the absorbance values after three pannings compared to the absorbance of a pre-panning aliquot of the *Nkuku*® library proved enrichment for phage displayed antibodies for all three FMD viruses (**Figures 1A–C**).

Phage displayed scFvs specific for FMDV were identified after a helper phage rescue of single bacterial colonies from the third round of panning and tested in ELISA. Both the phage displayed and soluble scFv formats were tested and the results are summarized in **Table 3**. A total of 94 clones each for SAT1/KNP/196/91 and SAT3/KNP/10/90 and 188 clones for A22 were screened. Twenty clones expressed phage-displayed scFvs specific to SAT1/KNP/196/91 with ELISA signals more than two-fold greater than that of the negative control. Furthermore, of these, seven clones secreted soluble scFvs that bound to SAT1/KNP/196/91. Sequencing of the seven clones revealed one unique binder for SAT1/KNP/196/91, designated SAT1scFv1 (**Table 4**), which had seven identical clones. Analysis of the 94 clones for SAT3/KNP/10/90 revealed three clones expressing phage-displayed scFvs specific to SAT3/KNP/10/90, all of which secreted soluble scFvs that bound to SAT3/KNP/10/90. Sequencing of these clones indicated two unique binders designated SAT3scFv1 and SAT3scFv2 (**Table 4**). In addition, of the 188 clones for A22, 25 clones expressed phage-displayed scFvs specific to A22, whilst 9 clones secreted soluble scFvs that bound to A22 and sequencing revealed nine unique binders for A22 designated A22scFv1 to A22scFv9 (**Table 4**). Interestingly, sequencing results revealed that SAT3scFv2 and A22scFv6 had an identical sequence in all three of the CDR's for the heavy and light chains and are essentially the same binder (**Table 4**). However, it must be noted that these biopannings were executed independently of each other and at different times and thus the possibility of cross-contamination is ruled out. The result inferred that the SAT3scFv2 and A22scFv6 binders recognize a conserved amino acid (aa) motif on

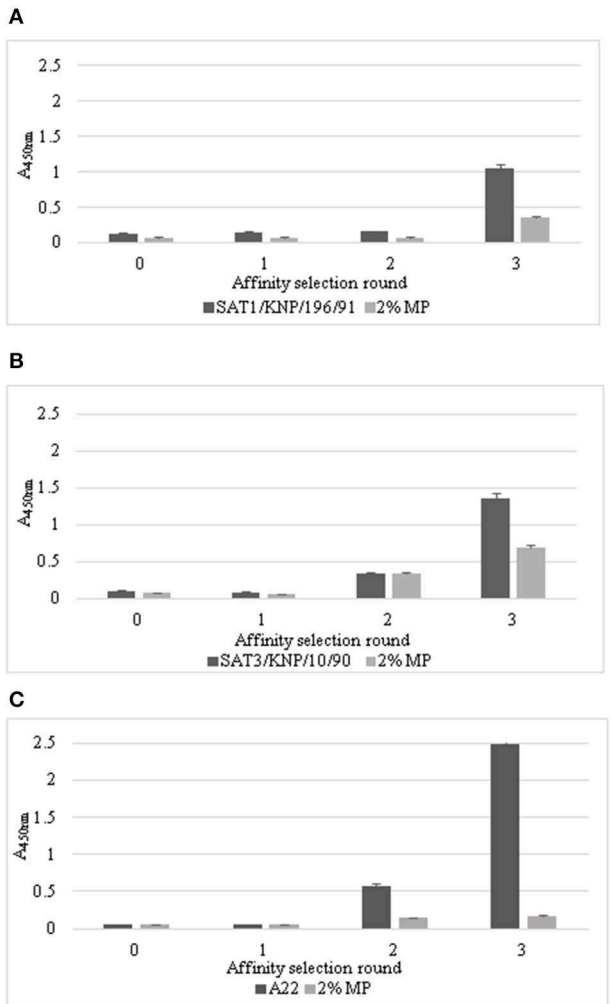

FIGURE 1 | Enrichment of phage-displayed scFvs for **(A)** SAT1/KNP/196/91, **(B)** SAT3/KNP/10/90, and **(C)** A22 using a polyclonal ELISA. Phage-displayed scFvs that bound to SAT1/KNP/196/91, SAT3/KNP/10/90, and A22 were eluted and enrichment of virus specific phage-displayed scFvs (black bars) was determined by a polyclonal phage ELISA of the outputs for three consecutive biopannings. The unpanned aliquot of the *Nkuku*® phage display library was a non-enriched control (selection round 0). The negative control used was 2% milk powder (labeled 2% MP, gray bars). The data are averages ±SD of three repeats.

both SAT3/KNP/10/90 and A22 viruses. The soluble scFvs were subsequently successfully purified by means of affinity chromatography and further characterized.

Binding Specificity of Soluble scFvs to FMDV

The specificity of the virus-specific soluble scFvs were determined by measuring their ability to bind to purified, complete 146S virions of viruses from serotypes A, SAT1, SAT2, and SAT3 in an ELISA. Negative controls were BHK-21 cell extract, 2% sucrose, and 2% milk powder (**Figure 2**). SAT1scFv1 phage-displayed binder cross-reacted with A22, SAT2/ZIM/7/83, and SAT3/KNP/10/90, however, the soluble scFv format did not exhibit any cross-reactivity (**Figure 2**). SAT3scFv1 phage-displayed and soluble scFv cross-reacted with A22 and SAT1/KNP/196/91 whilst there was borderline cross-reactivity with SAT2/ZIM/7/83 observed for the phage-displayed scFv (**Figure 2**). The amino acid sequence of the heavy and light chain complementary determining regions (CDRs) of SAT3scFv2 and A22scFv6 are identical and the cross-reactivity between SAT3scFv2 and A22 as well as A22scFv6 and SAT3/KNP/10/90 was observed with both the phage-displayed and soluble scFv (**Figure 2**). SAT3scFv2 phage-displayed scFv also cross-reacted with SAT1/KNP/196/91, whereas the soluble scFv did not cross-react (**Figure 2**). A22scFv5 soluble scFv showed cross-reactivity to A24 virus and for the phage-displayed scFv, borderline cross-reactivity was observed (**Figure 2**). There was no cross-reactivity observed with the A22scFv1, A22scFv2, A22scFv3, A22scFv4, A22scFv7, A22scFv8, and A22scFv9 binders with SAT1/KNP/196/91, SAT2/ZIM/7/83, SAT3/KNP/10/90, or A24 viruses. No cross-reactivity was observed with the reagents used for virus propagation and purification. The specificity investigations of the scFvs showed that the phage-displayed scFvs exhibited more prominent cross-reactivity when compared to the soluble scFvs. Due to this, further investigations for this study was continued with the soluble scFvs.

TABLE 3 | Screening for scFvs following three rounds of biopanning.

FMD serotype	Number of clones tested	Phage binders	Soluble scFv binders	Unique sequences/ binders
SAT1/KNP/196/91	94	20	7	1
SAT3/KNP/10/90	94	3	3	2
A22	188	25	9	9

Neutralization and Escape Mutant Investigations of the Identified scFvs

The ability of the soluble scFv's against SAT1, SAT3, and A22 to neutralize the SAT1/KNP/196/91, SAT3/KNP/10/90, or A22 viruses, respectively, *in vitro* was investigated. The SAT1scFv1 was unable to neutralize FMDV SAT1/KNP/196/91. Similarly, SAT3scFv1 and SAT3scFv2 binders were unable to neutralize the SAT3/KNP/10/90 virus. Additionally, A22scFv6, being essentially the same binder as SAT3scFv2, was unable to neutralize A22 virus *in vitro*. Nonetheless, three of the nine A22 soluble scFv binders, i.e., A22scFv1 (0.16 mg/ml), A22scFv2 (0.23 mg/ml), and A22scFv8 (0.18 mg/ml), were able to neutralize A22 *in vitro*. The neutralization titers ($TCID_{50}$) are indicated in **Table 5**.

FMDV A22 was serially passaged in the presence of soluble A22scFv1, A22scFv2, and A22scFv8 to select viruses from the A22 quasispecies population that escape neutralization by the soluble scFvs. Thus, the A22 viruses that escaped neutralization by soluble scFvs A22scFv1, A22scFv2, and A22scFv8 were designated scFv resistant virus (SRV) 1, 2, and 3, respectively. Following four consecutive passages under scFv pressure, the P1 nucleotide, and aa sequences were determined for SRV1,

TABLE 4 | Amino acid sequence alignment of the complementary determining regions (CDR) of the heavy and light chains of the SAT1/KNP/196/91, SAT3/KNP/10/90, and A22-specific soluble scFvs panned from the *Nkuku*® library.

scFv	Heavy Chain			Light chain		
	Complementary determining region			Complementary determining region		
	CDR1	CDR2	CDR3	CDR1	CDR2	CDR3
SAT1scFv1	SSHGMF	EITN––TGSYAAYGAAV	CAKSSYECTSSCWGNTGWID	SGDSSG––––YGYG	YNNNKRPS	GTED–GITDAGI
SAT3scFv1	SSNGMA	AISSRD–GSGTGYGSAV	CAKPVKGMY–––––––––ID	SGGTYYA–––––––	YDNTNRPS	GAYDSS–TYAGI
SAT3scFv2	SSFNMG	AINND––GGGTAYGSAV	CAKSVDDSWNV–––––DSID	SGGGSYAGS–YYYG	YDNTKRPS	GSYDSS–––GGI
A22scFv1	SSYSMQ	GIGS––DGSDTAYGAAV	CTKCGYGGS–GYCWYAGDID	SGGGNE–––––––YG	YWNDKRPS	GSYDSSA–––GI
A22scFv2	SSYEMQ	GIEN––DGSNPNYGAAV	CAKSAYGGSWGGYIPTDSID	SGG–SSS––––YYG	YDNTNRPS	GSFDSSTTV–GI
A22scFv3	SDYAMG	GIGTSADGSSTAYGAAV	CTRTGAAE––––––––––DID	SGG–SSS––––YYG	YANTNRPS	GSSDSTY––VGI
A22scFv4	SSHGMG	SISR––DSSYTDYGPAV	CTKSAGPYVNGDN––––––ID	SGGGRYAGNYYYYG	YSNNQRPS	GSADSNSTDGVT
A22scFv5	SDYGMS	EITND––DSWTGYGAAV	CAKNDYYSLF––––––––ID	SG––DSN––YYGYS	YDNDKRPS	GSADSSA–––VI
A22scFv6	SSFNMG	AINND––GGGTAYGSAV	CAKSVDDSWNV–––––DSID	SGGGSYAGS–YYYG	YDNTKRPS	GSYDSS–––GGI
A22scFv7	SSYGMG	GIEN––DGRYTGYGSAV	CAKDIYG–VGGGAFGADTID	SGG–SYS––––––YG	YDNTNRPS	GSIDSSY–V–GI
A22scFv8	SSYSMQ	GIGS––DGSDTAYGAAV	CTKCGYGGS–GYCWYAGDID	SGGGS–––––––YYG	YSNNQRPS	GSYDNSA–––GI
A22scFv9	SSYPMG	AISN––DGSYTGYGAAV	CAKDAYSYTTTGGWYVDEID	SGGGS–––––––YYG	YDNTNRPS	GGIDSTD–––AA

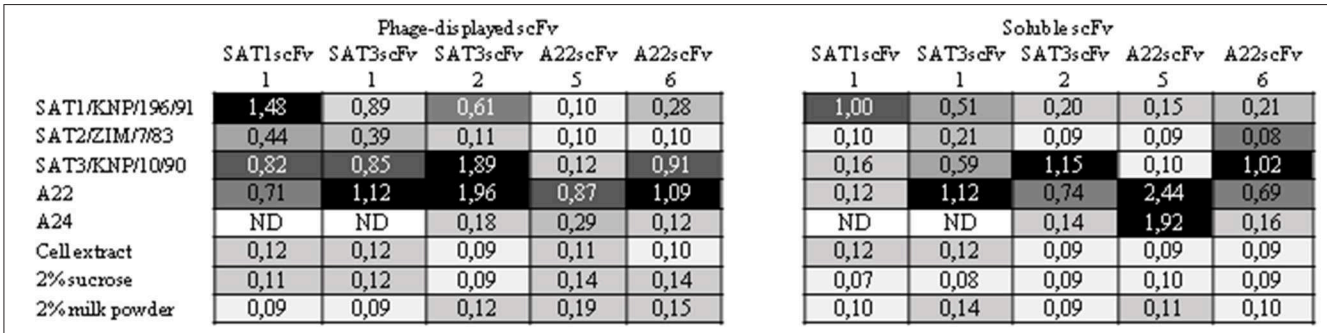

FIGURE 2 | Heat map representing color-coded ELISA A_{450nm} absorbance of the indirect ELISA testing the specificity of scFvs i.e., SAT1scFv1, SAT3scFv1, SAT3scFv2, A22scFv5, and A22scFv6. The remainder of the A22 scFv phage and soluble binders only reacted to A22 with no significant reaction to any of the other viruses or reagents (data not shown). Both the phage-displayed and soluble scFvs were tested against FMDV A22, A24, SAT1/KNP/196/91, SAT2/ZIM/7/83, and SAT3/KNP/10/90 SDG purified viruses as well as BHK-21 cell extract, 2% sucrose, and 2% milk powder (MP). An ELISA signal two-fold that of the 2% milk powder soluble scFv absorbance was considered a positive result.

TABLE 5 | The 50% tissue culture infectious dose ($TCID_{50}$) of A22 when neutralized by the A22 scFvs.

scFv*	Neutralization titer ($TCID_{50}$/50 μl)
A22scFv1	0.5
A22scFv2	0.63
A22scFv8	0.63

**Titer of A22 (without scFvs) was 1.25 $TCID_{50}$/50 μl.*

SRV2, and SRV3. Comparative analysis of the aa sequences of the SRVs compared to the parental A22 sequence indicated that SRV1 exhibited one aa substitution i.e., from a non-polar proline (Pro) to a polar serine (Ser) change at VP1 aa position 149 i.e., RGD+3 (Pro149➔Ser) (**Figure 3**). Interestingly SRV2 had no aa changes occurring in the P1 region, but SRV3 exhibited a single aa substitution of a leucine (Leu) to a phenylalanine (Phe) at position 150 of VP1 i.e., RGD+4 (Leu150➔Phe) (**Figure 3**). The aa substitutions for SRV1 and SRV3 occurred in the surface exposed and structurally flexible VP1 βG-βH loop, downstream of the RGD sequence.

The A22 virus was neutralized by three soluble scFvs and SRVs for A22, which showed a potential binding site for two of the scFvs in the GH-loop of the VP1 protein. The three CDRs of the H-chain of A22scFv1 and A22scFv8 are identical while the L-chain of A22scFv1 and A22scFv8 displayed different sequences (**Table 4**). Soluble scFvs for SAT1/KNP/196/91 and SAT3/KNP/10/90 viruses did not neutralize the respective viruses. Thus, the SAT1 and SAT3 scFvs were investigated for its potential use as diagnostic reagents in an ELISA.

SAT Virus-Specific scFvs as a FMDV Capturing Antibody in a Sandwich ELISA

To determine whether the SAT1 and SAT3 soluble scFv's retain the correct conformation to act as capturing reagents in a diagnostic sandwich ELISA, the soluble scFvs were coated

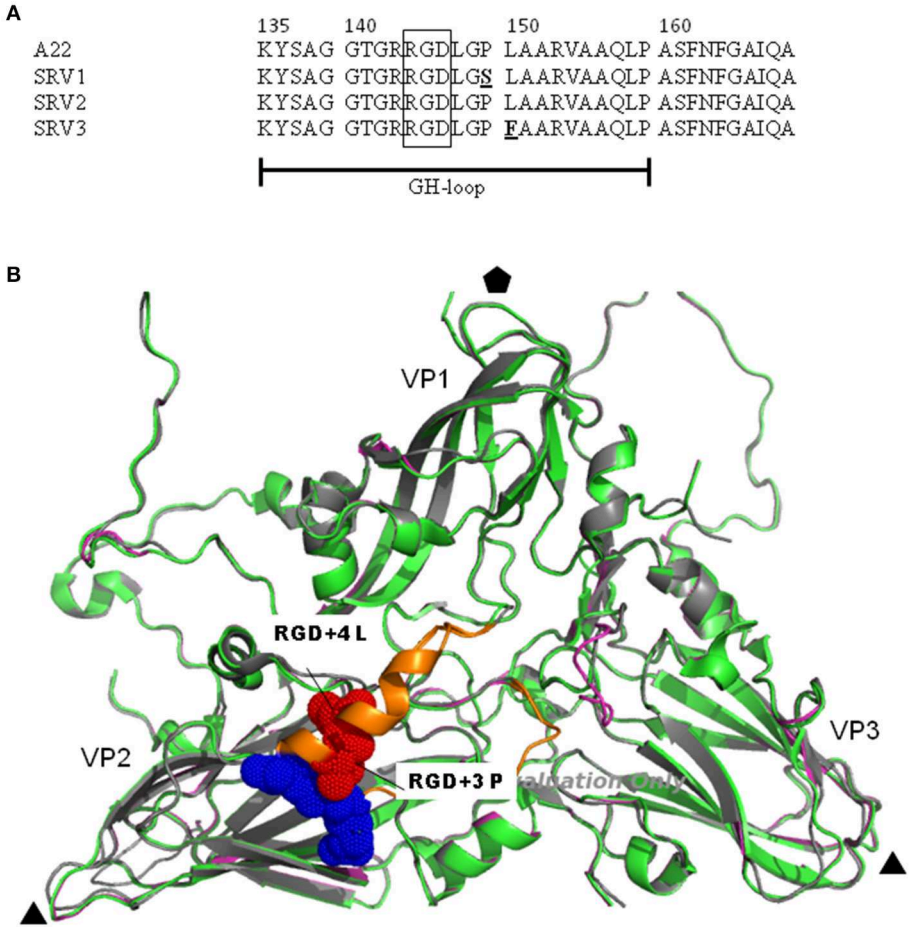

FIGURE 3 | (A) The A22 virus escape mutant substitutions are indicated in an aa alignment of the VP1 GH-loop of A22. The proline to serine (Pro149➔Ser) substitution at position 149 of VP1 for SRV1 and a leucine to a phenylalanine (Leu150➔Phe) substitution at position 150 of VP1 for SRV3 is shown in bold and underlined. The RGD motif is blocked. **(B)** The substitutions are shown as red dots on a cartoon model of the FMDV capsid proteins in a crystallographic protomer. The inferred 3-D structural model was rendered by Pymol v 1.8 (DeLano Scientific LLC). The VP1 GH-loop is absent from the A22 complete (PDB: 4GH4) and empty capsid (PDB: 5D8A) structures due to an instable loop conformation. However, the serotype O capsid structure (PDB: 1FOD) was reported with a VP1 GH-loop (47). The serotype A complete and empty capsid protomers (colored in green and magenta) were superimposed on the O protomer (in gray), showing the position of the VP1 GH-loop in orange and the receptor-binding RGD sequence as blue dots. The three-fold axis is depicted by the black triangles and the five-fold axis of the capsid by the black pentagon. The positions of the outer-capsid proteins, VP1, VP2, and VP3 are indicated.

directly onto maxisorp immunoplates. The antigen-binding activity of the immobilized soluble SATscFv1 was tested against a panel of PEG concentrated SAT1 ($n = 11$) viruses whilst soluble SAT3scFv1 and SAT3scFv2 was tested against a panel of SAT3 ($n = 12$) viruses (**Table 1**).

The results revealed that soluble SAT1scFv1 successfully captured the panel of SAT1 viruses tested as ELISA signals of A_{450nm} ≥0.48 and ≤1.68 were obtained (**Figure 4A**). Weak positive A_{450nm} of >0.4, <0.9 was observed when SAT1scFv1 captured SAT1/SAR/9/81, SAT1/ZIM/14/98, SAT1/KNP/10/03, SAT/NAM/272/98, and SAT1/SAR/2/10 (**Figure 4A**). Additionally, strong positive signals A_{450nm} >1, <1.68 for SAT1/KNP/3/03, SAT1/SAR/9/03, SAT1/ZAM/2/93, SAT1/SAR/33/00, SAT1/BOT/1/06, and SAT1/KNP/196/91 were observed when SAT1scFv1 was the capturing reagent (**Figure 4A**).

SAT3scFv1 and SAT3scFv2 soluble scFvs did not react efficiently with the SAT3 viruses when applied as a capturing reagent and displayed A_{450nm} signals not significantly higher than the negative controls. Weak positive results were obtained for SAT3scFv1 with SAT3/KNP/6/08 (A_{450nm} 0.6) (**Figure 4B**). SAT3scFv1 showed similar reactivity to SAT3/BOT/6/98 (A_{450nm} 0.31), SAT3/KNP/1/03 (A_{450nm} 0.31), and SAT3/ZIM/11/94 (A_{450nm} 0.40) viruses indicating this scFv may recognize the same epitope on the virion for these viruses (**Figure 4B**). Additionally, absorbance values of both the SAT3 scFvs in the capturing ELISA against the virus used for the panning i.e., SAT3/KNP/10/90 was <0.2.

Overall, the ELISA results using the scFvs as capturing reagents indicate that the soluble SAT1scFv1 was able to successfully bind to the polystyrene ELISA plate and react to viruses within the SAT1 serotype. The SAT3scFv1 and SAT3scFv2

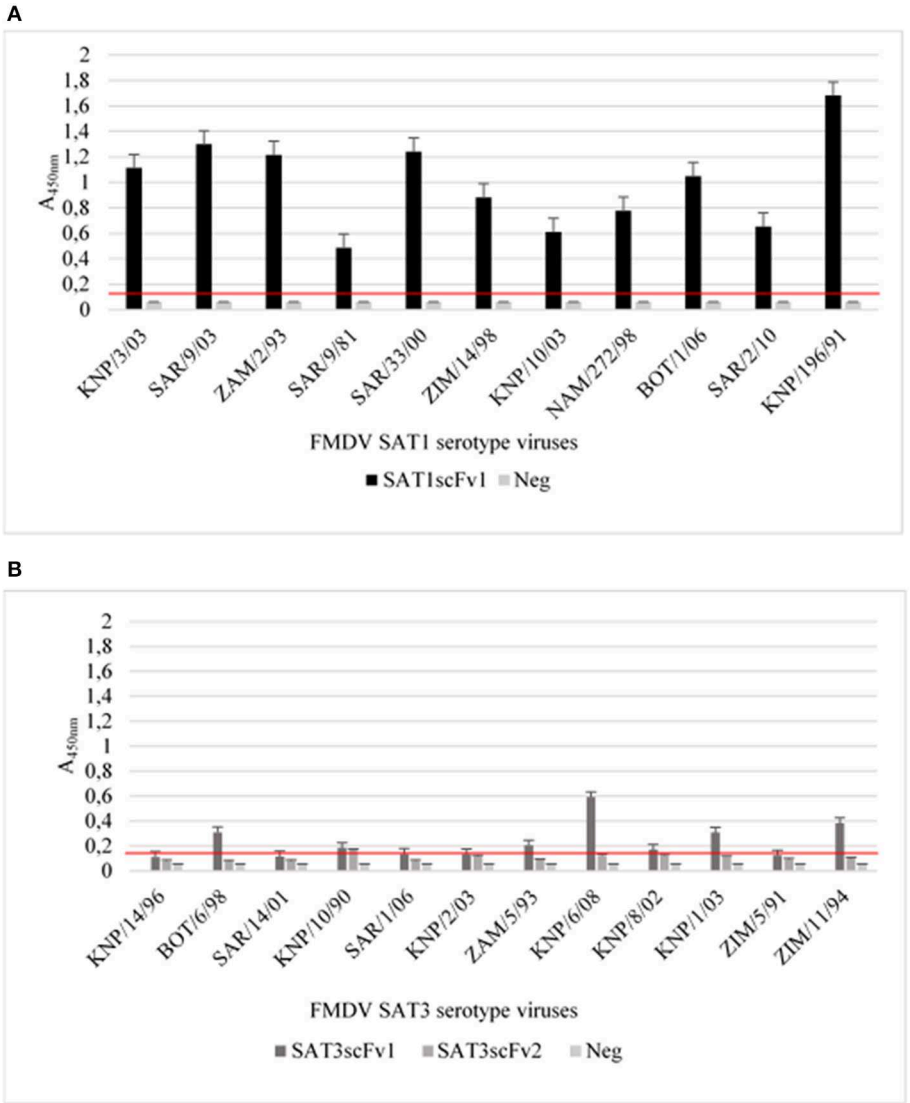

FIGURE 4 | A sandwich ELISA with soluble scFvs as capturing antibodies. The SAT1scFv1 was tested with a panel of PEG concentrated SAT1 viruses **(A)** and SAT3scFv1 and SAT3scFv2 was tested with the SAT3 viruses **(B)**. For the negative control (neg), 2% milk powder was included in the assays replacing the soluble scFvs coating the plate. The data are means ± SD of two independent experiments. An ELISA signal more than two-fold that of the negative control A_{450nm} was considered a positive result and the cut-off is indicated by a red line.

exhibited no or borderline reactivity with the SAT3 viruses tested. The low signals may be attributed to the conformational changes of the scFvs when binding to the ELISA plate. Furthermore, aa differences in the viral proteins may result in the variable ELISA signals observed.

SAT Virus-Specific scFvs as a FMDV Detecting Antibody in an ELISA

The soluble scFvs, SAT1scFv1, SAT3scFv1, and SAT3scFv2, were also applied as detecting antibodies in a sandwich ELISA. FMD virus was captured by polyclonal rabbit antiserum and the soluble scFvs was used to detect the 146S virus particles using the panel in **Table 1**. The standard diagnostic sandwich ELISA used for antigen detection (46) was performed concurrently as a comparison of the scFv ELISA performance.

Results showed that the diagnostic antigen detection ELISA was able to detect all viruses tested and produced positive ELISA signals (**Figures 5A,B**). The ELISA assay using SAT1scFv1 as a detecting antibody revealed two characteristic reactivity profiles against the panel of SAT1 viruses, i.e., (i) A_{450nm} >1.4 was observed for SAT1/SAR/9/03, SAT1/KNP/196/91, and SAT1/NAM/272/98 viruses and (ii) A_{450nm} absorbance values of ≥0.4, ≤0.82 for the remaining eight SAT1 viruses in the panel (**Figure 5A**).

The soluble SAT3scFv1 and SAT3scFv2 showed low absorbance signals for the SAT3 viruses tested (**Figure 5B**).

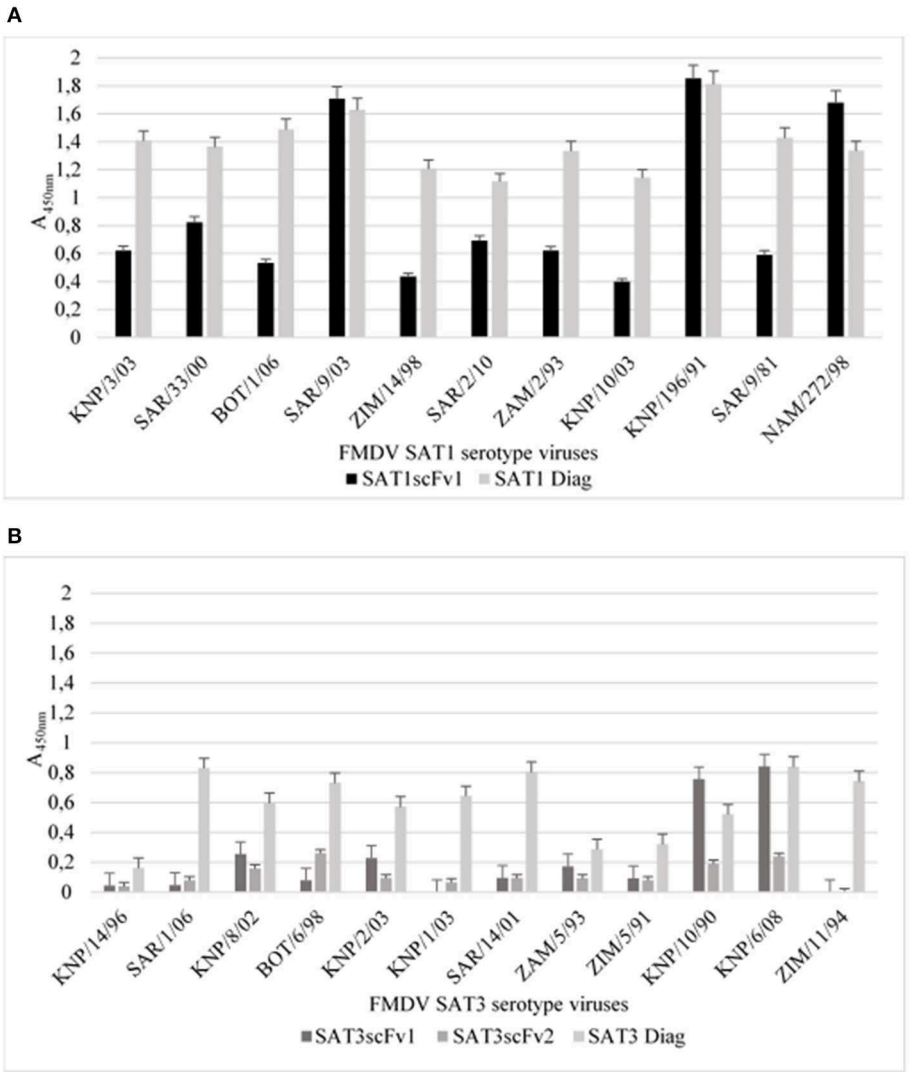

FIGURE 5 | Sandwich ELISA using soluble SAT1scFv1, SAT3scFv1, and SAT3scFv2 as detecting reagents. The standard diagnostic sandwich ELISA used for FMD antigen detection (46) [used as a positive control (SAT1 diag or SAT3 diag)] was adapted, where the detection antibody was replaced with a soluble SAT scFv. The ELISA was executed to determine the detecting potential of soluble SAT1scFv1 to PEG concentrated SAT1 viruses **(A)** and for SAT3scFv1 and SAT3scFv2 to PEG concentrated SAT3 serotype viruses **(B)**. For the negative control, 2% milk powder was included in the assays replacing the virus component. The negative control ELISA background signal was deducted when plotting the ELISA A_{450nm} result. The data are means ± SD of two independent experiments.

Absorbance signals (A_{450nm}) of 0.76 and 0.84 were observed for SAT3/KNP/10/90 and SAT3/KNP/6/08, respectively when SAT3scFv1 was the detecting antibody in the ELISA. All other A_{450nm} values were below 0.26 for the SAT3 viruses tested with SAT3scFv1 and SAT3scFv2 (**Figure 5B**).

Taken together, the results indicate that the two SAT3 specific scFvs showed poor potential as a detecting ELISA antibody whilst conversely, SAT1scFv1 showed good potential for incorporation as a detecting antibody in a diagnostic ELISA.

DISCUSSION

Serotype-specific serological tests for FMD detect antibodies against the structural proteins that are elicited by either vaccination or infection (48). Both the solid-phase competition (SPCE) and LPBE ELISAs for SAT1, SAT2, and SAT3 are OIE recognized and well established assays. Considering the high genetic diversity of the SAT-type viruses (2, 6, 26, 49), there is a continuous need for improvement of these assays as cross-reactivity has been noted with the SAT types using this assay. Additionally, for FMD vaccine matching where the antigenic variability of field virus strains is measured against current vaccine strains, the virus neutralization assay is utilized. This assay, however, is laborious and can cause a delay in decision making regarding FMD control measures. To address this shortcoming, utilizing MAbs or virus-specific scFv's for FMDV that recognize virus exposed antigenic epitopes in an ELISA format where results can be obtained timeously and accurately,

was explored. These scFv's or small recombinant MAbs can be produced in large quantities (50) and can also be beneficial in predicting epitopes, which can in turn be used in the design of improved FMD chimeric vaccines containing various antigenic sites that can elicit a wide immunological response or protection in vaccinated animals. To this end, phage display technology was explored.

The *Nkuku*® phage-display library has previously been panned on FMDV to obtain unique scFv binders for serotype SAT2 (25). Opperman et al. (25) has shown that one SAT2-specific soluble scFv neutralized SAT2/ZIM/7/83. This scFv interacts with a novel epitope at residue position 159 of VP1 and was applied in a scFv-based ELISA assay. We broadened this study by including FMDV serotype SAT1, SAT3, and A. Using naïve phage display libraries allows for the selection of recombinant antibodies where unique paratopes bind to exposed and complementary parts of the immobilized antigen. Thus, the possibility of obtaining antigen-specific binders would depend on the presence and accessibility of suitable surface-exposed structures of the antigen, which in this case, was the FMD virion. Another factor to consider when using phage display technology, is that the quality and the size of the naïve library plays an important role in the success of phage display (51), as paratopes that are not present within the library cannot be isolated.

In this study, the biopanning process with SAT1/KNP/196/91, SAT3/KNP/10/90, and A22 viruses resulted in unique FMDV-specific scFv binders i.e., one for SAT1, two for SAT3, and nine for serotype A. The nine A22 binders attained in this study is unique. Although none of the SAT scFv binders showed neutralization capability, three of the nine A22 binders exhibited neutralization. Of the three A22 neutralizing binders, two binders i.e., A22scFv1 and A22scFv8 had the same heavy chain sequences and only differed in the light chain region sequences. This characteristic is of interest as Hamers-Casterman et al. (52) showed that the V_H play a more important role in the binding of antibody fragments to antigens than V_L. In addition, investigations by Williamson and Matthews (53) showed that three neutralizing scFvs against pertussis toxin all had the same heavy chain sequences and were related. Thus, to obtain more neutralizing scFvs, one could modify only the heavy chain of non-neutralizing scFvs of differing V_{Ls} to be the same as their neutralizing counterparts.

For the generation of escape mutants for FMDV serotype A using the neutralizing scFvs, no aa changes were observed in the P1 region for SRV2, which may be because the mutation occurred in the minor population and due to Sanger sequencing, it was not detected. Additionally, the aa substitutions for SRV1 and SRV3 occurred in the surface exposed and structurally flexible VP1 βG-βH loop, downstream of the RGD sequence. The position is identical to the identified FMDV serotype A antigenic site I and furthermore, the βG-βH loop residues 140–160 have been shown to play an important role in antigenicity in most FMDV serotypes (15–17, 19, 54–57). Additionally, changes at the conserved aa leucine residue 150 has also been shown i.e., L➔P or L➔R on escape mutants pressured by using soluble integrins (58, 59). SRV1 and SRV3 alone did not solve the binding footprint of the A22scFv1 and A22scFv8 binders, respectively. It is reasonable to expect different binding footprints on the virion for the two scFvs even though they have a common binding site at VP1 aa position 149/150. For SRV1, there was a Pro to Ser aa change at VP1 position 149 (RGD +3 position) and for SRV3 the aa change occurred at VP1 position 150, RGD+4 i.e., from a Leu to a Phe. The residues succeeding the RGD motif are important for receptor recognition (58, 60) and the RGD is flanked on both sides by hypervariable sequences, which delivers a domain that is capable of adopting different conformations. Both residues are highly conserved in serotype A viruses and the substitution of amino acid residues at position 149 or 150 of MAR-viruses have been described for A_{10}, A_{12}, and A_{24} (15, 16, 57). Opperman et al. (25) showed with scFv neutralization investigations of FMDV SAT2/ZIM/7/83, an aa change at the base of the GH loop i.e., VP1 position 159 where there was an Arg to His change. Furthermore, a synthetic peptide ELISA confirmed VP1 aa 159 as an important residue in the epitope to which the SAT2 scFv binds (25). These investigations lead us to postulate that the epitope site for the A22scFv1 and A22scFv8 binding involves the VP1 aa position 149 and 150, respectively which is part of the antigenic sequence GDLGSLA for serotype A viruses (15). However, future investigations with SRV1 and SRV3, will be to derive a synthetic peptide from the predicted epitopic site and to confirm results with a synthetic peptide blocking ELISA.

A unique finding from this study is the result of two soluble, non-neutralizing scFvs, each from different FMDV serotypes but having the same heavy and light chain sequences i.e., SAT3scFv2 and A22scFv6. It is postulated that a common epitope between SAT3/KNP/10/90 and A22 resulted in the same soluble scFv from the antibody repertoire of the *Nkuku*® library. Neither of the two scFv's were able to neutralize the respective viruses and thus it was impossible to identify a binding site for the two scFv's on the viral capsid. Competing monoclonal antibody studies or capsid protein peptide libraries will be used to confirm the common or cross-reacting epitope between A22 and SAT3.

From the specificity analysis, the SAT1 and SAT3 soluble scFvs from this study bound to complete 146S virions of the virus used for biopanning i.e., SAT1/KNP/196/91 and SAT3/KNP/10/90, respectively. However, the phage displayed scFv formats did show cross-reactivity across the FMD serotype viruses tested. The SAT1 phage displayed scFv was found to bind to complete 146S virions of SAT2/ZIM/7/83 and SAT3/KNP/10/90 and similarly, the SAT3 phage-displayed scFvs were found to bind SAT1/KNP/196/91 and SAT2/ZIM/7/83 146S virions. Cross-reactivity of scFvs is not uncommon as in a study by Toth et al. (61), which obtained scFv clones against the potato leafroll virus where 7 clones did not cross-react with other luteoviruses whilst 4 clones did. Additionally, Toth et al. (61) proved that the cross-reacting scFvs are directed against continuous epitopes that are present on the coat proteins of certain related luteoviruses whereas the scFvs that did not show cross-reactivity, bound to discontinuous or conformation-dependant epitopes that are specific to potato leafroll virus. A major continuous FMDV epitope is located in the GH loop spanning VP1 residues around positions 140–160 (62, 63). Thus, the cross-reacting phage-displayed SAT1 and SAT3 scFvs from this study may possibly be recognizing continuous epitopes of the SAT serotype viruses and should be investigated further in this regard.

The SAT soluble scFvs, which had reduced cross-reactivity compared to the phage displayed scFvs, were further investigated for their possible use as diagnostic reagents in an ELISA format as a FMDV capturing and a detecting reagent. For both ELISA formats, the one SAT1 soluble scFv was able to produce high reactivity to the various SAT1 PEG concentrated viruses tested implying that there was little or no effect on its paratope when coated on polystyrene plates and was thus a good capturing reagent. However, the two SAT3 scFvs produced A_{450nm} signals just above the positive cut-off absorbance as a FMDV capturing reagent and a detecting reagent, which is not acceptable as a positive result for a diagnostic ELISA. It is vital for a capturing antibody in an ELISA assay to be efficiently immobilized onto the ELISA plate such that it is able to retain both the antibody conformation and the antigen-binding activity (64). Furthermore, important factors such as surface charge, hydrophobicity, co-adsorption of or exchange with surfactants, and other proteins play a role in determining stability and specificity of absorbed antibodies in ELISA assays (65, 66). These factors may have played a role to reduce the performance of the scFv ELISAs in this study. For conventional ELISAs where complete monoclonal or polyclonal antibodies are used, immobilization onto the ELISA plates occur via physical adsorption (66–69). Conversely, immobilization of small antibody fragments such as scFvs onto plastic surfaces causes unfavorable conformational changes to occur (66). Essentially, a hydrophobic interaction occurs in the linked V_H and V_L regions that forms a paratope resulting in a conformational change of the antigen-binding domain, which in turn results in decreased antigen-binding activity (66, 70, 71).

The analytical specificity of the SAT scFvs for the capturing and detecting SAT1 and SAT3 ELISAs showed that the soluble scFvs were specific for the respective serotype viruses tested and no cross-reactivity was observed. Additionally, the analytical sensitivity of both the capturing and detecting ELISAs for SAT1 and SAT3 scFvs against the SAT1 and SAT3 viruses, respectively, was found to detect all PEG concentrated viruses at a 1:8 dilution tested albeit the low A_{450nm} signals for the SAT3 scFv ELISAs. The SAT1scFv1 shows promise as a good detecting and immunocapture reagent due to the high reactivity for the SAT1 viruses tested. Studies have shown that when scFv fragments are utilized as a soluble protein and are not within the phage display system, low expression levels, or a low inherent affinity can occur (36). Also, the monomeric scFv fragments can have moderate binding affinities when binding to a large multivalent antigen like FMDV, which is in contrast to greater binding affinities that can be achieved by the multivalent display on the phage (72–74). To overcome the low SAT3 scFv ELISA signals, random mutations can be introduced in the gene coding for the scFv and the length of the linker within the scFv, increasing the bacterial expression of the scFvs and thus increasing the ELISA signal (41). Another approach is to stabilize the scFv to other proteins whilst retaining functionality. For example, fusion or linking to other proteins such as constant light chain domain (75), leucine zipper dimerization domain (76), Fc fragment (CH2 and CH3 domains) of mouse IgG1 (77), and alkaline phosphatase (78). Such approaches may be used in future studies to enhance the diagnostic potential of the SAT1 and SAT3 scFvs from this study.

This study has been beneficial to gain unique recombinant antibodies against FMDV SAT1, SAT3, and A serotype viruses. Although the SAT scFvs did not neutralize FMDV, the potential was shown as ELISA reagents, especially for SAT1scFv1. Further investigation and validations of SAT1scFv1 will be continued to improve ELISA absorbance signal.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

MC: majority of the experimental work, troubleshooting, result and data analysis, and manuscript composition and editing. PO: scFv expertise and training, experimental work, troubleshooting, result and data analysis, manuscript editing, and research grant acquisition. LR: serotype A experimental work, troubleshooting, result and data analysis, and manuscript editing. JFe and WW: scFv expertise advisor, Nkuku library, and manuscript editing. JFr: scFv column development, scFv purification training and expertise/advisor, and manuscript editing. ER: research grant acquisition, PI on grant at PIADC, project advisor, and manuscript editing. FM: research grant acquisition, PI on grant at ARC, project advisor, scFv expertise, and manuscript editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Use of IFN-Based Biotherapeutics to Harness the Host Against Foot-And-Mouth Disease

Gisselle N. Medina [1,2], Teresa de los Santos [1]* and Fayna Diaz-San Segundo [1]**

[1] Plum Island Animal Disease Center (PIADC), ARS, USDA, Orient Point, NY, United States, [2] Kansas State University, College of Veterinary Medicine, Manhattan, KS, United States

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals that severely constrains international trade of livestock and animal products. Currently, disease control measures include broad surveillance, enforcement of sanitary policy, and use of an inactivated vaccine. While use of these measures has contributed to eliminating foot-and-mouth disease virus (FMDV) from a vast area of the world, the disease remains endemic in three continents, and outbreaks occasionally appear in previously declared FMD-free zones, causing economic and social devastation. Among others, a very fast rate of viral replication and the need for 7 days to achieve vaccine-induced protection are the main limitations in controlling the disease. New fast-acting antiviral strategies targeted to boost the innate immunity of the host to block viral replication are needed. Here we review the knowledge on the multiple strategies FMDV has evolved to block the host innate immunity, with particularly focus on the past and current research toward the development of interferon (IFN)-based biotherapeutics in relevant livestock species.

Keywords: foot-and-mouth disease virus (FMDV), interferon (IFN), antivirals, biotherapeutics, IFN-α, IFN-γ, IFN-λ, IFN-ω

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****Correspondence:***
Gisselle N. Medina
gisselle.medina@usda.gov
Teresa de los Santos
teresa.delossantos@usda.gov
Fayna Diaz-San Segundo
fayna.diaz-sansegundo@usda.gov

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INTRODUCTION

The Disease: Foot-And-Mouth Disease

Foot-and-mouth disease (FMD) is one the most serious livestock diseases that affects cloven-hoofed animals including cattle, swine, sheep, and goats as well as numerous species of wild species (1). The disease displays high morbidity but is usually not lethal, except when it affects young animals that may develop myocarditis. Infected animals secrete copious amounts of virus particles before the onset of the clinical phase of the disease. Typical FMD clinical signs include fever and the appearance of vesicular lesions on the tongue, mouth, feet, and teats. Among ruminants that recovered from the disease, a relatively large number become asymptomatic virus carriers (2, 3), although it is not clear what is the contribution of these carrier animals to disease transmission in nature (4). The World Organization for Animal Health (OIE) lists FMD as a reportable disease and therefore, by law, participating nations are required to inform the organization about all FMD outbreaks. OIE member nations with reported cases of FMD are forbidden to engage in trading of FMD-susceptible animals or their products. Thus, the presence of FMD in a country can have severe economic consequences.

Different interventions to control an FMD outbreak include restriction of susceptible animal movement, slaughter of infected/contact animals, decontamination of infected and surrounding

premises, and vaccination. Vaccination is an option used mostly in countries in which FMD is endemic, but disease-free nations prefer to abstain from such practice. In general, FMD-free countries that occasionally opted to vaccinate to better contain the outbreak did slaughter all vaccinated animals to regain commerce rights faster as occurred in the 2001 outbreak in the UK and the Netherlands (5, 6). The current approved FMD vaccine consists of purified chemically inactivated virus [binary ethylenimine (BEI)-treated] formulated with oil-based or aluminum adjuvants that induces serotype-specific protection in approximately 7 days, and it is applied with a boosting protocol for ensuring long-term protection (7). While this vaccine has been successfully used for many decades leading to disease eradication of a vast area of our planet, challenges remain. FMD is endemic in most of Africa and Asia, and occasionally epizootics appear in South America or in nations that have been disease-free for many years, as it happened in the UK, the Netherlands, South Korea, Taiwan, and Japan (8). Novel vaccine technologies have been developed, but to this end, none of them has fully addressed the limitations of the commercially available vaccine or is currently approved for massive use (9, 10). Alternatives or additional therapeutics that could complement, or in some instances substitute for vaccination protocols, include the use of antivirals and biotherapeutics that act quickly prior to induction of vaccine-induced immunity. The development of such molecules requires a thorough understanding of the biology of the virus and its intricate interactions particularly, with the innate immune molecular and cellular mechanisms evolved by the host.

The Agent

Foot-and-mouth disease virus (FMDV) is a member of the *Aphthovirus* genus within the *Picornaviridae* family, and it is the etiologic agent of FMD (1). The virus contains a single-stranded RNA of positive polarity. Its genome of ∼8,500 nucleotides consists of a long open reading frame (ORF), flanked by a 5′ and a 3′-untranslated region (-UTR). The ORF encodes a polyprotein of about 2,300 amino acids which is processed by virus-encoded proteases. Processing results in the generation of precursors and mature protein products including: four structural [1A (VP4), 1B (VP2), 1C (VP3), 1D (VP1)] and ten non-structural (NS) proteins [L^{pro}, 2A, 2B, 2C, 3A, three distinct copies of 3B (VPg), $3C^{pro}$, and $3D^{pol}$]. Due to high genetic variability, FMDV is categorized in seven distinct serotypes, A, Asia-1, C, O, and Southern African Territories 1–3 (SAT 1–3), and numerous subtypes or topotypes. Upon infection, the virus spreads very rapidly usually achieving 100% morbidity. Depending on the route of entry, less than 10 tissue culture infectious doses are required to infect and cause disease in animals (11). In fact, FMDV is one of the fastest replicating RNA viruses in nature, taking as little as 3–4 h to induce cytopathic effects in susceptible tissue culture cells. One could envisage that during FMDV replication, almost every component of the virus must play a role in dampening interfering cellular responses to allow such rapid virus replication.

Innate Immunity and Interferon Activation

Early protection against viral infection is fundamentally mediated by the action of interferons (IFNs), the pillar molecules of the innate immune system (12–14). Expression of IFN is triggered by the recognition of molecular signatures, collectively named pathogen-associated molecular patterns (PAMPs), *via* cellular receptors, pattern recognition receptors (PRRs) that can distinguish "self from non-self" molecules (**Figure 1**). Binding of PAMPs to PRRs triggers a series of signal transduction events and posttranslational modifications (PTMs: phosphorylation, ubiquitination, ISGylation, etc.) that ultimately activate latent transcription factors to induce IFN transcription. Subsequently, secreted IFN proteins bind to specific receptors on the plasma membrane to activate, in an autocrine and paracrine manner, discrete and overlapping cellular signal transduction pathways. Depending on the cell type and affected tissue, over 500 specific IFN-stimulated genes (ISGs) may be induced, many of which display antiviral activity to control the viral infection (12, 15, 16). There are three families of IFNs based on the specific receptor usage: types I, II, and III (**Table 1**) (13, 43–50). Type I IFNs (i.e., IFN-α and IFN-β) signal through a heterodimeric receptor complex formed by IFNAR1/IFNAR2, type II IFN (IFN-γ) signals through the complex IFN-γR1/IFN-γR2, and type III IFNs bind the receptor complex IL-28Rα/IL-10Rβ. Despite the receptor differences, the three IFN families transduce signals through the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, and type I and type III IFNs induce redundant responses (**Figure 2**). Overall, the rapid production of IFN helps to limit viral replication while modulating other immune functions.

FOOT-AND-MOUTH DISEASE VIRUS IMPAIRS INNATE IMMUNITY MOLECULAR INTERACTIONS

Recognition of FMDV RNA by the host cell results in the establishment of a rapid antiviral state to limit and control infection. This selective pressure has allowed FMDV to evolve many strategies to ensure enhanced virulence and rapid infectivity. In general, RNA viruses can bypass the IFN response by blocking: (i) global cellular transcription and translation; (ii) IFN induction; and (iii) IFN signaling. Similarly to other RNA viruses, FMDV can also target IFN-independent antiviral responses mostly associated with cellular metabolic functions (i.e., autophagy, apoptosis, stress granule formation, etc.) that have been extensively described elsewhere (51, 52). In this section, we will summarize the current literature on studies conducted *in vitro* that explain how FMDV counteracts the host innate immune response at the molecular level, including RNA sensing, activation of adaptor/effector proteins, and regulation of signaling pathways by specific PTMs.

Block on Cellular Transcription and Translation

FMDV inhibition of cellular gene expression and protein synthesis during infection is mainly driven by the viral-encoded proteases: Leader (Lpro) and 3C. FMDV Lpro is a papain-like protease (PLP) that induces cleavage of the translation initiation factor eIF4G, including eIF4GI and eIF4GII (53, 54) to disable cap-dependent protein synthesis. Also, FMDV Lpro causes degradation of the transcription factor

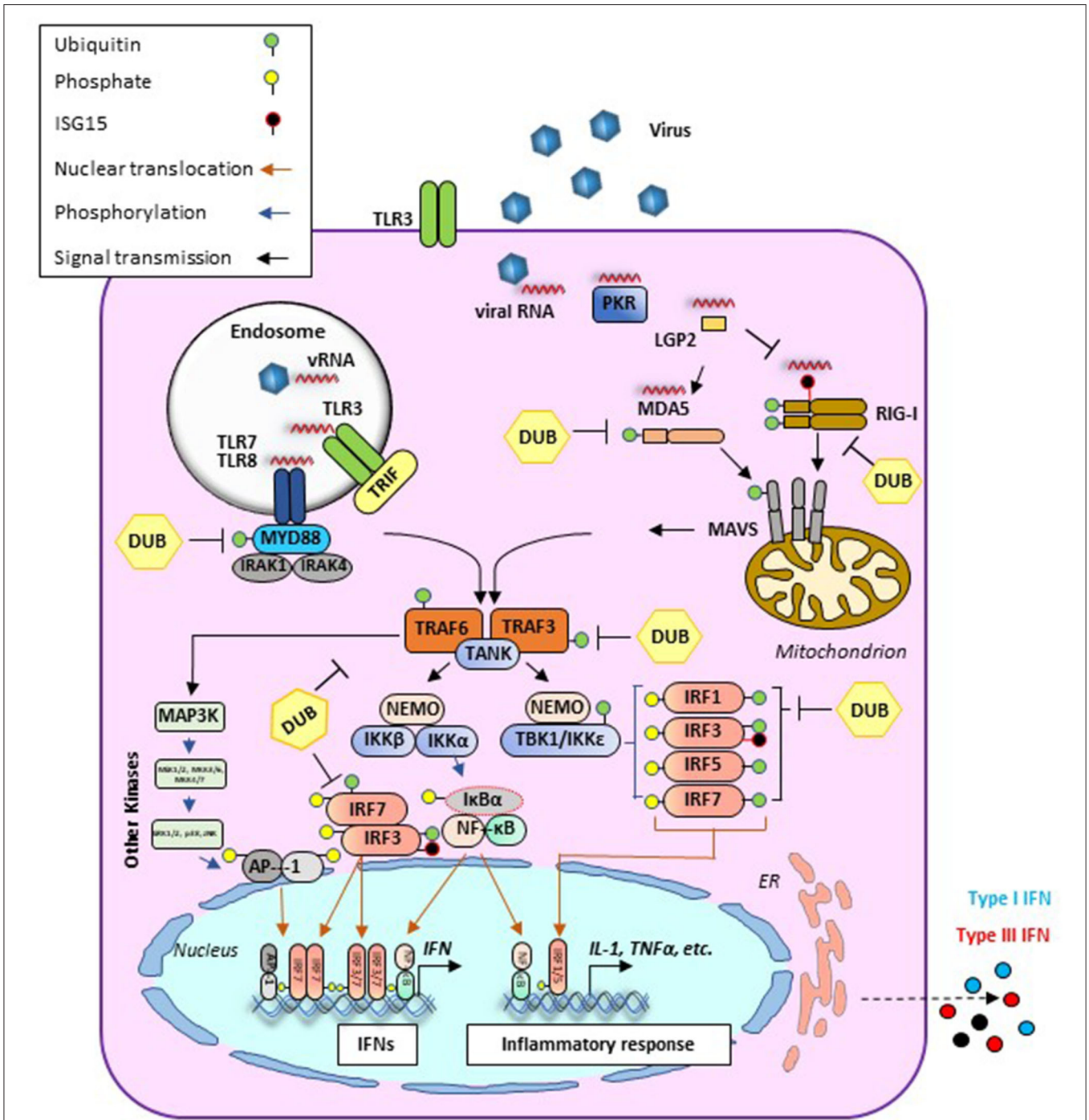

FIGURE 1 | Antiviral signaling pathways induced during viral infection. Cellular detection of microbial molecules known as pathogen-associated molecular patterns (PAMPs, i.e., viral RNA) is mediated by pattern recognition receptors (PRRs) including cytosolic RNA sensors (i.e., RIG-I, MDA-5, or LGP2) and/or membrane-bound TLRs. PAMP/PRR interaction activates signal transduction cascades (*black arrows*) that result in the production of IFN and inflammatory cytokines. RIG-I and MDA5 contain two caspase recruitment domains (CARD) and an RNA helicase domain. In the case of RIG-I, ubiquitination (*green circles*) is required for its effective activation. Activated signals from either RIG-I or MDA5 are transmitted downstream via the mitochondrial adaptor MAVS resulting in the formation of MAVS filaments. At this stage, different PTMs such as ubiquitination or ISGylation (*black circles*) can regulate their functions. Endosomal RNAs are detected by TLR3 or TLR7/8 which signal through adaptor proteins TRIF and MyD88, respectively. MyD88 uses other adaptors, IRAK1/4, to allow for interaction with TRAF proteins. In addition to their role as adaptor proteins, TRAFs also serve as E3 ubiquitin (Ub) ligases to regulate signaling. TRAF-mediated induction of poly-Ub is sensed by NEMO, thus recruiting downstream effector kinases such as TBK1 or IKK. These proteins form different signaling complexes (i.e., NEMO/TBK1 and NEMO/IKK), leading to phosphorylation (*blue arrows*) of transcription factors IRF3/7 (to a lesser extent IRF1 and IRF5 are also phosphorylated). IRF phosphorylation triggers dimerization and translocation

(Continued)

FIGURE 1 | (*orange arrows*) to the nucleus where they bind mainly to IFN promoters/enhancers. Alongside with this pathway, TRAF6-E3 ligases can activate MAPK3 and other kinases including ERK1/2 and JNK which phosphorylate the components of the AP1 heterodimer, allowing for translocation to the nucleus and binding to the IFNβ promoter/enhancer to activate transcription. Activated IKK also phosphorylates IκB, releasing NF-κB, which then translocates to the nucleus and binds at the IFNβ promoter. AP-1, activating protein 1; CARD, caspase activation and recruitment domain; DUB, deubiquitinase; ER, endoplasmic reticulum; IκB, inhibitor of KB kinases; IKK, IκB kinase; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; IRF, IFN regulatory factor; LGP2, laboratory of genetics protein 2; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene; MyD88, myeloid differentiation primary response protein 88d; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; PKR, protein kinase R; PTM, posttranslational modification; RIG-I, retinoic acid-inducible gene I; TANK, TRAF family member-associated NF-κB activator 1; TBK, TANK binding kinase; TLR, Toll-like receptor; TRAF, TNF receptor associated factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

TABLE 1 | Use of IFN-based therapies against FMDV.

Type	Recept.	Signal	Sub-type	Species	Milestone
Type I	*IFNAR1/IFNAR2*	*JAK1, TYK2*	*IFN-α/β*	Porcine/bovine	• Recombinant bacterial expressed IFN-α/β is a potent biotherapeutic against FMDV *in vitro* (17)
			IFN-α	Porcine	• Ad5 delivered poIFN-α protects swine against different serotypes of FMDV (18–20)
					• poIFN-α-protection correlates with enhanced tissue-specific innate immune cell infiltration in swine (21, 22)
					• poIFN-α protection correlates with upregulation of essential ISGs *in vitro* (23, 24)
			IFN-β	Porcine	• Ad5 delivered porcine poIFN-β protects swine against FMDV (20)
			IFN-δ	Porcine	• Bacterially expressed poIFN-δ8 significantly inhibits FMDV replication *in vitro* (25)
			IFN-ω7	Porcine	• *E.* coli produced poIFN-ω7 protects cells against FMDV (26)
			IFN-αω	Porcine	• Bacterially expressed IFN-αω added prior to infection resulted in a significant reduction in FMDV replication *in vitro* (27)
			IFN-τ	Ovine	• Ovine IFN-τ has antiviral effect against FMDV *in vitro* (28)
Type II	*IFNγR1 IFNγR2*	*JAK1, JAK2*	*IFN-γ*	Bovine	• Recombinant bovine IFN-γ reduced FMDV replication in BTY cell culture (29)
			IFN-γ	Porcine	• High dose of Ad5-poIFN-γ protects swine against FMD (30)
Type III	*IFN-λR1/IL-10R2*	*JAK2, TYK2*	*IFN-λ1*	Porcine	• Replication of FMDV in IBRS-2 cells is inhibited by treatment with the purified recombinant poIFN-λ1 (31)
			IFN-λ3	Bovine	• Inoculation with Ad5-boIFN-λ3 resulted in the induction of several ISGs in tissues of the upper respiratory tract (32) and protected cattle against challenge with FMDV (33)
				Porcine	• Ad5-poIFN-λ3 protects swine against challenge with FMDV (34)
IFN Combos			*IFN-α IFN-γ*	Porcine	• Use of a combination of Ad5-poIFN-γ and Ad5-poIFN-α (30) or Ad5-poIFN-αγ (35) showed an enhancement of the antiviral activity against FMDV in swine
Other			*Poly IC*	Porcine	• Double stranded (ds) RNA poly ICLC, in combination with Ad5-poIFN-α protected swine against FMDV (36)
			siRNA	Porcine	• Combination of Ad5-poIFN-αγ with Ad-3siRNA targeting FMDV NS coding regions blocked replication of all serotypes of FMDV *in vitro* (37)
			IRF7/3	Porcine	• Inoculation with Ad5-IRF7/3(5D) resulted in induction of IFN-α and fully protected mice and swine challenged with FMDV 1 day after treatment (38, 39)
			IRES	Porcine	• Use of synthetic IRES in combination with adjuvanted type-O FMD, improved immune response and protection against FMDV challenge (40)
IFN/vaccine combos			*IFN-α*	Porcine	• Use of a combination of Ad5-po-IFN-α and Ad5-A24 in swine resulted in complete protection after challenge (19)
			IFNα/γ	Porcine	• Ad5-poIFNα/γ co-administered with Ad5-siRNA targeting NS regions of FMDV, and a commercial inactivated FMD vaccine partially protected swine (41)
			IFN-λ3	Bovine	• Use of a combination of Ad5-bov-IFN-γ3 and Adt-O1M in cattle resulted in complete protection after aerosol challenge (42)

nuclear factor (NF)-κB and results in blockage of specific downstream signaling effectors (55, 56). Studies in porcine cells demonstrated that FMDV Lpro can promote its self-binding to the transcription factor activity-dependent neuroprotective protein (ADNP) and negatively regulate the activity of the IFN-α promoter (57). In contrast, chromatin changes that favor the upregulation of IFN and ISGs can inhibit FMDV replication (58). Interestingly, deletion or mutations in different domains of Lpro result in viral attenuation *in vitro* and *in vivo* (59–63). Furthermore, these studies have shown a strong type I IFN activity upon infection with different versions of FMDV Lpro mutants (23, 56, 61).

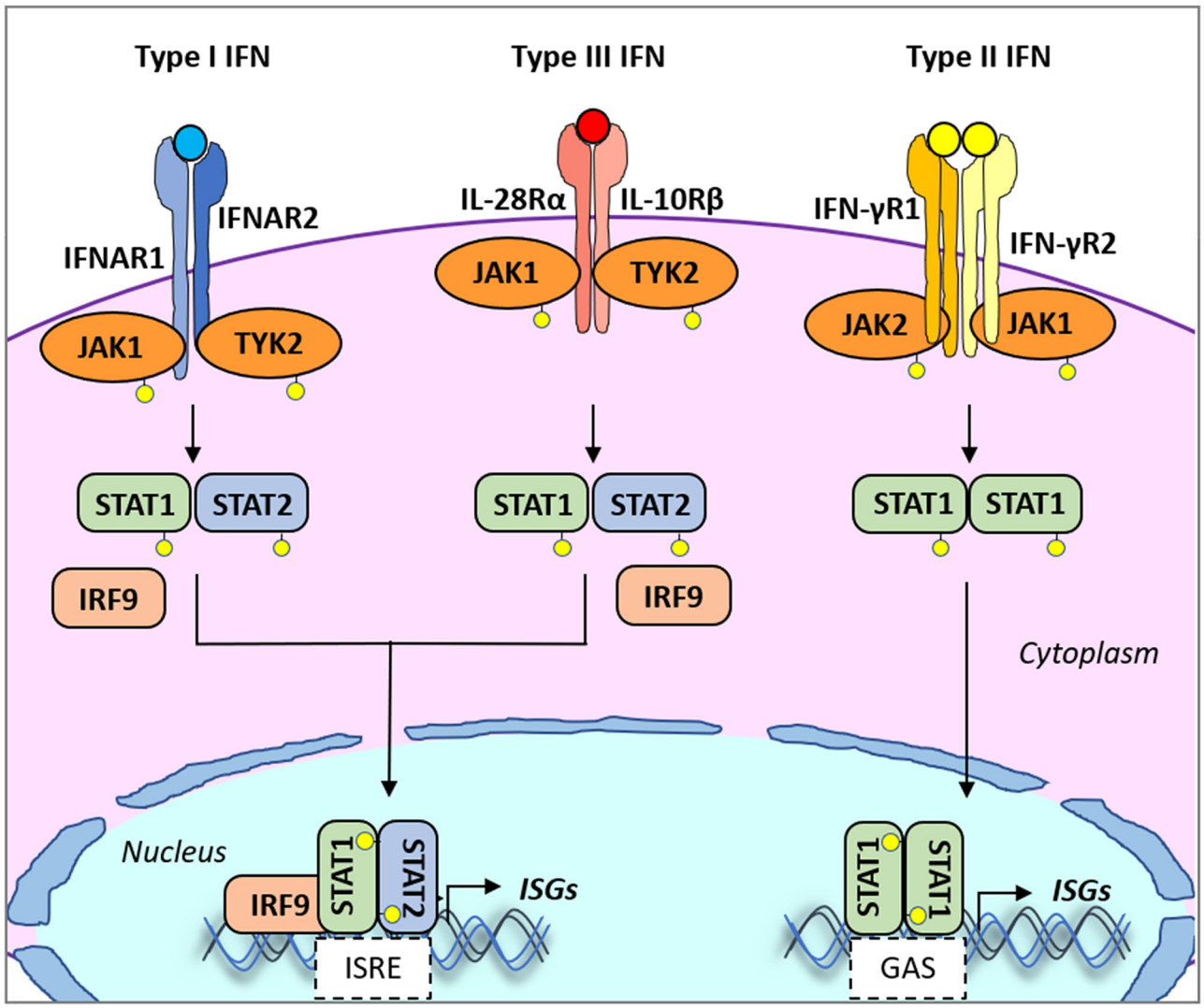

FIGURE 2 | Type I, II, and III interferon (IFN)-mediated signaling. All type I and type III IFN subtypes bind to respective receptors, IFNAR1/IFNAR2 and IFNLR1/IL10R2. These interactions trigger the phosphorylation of JAK1 and TYK2 kinases which in turn phosphorylate STAT1 and STAT2. JAK2 mediates type III IFN-dependent STAT phosphorylation. Phosphorylated heterodimers of STAT1/STAT2 bind to IRF9, forming the ISGF3G complex, which then translocates to the nucleus and binds to IFN-responsive elements (ISREs) present in the promoters of over 500 ISGs. Type II IFN binds to the heterodimeric IFNγR1/IFNγR2 receptor also inducing phosphorylation of JAK1/JAK2 kinases. In turn, mostly STAT1 is phosphorylated. Phosphorylated homodimers of STAT1 translocate to the nucleus and induce the expression of genes controlled by gamma-activated sequence (GAS)-dependent promoter sequences. IFNAR1/2, IFN alpha receptor1/2; IFNγR1/2, IFN-gamma receptor1/2; IFNALR1, IFN-lambda receptor 1; IL10R2, IL10 receptor 2; ISGs, IFN-stimulated genes; ISGF3G, ISG factor 3 gamma; JAK1/2, Janus kinase 1/2; STAT, signal transducer and activator of transcription.

Interruption of cellular translation during infection can also be mediated by FMDV 3Cpro, a chymotrypsin-like cysteine protease that similarly to Lpro targets eIF4G and the cap-binding complex eIF4A for cleavage, although these events occur later in the infection (64, 65). 3Cpro can also participate in the inhibition of host–cell transcription by cleaving histone H3 upon FMDV infection (66, 67).

Block on Interferon Induction

During infection, the initial event that leads to the production of IFN and pro-inflammatory cytokines is the recognition of viral RNA (**Figure 1**). Sensing of FMDV-RNA is mediated by MDA5 (68), a protein that belongs to a family of helicases known as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). Recent studies have shown that the interaction between RLRs (RIG-I and LGP2) and the FMDV proteins Lpro, 2B, and 3A interferes with the induction of type I IFN (69–72). Indeed, overexpression of either FMDV 2B or 3A resulted in the downregulation of RIG-I and MDA5 mRNA expression (69, 70). In contrast, upregulation of LGP2 transcripts has been observed during FMDV infection in porcine cells, despite a detectable reduction of LGP2 protein levels, presumably due to FMDV

Lpro-induced cleavage (71, 72). The apparent inconsistency between the levels of LGP2 mRNA and protein during FMDV infection may be explained by LGP2's ability to serve as a positive and negative regulator of RIG-I and MDA5 signaling, presumably affecting multiple steps of the IFN induction pathway (73). In addition to RLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), NOD1 and NOD2, also participate in the recognition of RNA. A study by Liu et al. (74) described the association of NOD2 with FMDV 2B, 2C, and 3Cpro to block innate immunity activation. Protein kinase R (PKR) is another recognized PRR that acts as an RNA sensor (75). Binding of RNA to PKR induces a conformational change that leads to autophosphorylation and activation (76). The primary target of activated PKR is the eukaryotic initiation factor 2 α subunit (eIF2α), whose phosphorylation results in the blockage of cellular protein synthesis, a relatively common process during viral infection (77). Although no direct interaction between FMDV RNA and PKR has been demonstrated, it has been reported that PKR activity modulates FMDV infectivity. In fact, in tissue culture experiments, depletion of endogenous levels of PKR using siRNA resulted in increased FMDV titers (17, 23). Furthermore, it has been recently shown that overexpression of autophagy-related ATG5-ATG12 proteins induces transcription of PKR and subsequent reduction of FMDV replication (78). These results suggest that PKR has a complex role as an RNA sensor but also as an antiviral agent during FMDV infection.

It has been demonstrated that FMDV also targets DExD/H-box RNA helicases, formally accepted as PRRs and modulators of the antiviral signaling pathway (79). *In vitro* experiments intending to analyze protein–protein interactions revealed the association between the RNA helicase DDX1 and FMDV 3D (80). Interestingly, these studies indicated that during FMDV infection in porcine cells, cleavage of DDX1 was detected, while overexpression of DDX1 resulted in the upregulation of IFN-β and other ISG mRNAs which correlated with virus inhibition (80). Other DExD/H-box RNA helicases such as RNA helicase H (RHA) are hijacked during FMDV infection and interact with FMDV 5'UTR, 2C, and 3A to facilitate virus replication (81).

Signaling pathways downstream from RNA sensing involve the activation of different adaptor and effector proteins. One of the pathways that lead to signal activation requires the formation of specific complexes such as NF-κB essential modulator (NEMO) and the kinase IKK, which bridges the activation of NF-κB and IFN regulatory factor (IRF) signaling pathways. It has been demonstrated that FMDV 3Cpro interacts with NEMO and induces its cleavage, resulting in impaired innate immune signaling (82). IRF-mediated signals driven by IRF-3 and IRF-7 can also be targeted by FMDV proteins. Specifically, overexpression of Lpro in PK-15 cells resulted in the downregulation of IRF-3 and IRF-7 protein levels and inactivation of IFN-β and IFN-λ1 promoter (31, 83).

Other factors involved in the activation of IFN include conventional PTMs such as phosphorylation and ubiquitination which ensure effective regulation of these signaling pathways (84). Also, different cellular deubiquitinases (DUBs) can reverse ubiquitination to control the intensity of the immune signaling response. Interestingly, it has been shown that FMDV Lpro can remove ubiquitin (Ub) molecules from several proteins required for IFN mRNA expression and those involved in the activation/repression of the IFN loop (85). This role became more evident by the observation that during infection, FMDV Lpro can cleave cellular substrates modified with the Ub-like molecule ISG15 (86). Furthermore, mutation of Lpro that impairs deISGylase/DUB function results in viral attenuation (87). In this regard, identification of FMDV targets for deubiquitination and deISGylation may contribute to elucidate the role of those factors in counteracting the innate response and develop novel countermeasures.

Block on Interferon Signaling

The ligand-mediated association of the specific IFN receptors promotes a signaling cascade that results in the phosphorylation of the receptor by the action of JAKs. These events result in the generation of docking sites for downstream adaptor and effector proteins including signal transducer and activator of transcription (STAT) proteins that associate with other factors and translocate to the nucleus inducing transcription of a plethora of ISGs (described above and in **Figure 2**). Although blockage of the JAK–STAT signaling pathway has not been reported during FMDV infection, overexpression of either FMDV 3Cpro or VP3 can inhibit this response. For instance, IFN-β-treated HeLa cells overexpressing FMDV 3Cpro suppressed IFN-stimulated promoter activities and induced proteasome- and caspase-independent protein degradation of karyopherin α1 (KPNA1), the nuclear localization signal receptor for tyrosine-phosphorylated STAT1 (88). This interaction inhibited the nuclear translocation of STAT1/STAT2, impeding maximal ISG promoter activity. In another study in HEK293T cells, overexpression of VP3 followed by co-immunoprecipitation revealed the association between VP3 and JAK1. FMDV VP3 also inhibited virus-triggered activation of the IFN-β promoter, leading to the decrease in transcription of ISGs presumably due to lysosomal-induced degradation of JAK1 (89). A yeast two-hybrid screen identified FMDV 2C in complex with N-myc and STAT interactor (Nmi), a protein known to augment immune function dependent on STAT-mediated transcription. Interestingly, such interaction resulted in the recruitment of Nmi to vesicular compartments followed by the induction of apoptosis in BHK-21 cells (90).

Evidently, FMDV proteins can also target crosstalk pathways induced by JAK/STAT signaling, and due to this versatility, understanding of these signaling events during FMDV infection is challenging.

FOOT-AND-MOUTH DISEASE VIRUS IMPAIRS INTERFERON-MEDIATED CELLULAR INNATE IMMUNE RESPONSES

Similarly to what happens *in vitro*, FMDV manipulates the early innate immune response *in vivo* to ensure a window of opportunity that favors viral replication and spread before

the onset of effective adaptive immunity required for virus clearance. During infection, FMDV interacts with a range of host cells including natural killer (NK) cells, dendritic cells (DCs), monocytes/Mϕ, and γδ T cells. All these cells play an important role in innate immune responses that trigger the production of large quantities of IFN and other cytokines which serve as autocrine agents (91–95).

Shortly after FMDV infection in swine, the number of circulating NK cells transiently decreases and the remaining NK cells show a dysfunctional lytic activity against target cells and a reduction of IFN-γ production (96). In parallel, FMDV blocks the ability of porcine DCs to mature into conventional DCs (cDCs) (97), dampening their response against Toll-like receptor (TLR) ligands (98). Another subset of porcine DCs, plasmacytoid DCs (pDCs), also referred to as the major professional systemic IFN-α producers, are also affected by FMDV (99, 100). During infection, partial depletion of pDCs in the peripheral blood has been detected, and the remaining pDCs are less capable of producing IFN-α in response to *ex vivo* stimulation by TLR ligands or virus (101). Similar to pDCs, FMDV infection reduces the production of IFN-α on Langerhans cells (LCs) (98), a distinct subset of tissue-resident DCs of the skin (102). It has also been suggested that porcine γδ T cells and Mϕ can serve as targets for FMDV infection in swine (103, 104), although the interplay between these cells and FMDV remains unclear.

Comparably to swine, FMDV infection in cattle triggers several early events in the innate immune system, although the effects are not exactly the same. For instance, bovine NK cells originated from FMDV-infected cows have an elevated cytotoxic function against bovine target cells *in vitro* (105). In addition, some subsets of cDCs are significantly decreased during the peak of viremia, while the expression of major histocompatibility complex (MHC) class II molecules on all bovine cDCs is reduced and the processing of exogenous antigen is impaired (106). Furthermore, during FMDV infection, the number of systemic mature bovine pDCs characterized by the expression of CD4+ and MHC class II+ is increased presumably to intensify a humoral response and T cell activation, while levels of immature CD4+ MHC class II-pDCs are declined (106). Examination of bovine γδ T cells revealed that these cells with the surface expression marker WC1+ show a transient activated phenotype and increased expression of IFN-γ (107).

FMDV also affects the innate immune response at the cytokine level in the natural host. *In vivo* cytokine profile analysis during the clinical phase of disease shows a systemic decrease of pro-inflammatory cytokines [IL-1β, IL-6, and tumor necrosis factor (TNF)-α] and an increase of the anti-inflammatory cytokine IL-10 and IFN-α (22, 33, 61, 101, 106). Most likely, these changes are related to the early T cell unresponsiveness and lymphopenia described in swine and cattle during FMDV infection (33, 102, 106, 108). Interestingly, a significant induction of inflammatory and antiviral factors at the local level is detected in cattle, in sites of abundant viral amplification, such as the nasal/oropharynx or vesicular lesions (109–111). A consistent upregulation of IFN-α, -β, -γ, and -λ mRNA in distinct microanatomical compartments of the nasopharyngeal mucosa, concurrent with occurrence of viremia, has also been detected in cattle (112). In contrast, studies in swine demonstrated that IFN expression in infected swine skin is inhibited (21). These differences may be due to the analysis of follicle-associated epithelium of the nasopharyngeal mucosa in cattle vs skin in swine or to the specific sampling technique used in each experiment. While in the cattle study laser-capture microscopy was used to focus only in areas of high FMDV replication, in the swine study, RNA was extracted from a piece of skin without discriminating between microanatomical compartments. Evidently, more studies are needed to elucidate the intricate interactions between FMDV and the innate immune system of specific animal hosts.

EFFECTIVE USE OF INTERFERON AGAINST FOOT-AND-MOUTH DISEASE VIRUS *IN VITRO*

Type I Interferon

The role of IFN in controlling FMDV replication was first proposed in 1962 when Dinter and Philipson demonstrated that calf kidney cells exposed to FMDV could become persistently infected and proposed this was a consequence of the induction of an IFN-like inhibitor present in the supernatant of infected cells (113). Later studies also suggested that swine leukocytes treated with phytohemagglutinin produced an inhibitor of FMDV replication with properties similar to IFN (114). It was not until 1999 that new studies demonstrated that the ability of FMDV to form plaques in cell culture correlated with the suppression of type I IFN (α/β) protein expression (115). These results were further supported by detection of IFN protein and antiviral activity in the supernatants of primary porcine, ovine, and bovine kidney cells infected with an attenuated FMDV mutant (leaderless) as compared to the supernatants of cells infected with wild-type (WT) virus. Later studies by the same group provided proof of concept on the use of recombinant bacterial expressed IFN-α/β as a potent biotherapeutic against FMDV (17). This approach was further developed by delivering recombinant porcine IFN-α/β using a replication-defective human Adenovirus 5 vector (Ad5-poIFN-α/β) (18). Infection of IBRS-2 cells with Ad5-poIFN-α/β resulted in secreted poIFN-α/β IFN protein detected as early as 4 h post-infection (hpi) and lasting for at least 30 h. Most important, expressed IFN protein displayed strong biological antiviral activity against FMDV. Follow-up studies by the same group showed that all FMDV serotypes are very sensitive to Ad5-delivered poIFN-α/β, and sterile protection could be achieved *in vivo*, highlighting the potential of this approach for the development into a broad biotherapeutic strategy to control FMDV replication (116).

In the last 10 years, advancements is genomics have led to the characterization of almost all type I IFN subtypes in the porcine and bovine genome (117–119), which are more numerous than those identified in primates and mice. This has revealed different functional genes and pseudogenes with diverse expression profiles and antiviral functions against different viruses, mostly in swine (118, 120, 121). In fact, a recent study

demonstrated that poIFN-ω7, known for its ability to induce the highest levels of antiviral activity when compared to other poIFN-ω subtypes, elicits an antiviral state against FMDV in IBRS-2 cells treated with the recombinant form of poIFN-ω7 produced in *Escherichia coli* (26). Other subclasses of type I IFN, known to be produced in swine and cattle, include IFN alphaomega (IFN-αω, also known as IFN-μ) and IFN delta (IFN-δ). Significant reduction in FMDV replication has been observed upon treatment of porcine cells with bacterially expressed IFN-αω or IFN-δ8 prior to viral infection (25, 27).

Recently, another member of type I IFN family, IFN-τ, which is only produced in ruminants, has been evaluated as an antiviral against FMDV (28). IFN-τ is a paracrine reproductive hormone secreted constitutively by trophoblasts and endometrial cells to increase the life span of the corpus luteum; however, production is not induced upon viral infection (122). While its secretion is restricted to ruminants, it has a broad-spectrum activity against various cross-species viruses. Interestingly, IFN-τ has 55% homology with the amino acids of IFN-α, which allows for binding to type I IFN receptors. The property of IFN-τ that makes it an interesting therapeutic candidate for the treatment of various viral diseases is its significantly lower toxicity as compared to other type I IFNs.

Type II Interferon

In contrast to type I IFN, the type II IFN family is composed of only one member, IFN-γ, which exerts its actions through a specific receptor, IFNGR1/IFNGR2. IFN-γ is weakly resistant to heat and acid, and it is able to activate leukocytes such as macrophages, and granulocytes, also exerting regulatory functions on T and B lymphocytes (123, 124). Indeed, production of IFN-γ is used as a tool to measure cell-mediated immune responses against FMDV in vaccinated cattle (125–127) and in swine (61). Interestingly, IFN-γ responses as measured by its ability to induce proliferation of CD4+ T cells correlate with a vaccine-induced protection and a reduction of FMDV persistence as it was shown for bovines inoculated with high doses of inactivated vaccine FMDV A Malaysia 97 (128). Therefore, the increase of the cellular immune response against FMDV seems to be comparable with the upregulation of IFN-γ at least in cattle (125, 127, 128).

One of the first experiments that examined the IFN-γ potential to inhibit FMDV replication was performed in bovine thyroid (BTY) cells. BTY cells were treated with different concentrations of recombinant bovine IFN-γ followed by infection with FMDV variants isolated from oropharynx cells collected from persistently infected bovines (29). Interestingly, IFN-γ pretreatment resulted in a significant reduction of viral RNA and FMDV proteins as measured by RT-PCR and ELISA, respectively. These results were further bolstered by experiments intended to provide insights on the molecular mechanism of the IFN-γ antiviral function against FMDV. Specifically, a transcriptomic analysis of FMDV-infected porcine kidney cells previously treated with IFN-γ revealed a significant upregulation of transcription factors (STAT1 and IRF1) involved in the regulation of diverse ISGs (129). By using the Ad5 vector strategy, it was also demonstrated that type II IFN displays antiviral activity against FMDV in porcine cells (19). Interestingly, significant enhancement of the antiviral effect against FMDV was observed by using a combination of Ad5-poIFN-γ and Ad5-poIFN-α. Furthermore, use of a dicistronic Ad5 vector that expresses both poIFN-γ and poIFN-α has shown enhanced antiviral activity in porcine cells (35).

Type III Interferon

The newest addition to the IFN families is the type III IFNs (IFN-λ1 or IL29, IFN-λ2 or IL28A, IFN-λ3 or IL28B, and IFN-λ4) which share signal transduction pathways of the type I IFN family albeit the use of a different cellular receptor, the IL-28Rα/IL-10Rβ heterodimer. In contrast to type I IFN receptors, which are expressed in almost all cell types, IL-28Rα is expressed in a tissue-dependent fashion such as epithelia (49, 50). In addition, downstream activation of IFN-λ-induced signals requires phosphorylation of STAT1 mediated by JAK2 (130). The first study that reported the antiviral function of IFN-λ against FMDV was conducted in bovine cell cultures (32). In this study, embryonic bovine kidney (EBK) cells treated with supernatants from cells previously transduced with Ad5-boIFN-λ3 protected cells from FMDV-induced cytopathic effects and correlated with enhanced upregulation of IFN and ISG mRNAs. Similarly, porcine cells could be protected against FMDV infection by pretreatment with recombinant porcine IFN-λ1 (poIFN-λ1) (31) or with supernatants of cells transduced with an Ad5-poIFN-λ3 (34). All together, these results demonstrated that FMDV is highly susceptible to the action of type III IFN.

EFFECTIVE USE OF INTERFERON AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN THE NATURAL HOST

Despite distinct induction of IFN and innate immune responses during FMDV infection in swine and cattle, spatial distribution of IFN is similar. In both species, *in vivo* detection of IFN occurs only after the virus has successfully replicated in the primary site and has spread systemically. In fact, similarly to what has been described *in vitro* (see previous section), the virus is very sensitive to the IFN antiviral effect *in vivo* (22). This property highlighted the potential use of these molecules as biotherapeutics against FMD, inviting new research to evaluate similar products against emerging animal diseases, a policy supported by the OIE. However, the use of IFNs in animals requires extensive testing in species of interest in order to evaluate the metabolic rate and potential adverse systemic effects of individual preparations (131–133). In this regard, although only in humans or animal models for human diseases, many approaches to change IFN's pharmacokinetic profile have been examined. These include the covalent modification of IFN with poly-ethylene-glycol (PEG) molecules (PEGylation) or the expression of recombinant IFN fused to Fc fragments of immunoglobulins. Evaluations of these modified IFNs have been tested for the treatment of multiple human diseases such as hepatitis B and C, multiple sclerosis, and cancer (134–137). Potential use of these new IFN-modified

platforms should improve its biotherapeutic function in the animal setting.

In this section, we summarize *in vivo* studies that evaluated the use of different platforms to deliver IFN or IFN inducers, alone or in combination, as a means to protect against FMD (**Table 1**).

Interferon Treatment Protects Swine Against Foot-And-Mouth Disease

The first IFN tested in swine for its antiviral activity against FMDV was poIFN-α, delivered with an Ad5 vector (18). Using this platform, swine intramuscularly (IM) inoculated with 10^9 pfu of Ad5-poIFN-α expressed relatively high levels of systemic antiviral activity detectable as early as 6 hpi and lasting for 72 h. These results correlated with complete protection against intradermal (ID) challenge with FMDV A24 at 24 h post Ad5-poIFN-α inoculation (18). Furthermore, complete protection lasted for 3–5 days, causing a delay in disease onset, reduced severity of clinical signs, and a significant reduction in viremia even when FMDV challenge was performed at 7 days post inoculation (dpi) or 1 day prior to the treatment (19). Extensive studies in swine using this vector or a modified proprietary version of Ad5 (Adt-poIFN, GenVec®) demonstrated that delivery of poIFN-β was also effective against challenge with FMDV at 1 dpi. Remarkably, depending on the administered Adt-poIFN dose, treated animals could be sterilely protected against FMD based on standardized parameters (20).

One of the advantages of using IFN against FMDV is the high likelihood for viral clearance regardless of the specific serotype (1, 138). In fact, swine experiments in which animals were inoculated with Ad5-pIFN-α and challenged intradermally (ID) 24 h later with different FMDV serotypes, A24, O1 Manisa or Asia, showed the same level of protection (20). Importantly, when the challenge was performed using a contact challenge, a route of inoculation that resembles the natural FMDV infection in swine (139–141), similar results were obtained (20).

Studies to understand the mechanisms of protection induced by type I IFN in swine demonstrated that protection of swine inoculated with Ad5-poIFN-α correlated with recruitment of partially mature skin DCs showing increased expression of CD80/86 and decreased phagocytic activity (21, 22). At the same time, an increase in the number of NK cells in draining lymph nodes was noticeable (21). These findings corresponded with upregulation of a number of ISGs, including PKR and 2′-5′-oligoadenylate synthase (OAS), which block FMDV replication in cell culture (17, 23). Other cytokines and chemokines, including monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and IFN inducible protein 10 (IP-10) which are involved in chemoattraction of DCs and NK cells (142), were also upregulated. Interestingly, using a mouse model for FMDV (143), it was shown that IP-10 is necessary for protection conferred by murine IFN-α (muIFN-α), since C57Bl/6-IP-10 knockout mice treated with muIFN-α prior to challenge were not protected against disease, whereas C57Bl/6-WT mice pretreated in the same way, were completely protected (24).

The effect of type II IFN has also been tested in swine using the Ad5 platform for delivery of IFN-γ (30). Animals IM inoculated with 10^{10} pfu of Ad5-poIFN-γ were protected against challenge at 1 dpi. Interestingly, enhanced antiviral activity was observed when a combination of Ad5-poIFN-α and Ad5-poIFN-γ was administered, allowing for Ad5-IFN vector dose sparing to fully protect swine against challenge with FMDV A24 at 1 dpi (30). More recently, Kim et al. (35) used a similar approach against FMDV O1 in swine. Enhancement of potency against FMD was observed upon treatment with an Ad5 vector that expressed bicistronically poIFN-α and IFN-γ, as compared to either IFN alone (35).

The type III family of IFNs also has an antiviral effect against FMDV *in vivo*. Swine inoculated with Ad5-poIFN-λ3 and exposed 1 day later to FMDV by contact exposure to infected swine were completely protected from clinical disease, with no detectable viremia, viral RNA, or virus shedding (34). Interestingly, protection was achieved even when systemic antiviral activity or upregulation of ISGs in peripheral blood mononuclear cells (PBMCs) were undetected. This was consistent with previous reports indicating that expression of the IFN-λ receptors (IFN-λR1) and sensitivity to IFN-λ are highest in epithelial tissues and not in leukocytes (144, 145).

Additional IFN-based therapeutics have been used *in vivo* in swine. These strategies were directed toward the use of synthetic nucleic acids that would mimic viral PAMPs or could interfere with the expression of specific viral genes without triggering the IFN response. In addition, construction of Ad5 vectors that deliver transcription factors or other antiviral factors involved in the production of IFN has been tested.

Use of nucleic acid-based molecules including the synthetic double-stranded polyriboinosinic-polyribocytidylic acid molecule stabilized with poly-L-lysine and carboxymethylcellulose (polyICLC) in combination with Ad5-poIFN-α protected swine against FMDV challenge as these animals developed the highest levels of antiviral activity along with detectable poIFN-α in the blood (36). This is in contrast with original studies done in pigs where intravenous inoculation of polyIC alone did not result in protection (146), highlighting the importance of the route of administration and immunity. Other studies have demonstrated that inoculation of mice with *in vitro*-transcribed RNAs mimicking some structural domains contained within the 5' and 3' non-coding FMDV UTRs can induce stable and robust production of systemic type I IFN (147). Moreover, the same group showed that delivery of a synthetic RNA, corresponding to 470 nt of the FMDV internal ribosome entry site (IRES), improves the immune response induced in mice in terms of timing, magnitude, and endurance of specific antibody titers (148). More recently, the same approach was evaluated in swine. Inoculation with the synthetic IRES transcript in combination with an adjuvanted type-O FMD vaccine resulted in an improved immune response and protection against FMDV challenge as compared to inoculation with the same vaccine alone (40). Interestingly, administration of this vaccine combination resulted in enhanced specific B and T cell-mediated immune responses as compared to suboptimal doses of the vaccine alone (40).

Additionally, Ad5 delivery of small interfering RNAs (siRNAs) targeting FMDV structural and NS coding regions protected swine against FMDV (149), even when animals were treated 3 days after the challenge (150).

Due to the high mutation rate inherent to RNA viruses (151, 152), use of antivirals can result in virus adaptability. Studies by Kim et al. (35, 37, 153) have proposed that the combination of antivirals including siRNA, viral polymerase inhibitors (i.e., ribavirin), and IFNs is better suited to minimize the generation of FMDV-resistant mutants. For instance, combination of Ad5-poIFN-α/γ with an Ad5 expressing three different siRNAs (Ad5-3siRNA) targeting FMDV NS coding regions (2B and 3C) was effective against all serotypes of FMDV in swine cells (37). Thus, a combined treatment with Ad5-poIFN-α/γ and Ad5-3siRNA could work as a fast-acting antiviral treatment to induce protection prior to the induction of vaccine-mediated adaptive immunity.

Another approach known to induce an early broad innate immune response is the use of replicon vaccine vector systems, such as the Venezuelan equine encephalitis virus (VEE) replicon particles (VRPs) (154). Treatment with this biotherapeutic platform results in the upregulation of a number of ISGs and the production of type I IFN protein (155) and has been tested successfully against FMDV *in vitro* and *in vivo* using a mouse model (24).

Baculovirus-based strategies have also proved successful in mice against FMD based on their robust IFN induction capacity. Molinari et al. (156) demonstrated that pretreatment of C57Bl/6 mice with a single injection of *Autographa californica* nuclear polyhedrosis virus (AcNPV) at 3 h or 3 days before FMDV challenge prevented animal death and decreased symptoms of disease and viremia. Further, treatment of mice with a combination of AcNPV and vaccine conferred early and full protection against lethal FMDV challenge (157).

More recently, a constitutively active transcription factor, IRF7/3(5D) fusion protein was explored as a means to induce innate responses against FMDV. *In vivo* delivery of IRF7/3 (5D) using the Ad5 vectored expression system resulted in potent induction of IFN-α and complete protection against FMDV in mice and swine (38, 39).

Interferon Treatment Protects Cattle Against Foot-And-Mouth Disease

Although the use of type I IFN using the Ad5 platform has been proven very successful in swine, preventive therapy only had limited efficacy in cattle. Inoculation of bovines with high doses of Ad5-poIFN-α or Ad5-bovine IFN-α (Ad5-boIFN-α) induced a relatively low level of systemic antiviral activity (100–200 U/ml), and challenge of these animals with FMDV A24 by intradermolingual (IDL) inoculation only resulted in a short delay and reduced severity of disease as compared to control animals (158).

In contrast, in preliminary experiments, the use of the type III IFN in bovine proved to be more successful than the use of type I IFN (32), although inoculation of cattle with Ad5-boIFN-λ3 resulted in low levels of systemic antiviral activity. Interestingly, induction of several ISGs was detected in tissues of the upper respiratory tract, known targets of FMDV. An enhanced effect in ISG upregulation was detected when animals were treated with a combination of Ad5 vectors expressing type I and III IFNs. Inoculation of cattle with high doses of Ad5-boIFN-λ3 followed by FMDV IDL challenge at 24 hpi resulted in a significant delay (6–12 days) and reduced severity of disease (33). Furthermore, a stronger effect was detected when treated cattle were challenged by aerosolization of FMDV using a method that best resembles the natural route of infection (140). No clinical signs of FMD, viremia, or viral shedding were found in the Ad5-boIFN-λ3-treated animals for at least 9 days post-challenge, and one of three inoculated animals remained free of disease during the entire experiment (33). These results indicated that boIFN-λ3 plays a critical role in the innate immune response of cattle against FMDV, and treatment with Ad5-boIFN-λ3 is an effective biotherapeutic approach to control FMD in bovines.

COMBINATION OF INTERFERON AND FOOT-AND-MOUTH DISEASE VACCINE AS AN APPROACH TO FULLY PROTECT LIVESTOCK AGAINST FOOT-AND-MOUTH DISEASE VIRUS

A complete control strategy would ideally include both, a rapid-acting approach to immediately limit disease spread, and a long-lasting preventive measure to protect livestock from further exposure to FMDV. Therefore, it is reasonable to consider that a combination treatment of IFN and vaccine would be the best strategy to control FMD. In proof-of-concept studies in swine, a combination of Ad5-poIFN-α and an Ad5 vaccine that delivers structural and capsid processing proteins of FMDV A24 (Ad5-FMD-A24) resulted in complete protection when animals were challenged at 1–5 dpi while a strong adaptive immune response was induced (19). Using a comparable platform, a combination of Ad5-boIFN-λ3 and Ad5-FMD-O1M had a similar performance in cattle. In this experiment, complete protection was achieved after animals were exposed to FMDV by aerosol (42). Remarkably, protection of animals treated with the combination occurred despite the absence of detectable neutralizing antibodies or antiviral activity in serum at the time of the challenge (42). Although not proved in this study, it is possible that the remaining antiviral activity at the mucosal level was able to block FMDV replication, as described for type III IFN during rotavirus infection (159). However, exploring other protective mechanisms such as cellular immunity should also be considered to understand this protection. Other strategies that have been explored *in vivo* include the simultaneous treatment with an Ad5 that delivers poIFN-α and FMDV VP1, but this study was performed in mice and it was not followed up with experiments in the natural FMDV host (160). More recently, You et al. (41) tested in swine the efficacy of the combined treatment with three antivirals, Ad5-poIFNα/γ co-administered with Ad5-siRNA, and a commercial inactivated FMD vaccine, however, only partial protection was observed

when challenge was performed at 1, 2, or 7 days post-vaccination (dpv) (41). All together, these results indicate that a combination treatment of IFN and vaccine is a desirable strategy that could be used to fully protect cattle and swine from FMD.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Over the past 20 years, considerable progress has been made in the development of IFN-based biotherapeutics to control FMD. The use of different delivery technologies, such as the Ad5 vector, highlighted the ability of IFN to confer protective immunity against FMDV in swine and cattle. Importantly, the identification of different cellular factors and cellular immune responses that are targeted during FMDV infection and affect the IFN system furnished our knowledge of FMDV virulence and pathogenesis. These discoveries permitted the development of new intervention strategies to improve IFN-based therapies such as proper selection of IFN type, evaluation of the route and site of inoculation, and utilization of synthetic IFN inducers that could act as potential adjuvants, augmenting the intrinsic biotherapeutic effect, and also improving FMD vaccine performance. Such strategies seem ideal for application in endemic regions to potentially reduce the number of exposed or at high risk of exposure animals. On the other hand, a similar strategy could be applied in the unfortunate event of outbreaks in FMD-free countries that opt for a vaccination-to-kill policy. In this case, by using an antiviral/vaccine combination approach, disease spread would be more limited, hopefully reducing the economic burden.

However, before IFNs could be used as a gold standard therapeutic agent against FMD, several considerations must be taken. For instance, metabolic rate of absorption and toxicity should be carefully evaluated to finely tune therapeutic doses for each animal species of interest. Study of specific IFN expression profiles and intrinsic antiviral activities in different tissues may also help to improve and optimize treatments for specific animal hosts.

Some of these shortcomings could be aided by selecting the right type and subtype of IFN, depending of the specific animal species of interest. In addition, novel advancements in protein engineering have demonstrated that IFN potency and bioavailability could be improved. In this regard, chemically modified IFN molecules (i.e., PEGylation) or other protein fusions deserve being evaluated as possible interventions for animal diseases. Finally, continuing studies to better characterize innate immune responses during FMDV infection *in vitro* and *in vivo* will help refine our understanding of the anti-FMDV properties of IFN and hopefully develop improved therapeutics for effective FMD control and disease eradication.

AUTHOR CONTRIBUTIONS

All authors contributed equally with literature searches, writing, editing, and approving the submission.

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Serological Evidence of Foot-and-Mouth Disease Infection in Goats in Lao PDR

Nagendrakumar B. Singanallur[1], Sonevilay Nampanya[2,3], Isabel MacPhillamy[2], Vilayvanh Soukvilay[3], Chattouphone Keokhamphet[3], Russell D. Bush[2], Syseng Khounsy[3], Navneet K. Dhand[2], Peter Windsor[2] and Wilna Vosloo[1]*

[1] Australian Centre for Disease Preparedness (Formerly Australian Animal Health Laboratory), CSIRO-Health and Biosecurity, Geelong, VIC, Australia, [2] Sydney School of Veterinary Science, University of Sydney, Camden, NSW, Australia, [3] National Animal Health Laboratory, Department of Livestock and Fisheries, Kounta, Vientiane, Laos

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***Correspondence:**
Nagendrakumar B. Singanallur
nagendra.singanallur@csiro.au

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Foot and Mouth Disease (FMD) causes significant economic loss in Lao PDR (Laos) and perpetuates the cycle of smallholder poverty mainly through large ruminant productivity losses, increased costs of production and potential limitations to market access for trade in livestock and their products. Goats are emerging as an important livestock species in Laos, and there is an increasing trend in the number of households with goats, often farmed alongside cattle and buffalo. Although an FMD susceptible species, very little is known about the role of goats in the epidemiology of the disease in Laos. A cross-sectional seroprevalence study was conducted by detecting antibodies to the non-structural proteins (NSP), an indication of a previous infection, and serotype-specific structural proteins (SP) that could be due to vaccination or infection. The study commenced in late 2017 and sera were collected from 591 goats in 26 villages of northern, central and southern Laos. For a subset of sera samples, paired oral swab samples were also collected by a simple random sampling method to detect the prevalence of FMD virus infection at the time of collection. The NSP seroprevalence in the provinces of Borkeo and Xayabouli in the north was 42 and 8%, respectively and in Khammoune in the center, it was 20%. In the other five provinces, Luang Namtha and Luang Prabang (northern Laos), Xieng Khouang and Savannaket (central Laos), and Champasak (southern Laos), the seroprevalence was close to zero. The multivariable analysis indicated that age ($p < 0.001$) was positively associated with animal-level seropositivity and males were less likely to be seropositive than females (OR: 0.29; 95%CI: 0.10–0.83; $p = 0.017$). Continued sero-surveillance for FMD in goats is recommended to improve our understanding of their role in the epidemiology of FMD in the region and to extend support to FMD control decisions, particularly regarding vaccination.

Keywords: transboundary animal diseases, foot and mouth disease, South East Asia, Lao PDR, small ruminants, seroprevalence

INTRODUCTION

Foot-and-mouth disease (FMD) is a major transboundary animal disease that is endemic in Southeast Asia, causing sporadic disease outbreaks mainly in large ruminants in the Lao People's Democratic Republic (Laos) (1, 2). The disease causes significant economic losses at both national and village levels and perpetuates the cycle of smallholder poverty through reduced animal productivity, increased cost of production, particularly from treatment costs (3, 4) and potentially, limitations to market access for trading in livestock and their products (5). The South East Asia and China FMD (SEACFMD) campaign has facilitated significant national and multilateral efforts to control FMD in the region over the past two decades (2). In partnership with SEACFMD, the Australian government funded the Stop Transboundary Animal Diseases and Zoonoses (STANDZ; 2011–2016) initiative providing important technical and financial contributions toward control of FMD in South East Asia (6). Routine FMD vaccination was a key component of the STANDZ initiative and involved the administration of 1.6 million doses of bivalent (serotype O and A) or monovalent (O) FMD vaccines to large ruminants in northern Laos between 2012 and 2016 (7). Due to the vaccine sourcing strategy conducted by the OIE vaccine banks, the vaccines are guaranteed to be high quality but may be produced by numerous different reputable manufacturers (8). The Japan Trust Fund also contributed vaccines to this program. The program targeted areas which were known to be high risk for virus transmission: areas with repeat outbreaks recorded and areas with extensive livestock trade. However, due to the lack of resources at the government level in Laos, these activities have not been continued and endemic FMD viruses (FMDV) continue to circulate in Laos, with exotic serotypes occasionally emerging (6, 9).

Goats are emerging as an important livestock species in smallholder production system in Laos with small holder livestock keepers turning away from cattle and buffalo husbandry (10) but the role of goats in the maintenance and transmission of FMD is not well-studied for this region. There is an increasing trend in the number of households with goats, often farmed alongside cattle and buffalo. Since the year 2000, the national Lao goat herd has been gradually increasing from 121,700 to 588,000 by 2017 (11). With market demands in China and Vietnam, there is increased migration of goats, along with large ruminants, through Laos in to these markets (10). Goats are rarely vaccinated for FMD in South East Asia, cattle, and buffalo are often vaccinated when donor supported official FMD vaccination programs occur in Laos (7). However, goats are occasionally vaccinated but only during an outbreak response. Several studies have shown the risk posed by FMD in small ruminants (sheep and goats) and their role in spreading the disease, acting as short-term reservoirs (12–14).

FMD has been recorded in cattle and buffaloes in Laos in the northern and central provinces between 2010 and 2017. The earliest reports in this decade were in 2010–11 in the northern provinces (1) and few outbreaks were reported in these provinces between 2013 and 2017 following widespread vaccination. The STANDZ program ceased in June 2016 and all routine vaccinations were stopped due to lack of funding. FMD outbreaks re-emerged in the northern provinces in late 2017 following cessation of the vaccination campaign in 2016. Occasional outbreaks have also been reported more recently in the central and southern provinces. A summary of the FMD outbreaks in Laos since 2011 is provided in **Supplementary Table 1**.

To determine the role of goats in the epidemiology of FMD in Laos, we used a cross-sectional seroprevalence study that identified antibodies to the non-structural proteins (NSP), an indication of a previous infection, and serotype-specific structural proteins (SP) that could be due to vaccination or infection. The present study aimed to estimate the seroprevalence of FMD in goats in Laos, using ten villages within the Australian Centre for International Agricultural Research (ACIAR) funded research projects on transboundary animal diseases ACIAR project AH/2012/067 (https://aciar.gov.au/project/ah-2012-067) and AH/2012/068 (https://aciar.gov.au/project/ah-2012-068) and an additional 16 non-project sites. These projects were a collaborative activity between the University of Sydney and the Department of Livestock and Fisheries, Laos and funded by the ACIAR (AH/2012/068). The projects aimed to improve smallholder livelihoods by improving transboundary animal disease risk management and enhancing biosecure beef production (ACIAR projects AH/2012/067 and AH/2012/068).

MATERIALS AND METHODS

Study Design

Laos has seventeen provinces; each further subdivided into districts with many villages. The study was conducted between September 2017 and March 2018 in eight provinces of Laos (**Figure 1**; **Supplementary Table 2**). Five of the selected provinces were involved in FMD vaccination campaigns through the STANDZ program; in the northern and central provinces between 2012 and 2016 (7) and the central and southern provinces since 2016 funded through the New Zealand FMD control program (15). The provinces in north were Borkeo (BK), Luang Namtha (LNT), Luang Prabang (LBP), and Xayabouli (XYL); central provinces included Xieng Khouang (XK), Khoummoune (KM), and Savannakhet (SVK) and one southern Province, Champasak (CPS). Three provinces, Luang Prabang, Xieng Khouang and Xayabouli had villages actively involved in the AH/202/067 project, Savannakhet had villages actively involved in the AH/2012/068 project, and all provinces had been included at various stages in either the STANDZ or New Zealand FMD control program. The different vaccination campaigns have used either a monovalent vaccine with only an O strain (probably O1 Manisa) or a bivalent vaccine with O and A strains (probably O1 Manisa or O3039 and A Malaysia 97) sourced from a commercial vaccine manufacturer in Europe.

In 2017, there were ∼588,000 goats in Laos (11). The sample size was determined with assumptions that the expected prevalence of FMD in the population was 0.005–0.01 with 95% confidence and population size >100,000 (16). In each village, 5–10 smallholder goat farmers ($n = 134$), who owned

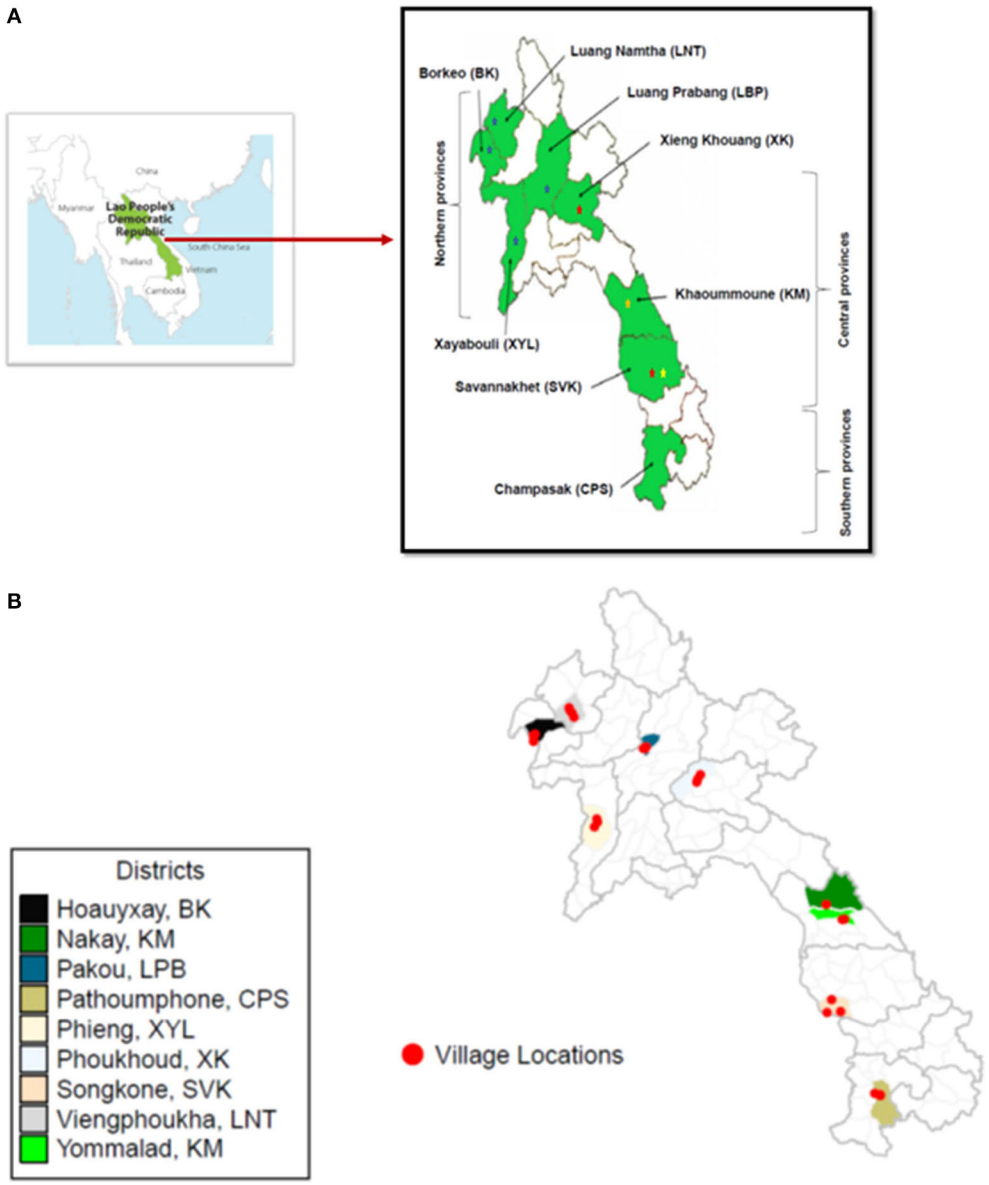

FIGURE 1 | Map of Lao PDR showing provinces in the Northern, Central, and Southern regions of the country where the goat samples were collected to study the seroprevalence of FMD in goats **(A)**. The stars represent previous outbreaks recorded in cattle and buffalos in the sample areas (blue = 2010–11; red = 2014, gold = 2016, and yellow = 2017). The locations of the villages are shown in **(B)**.

at least five goats were selected based on their willingness to participate in the survey. In each selected herd, 3–5 goats were randomly sampled ($n = 591$) resulting in a final number of 60–80 samples per province. The final study design consisted of 591 goats (445 does and 146 bucks) from 26 villages in 10 districts (**Supplementary Table 2**).

Sample and Data Collection

Blood samples were collected by jugular venepuncture, using disposable syringes (5 ml) with 21G needles. In the absence of a portable centrifuge, blood was allowed to clot inside the syringes at room temperature (~30°C) with the needle on, and the separated serum was poured into serum collection tubes within 2–3 h of collection. The serum containing vials were kept in an ice bath (4–8°C) and shipped to the nearest laboratory with a freezer for long term storage at −20°C. Finally, all samples were shipped on dry ice to the National Animal Health Laboratory (NAHL), Vientiane. In each province, oral swab samples ($n = 124$) were collected by randomly choosing a goat from each household (**Supplementary Table 1**) using GenoTube Livestock Swabs (Thermofisher, Australia). The advantage is the samples can be shipped dry without need for a transportation medium. At least 10 oral swabs were collected from each province and stored at 4–8°C until samples were transferred to NAHL. On arrival,

the swabs were transferred into lysis buffer, RNAeasy™ Mini kit (Qiagen, Germany) and stored at 4–8°C until further use.

Data were collected on animal related variables including age (in groups of <12, 12–24, and >24 months), body weight (kg) and sex (male/female) as well as grazing practices (free/forage/stall), co-grazing (yes/no); and occurrence of FMD and Orf in the last 2 years were recorded. There were no official records for vaccination of goats in any of the districts in the study area.

Laboratory Assays

Serological assays for antibodies to the NSP and SP of FMDV were performed using Prionics kits (NS ELISA Kit and serotype O, A, and Asia1 specific cELISA kits supplied in kind by M/s. Thermofisher Scientific, Australia) at the NAHL in Vientiane. All the assays were performed as per the manufacturer's instructions, and the samples declared as positive or negative based on the per cent inhibition (PI) values (PI > 50% was positive), for the NSP and serotype specific SP assays.

Total RNA was extracted from swab samples using the RNAeasy™ Mini kit (Qiagen, Germany) following the manufacturer's instructions. The purified RNA from each oral swab sample was tested with real-time RT-PCR for detection of FMDV genome (in duplicate), using an assay targeting the IRES region (17) and the Ag-Path ID One-Step RT-PCR reagents (Applied Biosystems, Australia). Reactions were performed on the IQ-cycler CFX96 (Biorad, Australia). Samples showing a Cq>38 were considered negative. Positive and negative reaction controls were included for each plate. Ribosomal 18S RNA (18S rRNA) was used as amplification controls for the real-time RT-PCR (18).

Statistical Analysis

Animals were classified as infected solely on the NSP result obtained; positive (1) or negative (0). The SP results were not considered for this classification due to the possibility that antibodies may be due to vaccination and not natural exposure. R 3.6.1 statistical software was used for data analysis (19); logistic regression analyses were conducted using the lme4 package (20). Univariable logistic regression was used to assess unconditional associations between potential risk factors (age, sex, weight, grazing and co-grazing practices and previous occurrence of FMD or Orf) and the outcome variable (NSP status; Positive or Negative). Grazing and co-grazing practices were coded at the farmer-level. Variables with a p-value <0.2 were shortlisted for the multivariable analysis. Correlations between the remaining variables were assessed using Cramer's V test with a cut-off of >0.30 (21). The geographic region and province/district/village level were assessed for significant difference with the Fisher's exact test.

A binomial logistic linear mixed model (LMM) was fitted for the multivariable analysis. Farmer, village, district and province were included as random effects to account for clustering, and the intraclass correlation (ICC) coefficient for each of these random terms was calculated based on the methodology described for ICC estimation from the random intercept logistic model (22). Clustering was deemed high for random effects that had an ICC greater the 0.3 (23). A backwards stepwise elimination approach was used until all variables had a p-value of <0.05 and were considered significantly associated with the outcome variable. Goodness-of-fit of the final regression model was assessed by calculating conditional R^2 for the final model ($R^2_{GLMM(c)}$) and the amount of variation in the data explained by the fixed effects was determined by calculating marginal R^2 for the fixed effects ($R^2_{GLMM(m)}$) (24). Estimated prevalence and confidence intervals were calculated using the prevalence package (v0.4.0) (25).

RESULTS

The details of the number of farmer households, villages, goats, mean age (±SD), and weight of goats (±SD) for each of the eight provinces from where the samples were collected are provided in **Supplementary Table 2**.

Sero-Prevalence

Prevalence analysis of only the NSP antibody assay results indicated a significant difference between the provinces ($p <$ 0.0005) with the highest number of positives in Borkeo (50%) and Xayabouli (12%) in the northern region, and Khammoune (27.5%) and Savannakhet (8.3%) in the central region (**Figure 1**, **Supplementary Table 1**, **Table 1**). Luang Prabang, Luang Namtha, Xieng Khouang, and Champasak had very low numbers of sero-positives, 0, 1.4, 1.3, and 1.3%, respectively. There was a significant difference in the seroprevalence between the three regions, i.e., north, central, and south ($p = 0.0006$), villages ($p =$ 0.0005) and districts ($p = 0.0005$).

Serotype specific ELISA kits were used to identify the proportion of animals that had antibodies to the SP of serotype O/A/Asia1 and compared that to the proportion of goats with antibodies to the NSP. A relatively low proportion of animals showed antibodies only to NSP (0–3.3%) (**Table 2**).

Amongst the northern provinces, goats in Borkeo showed a high seroprevalence to both NSP and serotype O (42%) and NSP, serotype O and A (6.6%), while Xayabouli had 8% seroprevalence to NSP and serotype O and 1.3% to NSP and serotype O and A (**Table 2**). Goats in the other two northern provinces, Luang Namtha and Luang Prabang, were seronegative to all three serotypes and 1.3 and 0% seropositive to NSP.

Of the three central provinces, goats in Khammoune had the highest seroprevalence to both NSP and serotype O (20%) followed by Savannakhet (3.3%) while these two provinces also had animals that were positive for NSP antibodies along with serotype O and A (5.0 and 1.7%, respectively). Xieng Khouang in central Laos and the southern province of Champasak did not have any goats with antibodies to serotype O, A, and Asia1. Some serum samples from Luang Namtha, Xieng Khouang and Champasak were NSP antibody positive but SP antibodies negative, the PI values for the NSP results were close to the cut-off value.

Some goats did not have antibodies to NSP but were positive for antibodies only to SP (2.5–18.7%) and in some cases, to more than one serotype (**Table 3**). In northern Laos, the province of Xayabouli had the highest percentage of animals showing

TABLE 1 | Results of apparent prevalence and true prevalence of FMDV non-structural proteins antibodies from sera collected in different sample locations in northern, central, and southern Laos between September 2017 and March 2018.

Province	District	Village	Samples tested	Number positive	Apparent prevalence (%) (95% CI)
Northern Laos			300	48	16.0 (11.8–20.1)
Borkeo			76	38	50.0 (38.7–61.2)
	Hoauyxay		76	38	50.0 (38.7–61.2)
		Houaytoum	25	9	36.0 (17.2–54.8)
		Namtoy	25	13	52.0 (32.4–71.6)
		Thongseng	26	16	61.5 (42.8–80.2)
Luang Namtha			74	1	1.4 (0.0–3.9)
	Viengphoukha		74	1	1.4 (0.0–3.9)
		Khampon	16	0	0.0
		Namkieng	25	1	4.0 (0.0–11.7)
		Phadeng	23	0	0.0
		Phoulad	10	0	0.0
Luang Prabang			75	0	0.0
	Pakou		75	0	0.0
		Hadkham	25	0	0.0
		Hadkor	25	0	0.0
		Somsanouk	25	0	0.0
Xayabouli			75	9	12.0 (4.6–19.3)
	Phieng		75	9	12.0 (4.6–19.3)
		Naboum	25	9	36.0 (17.2–57.8)
		Nongheung	25	0	0.0
		Pakthang	25	0	0.0
Central Laos			216	28	12.9 (8.5–17.4)
Khoummoune			80	22	27.5 (17.7–37.3)
	Nakay		33	10	30.3 (14.6–46.0)
		Oudoumsouk	28	6	21.4 (6.2–36.6)
		Phonpadpaek	5	4	80.0 (44.9–100)
	Yommalad		47	12	25.5 (13.1–38.0)
		Nadan	28	6	21.4 (6.2–36.6)
		Phonkeo	19	6	31.6 (10.6–52.5)
Savannakhet			60	5	8.3 (1.3–15.3)
	Songkone		60	5	8.3 (1.3–15.3)
		Bengkhamlai	19	1	5.2 (0.0–15.3)
		Sabouxay	19	1	5.2 (0.0–15.3)
		Xebanghieng	22	3	13.6 (0.00–27.9)
Xieng Khouang			76	1	1.3 (0.0–3.8)
	Phoukhoud		76	1	1.3 (0.0–3.8)
		Bong	25	0	0.0
		Naxay	26	1	3.8 (0.0–11.2)
		Phouvieng	25	0	0.0
Southern Laos			75	1	1.3 (0.0–3.9)
Champasak			75	1	1.3 (0.0–3.9)
	Pathoumphone		75	1	1.3 (0.0–3.9)
		Nakok	25	0	0.0
		Nalan	25	0	0.0
		Paktouay	25	1	4.0 (0.0–11.7)
Total			591	77	13.0 (10.3–15.7)

antibodies to SP of O (18.7%) with another 8% animals positive to both serotypes O and A (**Table 3**). Goats with antibodies to serotype O were found in all provinces. Antibodies to serotype Asia1 only were only in Champasak (1.3%) and together with serotypes O and A in Luang Namtha (1.3%) and Luang Prabang (1.3%) provinces.

No FMDV RNA could be detected in any of the 124 oral swab samples. All oral swabs, except for one, were positive for

TABLE 2 | Percentage of sera from goats seropositive for both FMDV non-structural proteins and structural proteins (serotype specific antibodies) collected in different provinces, districts and villages in northern, central, and southern Laos between September 2017 and March 2018.

	Northern Laos				Central Laos			Southern Laos
	BK	LNT	LBP	XYL	XK	KM	SVK	CPS
Total samples (*n*)	76	74	75	75	76	80	60	75
Only NSP antibodies	1.3	1.3	0	2.7	1.3	2.5	3.3	1.3
NSP and SP antibodies (%)	48.7	0	0	9.3	0	25	5	0
NSP & Serotype O only	42.1	0	0	8	0	20	3.3	0
NSP & Serotype A only	0	0	0	0	0	0	0	0
NSP & Serotype Asia1 only	0	0	0	0	0	0	0	0
NSP, Serotypes O & A	6.6	0	0	1.3	0	5.0	1.7	0
NSP, Serotypes O & Asia1	0	0	0	0	0	0	0	0
NSP, Serotypes A & Asia1	0	0	0	0	0	0	0	0
NSP, Serotypes O, A & Asia1	0	0	0	0	0	0	0	0

BK, Borkeo; LNT, Luang Namtha; LBP, Luang Prabang; XK, Xieng Khouang; XYL, Xayabouli; KM, Khoummoune; SVK, Savannakhet; CPS, Champasak.

TABLE 3 | Percentage of goats showing serotype specific antibodies in the absence of NSP antibodies indicating exposure to FMDV vaccines from sera collected in different provinces, districts and villages in northern, central, and southern Laos between September 2017 and March 2018.

	Northern Laos				Central Laos			Southern Laos
	BK	LNT	LBP	XYL	KM	SVK	XK	CPS
Total samples (*n*)	76	74	75	75	80	60	76	75
Only SP antibodies (%)	2.6	12	6.7	18.7	2.5	3.4	3.9	6.7
Serotype O only (%)	2.6	4	0	6.7	2.5	1.7	3.9	2.7
Serotype A only (%)	0	0	1.8	4	0	1.7	0	2.7
Serotype Asia1 only (%)	0	0	0	0	0	0	0	1.3
Serotypes O & A (%)	0	1.3	1.3	8	0	0	0	0
Serotypes O & Asia1 (%)	0	0	0	0	0	0	0	0
Serotypes A & Asia1 (%)	0	0	0	0	0	0	0	0
Serotypes O, A & Asia1 (%)	0	6.7	4	0	0	0	0	0

BK, Borkeo; LNT, Luang Namtha; LBP, Luang Prabang; XK, Xieng Khouang; XYL, Xayabouli; KM, Khoummoune; SVK, Savannakhet; CPS, Champasak.

the housekeeping gene, 18S rRNA (mean ± SD for Cp values was 27.9 ± 3.4) indicating successful extraction of total RNA and subsequent amplification of the housekeeping gene in the real-time RT-PCR (results not shown). This provides verification that the negative results are a true reflection of the virus status in the goats.

Univariable Binomial Logistic Regression Analyses

A total of seven variables were tested for associations with FMDV serological status based on NSP antibodies, three variables at the animal-level (age, sex, and weight) and four variables at the farmer-level (grazing practices, co-grazing, occurrence of FMD, and Orf); univariable odds ratios with 95% confidence intervals (95%CI) for these are provided in **Table 4**. Four variables returned a $p < 0.2$ and were considered for multivariable analysis. Sabouxay village (Songkone district, Savannakhet province) was the only village containing farmers that practiced co-grazing and those who did not. The remainder of the villages had either all or no farmers practicing co-grazing.

Multivariable Mixed-Effects Logistic Regression Analyses

The final model for FMDV serological status is presented in **Table 5**. Only goat-level variables remained in the final model. Goat age and sex were both significantly associated with seropositivity. Older goats (>12 months of age) had higher odds of being seropositive compared to those under 12 months. Male goats had lower odds than female goats to be seropositive. The conditional R^2 value for the overall model was 0.52; the marginal R^2 value for the fixed effects was 0.10, indicating that the fixed effects accounted for 11.3% of the variation in the data. The variances and interclass correlation coefficients (ICCs) for the four random effect terms are shown in **Table 5**. The data were highly clustered at the farmer and province levels.

DISCUSSION

Sound knowledge of the epidemiology of FMD in susceptible species in Laos is required to apply effective transboundary disease prevention and control measures. The epidemiology of FMD in large ruminants has been well-studied in the region

TABLE 4 | Descriptive and univariable binomial regression results for explanatory variables considered potential risk with FMDV serological status based on NSP antibodies, amongst 591 goats from 134 farmers surveyed in northern, central, and southern Laos between September 2017 and March 2018.

Variable	Categories	FMDV NSP status		Total	*p*-value[†]	Univariable Odds Ratio (95%CI)
		Negative (%)	Positive (%)			
Age[§]					<0.0001	
	≤12 months[‡]	249 (95)	13 (5)	262		
	13–24 months	170 (83)	36 (17)	206		12.88 (4.26–39.0)
	>24 months	95 (77)	28 (23)	123		18.11 (5.72–57.35)
Sex[§]					<0.0001	
	Female[‡]	376 (84)	69 (16)	445		
	Male	138 (95)	8 (5)	146		0.16 (0.06–0.42)
Weight (kg)					<0.0001	
	≤15[‡]	144 (94)	9 (6)	153		
	16–30	336 (84)	66 (16)	402		11.04 (3.80–32.05)
	>30	34 (94)	2 (6)	36		5.17 (0.58–46.19)
Grazing practices					0.104	
	Free grazing[‡]	443 (89)	55 (11)	498		
	Stall Fattening	21 (66)	11 (34)	32		0.56 (0.15–2.06)
	Forage grazing	50 (82)	11 (18)	61		6.76 (1.44–31.74)
Co-grazing with large ruminants					0.858	
	No[‡]	32 (56)	25 (44)	57		
	Yes	483 (90)	52 (10)	535		0.81 (0.08–8.56)
FMD has occurred in the village and district in the last 2 years					1.00	
	No[‡]	352 (88)	50 (12)	402		
	Yes	163 (86)	27 (14)	190		1.00 (0.05–18.6)
Orf has occurred in the herd/village/district in the last 2 years					0.690	
	No[‡]	295 (95)	16 (5)	311		
	Yes	220 (78)	61 (22)	281		0.64 (0.07–5.76)

[†] *GLM univariable binomial logistic regression model with farmer, village, district and province included as random terms.*
[§] *Variables included in the final multivariable model.*
[‡] *Reference category.*

TABLE 5 | Final multivariable mixed effects logistic regression model for FMDV serological status based on NSP antibodies, amongst 591 goats from 134 farmers surveyed in northern, central, and southern Laos between September 2017 and March 2018.

Variables	*β*	SE (β)	OR	95% CI (OR)	*p*-value
FIXED EFFECTS					
Intercept	−5.42	1.13			<0.0001
Age					<0.0001
≤12 months	–	–	1		
13–24 months	2.30	0.56	9.97	3.32–29.89	<0.0001
>24 months	2.54	0.59	12.68	3.99–40.30	<0.0001
Sex					0.017
Female	–	–	1		
Male	−1.24	0.54	0.29	0.10–0.83	0.023

Goodness-of-fit R_2-test statistic $R^2_{GLMM(c)} = 0.518$ $R^2_{GLMM(m)} = 0.10$; Intraclass correlation coefficient: Province = 0.60; District <0.0001; Village = 0.09; Farmer = 0.42.

(2). However, the role of small ruminants in the maintenance and transmission of FMDV in endemically infected countries has only recently received attention (13, 14, 26). Smallholder small ruminant production, particularly goats, has been emerging in Laos in recent years due to increasing regional demand, especially from China (10). However, goats are not routinely included in FMD vaccination campaigns, despite two major donor-funded FMD control programs in Laos.

This study is the first of this magnitude to report the seroprevalence of FMD in goats and the potential risk factors for FMD infection in Laos by detecting antibodies to the non-structural and structural proteins of the virus. Since goats are not routinely tested, there is a dearth of knowledge about the use of commercial serological assays for this species. In this study, commercial kits were used to determine the seroprevalence in goats and to test the application of these kits for goat sera.

The locations selected in this study have a history of routine FMD vaccination of the large ruminant populations as they are generally considered to harbor "hotspots and nodes" (areas of extensive animal trade) of FMD infection and therefore targeted to control the further spread of the disease (27). These vaccination activities likely result in a lower frequency of outbreaks, although may not necessarily stop FMDV transmission. As these are areas where FMD has historically been recognized in large ruminants, goats may be expected to have a higher seroprevalence than in those areas that have had historically had fewer outbreaks.

There is evidence in this study that some goats may have been vaccinated, with the highest proportion of animals with antibodies to the SP located in Xayabouli (18.7%) and Luang Namtha (12%). These goats had antibodies to various combinations of the serotypes in the absence of antibodies to the NSP. They could have originated from a neighboring country, where vaccination is routinely used in their national campaign for FMD control (28). It is also possible that the NSP response in these goats has decreased below detectable levels and reactions to more than one serotype could also be due to cross-reactions.

Vaccine-induced SP antibodies are only expected to remain above detectable levels for up to 6 months post-vaccination (29) and vary with the type of adjuvant (AlGel-Saponin or Oil adjuvant) used in the vaccine (30, 31). In naturally acquired FMDV infection, SP antibodies are also present. Some studies have suggested that the NSP antibodies persist for longer duration than the SP antibodies (32, 33). In fact, persistence of FMD antibodies (both SP and NSP) have been shown up to 3 years post-infection in one study (34). One study found SP antibodies to serotype A remaining at detectable level 833 days post-infection (33). Additionally, the data collection survey was unable to collect reliable life history information for the sampled animals, making it difficult to determine whether and where vaccines were administered in older animals.

The diagnostic specificity of the NSP assay used in this study is 99% in cattle (35), but the assay has not been validated for use in goats. Therefore, in the absence of clustering within a village or district, the positive samples could be the result of non-specific reactions. Another possibility of weak NSP antibody responses in some goats could be due to infection of goats leading to subclinical disease without overt clinical signs of FMD. Goats generally show mild clinical signs, and these results could indicate subclinical infection within the goat population (36).

As the provinces included in this study were purposively selected to determine if the goat population was infected in areas where outbreaks of FMD had occurred in large ruminants, selection bias was necessary, and when extrapolating the seroprevalence to the remainder of the country, caution is advised.

No antibodies to serotype Asia1 were detected in NSP positive animals, supporting the assumption that this serotype is no longer circulating in Laos (2). An outbreak in cattle was recorded in Myanmar in February 2017; the virus has been found to be closely related to samples collected in Bangladesh in 2013 (37). Although no other outbreaks caused by Asia1 have occurred prior to this study since 2006 in South East Asia (37), this serotype is still circulating in neighboring regions and with an increasingly naïve population, a reintroduction could lead to widespread outbreaks (38). Corresponding serology of the large ruminant population is required to confirm this hypothesis. Policymakers must ensure that strict biosecurity protocols are enforced to prevent the incursion of serotype Asia1 and other emerging serotypes of FMD into Laos.

Previously, free-grazing has been identified as a key risk factor for clinical FMD and NSP seropositivity in large ruminants (9). However, goats that were stall fattened were found to have a higher seroprevalence than those free-grazed (univariable OR 6.76; 95%CI 1.44–31.74). The specific differences of these practices warrant further investigation to ensure clear and consistent advice can be provided to farmers. Interestingly, in this study co-grazing was not found to be significant and goats that were co-grazed with large ruminants were marginally less likely to be seropositive (univariable OR: 0.81; 95%CI 0.08–8.56). Information was not collected on the intensity of farming for goats not co-grazed and further research is warranted to determine if there are difference in risks associated with smallholder, semi-commercial or commercial farms.

Orf outbreaks have previously been found to be incorrectly diagnosed as FMD outbreaks in Laos (10) and information on the presence of outbreaks was deemed relevant to collect. Orf outbreaks in the herd, village or district were not significantly associated with serostatus at the univariable level. However, there was a higher proportion of seropositive animals being present in an area that has had an Orf outbreak. This may warrant further investigation into factors that play a role in the spread of both diseases, and outbreak investigations are recommended to ensure the correct diagnosis is reached and appropriate control measures are implemented.

Older animals and females had higher odds of being seropositive. As females are generally retained for longer periods for breeding purposes, the likelihood they are exposed to circulating FMDV is increased, as it is for any older animal. This trend has been observed in other FMD serosurveys (14).

Cross-sectional serosurveys do not provide information regarding the temporality of disease occurrence and as a result, make it difficult to provide definitive information regarding risk factors (39). However, they do provide supporting evidence for further studies. A longitudinal serological study of proven FMD naïve animals investigating possible risk factors is recommended to determine management-related risk factors and further explore the relationship goats may play in FMDV circulation in mixed-species villages and farms. Alternatively, regular NSP antibody titer testing of targeted goat populations in recognized "hotspots" may also prove effective to further investigate the role goats play in transmission. As goats and pigs require less capital investment, they may be more likely to be present in the same villages depending on the overall socioeconomic status. It would be prudent to include pigs, goats and large ruminants in FMDV serosurveys to investigate the roles these species play in the circulation of FMDV at the village and district level in Laos (13, 14, 40). The high ICC at the province and farm level indicate the data were highly clustered with higher variance between clusters than within. This is not surprising at the farmer level as management of individual animals would be similar for each farmer and may differ between farmers. The low ICC at the

village level suggests that there is a high level of variation between management practices within each village. Further investigation is warranted at the farmer and provincial level to identify any unmeasured variables that may explain the FMD serostatus compared to the animal and farm level factors explored in this study. Further, investigation of importing behaviors and goat trade movements is increasingly important for Laos (10) and is likely to provide important information that may assist provincial and national FMD control measures.

CONCLUSIONS AND RECOMMENDATIONS

Seroprevalence to both NSP and serotype O in Borkeo, Xayabouli and Khammoune indicate the likelihood of FMDV transmission and raising the possibility that caprine outbreaks occurred and were unrecognized. In the other provinces, the seroprevalence was close to zero, and a careful analysis of the results showed that the sera that tested NSP positive were close to the cut-off value, suggesting these may be non-specific reactions. Based on these results, and in the absence of reported clinical disease and vaccination in goats, we conclude that at least two provinces in the north and one in the center had FMDV infection in goats in the recent past. The study confirmed the utility of the NSP antibody kits and other serological kits to detect antibodies against serotype O, A and Asia1 viruses, are valuable additions for FMD sero-surveillance in this region. It should be mandatory to include goats in sero-surveillance activities for FMD in Laos and presumably other countries in the region, particularly where large scale vaccination strategies in large ruminants are planned toward FMD control and establishment of FMD free zones by vaccination in South East Asia.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Sydney Ethics Committee (project no. 2015/765 and 2014/783, respectively) in compliance with State Acts and National Codes of Practice. Written informed consent for participation was not obtained from the owners because the samples were collected by staff of the National Animal Health Laboratory and the Department of Livestock and Fisheries, Lao PDR for their routine FMD sero-surveillance programs.

AUTHOR CONTRIBUTIONS

NS, SN, SK, PW, and WV contributed to the conception and design of the study. SN coordinated the field sampling. NS, VS, and CK performed the laboratory assays. IM and ND performed the statistical analysis. Funding was managed by RB, SN, SK, PW, and WV. NS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00544/full#supplementary-material

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Complex Circulation of Foot-and-Mouth Disease Virus in Cattle in Nigeria

Hussaini G. Ularamu[1], David J. Lefebvre[2], Andy Haegeman[2], Yiltawe S. Wungak[1], David O. Ehizibolo[1], David D. Lazarus[1], Annebel R. De Vleeschauwer[2] and Kris De Clercq[2]*

[1] FMD Laboratory, Viral Research Division, National Veterinary Research Institute (NVRI), Vom, Nigeria, [2] Sciensano, Scientific Direction of Infectious Diseases in Animals, Service for Exotic Viruses and Particular Diseases, Brussels, Belgium

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***Correspondence:**
David J. Lefebvre
david.lefebvre@sciensano.be

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Nigeria is a large densely populated country in West Africa. Most of its livestock is raised in a pastoralist production system with typical long distance migration in search of water and feed. As the demand for animal products largely exceeds the domestic production, large numbers of livestock are imported from neighboring countries without sanitary restrictions. In Nigeria, foot-and-mouth disease virus (FMDV) serotypes O, A, and Southern African Territories (SAT)2 are endemic for a long time. Clinical outbreaks of FMD due to serotype SAT1 are described again since 2015, after an absence of more than 30 years. Historically, outbreaks of FMD due to serotypes O, A, SAT1, and SAT2 were each time associated with trade of cattle entering Nigeria from neighboring countries. In the present study, tissue samples from 27 outbreaks of FMD were collected in Nigerian cattle from 2012 until 2017 in six different States and in the Federal Capital Territory. FMDV was isolated and serotyped and further characterized by VP1 sequencing and phylogenetic analysis to gain more knowledge on FMDV circulation in Nigeria. Half of the outbreaks were characterized as FMDV topotype O/EA-3, while outbreaks with other serotypes and topotypes were—in descending order—less prevalent: A/Africa/G-IV, SAT1/X, SAT2/VII, and O/WA. The high dynamics and omnipresence of FMD in Nigeria were illustrated in Plateau State where FMDV serotypes O, SAT1, and SAT2 were isolated during the course of the study, while at some point in the study, outbreaks due to FMDV serotype A were observed in three remote States. The genetic and phylogenetic analysis suggests a mixed origin of FMD outbreaks. Some outbreaks seem to be caused by sustained local transmission of FMDV strains present in Nigeria since a number of years, while other outbreaks seem to be related to recent incursions with new FMDV strains. The role of African buffaloes in the etiology of FMD in Nigeria is unclear, and sampling of wildlife is needed. The results of the present study suggest that systematic sample collection is essential to understand the complex concomitance of FMDV strains in Nigeria and essential to support the implementation of a vaccination-based control plan.

Keywords: foot-and-mouth disease virus, foot-and-mouth disease (FMD), Nigeria, VP1, phylogeny, topotypes

INTRODUCTION

Foot-and-mouth disease (FMD) is an acute viral infection in domestic and wild cloven-hooved animals. Viral replication causes fever and painful lesions in the mouth and on the feet resulting in lethargy, reduced feed intake, and lameness. Direct losses for farmers are due to reduced milk and meat production and to reduced draft power and transportation. Mortality may occur, usually in very young animals. Due to international trade restrictions, countries affected by FMD cannot export susceptible animals or products of these animals to countries free from FMD. As a result of direct losses and trade restrictions, FMD significantly contributes to food insecurity and poverty in endemic regions in Africa and Asia (1–3).

The foot-and-mouth disease virus (FMDV), an *Aphthovirus* in the family of the *Picornaviridae*, exists in seven different serotypes [A, O, C, Asia 1, Southern African Territories (SAT)1, SAT2, and SAT3]. It is a non-enveloped icosahedral virus consisting of four different structural proteins (VP1, VP2, VP3, and VP4) and a single-stranded positive sense RNA genome of ∼8.5 kb, which also encodes a number of nonstructural proteins that are expressed in the host cell during viral replication. The VP1 protein contains the most important neutralizing epitope, and the VP1 gene is mostly used for phylogenetic characterization because of its biological relevance and its heterogeneity. The VP1 gene is very well suited for routine monitoring of transboundary movements of FMDV (1, 4, 5).

Nigeria is a large, densely populated country in West Africa (surface 924,000 km^2, >200 million inhabitants) (6). Most of its agriculture is subsistence-oriented, and although there is an estimated population of 21 million cattle, 43 million sheep, 81 million goats, and 7 million pigs, the demand for meat products of these animal species largely exceeds the domestic production (7). Due to its low mortality compared to some other livestock diseases, FMD is not considered to be of the highest priority by Nigeria's competent authorities. Nevertheless, a recent study shows the high impact of FMD on the pastoral local dairy production system and on the food security and livelihood of the affected communities (8). There is no systematic surveillance for FMD in Nigeria and no control program, and only sporadic FMD notifications are made. Vaccination is not practiced except in a few established farms that have exotic cattle breeds which are more prone to severe clinical signs of FMD than domestic breeds (9). The Federal Republic of Nigeria is divided into 36 States plus the Federal Capital Territory (FCT) and, geopolitically, Nigeria is divided into six zones: North Central, North East, North West, South East, South South, and South West. About 90% of the cattle population and 70% of the sheep and goat populations are concentrated in the northern region of Nigeria (10).

FMD is considered endemic in domesticated livestock in Nigeria, and four different serotypes of FMDV are circulating at present: A, O, SAT1, and SAT2 (11–15). Remarkably, FMDV serotype SAT1 was never isolated from clinical cases of FMD in Nigeria in the period between 1981 and 2015 (12, 16). As in many other countries in sub-Saharan Africa, livestock movement is not controlled and FMD spreads in Nigeria due to unrestricted local and transboundary trade and due to the pastoral farming system which is characterized by long distance migration of livestock in search for greener pasture and watering points. Apart from trade practices and transhumance activities, other factors that contribute to the spread of FMD in Nigeria may be political conflict, which increases movements of nomadic people and their herds, conflict between arable farmers and pastoralists resulting from competition for resources, and the presence of game reserves as a possible source of infection for susceptible livestock (9, 13).

In the present study, samples from clinical cases of FMD in Nigerian cattle were collected from November 2012 until September 2017. The obtained FMDVs were characterized by virological and molecular techniques and compared to previously obtained and characterized viruses. As such, the aim of the study is to gain more in-depth knowledge on the circulation and distribution of FMDVs in Nigeria.

MATERIALS AND METHODS

Clinical Specimens

Epithelial tissue samples were collected from clinical cases of FMD in cattle in five States in Northern Nigeria (Bauchi, Benue, Kaduna, Nasarawa, and Plateau) within three geopolitical zones (North Central, North East, and North West), one State in South West Nigeria (Oyo) and in Abuja FCT between November 2012 and September 2017, as shown in **Figure 1**. The epithelial tissue samples were collected from un-ruptured and freshly ruptured vesicles and stored in vials containing in-house 5X-PSGA (penicillin, streptomycin, gentamycin, and amphotericin-B) diluted 1:1 with glycerol. Samples were transported on ice to the National Veterinary Research Institute (NVRI) and stored at −80°C until processing or shipment on dry ice to Sciensano. In both institutes, the samples were processed and FMDV was characterized based on the procedures described in the FMD Chapter of the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (17).

Primary Characterization

At NVRI, FMDV present in the tissue samples was characterized by virus isolation on the fetal goat tongue cell line ZZ-R 127 and a commercial antigen ELISA (IZSLER Biotech Laboratory, Brescia, Italy), as detailed previously (15). Thereafter, 81 duplicated tissue samples, originating from 27 outbreaks (17 in Plateau, three in Kaduna, two in Bauchi, two in Oyo, one in Abuja FCT, one in Benue, and one in Nasarawa), were selected and send to Sciensano for confirmatory analysis, VP1 sequencing, and phylogenetic characterization.

Confirmatory Analysis

The duplicated tissue samples were confirmed to be positive for FMDV by RNA extraction and real-time RT-PCR (rRT-PCR) using the "3D" and "5′-UTR" reference methods of Callahan et al. (18) and Reid et al. (19) as detailed previously (11), but with the addition of 5'-tails to the FMDV-specific primers to enhance the detection of FMDV as described by Vandenbussche et al. (20, 21).

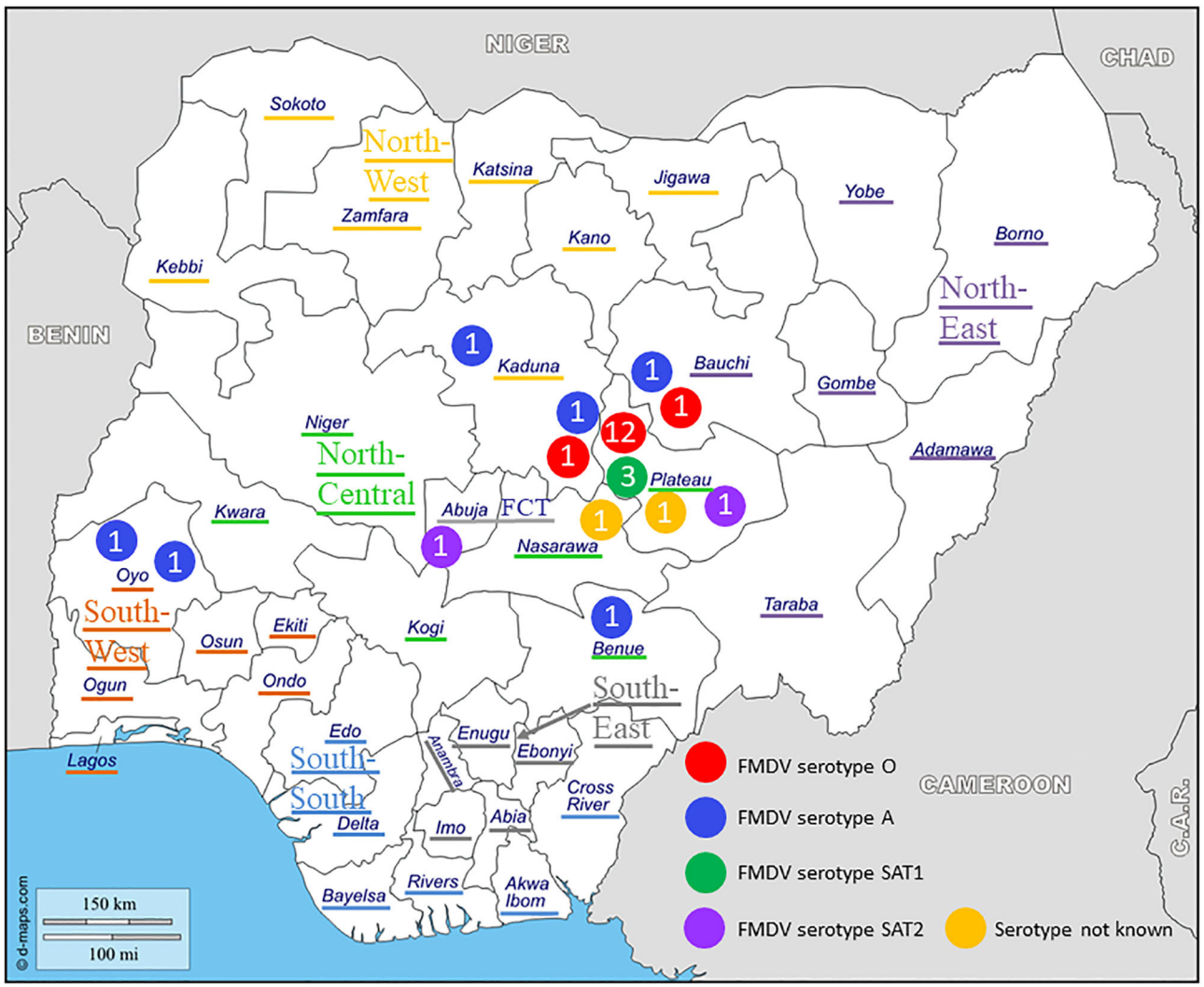

FIGURE 1 | Geographical distribution and serotypes of foot-and-mouth disease virus (FMDV) isolates collected in Nigeria between 2012 and 2017. The geopolitical zones and the Federal Capital Territory (FCT) are indicated.

Subsequently, virus isolation on porcine kidney cell line IB-RS-2 or ovine epithelial cell line OA3T was performed as detailed previously (11). In case of a positive result, as determined by cytopathic effect (CPE) formation, the FMDV present in the cell culture supernatant was serotyped by an in-house antigen ELISA detecting all seven serotypes of FMDV, as described in the OIE Manual (17) and as detailed previously (11).

From those outbreaks where the virus isolation yielded negative results despite the presence of high to moderate amounts of FMD viral RNA in the tissue samples (Ct-values < 30 as determined by rRT-PCR), attempts were made to rescue infectious FMDV from viral RNA. Briefly, BHK-21 cells (100 µl at a concentration of 5×10^6 cells/ml) in PBS were mixed in duplicate with 1 or 10 µl of RNA, respectively, in a cuvette (0.2 cm) and subjected to the exponential decay pulse protocol at a voltage of 150 V, a capacitance of 250 µF, and a resistance of 400 Ohm using a BioRad Gene Pulser Xcell electroporation system. Following electroporation, 1 ml of preheated (37°C) growth medium [minimum essential medium (MEM) with 20 µg/ml gentamicin and 1 µg/ml Fungizone] supplemented with 5% fetal bovine serum (FBS) was added to the cuvette. Subsequently, the resuspended electroporated cells were transferred into a 24-well cell culture plate containing 1 ml of growth medium and incubated in a CO_2 incubator at 37°C for 48–72 h and monitored daily for the formation of CPE. Further passages on cell culture were performed as detailed previously (11). In case of CPE, the FMDV was serotyped as described above.

VP1 Sequencing and Phylogenetic Analysis

From those outbreaks from where virus could be isolated or rescued by *in vitro* electroporation, at least one sample was sequenced and used for phylogenetic analysis. Briefly, the complete VP1 genomic region of FMDV present in the original sample was amplified by RT-PCR as described by Ayelet

TABLE 1 | Summary of foot-and-mouth disease virus sequences from Nigeria obtained in the present study.

Sample ID	State	Local Government Area	Collection date (d/m/y)	FMDV serotype/topotype	GenBank accession #
O/NIG/8/2013	Kaduna	Jema'a	27/06/2013	O/WA	MT239372
O/NIG/9/2013	Kaduna	Jema'a	27/06/2013	O/WA	MT239373
O/NIG/13/2014	Plateau	Jos South	23/03/2014	O/EA-3	MT239374
O/NIG/14/2014	Plateau	Jos South	09/06/2014	O/EA-3	MT239375
O/NIG/15/2014	Plateau	Jos South	18/07/2014	O/EA-3	MT239376
O/NIG/16/2014	Plateau	Barkin Ladi	20/07/2014	O/EA-3	MT239377
O/NIG/2/2015	Plateau	Jos South	14/09/2015	O/EA-3	MT239378
O/NIG/3/2015	Plateau	Jos South	14/09/2015	O/EA-3	MT239379
O/NIG/1/2017	Plateau	Barkin Ladi	03/08/2017	O/EA-3	MT185923
O/NIG/2/2017	Bauchi	Toro	05/08/2017	O/EA-3	MT185919
O/NIG/3/2017	Plateau	Jos South	25/08/2017	O/EA-3	MT185922
O/NIG/4/2017	Plateau	Jos South	28/08/2017	O/EA-3	MT185921
O/NIG/5/2017	Plateau	Mangu	31/08/2017	O/EA-3	MT185918
O/NIG/6/2017	Plateau	Jos-East	26/09/2017	O/EA-3	MT185920
A/NIG/08/2015	Bauchi	Toro	11/09/2015	A/Africa/G-IV	MG712579
A/NIG/09/2015	Bauchi	Toro	11/09/2015	A/Africa/G-IV	MT211640
A/NIG/01/2017	Oyo	Saki West	22/03/2017	A/Africa/G-IV	MT211641
A/NIG/02/2017	Benue	Makurdi	29/05/2017	A/Africa/G-IV	MT220002
A/NIG/04/2017	Kaduna	Kaura	04/07/2017	A/Africa/G-IV	MT228048
A/NIG/05/2017	Kaduna	Kaduna	21/09/2017	A/Africa/G-IV	MT228049
SAT1/NIG/5/2015	Plateau	Jos South	21/09/2015	SAT1/X	MT239384
SAT1/NIG/6/2015	Plateau	Jos South	27/11/2015	SAT1/X	MT239380
SAT1/NIG/7/2015	Plateau	Jos South	27/11/2015	SAT1/X	MT239381
SAT1/NIG/8/2015	Plateau	Jos South	02/12/2015	SAT1/X	MT239382
SAT2/NIG/7/2013	Abuja FCT	Abuja FCT	03/01/2013	SAT2/VII	MT239385
SAT2/NIG/1/2017	Plateau	Langtang North	14/09/2017	SAT2/VII	MT239383

et al. (22) and further processed, sequenced, and analyzed as described previously (11). The % VP1 nucleotide identity between isolates was calculated using the multiple sequence alignment tool in BLAST® at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic evaluation of the obtained FMDV VP1 regions were performed after a best fit model analysis. The resulting Bayesian information criterion (BIC) values were compared in combination with the obtained tree topologies of reference sequences in order to select the most optimal phylogenetic settings across the four FMDV serotypes included in this study. The evolutionary history was inferred by using the maximum likelihood (ML) method based on the Tamura-Nei model (23) and the neighbor-joining (NJ) method (24). Bootstrap analysis (1,000 replicates) was carried out for both methods (25) whereby branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. For the initial ML tree(s), the heuristic searches were obtained by applying the NJ method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions with <95% site coverage were eliminated. All calculations were performed in the MEGA6 software package (26). For the NJ analyses, the evolutionary distances were computed using the Tamura-Nei method (23) and the tree length was measured in substitutions per site. The rate variation among sites was modeled with a Gamma distribution. All ambiguous positions were removed for each sequence pair. Similar to the ML method, all calculations were done in MEGA6.

RESULTS

Virus Identification and Confirmation

At Sciensano, all 81 samples received from NVRI were confirmed to be positive for FMDV by rRT-PCR. Virus could be isolated from 55 out of 81 samples originating from 24 out of 27 outbreaks and from one more outbreak by *in vitro* electroporation. The presence of four different serotypes of FMDV in the set of samples (O, A, SAT1, and SAT2) was confirmed by the in-house antigen ELISA. The results of the different obtained FMDV sequences are summarized in **Table 1**.

Serotype O

Twelve outbreaks (four in Plateau State in 2014, two in Plateau in 2015, and five in Plateau and one in Bauchi in 2017) were characterized by antigen ELISA as FMDV serotype O and by VP1 sequencing and phylogenetic analysis as topotype O/EA-3. One more outbreak in Plateau (2014) was serotyped as O, but

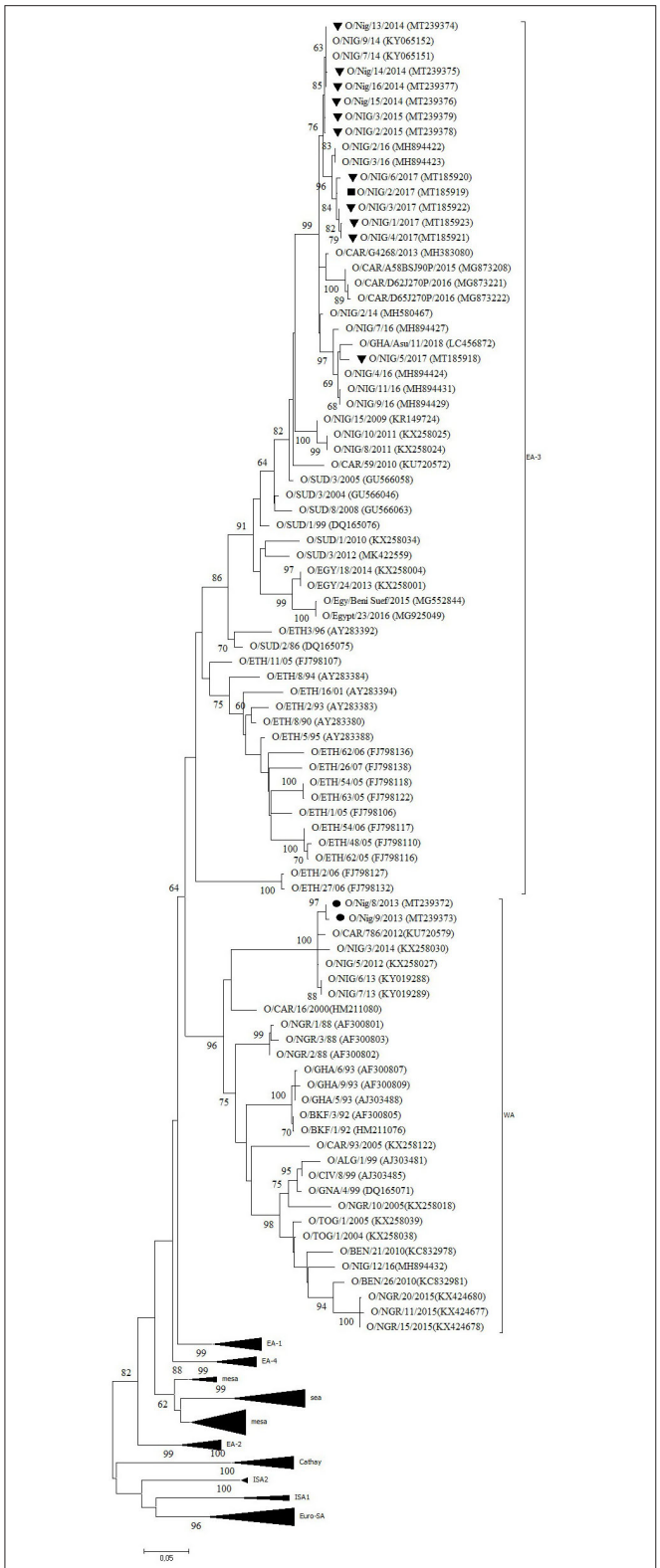

FIGURE 2 | VP1 phylogenetic tree for foot-and-mouth disease virus (FMDV) serotype O inferred using the maximum likelihood method based on the Tamura-Nei model. Branch lengths indicate the number of substitutions per site. Bootstrap values ≥60% are indicated at the nodes. Novel Nigerian FMDV from this study from Plateau is indicated with ▼, from Kaduna with ● and from Bauchi with ■.

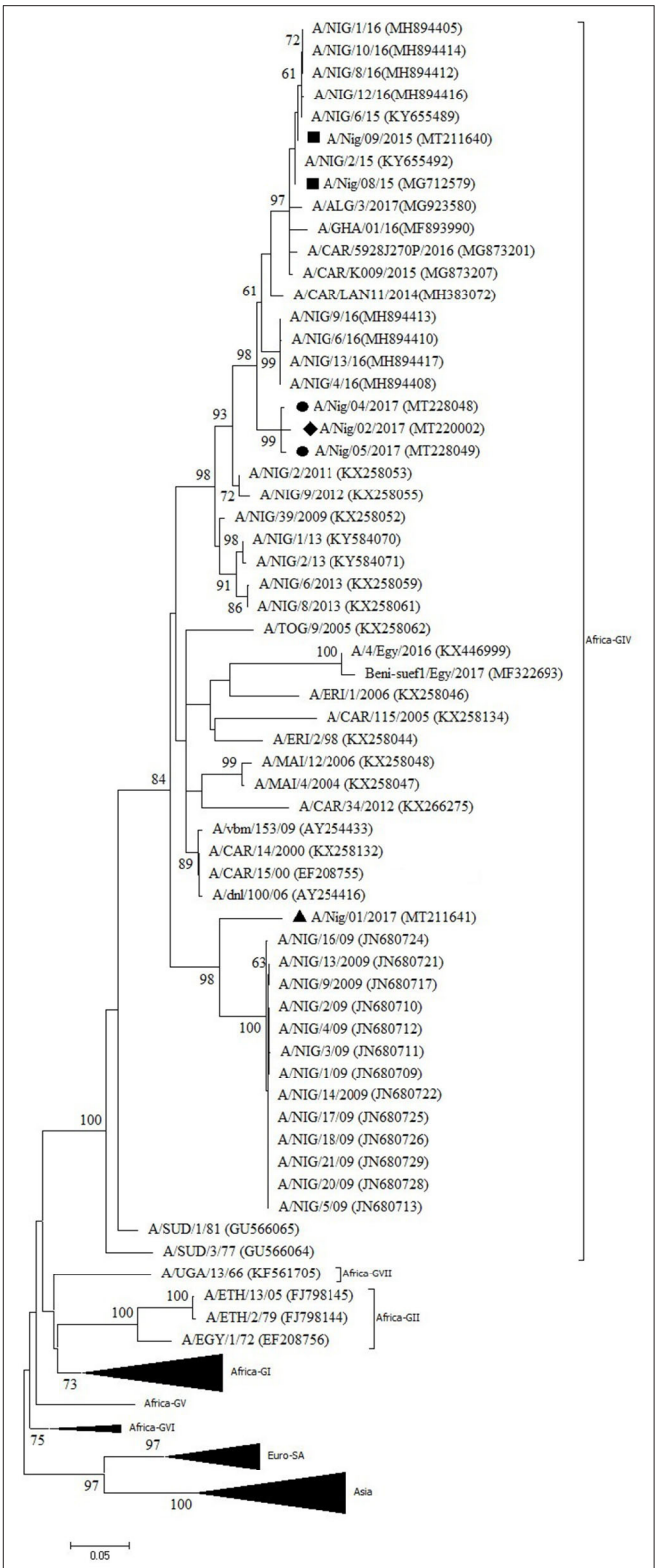

FIGURE 3 | VP1 phylogenetic tree for foot-and-mouth disease virus (FMDV) serotype A inferred using the maximum likelihood method based on the Tamura-Nei model. Branch lengths indicate the number of substitutions per site. Bootstrap values ≥60% are indicated at the nodes. Novel Nigerian FMDV from this study from Kaduna is indicated with ●, from Bauchi with ■, from Oyo with ▲ and from Benue with ♦.

a valid nt sequence could not be obtained. In the NJ and ML phylogenetic trees (**Figure 2**), the isolates from 2014 and 2015 clustered with other Nigerian isolates from 2014 (11). Most of the isolates from 2017 clustered with Nigerian isolates from 2016 (14) and grouped together with the majority of the Nigerian isolates from 2014 and 2015. Within this group, we observed ∼1% of difference in VP1 nt identity per year.

One isolate from Plateau (2017) clustered with other Nigerian isolates from 2016 (14) and with an isolate from Ghana (GHA, 2018), another country in West Africa, and with an isolate from 2014 from the Kachia Grazing Reserve in the neighboring Kaduna State, i.e., O/NIG/2/14 (13). Between this isolate from Plateau (2017) and the other isolates from 2017, we observed ∼95% VP1 nt identity.

The two isolates from an outbreak in Kaduna State (2013) were characterized by antigen ELISA as FMDV serotype O and by VP1 sequencing and phylogenetic analysis as topotype O/WA and clustered with other contemporary O/WA isolates from Nigeria and neighboring country Cameroon (CAR) (11, 15, 27).

Serotype A

One outbreak in Bauchi State in 2015 and four outbreaks in 2017 (two in Kaduna, one in Benue, and one in Oyo) were characterized by antigen ELISA as FMDV serotype A and by VP1 sequencing and phylogenetic analysis as topotype A/Africa lineage G-IV. One more outbreak in Oyo (2017) was serotyped as A, but a valid nt sequence could not be obtained. In the antigen ELISA, a frequent cross-reaction with serotype SAT1 was observed; on a single occasion, a cross-reaction with serotype SAT3. VP1 genomic sequences other than serotype A were however not found.

In the NJ and ML phylogenetic trees (**Figure 3**), the serotype A isolates from Bauchi (2015) clustered with other Nigerian isolates from 2015 and 2016 (11, 14) with >99% VP1 nt identity. A highly similar VP1 nt identity was also observed with isolates from neighboring country CAR and GHA from 2015 and 2016 (28, 29). The isolates from Benue and Kaduna (2017) clustered separately and had 96% VP1 nt identity with Nigerian isolates from 2016 (14). The isolate from Oyo (2017) clustered with Nigerian isolates from 2009 (30) with 91% VP1 nt identity with these isolates. This isolate from Oyo (2017) had 87% VP1 nt identity with the isolates from Benue and Kaduna (2017).

Serotype SAT1

Three outbreaks in Plateau State between September and December 2015 were serotyped as FMDV SAT1 and further characterized as topotype X. The isolates showed >99% VP1 nt identity with other Plateau isolates (2015–2016) and >98% VP1 nt identity with Cameroonian isolates from 2016 (12, 14). In the NJ and ML phylogenetic trees (**Figure 4**), the isolates from CAR clustered separately of those from Nigeria, as observed before (14).

Serotype SAT2

Two outbreaks, one in Abuja FCT (2013) and one in Plateau State (2017) were serotyped as FMDV SAT2 and further characterized as topotype VII. In the NJ and ML phylogenetic trees (**Figure 5**), the isolate from 2017 clustered with isolates from 2014 isolated in the neighboring State of Bauchi (14) with 97% VP1 nt identity and with viruses isolated in the neighboring countries CAR and Chad (CHD) in 2015 and 2016, respectively, and in GHA in 2018, with 94–96% VP1 nt identity (28, 31). For the isolate from Abuja FCT (2013), only a partial VP1 sequence was obtained. This isolate showed 95% VP1 nt identity with the isolate from Plateau (2017) and clustered with isolates from neighboring country CAR from 2012 (32, 33) as well as from Libya (LIB) in North Africa from 2012 (34). Both isolates from this study branched separately from a group of Nigerian viruses isolated from 2007 to 2013 and had ≤90% VP1 nt identity with this group. Another isolate from Nigeria from 2013, i.e., SAT2/Nig/5/13 (11), clustered with isolates from Libya from 2003 (34).

DISCUSSION

In the present study, samples were collected from clinical cases of FMD in cattle in Nigeria during a 5-years period. The obtained FMDVs were characterized and compared with previously characterized viruses from Nigeria and neighboring countries. From ∼two thirds of the samples, live virus could be isolated on cell cultures, despite the presence of high quantities of FMD viral RNA in most of the other third of the samples. For two outbreaks from 2012, it was not possible to obtain virus or RNA of sufficient quality to allow further characterization. Serotypes O, A, SAT1, and SAT2 were detected by antigen ELISA and later on confirmed by VP1 sequencing. In some cases, a reaction to a second FMDV serotype was observed in the antigen ELISA, particularly to serotype SAT1 in samples positive for serotype A. Although this may suggest a dual infection with two virus strains of different serotypes, we did not identify serotype SAT1 VP1 genomic sequences in samples positive for serotype A.

Two thirds of the investigated outbreaks were in Plateau State (North Central region) where the National Reference Laboratory for FMD is located. And all but two of the outbreaks were in Plateau and surrounding States which are located in the subhumid region of Nigeria and where almost 50% of Nigeria's cattle population can be found (10). Nevertheless, it would be beneficial if more samples could be obtained from regions with lower livestock densities, as well as more samples from small ruminants and wildlife, to get a more complete view on the circulation and distribution of FMDV in Nigeria. This will also be necessary to support a future vaccination-based control plan.

In the present study, three different serotypes of FMDV were observed in 3 years of time in Plateau State: serotype O in 2014, 2015, and 2017, serotype SAT1 in 2015, and serotype SAT2 in 2017. In the same period, serotype A was observed in the neighboring States of Bauchi and Kaduna. Highly remarkable is that in September 2015, in <2 weeks of time, clinical cases of FMD due to serotypes O and SAT1 were observed in Local Government Area (LGA) Jos South in Plateau and due to serotype A in LGA Toro in Bauchi, with both LGAs in close proximity (<100 km). In 2017, serotype A was observed in three remote States [Kaduna (North West), Benue (North Central), and Oyo (South West)] which are separated by several

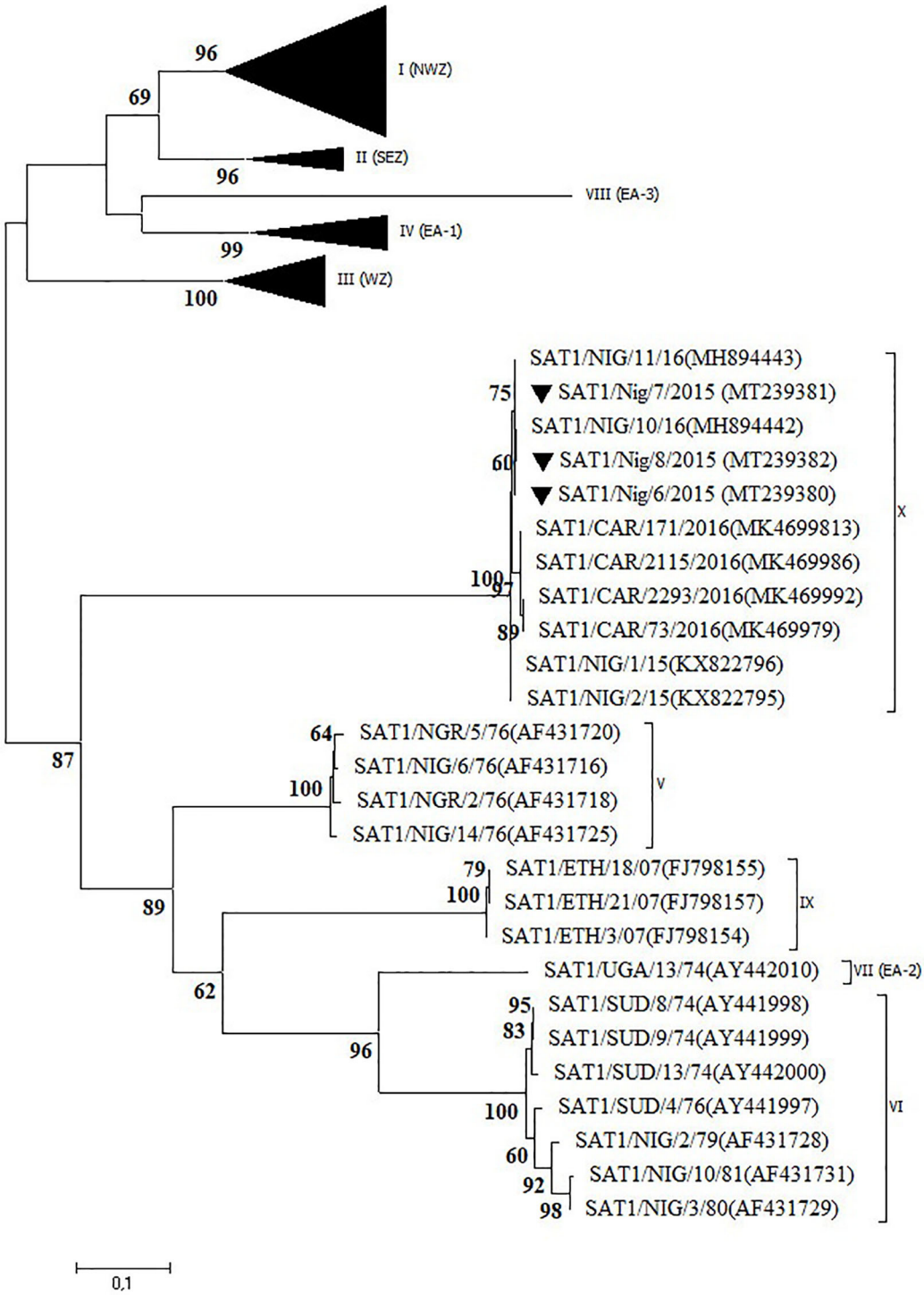

FIGURE 4 | VP1 phylogenetic tree for foot-and-mouth disease virus (FMDV) serotype Southern African Territories (SAT)1 inferred using the maximum likelihood method based on the Tamura-Nei model. Branch lengths indicate the number of substitutions per site. Bootstrap values ≥60% are indicated at the nodes. Novel Nigerian FMDV from this study from Plateau is indicated with ▼.

hundreds of kilometers. It has previously been shown that movement of infected livestock is the most important factor in the spread of FMD within endemically infected regions (2, 35, 36). Consequently, the results of the present study suggest that due to the high number of long distance and transboundary cattle movements, FMDV is highly dynamic and widely distributed

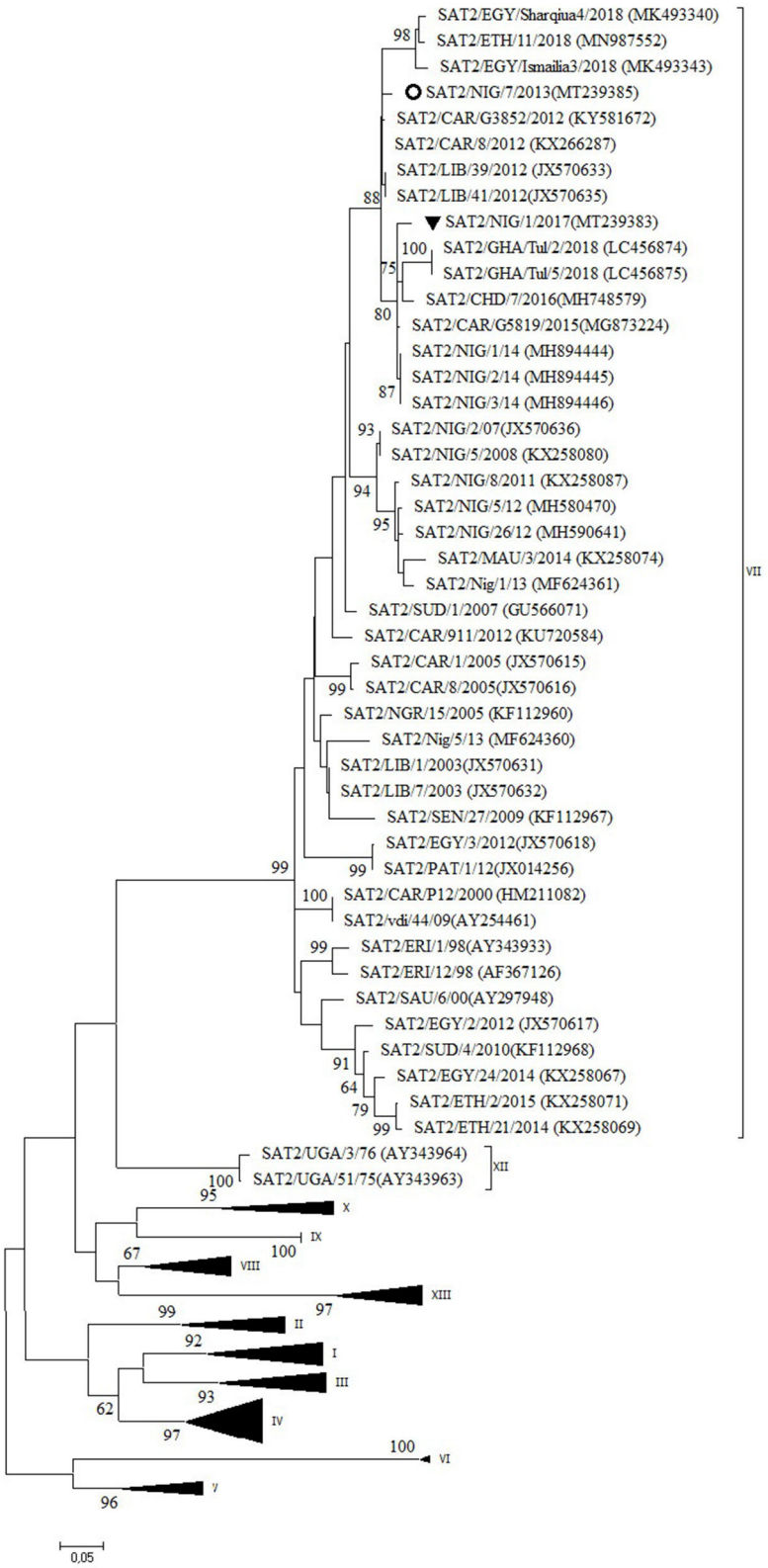

FIGURE 5 | VP1 phylogenetic tree for foot-and-mouth disease virus (FMDV) serotype Southern African Territories (SAT)2 inferred using the maximum likelihood method based on the Tamura-Nei model. Branch lengths indicate the number of substitutions per site. Bootstrap values ≥60% are indicated at the nodes. Novel Nigerian FMDV from this study from Plateau is indicated with ▼ and from Abuja FCT with ○.

in Nigeria and illustrate the complex concomitance of FMDV strains in Nigeria.

Half of the investigated outbreaks were FMDV topotype O/EA-3. Based on the phylogenetic trees and nucleotide sequence alignment, the data of the present study suggest the continued circulation in 2017 in Nigeria of the FMDV topotype O/EA-3 virus lineage described by Ehizibolo et al. (11) in 2014 with ∼1% VP1 nt change per year. This confirms previous observations from Bertram et al. (32) who suggested a pattern of continuous transmission of FMDV topotype O/EA-3 in the West African region. This % of change in VP1 nucleotide identity is in agreement with previous observations made by Knowles and Samuel (37). The data of the present study also suggest that two different sub-lineages of FMDV topotype O/EA-3 were circulating in Nigeria in 2017, in line with previous observations made by Ehizibolo et al. (14) in 2016. The data of the present study do not allow to conclude whether this is a result of two separate introductions of FMDV topotype O/EA-3 into Nigeria or a result of local virus evolution from a common ancestor. Although in the present study FMDV topotype O/WA was not isolated after 2013, it should be noted that this topotype was isolated in Niger, a country which borders to the north of Nigeria, in 2015 (38) and on a cattle market in Plateau State in 2016 (14). This suggests that the FMDV topotype O/WA continues to circulate in the region despite the abundant presence of clinical outbreaks caused by FMDV topotype O/EA-3.

Two different sub-lineages of FMDV topotype A/Africa lineage G-IV were isolated in Nigeria in 2017, respectively, in Oyo (South West region, bordering Benin) and in Benue and Kaduna (North Central and North West regions, respectively). Based on the available sequence information, the virus isolated in Oyo suggests continued circulation in Nigeria of a virus sub-lineage previously reported in 2009, with 1% change in VP1 nt identity per year, although it seems remarkable that this virus sub-lineage was not detected in Nigeria between 2009 and 2017. It should also be noted that in Benin, a country which borders to the west of Oyo State, the FMD serotype A viruses isolated in 2010 were characterized as topotype A/Africa lineage G-VI while at that time topotype A/Africa lineage G-IV was present in Nigeria (39). The viruses isolated in Benue and Kaduna in the present study may be the result of a new introduction into Nigeria in 2017 as this virus strain did not seem to circulate in Nigeria during the previous years, or at least was not detected.

The present study confirms the occurrence of a newly discovered FMDV topotype SAT1/X (12) in Nigeria in 2015 after an absence of clinical cases of FMD caused by serotype SAT1 for more than 30 years. The latter was further confirmed in the present study by serological testing of 300 samples of sheep and goat and 38 samples from wildlife obtained in the period 2009–2015. None of these 338 serum samples reacted with FMDV serotype SAT1 in the in-house solid-phase competition ELISA (17) performed at Sciensano, whereas antibodies against serotypes A, O, and SAT2 were observed (data not shown). It should however be noted that Dhikusooka et al. (40) could isolate FMDV serotype SAT1 from probang samples from young, healthy, unvaccinated cattle in Uganda. This FMDV strain differed significantly from other SAT1 FMDV strains previously isolated from cattle or buffalo in the same region. This suggests that at least some SAT1 FMDV strains can circulate in cattle herds without giving rise to clinical symptoms. Bastos et al. (41) have described the African buffalo (*Syncerus caffer*) as a reservoir host for the maintenance of FMDV serotype SAT1 and as a source of infection for domestic livestock in Southern Africa. To our interpretation, the role of African buffalo in the etiology of FMD in domestic livestock in West Africa is unclear (42). Two subspecies of the African buffalo, the West African Savannah buffalo (*S. caffer brachyseros*) and the African forest buffalo (*S. caffer nanus*), reside in the respective subhumid and humid border regions between Nigeria and CAR (42). A population of the West African Savannah buffalo is also present in the Yankari National Park in Bauchi (43). This national park is an interface of 2,250 km^2 between wildlife, domestic animals, and humans and is surrounded by villages populated by farmers and herders. It is located at ∼150 km from the first described outbreaks of FMDV topotype SAT1/X in 2015 in LGA Jos South in Plateau, but no studies have ever been conducted to detect FMDV in buffalo or other wildlife species in the Yankari National Park.

The data of the present study suggest the continued circulation in 2017 in Nigeria of the FMD SAT2/VII/Lib-12 virus lineage previously observed in Nigeria in 2014 (14) with ∼1% VP1 nt change per year, with a concomitant circulation of this virus lineage in neighboring countries. The data also suggest that three different lineages of FMDV topotype SAT2/VII circulated in Nigeria in 2013. The isolate from Abuja FCT from 2013 seems to be the earliest description of the FMD SAT2/VII/Lib-12 virus lineage in Nigeria, which seems to have become the dominant SAT2/VII virus lineage in Nigeria since then. Similarly, a pattern of repeated introductions of different FMD SAT2/VII virus lineages was observed in neighboring country CAR in the period 2010–2014 (32). The establishment of the FMD SAT2/VII/Lib-12 virus lineage from Libya in Nigeria is another example of the epidemiological link of FMDV that exists between West Africa and North Africa as more recent examples have shown the incursion of FMDV topotypes A/Africa/G-IV and O/EA-3 from West Africa into Algeria in 2017 and 2018, respectively (44, 45).

Taken together, these results indicate that the epidemiology of FMD in Nigeria is dynamic and complex and probably results from a combination of sustained local transmission of present FMDV strains and the incursion of new FMDV strains into Nigeria. This is similar to previous observations made in neighboring country CAR (46). It has been alleged that the incursion of most of these new FMDV strains results from trade of cattle entering Nigeria from neighboring countries (9).

In conclusion, in the present 5-years study conducted in Nigeria in the period 2012–2017, we isolated FMDV of topotypes O/EA-3, O/WA, A/Africa/G-IV, SAT1/X, and SAT2/VII from clinical cases in cattle and compared them with previously obtained FMDVs from Nigeria and neighboring countries. The results of our study suggest that the presence of these FMDVs result from sustained local transmission of FMDV strains present in Nigeria since a number of years ago and from repeated introductions into the country of new FMDV strains with

shorter periods of sustained transmission. The epidemiology of FMD in Nigeria is complex, and more studies, including studies in wildlife, are needed to support the implementation of control programs.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the present study are available from the authors upon reasonable request. The nucleotide sequences obtained in the present study are accessible in GenBank under the numbers indicated in **Table 1**.

ETHICS STATEMENT

Ethics approval was not required as per institutional guidelines and local legislation as the samples were collected as part of routine investigation of disease outbreaks.

AUTHOR CONTRIBUTIONS

HU conceived and designed the study, coordinated laboratory analyses at NVRI, and helped to draft the manuscript. DJL coordinated laboratory analyses at Sciensano, analyzed the data, and drafted the manuscript. AH performed the sequencing studies and the phylogenetic analysis. YW and DDL participated in the design of the study and contributed to sample acquisition. DE helped to coordinate the study and contributed to sample acquisition. AD coordinated safe sample shipment and helped to coordinate the study. KD coordinated and helped to conceive and design the study. All authors contributed to manuscript revision and read and approved the submitted version.

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REVIEW
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Advances in the Diagnosis of Foot-and-Mouth Disease

Chuan Loo Wong[1], *Chean Yeah Yong*[1,2], *Hui Kian Ong*[3], *Kok Lian Ho*[3] *and Wen Siang Tan*[1,2]*

[1] *Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Malaysia,* [2] *Laboratory of Vaccines and Biomolecules, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia,* [3] *Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia*

Foot-and-mouth disease (FMD) is a devastating livestock disease caused by foot-and-mouth disease virus (FMDV). Outbreaks of this disease in a country always result in conspicuous economic losses to livestock industry and subsequently lead to serious socioeconomic damages due to the immediate imposition of trade embargo. Rapid and accurate diagnoses are imperative to control this infectious virus. In the current review, enzyme-linked immunosorbent assay (ELISA)-based methods used in FMD diagnosis are extensively reviewed, particularly the sandwich, liquid-phase blocking, and solid-phase competition ELISA. The differentiation of infected animals from vaccinated animals using ELISA-based methods is also highlighted, in which the role of 3ABC polyprotein as a marker is reviewed intensively. Recently, more studies are focusing on the molecular diagnostic methods, which detect the viral nucleic acids based on reverse transcription-polymerase chain reaction (RT-PCR) and RT-loop-mediated isothermal amplification (RT-LAMP). These methods are generally more sensitive because of their ability to amplify a minute amount of the viral nucleic acids. In this digital era, the RT-PCR and RT-LAMP are progressing toward the mobile versions, aiming for on-site FMDV diagnosis. Apart from RT-PCR and RT-LAMP, another diagnostic assay specifically designed for on-site diagnosis is the lateral flow immunochromatographic test strips. These test strips have some distinct advantages over other diagnostic methods, whereby the assay often does not require the aid of an external device, which greatly lowers the cost per test. In addition, the on-site diagnostic test can be easily performed by untrained personnel including farmers, and the results can be obtained in a few minutes. Lastly, the use of FMDV diagnostic assays for progressive control of the disease is also discussed critically.

Keywords: foot-and-mouth disease virus (FMDV) diagnosis, complement fixation test (CFT), virus neutralization test (VNT), enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription-loop-mediated isothermal amplification (RT-LAMP), reverse transcription-recombinase polymerase amplification (RT-RPA), lateral flow device (LFD)

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***Correspondence:**
Wen Siang Tan
wstan@upm.edu.my

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INTRODUCTION

Foot-and-mouth disease (FMD) is a contagious vesicular disease caused by foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family. The virus infects a wide range of wild and domesticated cloven-footed mammals. An accidental introduction of FMDV in a susceptible population can result in an abrupt outbreak of the disease, leading to a massive economic loss.

Immediate actions are usually taken in response to an FMD outbreak to secure a differential and definitive diagnosis and to prevent further spread of the disease (**Figure 1**). To complement the vaccination and stamping out policies, early FMD detections in cloven-hoofed animals using current available diagnostic tools have been widely employed to counter this highly scrutinized agent.

Generally, a suspected case of FMD can be identified based on observations of clinical signs. Severity of the symptoms in animals is affected by many factors, such as the species and age of the animals, virus strains, dosage of exposure to FMDV, and the host immunity. The symptoms are generally more severe in cattle and intensively reared swine (high-density indoor-rearing in straw-lined sheds or group-housing), as compared to goats

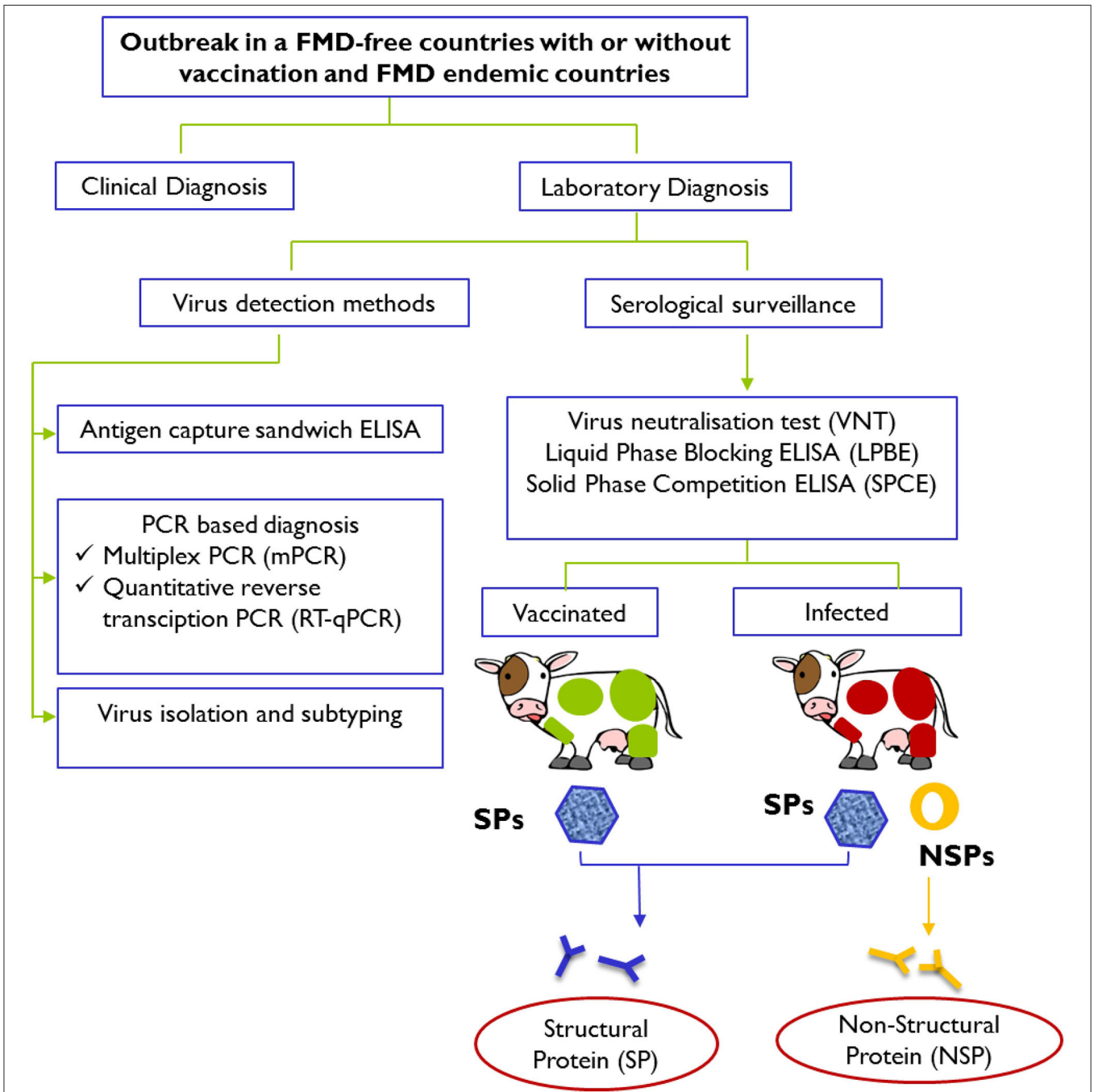

FIGURE 1 | Schematic representation of laboratory tests for determining evidence of infection with FMDV after an outbreak in FMD-free countries with or without vaccination and FMD endemic countries. Laboratory confirmation of a presumptive diagnosis of FMD involves detection and identification of viral materials in animals' samples or presence of specific antibodies against structural proteins (SPs; presence in both vaccinated and infected animals) and specific antibodies against non-structural proteins (NSPs; presence in infected animals only) in serum samples. Diagnostic procedures for FMD can be found in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019* (https://www.oie.int/standard-setting/terrestrial-manual/access-online/).

and sheep (1, 2). Typically, FMDV-infected animals will develop lesions on the tongue, muzzle, oral cavity, coronary bands, and teats. Other symptoms frequently observed include fever, loss of appetite, weight loss, hypersalivation, depression, growth retardation, and severe decrease in milk production, which could persist after recovery (2). However, diagnoses based on clinical symptoms are highly unreliable, because several other diseases share similar symptoms as FMD, which include swine vesicular disease (SVD), vesicular stomatitis and vesicular exanthema. Swine are vulnerable to vesicular stomatitis, SVD, and FMD, whereas cattle are vulnerable to vesicular stomatitis and FMD, all of which could not be distinguished based on clinical symptoms (3–5). Hence, confirmatory laboratory diagnosis of any suspected FMD case is vital.

Conventional techniques such as complement fixation test (CFT), virus isolation test, virus neutralization test (VNT), and enzyme-linked immunosorbent assay (ELISA) are routinely used to detect FMDV in clinical samples. As virus isolation tests, CFT and VNT are well-established and often used as standards in development of new detection assays; thus, they will not be discussed in detail in this article. Advancement in molecular techniques accelerates rapid and accurate diagnoses of FMDV through detection of the viral RNA. In this article, the most recent advancements in reverse transcription-polymerase chain reaction (RT-PCR) and RT-loop-mediated isothermal amplification (RT-LAMP)-based methods are thoroughly reviewed. Lastly, the roles of lateral flow immunochromatographic (LFI) test strips in FMDV diagnosis are also discussed.

NUCLEIC ACID DETECTION METHODS

Nucleic acid detection methods are molecular-based techniques used to detect the presence of viral nucleic acids. As these methods involve amplifications of viral nucleic acids, they have higher sensitivity compared to serological methods. In addition to detection of FMDV, primers used for FMDV serotyping have also been developed (6). As FMDV is an RNA virus, RT is required before the targeted viral nucleic acid can be amplified. Two of the most common nucleic acid detection methods used to detect FMDV are RT-PCR and RT-LAMP. Although the detection and typing of FMDV using microarray was also reported (7–9), its usage is highly limited, possibly due to the high operating cost. **Table 1** summarizes some recent studies on molecular diagnostic assays for the detection of FMDV.

Reverse Transcription-Polymerase Chain Reaction

Detection of FMDV using RT-PCR was first reported by Meyer et al. (32), in which a conserved region in the viral genome encoding the RNA polymerase was amplified and analyzed using agarose gel electrophoresis and further confirmed by restriction enzyme digestion or Southern blotting. Höfner et al. (33) also demonstrated the detection of FMDV in clinical samples using primers targeting the 1A and 2A/2B conserved regions, amplifying the whole viral capsid coding region. Nucleotide sequencing of the amplified region can directly aid in the study of viral epidemiology. In a separate study, Laor et al. (34) showed that the primers targeting the RNA polymerase coding region could detect FMDV of different isolates, whereas another primer set targeting the variable region of VP1 was capable of differentiation detection. Dill et al. (10) developed a universal RT-PCR, which is rapid and cost-effective in generating the genome sequences of all FMDV serotypes, allowing immediate virus genotyping, phylogenetic analysis, and epidemiological studies of FMDV. Most recently, a primer set, namely, FM8/9, which targets the conserved region of 3D domain has also been reported to be $10^{0.6}$- to $10^{3.8}$-fold more sensitive than the 1F/R primer set as suggested in the OIE manual (11).These agarose gel electrophoresis-based methods have since laid the basis for the modern RT-PCR and RT-LAMP detection methods.

To date, many improved versions of RT-PCR have been employed for the detection of FMDV. To simultaneously screen for the presence of multiple viruses, multiplex RT-PCR, which uses multiple primer sets in a single reaction, has been developed. Lung et al. (9) demonstrated the use of multiplex RT-PCR for simultaneous detection and differentiation of FMDV serotypes and other vesicular disease viruses, including vesicular stomatitis virus (VSV), swine vesicular disease virus (SVDV), and vesicular exanthema of swine virus (VESV). When the multiplex RT-PCR is used in conjunction with slide microarray, the sensitivity improved by at least one log unit. However, the sensitivity was reported to be comparable but no better than real-time RT-PCR (RT-qPCR). Similarly, Erickson et al. (22) also used multiplex RT-PCR coupled with a more advanced, automated electronic microarray assay for simultaneous detection and differentiation of several swine viruses, including FMDV and other viruses such as SVDV, African swine fever virus, porcine circovirus type 2, porcine respiratory and reproductive syndrome virus, VESV, and classical swine fever virus. Although it is convenient to detect multiple viruses of different serotypes in a single reaction, careful design, and testing of primers are needed to achieve desirable assay sensitivity and specificity, as multiplex RT-PCR assays have been reported to have better sensitivity upon removal of certain primers from the pool (22).

Real-time quantitative PCR (qPCR)-based analyses coupled with fluorescent-emitting compounds have been used to measure the number of amplicons during an amplification process in real time (35). Generally, there are two types of fluorescent-emitting compounds used in qPCR: (i) non-specific intercalating dye such as SYBR green and (ii) specific reporter probes with fluorochromes attached to specific oligonucleotide sequences, such as fluorescence resonance energy transfer (FRET) and Taqman probes. These compounds have been used in RT-qPCR for detection of FMDV [FRET- (36); TaqMan- (37, 38); SYBR green- RT-qPCR (17, 39)]. While SYBR green is more economic, TaqMan and FRET probes have the advantage as signals generated from unspecific PCR are negligible. The most common target sequences for the detection of FMDV with RT-qPCR include 3D and 5′ UTR sequences (37, 38), in which the addition of 5′-tails to the primers targeting 3D and 5′ UTR sequences was reported to enhance the detection of FMDV (16). As in RT-PCR, RT-qPCR has also been

TABLE 1 | Molecular diagnostic assays for detection of FMDV infection.

Methods	Description	Detection limit	Tested clinical samples	References
RT-PCR	Amplification of genome fragments from IRES to the end of the ORF, followed by the Sanger sequencing for serotyping	• 5 $\log_{10}$ dilution of viral RNA of FMDV O/MOG/7/2010	Bovine saliva, porcine podal vesicle, ovine saliva, caprine serum samples	(10)
	An RT-PCR based on a novel primer (FM8/9) targeting the 3D region of the FMDV	• $10^{0.2}$ to $10^{-2.8}$ $TCID_{50}$/mL of FMDV depending on the strains	Serum, and saliva samples from pigs and cows	(11)
RT-iiPCR	Utilizes a commercially available compact, portable POCKIT™ Nuclei Acid Analyser (GeneReach, USA) for rapid (<2 h) detection of all seven serotypes of FMDV. Coupling to the taco™ mini extraction kit (GeneReach, USA), the detection assay detected 63 different FMDV strains representing all seven serotypes. Detection of the FMDV RNA from vesicular fluid samples is possible without nuclei acid extraction	• ≥9 copies of *in vitro*–transcribed FMDV O1 Manisa/69 3D RNA	Nasal and oral swabs from calves, sheep, and piglets, oral fluid, epithelial tissues, and vesicular fluids from piglets	(12)
RT-ddPCR	Targets FMDV 3D region based on the water-oil emulsion droplet technology for partition of nanoliter droplets containing viral cDNA. This assay enables absolute quantification of the FMDV RNA without the need of reference standards. Detects serotypes O, A, and C	• $10^{1.4}$ $TCID_{50}$/mL and 26.5 copies of viral RNA determined using FMDV A24 Cruzeiro and a plasmid containing the 3D-FMDV sequences, respectively	Epithelium and esophageal–pharyngeal fluids of bovine origin	(13)
RT-RPA	An assay based on isothermal DNA amplification and the use of combinatory enzymes and proteins. The assay was reported to detect 3D RNA of FMDV within 4–10 min	• 1,436 copies of *in vitro*–transcribed FMDV 3D RNA • Diagnostic sensitivity: 98%	Heart, blood, serum, milk, saliva, and vesicular samples from cattle, buffaloes, and sheep	(14)
RT-qPCR	A multiplex assay targeting the VP1 region of FMDV for specific and simultaneous detection of FMDV of O, A, and Asia 1 circulating in the Middle East	• 1.78 to 2.74 copies of *in vitro*–transcribed FMDV RNA depending on serotypes	Vesicular epithelium, saliva, and heart tissue homogenates from animals	(15)
	Addition of 5′-tails to the primers targeting 3D and 5′ UTR region of FMDV was demonstrated to enhance detection of FMDV. The RT-qPCRs using the tailed forward and reverse primers targeting 3D and 5′ UTR were performed in parallel in a triplex one-step protocol. Both assays detected all seven serotypes of FMDV with enhanced overall performance	• The detection limit of RT-qPCR (with tailed primers) targeting 3D and 5′ UTR of FMDV are −0.72 and −0.35 $\log_{10}$ $TCID_{50}$/mL of FMDV O1 Manisa, respectively	Serum samples from cattle, pigs, and sheep	(16)
	An RT-qPCR assay based on the SYBR green I dye for detection of FMDV of all seven serotypes. Primers used in this assay were carefully selected using multiple *in silico* approaches to enhance amplification efficiency	• 1–10 copies/μL of *in vitro*–transcribed FMDV RNA depending on the target regions	Epithelium and vesicular fluid samples from cattle	(17)
	A pen-side, fully automated diagnostic tool (Enigma MiniLab®) which integrate both nucleic acid extraction and downstream RT-qPCR for rapid detection (<1.5 h) of FMDV. The assay was shown to produced comparable results to the standard RT-qPCR assay recommended by the OIE	• 10^{-5} to 10^{-6} dilution of the FMDV O/UAE 2/2003 stock depending on the nuclei acid extraction kits.	Saliva and epithelium of bovine, porcine, and ovine origin. Milk (from bovine) spiked with FMDV	(18)

(Continued)

TABLE 1 | Continued

Methods	Description	Detection limit	Tested clinical samples	References
	A field-deployable RT-qPCR-based diagnostic system (Biomeme two3^{TM}) with Biomeme proprietary nucleic acid extraction kit (M1) for rapid detection of FMDV, within 30–60 min. This system was reported to detect isolates representing six serotypes (O, A, Asia 1, SAT 1, SAT 2, and SAT 3) of FMDV. Detection of FMDV RNA in various samples was possible without nucleic acid extraction steps, but at lower sensitivity	• 10^{-4}, 10^{-3}, 10^{-2}, 10^{-5}, 10^{-3}, and 10^{-3} dilutions of FMDV O, A, Asia 1, SAT 1, SAT 2, and SAT 3 stocks, respectively	Serum, vesicular fluid, tissue suspension, oral fluid, oral, and nasal swab samples from sheep, pigs, and cattle	(19)
	This study compared the performance of two commercially available one step RT-qPCR systems, TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®) and Superscript III Platinum® One-Step qRT-PCR Kit (Invitrogen^{TM}) in detection of FMDV RNA from milk samples, a non-invasive alternative for detection and typing of FMDV	• The detection limit of Superscript III Platinum® One-Step qRT-PCR Kit and TaqMan® Fast Virus 1-Step Master Mix are 10^{-6} and 10^{-5} dilutions of FMDV A/KEN/6/2012, respectively	Serum, milk, vesicular epithelium or fluid samples of bovine origin	(20, 21)
RT-PCR-Microarray	This assay was capable of detecting and serotyping FMDV and VSV in addition to detecting VESV and SVDV. Multiplex RT-PCR was able to detect viruses representing all seven serotypes of FMDV. Typing of the FMDV was achieved by slide microarray containing serotype-specific probe	• The detection limit of multiplex RT-PCR and microarray are 46 and 4.6 $TCID_{50}$/mL of FMDV O1 Manisa, respectively • The diagnostic sensitivity and specificity: 92.6 and 100%, respectively	Oral swabs from calves	(9)
	This assay detects and differentiates FMDV, SVDV, VESV, ASFV, CSFV, PRRSV, and PCV2. Samples are amplified with multiplex RT-PCR and then applied to automated electronic microarray assay. This approach is less laborious and utilizes a single instrument that integrates and automates capture probe printing, hybridization, washing, and reporting on a disposable electronic microarray cartridge	• The detection limits of multiplex (seven-plex) RT-PCR and microarray for ASFV, PCV2 and PRRSV were reported to be at a range between one copy in PCR and 10 copies in microarray, respectively, followed by SVDV, CSFV and VESV at approximately 10 copies in PCR and 100 copies in microarray and >100 copies for FMDV in PCR and >10,000 copies in microarray	Biological material spiked with viruses, serum, and nasal and oral swabs from pigs	(22)
RT-LAMP	An assay for rapid detection (within 45 min) and typing of FMDV of serotype Asia 1. This assay targets the conserved region of VP1 sequence of FMDV serotype Asia 1	• NA	Infected pig samples (not specified)	(23)
	An assay for rapid detection and typing of FMDV of serotype C. This assay targets the conserved region of VP1 sequence of FMDV serotype C and can be completed in an hour	• 0.325 ng/mL RNA template harvested from cell culture infected with FMDV of serotype C	Infected cells and blood samples from animals	(24)

(Continued)

TABLE 1 | Continued

Methods	Description	Detection limit	Tested clinical samples	References
	A multiplex RT-LAMP assay that utilizes combined primer sets from different individual RT-LAMP assays to compensate high sequence variability of FMDV. The assay was demonstrated to be superior or at least as good as individual RT-LAMP assay	• Detection limit: 10^{-3} dilution of RNA template harvested from FMDV O1 Manisa TUR/8/69 infected epithelial suspensions • Diagnostic sensitivity and specificity: 98.0 and 98.1%, respectively	Epithelial suspension and esophageal–pharyngeal fluid samples from animals	(25)
	RT-LAMP assay utilizes swarm primers in addition to the standard six primers for improved sensitivity. This assay was demonstrated to specifically detect *VP3* gene of FMDV (O serotype)	• 10^2 $TCID_{50}$/mL or 10^3 copies/μL of *in vitro*–transcribed O/Andong/KOR/2010 3D RNA	Serum, saliva, and epithelial tissue samples from animals	(26)
	A real-time RT-LAMP assay targeting the 3D region for rapid detection of FMDV serotypes A, O, and Asia 1. It uses hydroxyl naphthol blue (HNB) dye for visual detection of positive sample in addition to the turbidity change	• 4.2×10^{-4}, 2×10^{-6} and 1.1×10^{-4} $TCID_{50}$/mL for FMDV serotypes O, A and Asia1, respectively	Tongue epithelium and semen samples from infected bulls	(27)
	The study evaluated RT-LAMP assay that utilized lyophilized reagents for detection of FMDV. Lyophilized reagents were shown to have no negative impact on amplification	• 10 copies/μL of *in vitro*–transcribed O/UKG/35/2001 3D RNA	Epithelial tissue, serum, and esophageal–pharyngeal fluid samples of bovine origin	(28)
	An RT-LAMP assay targeting the 3D region for rapid detection of FMDV. This assay targets the VP1 region of FMDV for specific detection of FMDV serotypes A, O, and Asia 1	• 10 copies of DNA	NA	(29)
Ag-RT-LAMP	An assay that utilizes FMDV genotype-specific IgG immobilized on a tube to capture the virus prior to RT-LAMP amplification. The assay can be completed within 3 h but was negatively affected by high viral load in the samples	• 0.58 x 10^2 copies of FMDV O/Akesu/58	Vesicle fluid samples from cattle	(30)
qRT-LAMP	A real time RT-LAMP assay targeting the 3D region for rapid detection of FMDV. This assay also targets the VP1 region of FMDV for specific detection of FMDV serotypes A, O, and Asia 1	• 10^{-5} dilution of FMDV RNA template harvested from infected epithelial suspensions • The detection limits of FMDV serotypes Asia 1 and O is 10^{-3} $TCID_{50}$/mL, and 10^{-5} $TCID_{50}$/mL for serotype A	Epithelial suspension, tongue, and foot epithelium from animals	(29, 31)

RT-PCR, reverse transcription-polymerase chain reaction; RT-iiPCR, reverse transcription-insulated isothermal polymerase chain reaction; RT-ddPCR, reverse transcription-droplet digital polymerase chain reaction; RT-RPA, reverse transcription-recombinase polymerase amplification; RT-qPCR, real-time reverse transcription-polymerase chain reaction; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; Ag-RT-LAMP, antigen-capture reverse transcription-loop-mediated isothermal amplification; qRT-LAMP; real-time reverse transcription-loop-mediated isothermal amplification; IRES, internal ribosome entry site; ORF, open reading frame; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; FMDV, foot-and-mouth disease virus; SVDV, swine vesicular disease virus; VESV, vesicular exanthema of swine virus; ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine respiratory and reproductive syndrome virus; PCV2, porcine circovirus type 2; VSV, vesicular stomatitis virus; $TCID_{50}$, 50% tissue culture infectious dose; UTR, untranslated region; IgG, immunoglobulin G; NA, data not available.

exploited for multiplex detection. Reid et al. (15) developed a multiplex RT-qPCR assay using primer/probe sets targeting the FMDV VP1 coding region for detection and differentiation of FMDV serotypes O, A, and Asia 1 circulating in the Middle East. Nonetheless, when compared to single RT-qPCR, multiplex RT-qPCR tends to produce false-negative results due to mismatch in the probe-binding regions, suggesting that a higher sequence identicality is required for application in

multiplex system. This complexity has limited the usage of this technique.

Although highly reliable, RT-qPCR often requires laboratory setting with a qPCR thermocycler, a cost factor that limits its usage in the field. To overcome such limitation, on-site devices capable of performing FMDV diagnosis in the field have been developed, such as the fully automated cartridge-based RT-qPCR diagnostic system, Enigma MiniLab® (18). Another handheld RT-qPCR device, Biomeme two3™ (two3) has also been developed and evaluated as a field-deployable platform for FMDV diagnosis, in which the sensitivity was shown to be almost comparable to RT-qPCR using the ABI7500 platform (19). The RNA samples can be extracted with an on-site RNA extraction kit such as Biomeme M1 Sample Prep™ cartridge kit, a method that is dispensable of laboratory equipment and chemicals such as microcentrifuge, alcohol, phenol, and chloroform (19).

Another modified version of RT-PCR known as the RT-insulated isothermal PCR (RT-iiPCR) assay was also developed for qualitative detection of FMDV (12). Unlike traditional PCR that requires cycles of multiple temperatures, RT-iiPCR utilizes a temperature gradient generated from a thermal convection from a single heating source, which hastens the detection process (12). Additionally, RT-recombinase polymerase amplification (RT-RPA) has also been used for detection of FMDV (14, 40). The RT-RPA uses three specific proteins: recombinase allows primer annealing to double-stranded DNA; single-stranded DNA-binding protein stabilizes primer binding; and strand-displacing DNA polymerase. As for real-time detection, another method based on water-oil emulsion droplet technology for partition of nanoliter droplets containing viral cDNA, known as the RT-droplet digital PCR (RT-ddPCR) was established for the absolute quantitation of FMDV RNA in epithelial and esophageal-pharyngeal fluid samples from FMDV-infected cattle (13).

Generally, the FMDV RNA for diagnostic purposes could be obtained from specimens, which include (i) swabs of oral, nasal, and lesion; (ii) epithelial tissue suspensions; and (iii) oral or vesicular fluid. In a recent study, Goller et al. (18) explored milk as a non-invasive sample for FMDV surveillance using RT-qPCR. They demonstrated that the RT-qPCR on milk sample was capable of detecting FMDV RNA 18 days after contact, which is later than the viral RNA detected in serum samples, suggesting milk as a feasible sample for FMD surveillance (18, 21). In addition, EDTA-treated blood samples have also been explored as a source of the viral RNA for the diagnosis of FMDV using RT-qPCR, owing to the samples' stability during transportation, as well as the ease of sample processing at the diagnostic laboratory (41). Fontél et al. (41) reported that the diagnostic assay using EDTA-treated blood samples was ∼10 times less sensitive than that of serum samples. However, the study used double the volume of serum than that of EDTA-treated blood samples for RNA extraction, of which the serum contains almost double the virus concentration with the same sample volume as the red blood cells were spun off during centrifugation. This gave rise to an uneven amount of virus/volume in each sample type, with serum containing nearly four times the amount of virus, thereby resulting in a difference in cycle threshold (Ct) value of ∼2, assuming that both types of samples were equally good for RT-qPCR detection. The results obtained by Fontél et al. (41) showed that the Ct value for serum was three to four times lower than that of EDTA-treated blood, in which the actual difference should be only 1- to 2-fold lower. Therefore, EDTA-treated blood samples, as initially proposed by Fontél et al. (41), may still remain a viable option for FMDV diagnosis.

Reverse Transcription-Loop-Mediated Isothermal Amplification

Unlike PCR that requires cycles of different temperatures for amplification of DNA, LAMP is a method capable of amplifying DNA at a single temperature at around 60° to 65°C. It was first invented by Notomi et al. (42), and the method was demonstrated to be highly sensitive and specific. In general, the method involves the use of at least four primers and a DNA polymerase with high strand displacement activity. Two of the primers form loop structures at their respective 5′ ends, and the other two primers play the role of displacing the loop-forming strands from the template at the loop regions as they are being synthesized by DNA polymerase with high strand displacement activity. Once a double-stranded product with both ends capable of forming loop structures is synthesized, it then functions as a template for infinite amplification of the DNA, provided there are still loop-forming primers and dNTPs available. Loop-mediated isothermal amplification is capable of producing 10^9 copies of DNA in less than an hour (42), which probably exceeds the speed of PCR amplification, as no denaturation of double-stranded DNA is required. Another significant difference between LAMP and PCR is their amplification products. While PCR generates high copy numbers of identical products, LAMP generates a mixture of stem-loop DNAs, which can be observed through visual detection, based on the formation of insoluble magnesium pyrophosphate, which can be detected by simple turbidimeter or visual turbidity (43).

As FMDV is an RNA virus, RT-LAMP is needed for detecting the FMDV genomic sequence. Before the double-stranded DNA template with both loop-forming ends can be generated, a loop-forming primer targeting the positive-sense RNA template accompanied by reverse transcriptase is required to generate the RNA-DNA hybrid, which will then be displaced by another primer targeting the RNA template at the loop-forming region of the DNA strand through the displacement activity of the DNA polymerase. The antisense viral DNA will then function as a template for LAMP as described above.

RT-LAMP was first employed for FMDV diagnosis by Dukes et al. (44), targeting the FMDV *3D RNA polymerase* gene, and the products of amplification can be visually inspected for turbidity, analyzed using agarose gel electrophoresis, or monitored in real time through addition of fluorescent dyes. Three years later, Li et al. (45) used RT-LAMP for detection of FMDV by targeting a conserved region within the FMDV *polyprotein* gene (3D), at positions 7,905–8,094 of FMDV O isolate o1bfs46 iso46 (GenBank accession no. AY593816). Thereafter, detections of FMDV serotypes C and Asia 1 using specific primers have

also been reported (23, 24). Generally, for detection of FMDV regardless of serotypes, RT-LAMP requires a longer conserved region for primer design compared to RT-PCR and RT-qPCR, as RT-LAMP requires four to six primers to function. Although the target gene selected for detection is highly conserved, some degree of differences exists between different FMDV isolates (25), which will result in mismatch of nucleotides between primers and the target gene, thereby lowering the assay's sensitivity.

As FMDV genomic sequences vary widely between different serotypes, primers used for the detection of one particular serotype may not detect FMDV of another serotype. Therefore, Yamazaki et al. (25) developed a multiplex RT-LAMP, which contains multiple primer sets targeting the 3D conserved regions for the detection of FMDV regardless of serotypes, in which the assay's sensitivity and specificity were reported to be up to 98.0 and 98.1%, respectively. In each multiplex reaction tested, a combination of two primer sets was used. Unlike RT-PCR, RT-LAMP uses primer sets containing around six oligonucleotides with overlapping sequences. As multiplex RT-LAMP involves mixing of primer sets for simultaneous detection of different target genes, its application could be limited by the number of primer sets that can be used in a single reaction, as increase in varieties of primer sequences may increase the rate of unspecific binding, thereby affecting the assay's specificity. In addition, antigen capture RT-LAMP (Ag-RT/LAMP) assay has also been reported to be capable of detecting and serotyping FMDV. An anti-FMDV immunoglobulin G (IgG) that interacts with the VP1 epitope was immobilized on a tube to capture FMDV of various genotypes, and subsequently the viral 3D conserved region was amplified using RT-LAMP amplification (30). Although this method provides an alternative for the differential detection of FMDV using RT-LAMP, the method is heavily dependent on the efficiency of antibody used to capture the target virus. In addition, this method requires a longer time for completion compared to normal RT-LAMP, as extra incubation time is needed for capturing of target virus by antibody. In addition, the specificity of Ag-RT/LAMP has also been reported to be affected by high viral loads in samples of interest.

In a separate study, RT-LAMP was used together with a lateral flow device (LFD) for the detection of FMDV in minimally processed (without RNA extraction) samples (46). Although the coupling of LFD did not increase the assay's sensitivity, it allowed an easier interpretation of the test results compared to visual detection of turbidity from magnesium pyrophosphate, or color changes of double-stranded DNA-staining dyes. The combined use of RT-LAMP and LFD will be discussed in more detail under the subsection chromatographic strip test. For FMDV detection, RT-LAMP has been demonstrated to have an analytical sensitivity of 10-folds lower than that of the conventional RT-PCR, which is 10-folds lower than that of RT-qPCR method (26). To increase the sensitivity of RT-LAMP, a recent study that used swarm primers to improve the accessibility of DNA to standard LAMP primers was deployed for rapid and accurate diagnosis of FMDV from the pool-1 region (26). At a high concentration, swarm primers anneal to the double-stranded cDNA at higher rate, thereby exposing the inner primer annealing sites, facilitating the binding of the RT-LAMP primers, which results in a faster amplification rate, as well as more RT-LAMP products. Overall, the swarm primer-based RT-LAMP (sRT-LAMP) has been demonstrated to have analytical sensitivity of 10-folds higher than the conventional RT-PCR, which is comparable to RT-qPCR for FMDV detection.

To date, real-time RT-LAMP (qRT-LAMP) is available for the detection and differentiation of FMDV serotypes O, A, and Asia 1 (47). Unlike RT-qPCR, qRT-LAMP for FMDV detection could not produce accurate quantitative results, as measurements of magnesium pyrophosphate by turbidity or DNA by fluorogenic dye are directly proportional to the size of LAMP products. As LAMP products are of many different sizes, these methods could not truly reflect the number of replication cycle as in RT-qPCR. Recently, real-time detection and monitoring of LAMP using self-quenching and dequenching fluorogenic probes for direct quantification of LAMP products have been developed (48). However, this method has yet been applied for the detection of FMDV. RT-LAMP was used for detecting and serotyping FMDV in India and Pakistan, in which the results were reported to be comparable to RT-qPCR (27, 29, 31). Another study in Africa demonstrated that RT-LAMP outperformed RT-qPCR in the detection of FMDV (49), although further testing with a bigger sample size and a wide variety of serotypes is needed to support the finding. However, without the need of sophisticated devices as in RT-qPCR, RT-LAMP represents a potential method to be used for on-site diagnoses of FMDV. To further encourage the use of RT-LAMP in laboratory and field settings, Howson et al. (28) demonstrated the use of lyophilized reagents for RT-LAMP (Enigma Diagnostics Limited) and RT-qPCR (OptiGene Limited), in which lyophilization greatly improved the storage stability of test reagents without jeopardizing the assays' performance.

SEROLOGICAL METHODS

Serological methods detect the presence of viral antigens or antibodies in serum or other body fluid samples. Apart from detecting the viral genome 1 to 2 days postinfection in oral fluid and serum samples (50, 51), FMD diagnosis can also be confirmed through the detection of anti-FMDV antibodies in serum samples. As infection by FMDV will often result in the production of antibodies against the viral antigens (detectable ∼4 days postinfection in cattle sera) (50), detection of these antibodies can therefore indicate the presence of current or past infection. Some of the serological detection methods include VNT, solid-phase competition (SPC) ELISA, and liquid-phase blocking (LPB) ELISA. When ELISA-based methods are used to detect antibodies against both the structural proteins (SPs) and non-structural proteins (NSPs), it is capable of differentiating the infected from vaccinated animals (DIVA), which will be discussed in detail in *Enzyme-Linked Immunosorbent Assay*. **Table 2** summarizes recent studies on ELISA-based FMDV diagnostic assays.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay, pioneered by Engvall and Perlmann (85), is an analytical method commonly used

TABLE 2 | Enzyme-linked immunosorbent assay (ELISA)-based methods for FMDV diagnosis.

Methods	Description	Diagnostic sensitivity	Diagnostic specificity	Tested clinical samples	References
Indirect ELISA	Fusion of FMDV VP1 to capsid protein of bacteriophage T7 that served as coating antigen reacted with the vaccinated and positive infected bovine sera. A highly conserved shorter VP1 was later fused to the capsid protein of T7 and was demonstrated to be a suitable diagnostic reagent for identification of antibodies directed against this region	92–100%	75–87.5%	Serum samples of bovine origin	(52, 53)
	An assay that utilizes a multiple-epitope protein (B4) comprising the G-H loops of VP1 from three topotypes of FMDV serotype O as diagnostic antigen. The assay successfully detected serum antibodies against FMDV serotype O in vaccinated pigs	95.9%	96.7%	Serum samples from pigs	(54, 55)
	An assay that utilizes baculovirus-expressed recombinant 3ABC of FMDV as coating antigen for detection of 3ABC-specific antibodies in FMDV-infected animals	95.8%	97.45%	Serum samples of bovine origin	(56)
	A negative marker virus was produced by deleting amino acid residues 93–143 of the 3A and 10–37 of 3B of FMDV. ELISA developed to target the deleted region was reported to allow DIVA	95.5%	96%	Serum samples from cattle and buffaloes	(57)
	A negative marker virus was produced by deleting amino acid residues 87–144 in 3A, and the whole $3B_1$ and $3B_2$ of FMDV. ELISA developed to target the deleted region was reported to allow DIVA	96%	97.1–100%	Serum samples of bovine origin	(58)
Sandwich ELISA	Monoclonal and polyclonal antibodies against conserved structural protein fragment 1AB′ of FMDV were used as capture and detection antibodies, respectively, for serotype-independent detection of FMDV	NA	NA	NA	(59)
	Monoclonal antibodies and chicken IgY against 146S antigen of FMDV were used as capture and detection antibodies, respectively, for detection of FMDV of serotypes O, Asia 1, and A	98.87%	100%	Tongue epithelial samples and tissue culture fluids	(60)
	An assay that utilizes baculovirus-expressed recombinant structural proteins of FMDV as diagnostic antigen for specific detection of antibodies against FMDV serotype Asia 1	NA	99.7%	Serum samples from cattle, pigs, and goats	(61)
	A monoclonal antibody was used as detection antibody for serotyping of FMDV serotype O	100%	100%	NA	(62)
	A recombinant antibody fragment, single-chain variable fragment (scFv) was used as detection antibody for the detection of FMDV-specific IgA in salivary samples	NA	NA	Saliva samples from cattle, buffaloes, sheep, goats, and canines	(63)
	A recombinant integrin αvβ6 and serotype-specific monoclonal antibodies were used as antigen-trapping and detection reagents, respectively, for identification and serotyping of FMDV	97.9%	96%	Positive cell-culture supernatants	(64, 65)
	A truncated bovine integrin αvβ6 was used as a universal trapping reagent in a sandwich ELISA for all FMDV serotypes. When coupled to serotype-specific monoclonal antibodies, the integrin can be employed to detect viruses representing all seven FMDV serotypes	NA	NA	Infected cell lysate	(66)
	A recombinant, bacteria-expressed, conserved region of 3ABC and a monoclonal antibody were used as diagnostic antigen and capture antibody in the assay for differentiation of infected animals from vaccinated animals	98.4%	100%	Serum samples of swine origin	(67)
	An assay that utilizes the bacteria-expressed truncated 3ABC of FMDV SAT 2 serotype as diagnostic antigen for detection and differentiation of FMDV SAT serotype–infected animals from vaccinated animals	76%	96%	Serum samples of bovine origin	(68)
	A negative marker virus with partial deletion in the VP1 G-H loop was generated. ELISA targeting deleted region was suggested to allow differentiation of infected from vaccinated animals	NA	NA	Serum samples of bovine origin	(69, 70)

(Continued)

TABLE 2 | Continued

Methods	Description	Diagnostic sensitivity	Diagnostic specificity	Tested clinical samples	References
LPB-ELISA	An assay utilizes two neutralizing monoclonal antibodies specific against FMDV serotype O as trapping and detection antibodies. Results generated from the assay correlated well with the results of VNT	NA	99.7–100%	Serum samples of bovine and porcine origins	(71)
	An assay for detecting antibodies against FMDV based on single dilution of the serum. Antibody titers against FMDV of serotypes O, A, C, and Asia 1 could be extrapolated from a linear regression curve generated with reference standards	NA	NA	Serum samples from cattle	(72, 73)
	An assay utilizes baculovirus-expressed recombinant structural proteins of FMDV as diagnostic antigen for specific detection of antibodies against FMDV serotype A	NA	98.5–99%	Serum samples from cattle, pigs, and goats	(74)
	Field application of an assay utilizing recombinant structural proteins of FMDV as diagnostic antigen for specific detection of antibodies against FMDV serotype A	84%	97%	Serum from beef, dairy, and deer farms	(75)
	VHHs specific to 146S antigen of FMDV serotypes O, A, and Asia 1 were used as trapping antibodies in LPB-ELISA. The assay produced results that correlate well to routine LPB-ELISA, which uses coating antibodies from rabbits	NA	NA	Serum samples of bovine origin	(76)
SPC-ELISA	An assay for detecting antibodies against FMDV antigen (146S). The assay was demonstrated to successfully detect antibodies against FMDV of A, C, SAT 1, SAT 2, SAT 3, and Asia 1 serotypes in infected samples	NA	99.41–99.9%	Serum samples from cattle, sheep, and pigs	(77)
	A commercially available kit based on SPCE-ELISA for detection of antibody against FMDV serotype O was reported to produce high false-positive rate	NA	NA	Serum samples from pigs	(78)
	An assay that utilizes bacterial-expressed recombinant capsid polyprotein as diagnostic antigen for specific detection of antibodies against FMDV serotype O	99%	100%	Serums samples from cattle, buffaloes, and goats	(79)
	An assay that utilizes bacterial-expressed virus-like particles of FMDV as diagnostic antigen for detection of antibodies against FMDV serotype O. The assay produced results comparable to commercially available kits	96%	100%	Serum samples of bovine, pig, and sheep origins	(80)
	Two serotype-specific monoclonal antibodies targeting the conserved VP2 regions of FMDV serotype A were used as competing antibodies in SPC-ELISA. The test detected antibodies directed against FMDV serotype A, and the results were comparable to VNT	99.3%	99.7%	Serum samples of bovine, porcine, and ovine origins	(81)
	The bacterial-expressed, recombinant 3ABC of FMDV and VHHs were used as diagnostic antigen and competing antibodies in SPC-ELISA for detection and differentiation of FMDV-infected animals from vaccinated animals	94%	97.67%	Serum samples from cattle	(82)
Microchip-based ELISA	An assay that involves immobilization of the recombinant 3ABC polyprotein to microbeads followed by immunoreaction with the 3ABC-specific antibodies in the test sera, and detection with thermal lens microscopy–based on the enzymatically colorimetric reaction between HRP-labeled antibody and the corresponding substrate	NA	NA	Serum samples from cattle and swine	(83, 84)

ELISA, enzyme-linked immunosorbent assay; LPB-ELISA, liquid-phase blocking enzyme-linked immunosorbent assay; SPC-ELISA, solid-phase competitive enzyme-linked immunosorbent assay; VNT, virus neutralization test; FMDV, foot-and-mouth disease virus; HRP, horseradish peroxidase; scFv, single chain variable fragment; VHHs, variable heavy chain antibody fragments; IgY, immunoglobulin Y; NA, data not available.

for qualitative and quantitative analyses. Current ELISA is a modified version of radioimmunoassay techniques, which was first described by Coons et al. (86), in which an antigen is immobilized on a solid phase either directly or indirectly to capture a targeted antibody, which is then reported through a secondary antibody conjugated to an enzyme, where signals will

be generated in the presence of its corresponding substrate. In general, ELISA is categorized into direct, indirect, sandwich, and competitive ELISA (87). Currently, ELISA is one of the most common approaches in detection of FMDV in addition to the virus isolation, VNT, and PCR-based techniques (88). According to a report from the Regional Reference Laboratory for FMD in South East Asia, more than 13,000 ELISAs were performed compared to 304 VNTs and 790 PCR-based assays for diagnosis of FMD in 2017 (89).

Detection of FMDV specific antibodies in vaccinated bovine sera using an indirect ELISA was first reported by Abu Elzein and Crowther (90), in which the test sera from cattle reacted with the FMDV coated on the microtiter plate followed by detection with anti-bovine antiserum conjugated to an enzyme. Subsequently, the same research group demonstrated the capability of a sandwich ELISA in detecting and quantifying FMDV with 50 to 100 times higher sensitivity than CFT (91). A double-sandwich ELISA method developed by Roeder and Le Blanc Smith (92) further improved the sensitivity of FMDV detection with 125 times higher than that of CFT. Unlike CFT, specific detection of FMDV using the sandwich ELISA was reported to be unaffected by the presence of 12S antigen (93) and procomplementary or anticomplementary factors in the samples (88). Moreover, the sandwich ELISA allows direct assessment of samples without virus isolation, and it is generally more cost-effective than CFT because of the lower amount of sera required per test (88). In addition, ELISA is not affected by the variation in tissue culture susceptibility (88). Comparative studies via repeated testing of sera also indicated that ELISA was more reproducible than VNT, and their results could be generated within a day compared to VNT, which normally took more than 3 days (94).

Measurements of antibody titers using ELISA involve passive absorption of an antigen to a solid phase support, particularly the microtiter plate wells. Several studies have indicated that passive absorption of an antigen to a solid support either directly or indirectly via trapping antibodies may distort the conformation of the antigen. The conformation of FMDV antigen was previously reported to be altered with the exposure of internal viral proteins following a non-covalent binding to a PVC plate in an indirect ELISA (95–97). To resolve this problem, an LPB-ELISA was developed for the determination of antibody reactivity to the 146S antigen in its most native conformation (97). In this assay, the 146S antigen and test sera were mixed and incubated before being transferred to a plate precoated with serotype-specific anti-FMDV antisera (94). Attributed to the good reproducibility, faster results, and good correlation with VNT, LPB-ELISA quickly replaced VNT in FMD routine screening (98). Liquid-phase blocking ELISA was also reported to be the best strategy in differentiating the antigenic differences between FMDV strains (99). Nevertheless, the LPB-ELISA was shown to produce some degree of false-positive results and may require VNT for additional verification in some of the low-positive LPB-ELISA results (98). Conventionally, to measure the antibody titer by endpoint titration using LPB-ELISA requires the serum to be serially diluted, which is more laborious and more prone to error. To overcome this problem, a single-dilution LPB-ELISA was previously developed to measure the FMDV-specific antibody titer in serum. This method is based on a linear regression curve generated with reference standards to extrapolate the antibody titers of the sera tested (72, 73).

An SPC-ELISA was also developed for detecting FMDV (98). This method is based on the competition between the antibodies in the sera tested and the serotype-specific guinea pig anti-FMDV antibodies (98). Although both the LPB-ELISA and SPC-ELISA were shown to have similar sensitivity and limit of detection, the specificity of SPC-ELISA was reported to be higher, offering an improved FMDV-specific antibody detection method for mass screening (98). Paiba et al. (100) also demonstrated that SPC-ELISA was more sensitive than VNT for early serological detection of FMDV infection in cattle and sheep, although opposite findings were observed when tested in pigs. In addition, the sensitivity of SPC-ELISA is less affected by the strains of FMDV used in the assay, whereas VNT sensitivity could reduce significantly if heterologous virus is employed (100). Solid-phase competition ELISA was reported to be able to detect antibodies against six non-O serotypes of FMDV (A, C, SAT 1, SAT 2, SAT 3, and Asia 1) with specificity ranging from 99.4 to 99.9% and sensitivity comparable to LPB-ELISA and VNT (77). On the other hand, serotype specificity of the SPC-ELISA was evaluated against different reference sera representing six FMDV serotypes (O, A, Asia 1, SAT 1, SAT 2, and SAT 3). The SPC-ELISA detected all the reference sera correctly but not the FMDV serotype SAT 3-positive serum. Similarly, VNT also produced a borderline positive response on this sample, suggesting that the sample might be degraded. In addition, cross-reaction in SPC-ELISA between FMDV serotypes A and Asia 1-positive samples was observed (77). Solid-phase competition ELISA kits for detection of specific SPs of FMDV of different serotypes were commercially available. Nevertheless, one recent study reported that the sensitivity of the SPC-ELISA kit for specific detection of FMDV serotype O was lower, and it produced more false-positive results as compared to LPB-ELISA and VNT (78).

To improve the performance of ELISA in FMD diagnosis, many modifications have been made, primarily focusing on the development of new coating antigens and new monoclonal antibodies (mAbs) as trapping or detection antibodies. Majority of the ELISA-based assays involve inactivated FDMV antigens in the diagnostic process. However, the production of these inactivated antigens still requires handling of live virus in high-containment laboratories (101). Along with the advancement in recombinant DNA technology, coating antigens can be produced in a safer alternative. Recombinant SPs of FMDV serotypes O, Asia 1, and A were generated via the baculoviral expression system and used as diagnostic antigens in LPB-ELISA (61, 74). These recombinant LPB-ELISA assays exhibited specificity and sensitivity comparable to VNT (74). When this method was applied in the field during an FMDV serotype A outbreak in Korea in 2010, its specificity and sensitivity were reported to be 97 and 84%, respectively (75). The SPs VP1, VP2, VP3, and VP4 are the secondary cleavage products of a capsid precursor polyprotein (P1) of FMDV (102). Biswal et al. (79) produced a recombinant capsid polyprotein (rP1) and

employed it as a diagnostic antigen in SPC-ELISA for detection of FMDV serotype O. Solid-phase competition ELISA based on rP1 demonstrated 100% specificity and 99% sensitivity. In addition, virus-like particles of FMDV serotype O (80) were also produced and used as a diagnostic antigen in SPC-ELISA. The specificity and sensitivity of this test were 100 and 96%, respectively (80). Interestingly, Wong et al. (52) genetically fused the capsid protein of T7 bacteriophage with the VP1 of FMDV and demonstrated that the recombinant protein, when served as the coating antigen in an indirect ELISA, could react with the vaccinated and positive infected bovine sera, suggesting its potential application in FMD diagnosis. Wong et al. (53) further delineated the VP1 sequence of FMDV to 12-amino-acid residues using amino acid sequence alignment, homology modeling, and phage display, in which the chimeric phage T7 displaying $VP1_{159-170}$ epitope was demonstrated to have an improved sensitivity of 100% in a phage-based ELISA. Recently, a multiple-epitope protein (B4) comprising the G-H loops of VP1 from three topotypes of FMDV serotype O was developed as a potential vaccine candidate (54, 103). When the B4 was employed as a coating antigen in an indirect ELISA, it detected antibodies against FMDV serotype O in pigs with specificity and sensitivity up to 96.7 and 95.9%, respectively. These results were also reported to correlate well with the LPB-ELISA (55).

Serotyping and identification of FDMV based on sandwich ELISA normally use rabbit and guinea pig polyclonal antibodies as capture and detection antibodies, respectively. However, there are some disadvantages of using these polyclonal antibodies in ELISA, including batch-to-batch variation, inconsistent yield of antibodies, and limited serum samples collectable from individual animals (60). van Maanen (104) demonstrated the use of mAbs in ELISA for identification of three FMDV serotypes (A10, O1, C1). This mAb-based ELISA (mAb-ELISA) was shown to be sensitive, specific, and more reproducible than VNT. In the same year, Smitsaart et al. (105) developed a competition ELISA using an mAb that binds to the 12S protein subunit. This assay successfully detected six of the seven serotypes of FMDV with a sensitivity higher than that of CFT (105). More mAbs were later developed and utilized as trapping and/or detection antibodies in ELISA for FMDV detection (59, 62, 106–109). Veerasami et al. (60) also produced mAbs and chicken IgY specifically against the 146S antigen of three FMDV serotypes (O, Asia 1, and A) and used them in ELISA as capture and detection antibodies, respectively. There are several advantages in using chicken IgY in ELISA for FMD detection including minimal or no cross-reaction with mammalian IgG, complete absence of non-specific binding, and elimination of the need for cross-species immunoabsorptions due to the phylogenetic differences between birds and mammals (60). This method produced results comparable to the routine ELISA and RT-qPCR in FMDV serotyping (60). Another two mAbs that bind specifically to VP2 protein of FMDV serotype A were generated and employed as competing antibodies in SPC-ELISA. These mAbs interact with the VP2 protein, which is more conserved, thus offering a distinct advantage over another similar assay, which targets the more variable VP1 protein of FMDV serotype A (74, 81). This assay demonstrated specificity and sensitivity of 99.7 and 99.3%, respectively (81). In addition, two neutralizing mAbs, namely 72C1 and 65H6, which were raised against the FMDV O/JPN/2000 strain, were previously employed in LPB-ELISA as trapping and detection antibodies, respectively. This modified LPB-ELISA produced results that correlated well with VNT and demonstrated specificity of 100 and 99.7% in negative bovine and swine sera, respectively (71).

Apart from mAbs, recombinant antibody fragments such as the single-chain variable fragments (scFvs) were also used as detection antibodies in sandwich ELISA to detect FMDV-specific IgA in salivary samples from vaccinated and infected cattle (63). In addition, the variable heavy chain antibody fragments (VHHs) from camels have been explored for FMD diagnostic applications (76). The VHHs are composed of two heavy chains, but lack the light chains and CH1 domain present in conventional antibodies (110). Dash et al. (76) produced VHHs that bind specifically to 146S antigen of FMDV serotypes O, A, and Asia 1 and used them as trapping antibodies in LPB-ELISA. This modified LPB-ELISA yielded results that correlate well to routine LPB-ELISA, which uses coating antibodies from rabbits (76). The FMDV-specific VHHs could be produced with bacterial expression system, offering batch uniformity, and thus lower the production cost (111).

All field isolates of FMDV initiate infection using arginine-glycine-aspartic acid-binding integrins as the cell receptors (66). This knowledge was leveraged for the development of FMD diagnostic tools. A recombinant integrin αvβ6 was previously produced as an antigen-trapping reagent in a sandwich ELISA for FMDV diagnosis (64). When the serotype-specific polyclonal and mAbs were used as the detection antibody, the sensitivity of these methods was reported to be 98.1 and 97.9%, respectively. Nevertheless, the latter demonstrated superior serotypic specificity (96%) to that of the former (61.5%) (65). Later, Shimmon et al. (66) also generated a truncated bovine integrin αvβ6 as a universal trapping reagent in a sandwich ELISA for FMDV detection. Serotype specificity of sandwich ELISA assays based on the integrin αvβ6 (αvβ6-ELISA) was evaluated against FMDV-positive sera representing all seven serotypes. Depending on the serotype specificity of the mAb used for detection, little to no cross-reactivity was observed. Additionally, different sensitivities were observed when the αvβ6-ELISA was tested against different FMDV strains within the same serotypes (65, 66).

Differentiation/Discrimination of Infected From Vaccinated Animals

Exposure of animals to inactivated or live FMDV during vaccination or infection induces antibodies specific to the SPs. Therefore, a detection method targeting the SPs of FMDV alone cannot differentiate between the infected and vaccinated animals. Although the SPs and NSPs of FMDV are immunogenic, only the SPs serve as the main immunogen for the induction of protective responses (112, 113). Thus, the elimination of the NSPs from the inactivated FMDV vaccine could enable DIVA via differential detection of NSP-specific antibodies in animals infected with FMDV (114, 115). With some modifications, conventional ELISA

methods have been adopted for the detection of NSPs of FMDV. Different NSPs of FMDV including 3ABC, 3AB, 3A, 3B, 3D, 2C, and 2B proteins have been employed in the establishment of NSP-based ELISAs.

Among the NSPs of FMDV, 3ABC polyprotein is reported to be the most antigenic and the most reliable marker for DIVA. Various formats of ELISA based on the 3ABC polyprotein were developed, including the LPB-ELISA, SPC-ELISA, and direct/indirect sandwich ELISA, all of which demonstrated good sensitivity, specificity, and capability for DIVA in various animals (56, 67, 68, 116–125). Enzyme-linked immunosorbent assays based on 3ABC have some added advantages over other NSPs including superior longevity of anti-3ABC antibody in infected animals compared to 2C, 3A, 3D, and Lb, and all infected cattle were shown to develop 3ABC-specific antibody at some points following the infection. Seroconversion to 3ABC in infected cattle was observed at 11 days postinfection, and the antibody remains detectable to the end of the experiments (301 days postinfection). Furthermore, repeated vaccination (fewer than five vaccinations) of the cattle with FMDV vaccine did not induce any antibody response against 3ABC polyprotein, in contrary to 3D protein (126). An agar gel immunodiffusion test was previously developed to detect 3D-specific antibodies in the sera of cattle, sheep, goats, and pigs for DIVA (127), but was found to have low sensitivity and specificity and was later replaced with LPB-ELISA (128). The conventional ELISA based on NSPs of FMDV uses partially purified antigens from infected cell cultures as diagnostic antigens, which require handling of live virus, posing risk of accidental virus escape from laboratories, and these partially purified antigens often lack batch-to-batch uniformity (129). Enzyme-linked immunosorbent assay based on the NSPs produced from either bacterial or baculoviral expression systems overcomes these concerns without compromising the sensitivity and specificity of the test (126, 130). Virus-like particles such as the tymovirus-like particles were also engineered to display 3B1, 3B2, 3AB, 3D, and 3ABD of FMDV and used as coating antigens in an indirect ELISA for DIVA (131). Variable heavy chain antibody fragments were also employed as competing antibodies for NSPs in SPC-ELISA and demonstrated high diagnostic specificity and sensitivity in detecting NSP-specific antibodies (82).

To further simplify and speed up the ELISA process for detecting FMDV-infected animals, the microchip-based ELISA was developed. This assay involved immobilization of the 6x-His tagged recombinant 3ABC polyprotein to microbeads with nickel (II) chelating chemistry, followed by immunoreaction with the 3ABC-specific antibodies in the test sera and detection with thermal lens microscopy based on the enzymatically colorimetric reaction between HRP-labeled antibody and the corresponding substrate. This method was demonstrated to be capable of detecting anti-3ABC antibodies in infected swine and cattle sera with good sensitivity and reproducibility. This assay is much faster (within 25 min) and requires lower serum volume (83, 84). Apart from the microchip-based ELISA, a chemiluminescence immunoassay (CLIA) was also developed for rapid identification of the anti-NSP antibodies. Chemiluminescence immunoassay was reported to simultaneously detect antibodies against 3ABC and 2C proteins of FMDV in experimentally infected pigs with sensitivity and specificity comparable to the commercial kits. This method produced results within 15 min, a remarkably short analysis time compared to other standard ELISA methods (132). Chemiluminescence immunoassay was later applied in the field for DIVA in bovines by simultaneously detecting 3A- and 3B-specific antibodies in the serum samples. In this field test, CLIA was reported to have concordance rate of 88.1% with the commercial PrioCHECK® FMDV NSP ELISA kit and produced no false-positive result in sera collected from bovine that had been vaccinated less than five times and low false-positive results in sera collected from bovine that had been vaccinated up to 10 (<2.2%) and 15 times (<6%) (133). Chemiluminescence immunoassay that enables simultaneous detection of two different antibodies against different NSPs of FMDV is advantageous over ELISA method, which detects only a single anti-NSP antibody. To ensure accurate diagnosis, retesting positive samples by detecting other antibodies against NSPs is a preferred measure (134).

Foot-and-mouth disease virus vaccines based on inactivated virus may contain a trace amount of FMDV NSPs, which could lead to the production of antibodies against the NSPs upon multiple vaccinations, which affect DIVA diagnosis (114, 130, 135). Negative marker vaccines that protect animals from FMDV infection while allowing DIVA were developed via removal of NSPs, which were used as markers for DIVA (57, 58, 136, 137). Alternatively, non-replicating FMDV virus-like particle was explored by Grubman (138) as a marker vaccine. While most negative marker vaccine developments involve deletion of NSPs, a few studies deleted part of the SPs, particularly the VP1 G-H loop, as the antibodies against G-H loop were demonstrated to be inefficient to provide a good protection (69, 70, 139, 140).

CHROMATOGRAPHIC STRIP TESTS

Fast detection and accurate identification of FMDV allow effective FMD surveillance and responses by imposing suitable controls and prevention strategies in case of an FMD outbreak. To date, typical assays for FMDV diagnosis such as virus isolation combined with antigen ELISA and RT-qPCR have been employed in FMDV reference laboratories (141). Despite the reliable and accurate diagnoses of FMDV, these diagnostic assays rely heavily on the availability of high-throughput equipment and highly trained personnel. Furthermore, the poor quality of the samples that resulted from the transport of materials from a field to a laboratory may obstruct or delay the early diagnosis of the disease. Thus, alternatives such as isothermal assays and dipsticks assays (also known as chromatographic strip tests) could serve as promising diagnostic methods in the field for a prompt FMD detection to allow timely implemented control measures. Reverse transcription-RPA (14, 40), RT-LAMP (23, 25, 29, 46, 47), and nucleic-acid sequence-based amplification (142) have been used to detect FMDV. A combination of dipsticks assays with RT-LAMP and RT-RPA has also been used for virus serotyping in field samples (14, 28, 46). Nevertheless, a drawback of the LAMP assay is that it involves the use of a few sets of intricate

primers, while the RPA products require an electrophoresis setup and a fluorescent probe. Hence, a portable, rapid, and accurate detection method is still prominent for initial diagnosis of FMDV.

A chromatographic strip test such as LFI is a well-established fast paper-based analytical platform for detection and quantification of analytes. It is a simple and inexpensive point-of-care (POC) diagnosis without the need of elaborating sample preparations and sophisticated instruments (143). This has led to the increased applications of LFI assay in multiple field conditions where rapid screening is required. **Table 3** summarizes LFI assays for FMDV diagnosis. A typical LFI strip normally consists of overlapping membranes that are mounted on a backing card. A liquid sample containing the analyte of interest moves through the cellulose membrane by a capillary force and is captured by the attached molecules that interact with the analyte along the membrane. In this context, a colored or fluorescent particle conjugated with an antibody that interacts specifically with the target analyte is used as the tracer for the development of signal (157). This LFI assay has been widely used for the diagnosis of infectious diseases (158–161) and detection of bioactive molecules (162, 163). Without the need of specific instruments, LFI strip test is a low-cost diagnostic method, which is easy to perform, giving straightforward results in a very short time. Lateral flow immunochromatographic strip tests have been used intensively for the detection of serotype-specific FMDV such as type-O (144, 149), -A (144, 145), -Asia 1 (144, 150, 164), and -SAT 2 (147). Likewise, LFI strips used for the detection of non-serotype-specific FMDV have also been reported (147, 151, 165, 166). However, one of the drawbacks for this non–serotype-specific LFI assay is the restricted usage of these strips in endemic countries, where rapid identification is essential for disease control (167, 168).

Most of the LFI strips detect FMDV SPs, but detection of specific antibodies against FMDV SPs (149) and NSPs (148, 152) has also been performed. Unlike strips that detect SPs, detections of antibodies against SPs are often performed to identify the vaccination status of animals, whereas detections of antibodies against NSPs are used to identify animals that have been infected by FMDV. Yang et al. (149) developed a lateral flow test strip using the recombinant VP1 protein for specific detection of antibodies against FMDV serotype O. Similar to ELISA, LFI test strips that are able to detect antibodies against SPs are unable to differentiate whether an animal that tested positive is vaccinated or infected. Therefore, test strips that detect antibodies against NSPs are required for the purpose of DIVA. Chen et al. (148) used recombinant 3ABC protein of FMDV serotype O for the detection of anti-NSPs antibodies in porcine. Although the NSPs of FMDV are highly conserved among all FMDV serotypes, only the samples of serotype O were tested. Later on, Wu et al. (152) developed an LFI test strip based on the recombinant 2C′3AB protein of FMDV serotype O, in which 3C was removed because of its low immunogenicity and replaced by part of 2C protein, which was fused to the N-terminus of 3AB. Despite the high sensitivity and specificity of the test, the serotypes of positive and vaccinated serum samples tested were not reported. The LFI strip technology has also been proposed for use in DIVA, but its practical usage in DIVA has yet been reported.

While most of the LFI strips utilize rabbit and guinea pig polyclonal sera, respectively, as the capture and detection antibodies, the usage of mAb as the capture and detection antibodies for FMDV detection in the LFI strip tests has also been developed to improve the efficiency of diagnosis (146, 165, 166, 169). For this purpose, strips are specifically designed for each antigen in order to increase the accuracy, sensitivity, and consistency of the assay. Reid et al. (165) reported that an equivalent sensitivity (100%) to the conventional antigen ELISA was observed in both the clinical samples from animals infected experimentally and in cell culture supernatant using the Clearview^{TM} chromatographic strip test technology with mAb isotype IgG1, designated as Cla. The mAb Cla displayed high reactivity against FMDV serotypes O, A, C, and Asia 1 and no cross-reactivity with SVDV. Utilization of the mAb approach, in which specific mAbs were used as the capture antibody, and serotype-independent mAbs were employed as the detection antibody, produced a new generation of the generic Rapid Assay Device (gRAD) for the detection of FMDV serotypes O, A, and Asia 1 (153). The gRAD, which is currently commercially available, has been shown to achieve a sensitivity similar to that of the double antibody sandwich ELISA for viral antigen detection with a detection limit of 2.55 to 6.3 $\log_{10}$ $TCID_{50}$/mL of 10% tissue suspension from epithelial lesions in a process that took only 10 min (153). Another commercially available LFI strip known as the Svanodip FMDV-Ag LFD by Boehringer Ingelheim Svanova (Sweden) can also be used to detect all the seven serotypes of FMDV antigens based on IF10 mAbs.

As serotype-specific LFI strips can only detect one FMDV serotype at a time (170), thus development of a multiplex platform for simultaneous detection of multiple FMDV serotypes will undoubtedly enhance the usage of the LFI strips in the field. A multiplex-LFI strip test for detecting Hantavirus in humans was developed by Amada et al. (171). The first study describing the development of a multiplex-LFI strip test for detecting FMDV serotypes O, A, and Asia 1 was reported by Yang et al. (151). Following this report, Morioka et al. (154) successfully developed another multiplex FMDV LFI strip based on mAbs that can detect all the seven serotypes and concurrently distinguish serotypes O, A, C, and Asia 1. The developed multiplex-LFI strip had a sensitivity ranging from 10^3 to 10^4 of a 50% tissue culture infectious dose ($TCID_{50}$) of each FMDV strain, comparable to the commercial product, Svanodip FMDV-Ag LFD, which can detect all the seven serotypes of FMDV, but is not able to serotype them.

Recently, a combination of LFI assay and other technologies, such as PCR (172), RT-LAMP (173), RT-RPA (155, 174–176), and quantum dots (177), for the diagnosis of animal pathogens has also been explored. Therefore, the current approach in the development of a desirable FMD diagnostic test typically involves the incorporation of two assays such as RT-LAMP-LFD (46) and RT-RPA-LFD (155, 156). Waters et al. (46) modified an existing FMDV RT-LAMP assay to allow detection of LAMP products with LFD by labeling the FIP/BIP at the 5′ terminus with fluorescein (Flc) and biotin (Btn). This RT-LAMP-LFD assay produced concordant results as compared to those obtained using RT-qPCR with a positive detection of FMDV RNA when the FMDV spiked 10% epithelium suspensions diluted to a range

TABLE 3 | Lateral flow immunochromatographic (LFI) assays for FMDV diagnosis.

Methods	Description	Diagnostic sensitivity	Diagnostic specificity	Tested clinical samples	References
LFD	LFDs using guinea pig serotype–specific capture antibody-gold conjugate were produced for rapid detection of FMDV serotypes O, A, and Asia 1. Goat anti–guinea pig antibody and specific antibodies against FMDV serotypes O, A, and Asia 1 were blotted on nitrocellulose membrane as control line and test line, respectively	88.3–88.7%	97.1–98.2%	Vesicular epithelia and fluid from animals	(144, 145)
	LFD for detection of FMDV serotypes O, A, Asia 1, and C. A non-neutralizing monoclonal antibody that cross-reacts with the FMDV serotypes O, A, Asia 1, and C was labeled with colloidal gold for detection. The test and control lines contained the immobilized monoclonal antibody specific against the antigens, and rabbit anti–mouse antibody, respectively	87.3%	98.8%	Epithelial suspensions	(146)
	LFD based on the use of a monoclonal antibody, namely Mab 2H6 specific against FMDV serotype SAT 2 was developed. The device detected a wide range of FDMV strains within the SAT 2 serotype	88%	99%	Vesicular epithelial suspensions	(147)
	LFD based on recombinant 3ABC to detect anti-NSP antibodies in infected swine	96.8%	98.8–100%	Serum samples from swine	(148)

Methods	Description	Detection limit	Tested clinical samples	References
LFD	LFD generated to detect the antibodies directed against VP1 of FMDV serotype O. The VP1 was conjugated to colloidal gold as detector, while the capturing staphylococcal protein A and swine anti-FMDV antibody were blotted on nitrocellulose membrane for the test and control lines, respectively	• 1:1,280 dilution of a known titer FMDV serotype O-specific antibody	Serum samples of swine origins	(149)
	Two monoclonal antibodies, namely 1B8 and 5E2 specific against FMDV serotype Asia 1 were involved in the assay. 1B8 was labeled with colloidal gold and used as detector, whereas 5E2 and goat anti–mouse antibody were blotted on the nitrocellulose membrane as the test and control line, respectively	• 10^{-5} dilution of Asia1/JSL/05 ($1 \times 10^{7.2}TCID_{50}$/50 μL)	Vesicular epithelial suspensions from the field	(150)
	A multiplex LFD for simultaneous detection and identification of FMDV serotypes O, A, and Asia 1. A cocktail of gold-labeled monoclonal antibodies reacted to the test samples in a separate tube. The multiplex LFD device was dipped into the mixture samples and FMDV of each serotype was detected by the serotype-specific antibodies on the three test lines. The control line contained anti–mouse antibody	• 17–7,200 viral particles	Tissues suspensions (tongues, foot lesion, coronary band, and heart) and swabs collected from ruptured lesions of the infected animals	(151)
	LFD utilized for the detection of antibodies against recombinant NSP (part of the 2C fused to 3AB) of FMDV. The recombinant NSP was labeled with colloidal gold for use as detector. The test and control lines contained the recombinant NSP antigen and rabbit antirecombinant NSP antibody, respectively.	• 1:32 to 1:64 dilution of sera samples	Serum samples from pigs, cattle, sheep	(152)
	LFD that utilizes serotype-specific biotinylated monoclonal antibody as capture antibody and serotype-independent monoclonal antibody labeled with	• 2.55–6.3 $\log_{10}TCID_{50}$/mL of FMDV	Vesicular fluid and epithelial samples, and swabs	(153)

(Continued)

TABLE 3 | Continued

Methods	Description	Detection limit	Tested clinical samples	References
	colloidal gold for detection. The test and control lines contained the biotin-binding protein and anti–mouse antibody, respectively. This assay detected FMDV serotypes O, A, and Asia.		collected over the lesion areas from animals	
	A multiplex LFD that detected all seven serotypes of FMDV and concurrently distinguished serotypes O, A, C and Asia 1. A serotype-independent monoclonal antibody, 1H5, was labeled with colloidal gold for detection. Each serotype-specific monoclonal antibody and 1H5 were blotted on different test lines on nitrocellulose membrane as capture antibodies. The control line contained the anti–mouse antibody	• 10^3 to 10^4 $TCID_{50}$ of FMDV	Vesicular fluids, vesicular epithelial emulsions and oral and/or nasal swabs from pigs	(154)
RT-RPA-LFD	A combination of RT-RPA and lateral flow dipstick for detecting and serotyping FMDV O, A, and Asia 1. The probes and primers used in RT-RPA were labeled with fluorescein and biotin, respectively, to enable detection in LFD.	• 50 copies of viral RNA	Vesicular material, saliva, aerosol, esophageal–pharyngeal fluid, blood, and nasal swab samples from animals	(155)
	RT-RPA-LFD assay performed without equipment but body heat (in a closed fist). The assay detected FMDV serotypes O, A, and Asia in 17 min. The probes and primers used in RT-RPA were labeled with fluorescein and biotin, respectively, to enable detection in LFD	• 100 copies of *in vitro* transcribed FMDV RNA	Vesicular fluid and epithelial tissue samples collected from pigs. Serum samples of bovine origin	(156)
RT-LAMP-LFD	RT-LAMP coupled to LFD for improved detection of FMDV. The primers used in RT-LAMP were labeled with fluorescein and biotin to enable detection in LFD. This assay enables detection of FMDV without nuclei acid extraction step	• 10^{-5} dilution of FMDV-infected epithelial suspensions	Epithelial suspension and air samples from pig, cattle, and sheep	(46)

LFD, lateral flow device; RT-RPA, reverse transcription–recombinase polymerase; RT-RPA-LFD, reverse transcription–recombinase polymerase amplification-lateral flow device; RT-LAMP, reverse transcription–loop-mediated isothermal amplification; RT-LAMP-LFD, reverse transcription–loop-mediated isothermal amplification-lateral flow device; NSP, non-structural protein; RNA, ribonucleic acid; $TCID_{50}$, 50% tissue culture infectious dose.

of 10^{-5}. The RT-LAMP-LFD assay also showed 10^4 times more sensitive in detecting FMDV than most of the FMD-specific antigen lateral flow devices. Hence, this assay not only resolved the problem of relatively low analytical sensitivity encountered by most LFD used in the field, but it also detected FMDV RNA in the raw epithelial suspension (in the absence of RNA extraction) by only diluting the samples with nuclease-free water and incubating the mixture using a water bath set at 60°C for RT-LAMP amplification. With its ideal characteristics, this LFD assay serves as a "proof of concept" for the future use of LAMP in the development of a pen-side assay for FMDV. However, the difficulty in designing the four to six primers needed in RT-LAMP, especially in a virus like FMDV that exhibits a high mutation rate during its replication, hinders the usage of RT-LAMP-LFD assay. In addition, the incubation for RT-LAMP for 45 to 60 min is disadvantageous compared to an RT-RPA approach with a run time of only 4 to 10 min (14). As described earlier, as an isothermal DNA amplification method, RPA has been widely used in the detection of different pathogens. Wang et al. (155) established a combination method of RT-RPA and lateral flow dipstick (RT-RPA-LFD) for detecting and serotyping of FMDV in the field. They constructed a recombinant vector, pcDNA3.1-2B, containing the *2B* gene of FMDV, and amplified it with RT-RPA using specific primers and a probe within 20 min. The newly established FMDV RT-RPA-LFD assay has a higher sensitivity, up to 10 copies as compared with the previous FMDV RT-RPA assays (14, 40) with sensitivity limited to 100 RNA copies. Furthermore, this RT-RPA-LFD assay only requires a thermos metal bath at 38°C unlike other previous RT-RPA assays, which need a sophisticated instrumentation, and the RPA amplicons can be detected by LFD within 5 min. The RT-RPA-LFD is a promising POC diagnostic test for FMDV as it reacts with the FMDV reference strains, including serotypes O, A, and Asia 1, and with no cross-reactivity with other viral pathogens from cattle, which had similar vesicular lesions and clinical symptoms. At the same time, another FMDV RPA-LFD assay that targets the *VP1* gene was also developed by the same research group (178). VP1 protein has been widely used to determine the genetic relationships between different strains of FMDV because of its high genetic heterogeneity (179).

Therefore, primers and probes specific for serotypes O, A, and Asia 1 of FMDV were designed based on the alignment of the VP1 nucleotide sequences. The detection limits of these assays were three copies of plasmid DNA or 50 copies of viral RNA with 98.41% concordance between the RT-RPA-LFD and RT-qPCR assays. The development of this serotype-specific RT-RPA-LFD assay provides a rapid, sensitive, and specific method for differentiation of FMDV serotypes A, O, or Asia 1. On the other hand, an equipment-free FMDV RPA-LFD specifically designed for the *3D* gene was also developed by Liu et al. (156). They performed the assay by incubating the reaction tubes in a closed fist using body heat for 15 min. The developed RPA-LFD was capable to detect FMDV serotypes O, A, and Asia 1 using 10 ng viral RNA and DNA as templates with no cross-detections observed. The analytical sensitivity was equivalent to RT-qPCR with 100 copies of *in vitro*–transcribed FMDV RNA per reaction. One of the benefits in their work is the instant utilization of FMDV RNA as the template in the RPA-LFD without the need to reverse-transcribe the viral RNA into cDNA as required in other RPA assays. This rapid, visible and equipment-free method makes FMDV RPA-LF assay ideal for reliable detection of FMDV in an underequipped laboratory and at point of need, especially in low-resource settings.

FMD-DIAGNOSTIC ASSAYS FOR THE PROGRESSIVE CONTROL OF FMD

Over the past decades, livestock industry has developed remarkably, contributing 40% of the global value of agricultural output and, sustaining the food security of almost 1.3 billion people (180, 181). However, outbreaks of animal diseases remain a major concern that threatens the livestock industry. Foot-and-mouth disease, as one of the most significant animal diseases, poses a severe constraint on the reduction of poverty in countries where this disease is endemic and more prone to food insecurity. Contingency plans for an FMD emergency enable rapid detection of the virus before it progresses to an epidemic outbreak (182). Current laboratory approaches for FMD diagnosis are generally based on assays that exploit the clinical windows of infected animals. The diagnostic window is typically 2 to 14 days with an early observation of clinical signs from vesicular lesions. Rapid confirmation includes assays that aim to detect FMDV in vesicular epithelium and vesicular fluid from clinical lesions, as well as in the blood and mucosal swabs from the active surveillance of infected animals in preclinical cases. Furthermore, FMDV-specific antibody responses can also be detected by serological assays in animals exposed to and recovered from FMDV.

Foot-and-mouth disease diagnosis is performed at two levels: (i) in the field/local and (ii) in the central laboratory. If there is a suspected case of FMD in the field, a quick diagnosis is performed by the FMD diagnostic specialist in order to implement immediate control or biosecurity measures. Clinical examinations and collection of suspected animal's history are performed for epidemiological and disease prevalence investigations. In addition, a range of specimens that might be included in the differential diagnosis is collected and transported back to the regional or central laboratory for further examination. These specimens consist of (i) oral swabs from ruptured lesions; (ii) nasal swabs from lesion less than a week old, where vesicular material is not available; (iii) vesicular fluid from unruptured vesicles; (iv) epithelium from ruptured tissues, placed in a neutral buffer phosphate saline with 50% glycerol; and (v) blood specimens from suspected cases. Although proband samples are not recommended for the first-line diagnostic tests, the oropharyngeal fluid is collected if no fresh lesions are detected. All samples in the ideally leakproof transport containers are labeled and stored in an insulated cool box with a submission form with case history sealed in an external disinfectant see-through bag with photographs of infected animals. The assessment of the situation on the field, and steps taken to secure a confirmatory diagnosis must be immediately reported to the state or regional and central veterinary officers for further advices regarding the disease control strategies.

The subsequent diagnosis of FMD generally depends on the laboratory testing, which includes live virus isolation from tissue culture coupled with the identification of the viral antigen by ELISA or detection of the viral nucleic acid by RT-PCR. Detection of elevated FMD-specific antibodies by ELISA or VNT may also aid in indicating a recovery from the virus infection. These diagnostic assays were performed at a regional or central laboratory to prescribe appropriate control measures based on the confirmation of a definitive diagnosis (168). Hence, these tests should be highly sensitive and specific to provide a differential diagnosis. In the central laboratory, virus and its viral components can be detected with various diagnostic assays. These assays include VI, Ag-ELISA, multiplex RT-PCR, RT-qPCR, and nucleotide sequencing. In addition, antiviral antibodies against SPs can also be detected using VNT, LPB sandwich ELISA, and SPCE-ELISA, whereas antibodies against NSPs can be detected using 3ABC-ELISA. The detailed diagnoses performed at the central laboratory enable the confirmation of disease, serotyping of virus, molecular epidemiology, and phylogenetic analysis and lastly determined the most relevant vaccine matching strains to control an outbreak (183). The performance of all these assays varies in terms of sensitivity, specificity, and time required. The speed of a definitive diagnosis would vary depending on the distance of the samples being transported from the field to an appropriate laboratory. Thus, a network of international reference laboratories and collaborating centers is essential for handling of specimens in the event of a large outbreak, for the purpose of both surveillance and rapid diagnosis. Scalability and cost of each assay must also be taken into consideration especially in FMD endemic and underdeveloped countries. The establishment of centralized facilities for testing, together with the implementation of quality control systems, have improved significantly the assays for routine diagnostic purposes.

More recently, portable tests or POC diagnostics, such as LFD, mobile PCR, and isothermal assays, have been developed to increase the applicability of these assays in multiple field conditions where rapid screening is of paramount importance. Even though LFD can be operated by "non-specialist," the usage

of this portable test may be restricted by its low-throughput assay performance. A commercially available LFI strip known as the Svanodip FMDV-Ag LFD by Boehringer Ingelheim Svanova (Sweden) was reported to show similar assay performance to laboratory-based Ag-ELISA when it was applied on the field during the 2007 UK outbreaks (166). The deployment of LFD on field remains advantageous for FMD endemic countries as compared with portable RT-qPCR in terms of production cost (25, 184). Although these simple-to-use POC tests offer a rapid result that can support the local decisions, they are also limited by the cost-benefit analysis. In conclusion, the deployment of these portable tests on the field will be taken into consideration after their characteristics have been thoroughly evaluated in terms of test performance, speed, cost, simplicity, and robustness.

The control of FMD varies among countries, depending on the FMD status. As the FMD control in FMD-free countries emphasizes on reducing the risk and impact of the virus incursions from both neighboring and trade-partner countries, the control policies in FMD-free countries have been based on depopulation of infected and in-contact animals, together with restrictions on movement of animals and their products. Early detection followed by surveillance is crucial. In order to regain the international trading rights, FMD-free countries are required to identify the remaining sources of infection and to demonstrate that they are free of the disease. On the other hand, FMD control in endemic countries is implemented by diagnoses, surveillance, and regular mass vaccinations. Most importantly, there is a continuous need for an up-scaling of improved quality vaccines with longer-lasting protection at a lower cost (185). In this context, serological assays including ELISA for detection of antibodies against FMDV SPs and NSPs are used. The former is useful to measure the vaccine efficacy, and the latter is generally used to establish prevalence and to monitor virus circulation as it can detect the presence of the infection regardless of the vaccination status of the animals (186, 187). The SP tests are serotype specific. Therefore, virus and antigen closely related to the field strain are selected to be used in ELISA for optimal sensitivity. To date, the commercially available PrioCHECK® FMDV type-specific products by Prionics can only detect anti-SP antibodies of 3 FMDV serotypes: O, A, and Asia. Hence, determination of the serotype involved in field outbreaks is important for a proper control of the disease. On the other hand, the use of NSP tests in FMD endemic countries is complicated by the fact that the vaccinated animals may seroconvert after repeated vaccinations. Anti-NSP antibody responses may also be delayed in cases of subclinical or mild clinical infections following routine vaccinations. Moreover, anti-NSP antibodies can persist for a long period and may not indicate a recent FMDV infection (134, 188).

The breakthrough of molecular diagnostics along with the development of pen-side devices has allowed the determination of the FMDV serotypes. For endemic countries, the use of LFD is more favorable. A routine screening with an LFD device has been viewed as a rapid and economical tool to determine incidences of the infection in countries where the emergence rate of FMDV is high, under limited-resources veterinary settings. Therefore, rapid action is needed to minimize the virus spread. As mentioned earlier, serotype-specific LFI test strips can be used for rapid detection and identification of various FMDV serotypes (144, 145, 147, 149, 150, 164). By identifying these FMDV serotypes, appropriate commercial FMD vaccine can be applied to the animal population to minimize the loss of productivity in majority of the smallholder and commercial farmer settings in endemic countries. As for FMD-free countries, confirmatory tests such as ELISA and RT-qPCR are more desirable. Due to the occurrence of FMDV is relatively much lower in these countries, confirmation of the disease is more important than rapid identification of the virus to avoid unnecessary culling of suspected animals. Setup of RT-qPCR in the regional laboratories in these more developed countries, which are typically FMD-free, can increase the diagnostic capacity and subsequently reduce the sample shipping times during a sudden outbreak. The FMD outbreak confirmation along with the virus typing and characterizations enables the study of the virus lineage and routes of transmission, which will provide substantial information for epidemiology study in the effort to control the spread of FMD.

CONCLUSIONS

The deployment of diagnostic tools to rapidly identify and confirm initial clinical symptoms of an infection is prerequisite in any epidemic disease control strategy, particularly when it comes to the prevalence of the FMDV in a livestock population. As the FMDV infection is clinically indistinguishable from infections resulting from other similar vesicular disease viruses, early diagnosis is critical for efficient disease control. Various diagnostic methods ranging from conventional such as virus isolation and competitive- and blocking-antigen ELISA to molecular-based methods such as RT-PCR and RT-LAMP have been developed over the years. Although ELISA-based methods have good diagnostic sensitivity and specificity, molecular detection methods have the advantage of higher analytical sensitivity for the detection of minimal viral RNA. Despite these accurate and reliable FMDV assays, researchers have been developing alternatives methods that allow for pen-side testing in an attempt to overcome some of the practical challenges such as tedious procedures and the availability of an equipped laboratory setting with trained field personnel. Development of lateral flow devices and integration of the portable RT-PCR, RT-LAMP, and RT-RPA with LF technologies have been made to increase the sensitivity of FMDV detection. Nevertheless, translations of these assays from laboratories to practical applications in the field remain limited, and various technical and cost issues need to be addressed to develop a more flexible and affordable diagnostic tools that can be widely used for FMDV detection.

AUTHOR CONTRIBUTIONS

CW, CY, and HO wrote the manuscript. CW, CY, HO, KH, and WT approved its final version. All

authors contributed to the article and approved the submitted version.

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Genetic Basis of Antigenic Variation of SAT3 Foot-And-Mouth Disease Viruses in Southern Africa

Lorens Maake[1,2], *William T. Harvey*[3], *Lia Rotherham*[1], *Pamela Opperman*[1,4], *Jacques Theron*[2], *Richard Reeve*[3] *and Francois F. Maree*[1,2]*

[1] *Vaccine and Diagnostic Development Programme, Onderstepoort Veterinary Institute, Agricultural Research Council, Pretoria, South Africa,* [2] *Department of Biochemistry, Genetics and Microbiology, Faculty of Agricultural and Natural Sciences, University of Pretoria, Pretoria, South Africa,* [3] *Boyd Orr Centre for Population and Ecosystem Health, Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom,* [4] *Department of Animal Production Studies, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa*

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***Correspondence:**
Francois F. Maree
mareef@arc.agric.za

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Foot-and-mouth disease (FMD) continues to be a major burden for livestock owners in endemic countries and a continuous threat to FMD-free countries. The epidemiology and control of FMD in Africa is complicated by the presence of five clinically indistinguishable serotypes. Of these the Southern African Territories (SAT) type 3 has received limited attention, likely due to its restricted distribution and it being less frequently detected. We investigated the intratypic genetic variation of the complete P1 capsid-coding region of 22 SAT3 viruses and confirmed the geographical distribution of five of the six SAT3 topotypes. The antigenic cross-reactivity of 12 SAT3 viruses against reference antisera was assessed by performing virus neutralization assays and calculating the r_1-values, which is a ratio of the heterologous neutralizing titer to the homologous neutralizing titer. Interestingly, cross-reactivity between the SAT3 reference antisera and many SAT3 viruses was notably high (r_1-values >0.3). Moreover, some of the SAT3 viruses reacted more strongly to the reference sera compared to the homologous virus (r_1-values >1). An increase in the avidity of the reference antisera to the heterologous viruses could explain some of the higher neutralization titers observed. Subsequently, we used the antigenic variability data and corresponding genetic and structural data to predict naturally occurring amino acid positions that correlate with antigenic changes. We identified four unique residues within the VP1, VP2, and VP3 proteins, associated with a change in cross-reactivity, with two sites that change simultaneously. The analysis of antigenic variation in the context of sequence differences is critical for both surveillance-informed selection of effective vaccines and the rational design of vaccine antigens tailored for specific geographic localities, using reverse genetics.

Keywords: foot-and-mouth disease (FMD), Southern African Territory (SAT) type 3, antigenic, cross-reactivity, antigenic matching, phylogeny, virus neutralization test

HIGHLIGHTS

- Phylogenetic relationships of the capsid-coding region of SAT3 viruses confirmed the geographical distribution of the southern African topotypes.
- Cross-reactivity between SAT3 reference antisera and SAT3 viruses is notably high.
- Avidity could explain some of the higher cross-reactivity observed.
- Unique amino acid residues may be associated with a change in cross-reactivity.

INTRODUCTION

Foot-and-mouth disease (FMD) continues to be a major burden for livestock owners in endemic countries (1). The occurrence of FMD negatively impacts on the livelihoods of local farmers due to its effects on productivity, food insecurity and losses of income, but also have damaging consequences on international trade in livestock and animal products. The disease is widely distributed in Africa, Asia, and South America where FMD is regarded as endemic. FMD outbreaks particularly affect vulnerable individuals, such as women and children since ∼75% of livestock in Africa are raised under the communal smallholder, communal-grazing or pastoral systems that sustain livelihoods of these groups (2, 3). Controlling FMD at its source is therefore a shared interest between endemic and free countries (4). The epidemiology of FMD in sub-Saharan Africa is unique due to the presence of the South African Territories (SAT) serotypes that are almost exclusively endemic, and its continuous maintenance in wildlife (5–7). Therefore, FMD control in livestock is dependent, in part, on an understanding of pathogenesis, persistence, and transmission from African buffalo (*Syncerus caffer*) (7).

Clinically indistinguishable FMD viruses (FMDV) belonging to the SAT serotypes are maintained in buffalo, but differ from each other with respect to their geographic distribution, incidence, outer capsid-coding sequence and antigenicity. SAT2 is the most widely distributed in Africa and is also the serotype most often associated with outbreaks in cattle in southern Africa, followed by SAT1 and then SAT3 (8–10). However, viruses of the SAT1 serotype is most frequently isolated from buffalo (7, 11). Viruses belonging to the SAT3 serotype have the most restricted distribution and essentially occur in southern Africa and in the south-western region of Uganda (12, 13). The SAT3 serotype is also less frequently detected in African buffalo (12). In South Africa, in the Kruger National Park, a SAT3 outbreak occurred in 1958/59 where it involved wildebeest (*Connochaetes taurinus*), kudu (*Tragelaphus strepsiceros*) and sable antelope (*Hippo tragus niger*) (14) and was also detected in Mozambique. Other outbreaks were detected in cattle in Limpopo (Giyani) during 1979/80 (15), in Phalaborwa during 2002 affecting buffalo, in Thulamela during 2006 and in the Kruger National Park (Pafuri) during 2008 affecting impala (*Aepyceros melampus*) as well. The 1979/80 outbreak in Giyani lasted for 9 months and was the longest SAT3 outbreak reported to date (9). Neighboring southern African countries also experienced SAT3 outbreaks during similar times with the most recent outbreaks reported in livestock in Namibia in 2011, Zimbabwe in 1999 and 2013, Zambia in 2015 and 2017, and Mozambique in 2016–2017 (Records of the OIE).

Phylogenetic reconstruction of the partial VP1-coding nucleotide sequence from SAT3 viruses has revealed at least six (I–VI) distinct topotypes. Amongst them, topotypes I–IV occur in southern Africa, whereas topotypes V and VI are unique to Uganda (12, 13). The SAT3 viruses belonging to different topotypes differed by 20% or more in complete nucleotide sequence alignments of the VP1-coding region (12). Studies comparing genetic variation and serological cross-reactivity have shown that SAT1 and SAT2 viruses from different topotypes are generally antigenic poorly related (16, 17). However, similar studies have not yet been undertaken for SAT3 viruses.

Studies focusing exclusively on SAT3 viruses are lacking. Limited studies have been performed to determine the genetic diversity of SAT3 viruses, but these studies were primarily based on partial VP1 sequences. Here, we assessed the intratypic SAT3 genetic variation of the VP1, VP2, VP3, and VP4 capsid proteins and antigenic cross-reactivity within the southern African SAT3 viruses. The analysis of antigenic variation is critical to allow proper vaccine selection or the design of vaccine antigens tailored for specific geographic localities, using reverse genetics.

MATERIALS AND METHODS

Cells and Viruses

Instituto Biologico Renal Suino-2 cells (IB-RS-2) and primary pig kidney (PPK) cells were maintained and propagated in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Delta Bioproducts) and a 1 μg/ml amphotericin B and 0.5 mg/ml gentamycin mixture (Gibco) (18).

Twelve SAT3 viruses, collected from buffalo or cattle during 1990–2010 in southern Africa, were sequenced and used for genetic and antigenic analysis and an additional ten SAT3 P1 sequences available in GenBank were included for the genetic analysis. The viruses form part of the virus databank of the Agricultural Research Council-Onderstepoort Veterinary Research Institute (ARC-OVR), Transboundary Animal Diseases (TAD) Biosafety Level 3 (BSL-3) laboratory (South Africa). The species, which the viruses were isolated from, the country of origin, and year of isolation are summarized in **Table 1**. The viral isolates were initially passaged on PPK cells, prior to propagation on IB-RS-2 cells and harvested when maximum cytopathic effect (CPE) was observed or after 48 h. All viruses were titrated to determine the tissue culture infectious dose at 50% ($TCID_{50}$). Virus growth medium (VGM) was prepared with RPMI supplemented with 5% (vol/vol) FBS and 1% (vol/vol) antibiotics/antimycotic mixture (Gibco). The SAT3/KNP/10/90, SAT3/SAR/1/06 (topotype I), and SAT3/BOT/6/98 (topotype II) viruses were selected as reference material for the preparation of antisera.

Virus Titrations

The viral titers were determined in flat-bottomed microtiter plates (Nunc). Briefly, 0.5 $\log_{10}$ dilutions of the virus stocks

TABLE 1 | List of SAT3 viruses used in the current study including species of isolation, passage history, year of isolation, and country of isolation.

SAT3 virus	GenBank accesion numbers	Species	Passage history	Year	Country of isolation	References
KNP/10/90	AF286347	Buffalo	PK1RS2	1990	South Africa	This study
KNP/2/03	MK415738	Buffalo	PK1RS2	2003	South Africa	This study
KNP/6/08	MK415735	Buffalo	PK1RS3	2008	South Africa	This study
KNP/14/96	MK415741	Buffalo	PK1RS2	1996	South Africa	This study
KNP/8/02	MK415739	Buffalo	PK1RS1	2002	South Africa	This study
KNP/1/03	MK415737	Buffalo	PK1RS2	2003	South Africa	This study
KNP/1/08	MK415734	Buffalo	PK1RS2	2008	South Africa	(7)
SAR/57/59	AY593850	–	–	1959	South Africa	(19)
SAR/14/01	MK415740	Buffalo	PK1RS2	2001	South Africa	This study
SAR/1/06	MK415736	Buffalo	PK1RS2	2006	South Africa	This study
ZIM/4/81	KX375417	–	–	1981	Zimbabwe	(20)
ZIM/6/91	KM268901	–	–	1991	Zimbabwe	(21)
ZIM/11/94	MK415743	Buffalo	PK1RS5	1994	Zimbabwe	This study
BOT/6/98	MK415742	Buffalo	PK1RS2	1998	Botswana	This study
KEN/11/60	AY593852	–	–	1960	Kenya	(19)
BEC/20/61	AY593851	–	–	1961	Botswana	(19)
BEC/1/65	AY593853	–	–	1965	Botswana	(19)
ZIM/5/91	MK415745	Buffalo	PK1RS5	1991	Zimbabwe	This study
ZAM/5/93	MK415744	Buffalo	PK1RS2	1993	Zambia	This study
ZAM/4/96	DQ009741	–	–	1996	Zambia	(16)
UGA/2/97	DQ009742	–	–	1997	Uganda	(16)
UGA/1/13	KJ820999	–	–	2013	Uganda	(13)

were titrated into 96-well microtitre plates (Nunc), followed by addition of 3×10^5 IB-RS-2 cells per well. Plates were incubated at 37°C with continuous CO_2 influx. At 72 h post-inoculation the remaining intact cells were stained with 1% (wt/vol) methylene blue in 10% (vol/vol) formalin. The plaques were counted to calculate virus titers, which were expressed as tissue culture infectious dose 50% ($TCID_{50}$) according to the method of Kärber (22).

RNA Extraction, cDNA Synthesis, PCR Amplification, and Sequencing

Viral RNA was extracted from infected cell culture supernatant using the QIAamp viral RNA mini extraction kit (Qiagen) and used as template for cDNA synthesis (23). First-strand cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System (Invitrogen) and the genome-specific oligonucleotide 2B (24) following the manufacturer's recommendations. The FMDV ca. 3.0 kb Leader/capsid-coding region was PCR amplified using the Expand High Fidelity PCR system (Roche) and flanking oligonucleotides NCR (5′-TAACAAGCGACACTCGGGATCT-3′) and WDA (5′-GAAGGGCCCAGGGTTGGACTC-3′) (25). Amplicons were purified from an agarose gel with the QIAquick® Gel Extraction Kit (Qiagen). Sequencing of the amplicons was performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Perkin Elmer Applied Biosystems) and resolved on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The sequences were assembled using Sequencher 5.1 (GeneCodes). The GenBank accession numbers of the capsid-coding sequences are shown in **Table 1**. The nucleotide sequences were aligned using CLUSTAL_X (26) and phylogenetic trees were constructed using MEGA (27).

Preparation of Bovine Serum

Convalescent sera were obtained from cattle infected with the respective SAT3 reference viruses (SAT3/KNP/10/90, SAT3/SAR/1/06, and SAT3/BOT/6/98), 28 days post-infection (dpi). Groups of five cattle were inoculated intradermoligually with 1 ml of 10^4 $TCID_{50}$ per ml of either of the reference viruses. Cattle were housed in the biosafety level 3 stables at the ARC-OVR, TAD. All procedures were approved by the ARC-OVR Animal Ethics Committee (Ethics approval number AEC18.11) according to national animal welfare standards and performed with the permission of the Department of Agriculture, Forestry, and Fisheries (Act 35 of 1984).

Sera collected from each infected cattle were inactivated at 56°C for 30 min. Inactivated sera from the five cattle for each group were pooled and the pooled sera were used in subsequent experiments.

Virus Neutralization Test

Antigenic cross-reactivity of FMDV against the convalescent animal sera was determined using the virus neutralization test (VNT) according to the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (28). Briefly, the test serum was diluted 2-fold in VGM using 96-well microtitre (Nunc) plates, starting with a 1/8

dilution, and mixed with a virus suspension containing ∼100 $TCID_{50}$ per well. After 1 h of incubation at 37°C, 3×10^5 IB-RS-2 cells were added to each well and incubated for a further 72 h at 37°C in a humid atmosphere containing 5% CO_2. Cell-only controls were added to each plate and a virus titration control and positive serum control (cells, virus, and positive reference serum) were performed on each day. Plates were analyzed microscopically and colorimetrically for CPE and 50% end-point serum titers were calculated according to the method of Kärber (22). Virus neutralization titers were expressed as the $\log_{10}$ of the reciprocal serum dilution that protected the cells in 50% of the inoculated wells. All VNTs were performed at least three times. One-way antigenic relationships (r_1-value) of the field virus isolates relative to the reference viruses were calculated, and expressed as the ratio between heterologous and homologous serum titer. The criteria of the OIE Manual (28) were applied for interpreting the antigenic relationships. Briefly, r_1-values between 0 and 0.29 indicated significant antigenic variation from the reference viruses, and values of ≥0.30 demonstrated that the reference and field viruses are sufficiently antigenically similar.

Virus Purification

BHK-21 cell were seeded, based on cell counts performed using a haemocytometer and tryphan blue staining, into 8×750 cm^2 plastic roller bottles (Corning) to obtain confluent monolayers. Confluent BHK-21 cell monolayers were infected at an multiplicity of infection (MOI) of 5–10 pfu/cell with SAT3/KNP/10/90, SAT3/BOT/6/98, SAT3/SAR/1/06, SAT3/KNP/14/96, or SAT3/SAR/14/01 in Glasgow's Minimal Essential Medium (GMEM) supplemented with 10% (vol/vol) tryptose phosphate broth (TPB), 3% (vol/vol) lactalbumin hydrolysate solution, 1% (vol/vol) FBS, 1% (vol/vol) antibiotic-antimycotic solution, and 25 mM HEPES buffer. Following incubation for 14–16 h at 37°C, the cells were lysed by addition of 10% (vol/vol) Nonidet P-40 and 0.5 M EDTA (pH > 7.4). The virus particles were recovered and concentrated from the lysed cell supernatants as described by Opperman et al. (29). The 146S virus particles were purified on a 10–50% (wt/vol) sucrose density gradient (SDG), prepared in TNE buffer (50 mM Tris [pH 7.5], 150 mM KCl, 10 mM EDTA), as described previously (30). Peak sucrose fractions corresponding to 146S virion particles were pooled and the amount of antigen was calculated (31).

Single Dilution Avidity ELISA (sd A-ELISA)

The protocol was adapted from Lavoria et al. (32). Briefly, Maxisorp ELISA plates were coated, in duplicate, overnight at 4°C with 200 ng of sucrose density gradient (SDG)-purified virus in 50 mM carbonate/bicarbonate buffer (pH 9.6). The plates were washed with phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween-20 (PBS-0.05%T) and blocked at 37°C for 1.5 h with blocking buffer [PBS, 20% (vol/vol) FCS, 0.002% (wt/vol) thimerosal, and 0.1% (wt/vol) phenol red] and washed. The reference sera were diluted 1:40 in blocking buffer, added to the plates and incubated at 37°C for 1 h. The negative control sera consisted of a pool of five negative bovine sera. The plates were washed three times with PBS-0.05%T and then 4 M urea in PBS was added to one plate and PBS was added to the remaining plate. Following incubation at room temperature for 20 min, the plates were washed again before the FMDV-specific antibodies were detected with horseradish peroxidase-labeled anti-bovine conjugate (Sigma-Aldrich), diluted 1:20,000 in blocking buffer (29). The ELISA plates were developed using a substrate/chromogen solution, consisting of 4 mM 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1 M citric acid monohydrate, 0.1 M tri-potassium citrate; pH 4.5) and 0.015% (vol/vol) of H_2O_2. The color reaction was stopped after 10 min with 1 M H_2SO_4 and the optical density (OD) was read at 450 nm using a Labsystems Multiscan Plus photometer. Mean OD values of samples and controls were corrected by subtracting mean blank OD values (cOD). The avidity index (AI) was calculated as described previously (32). Briefly, AI% = (cOD sample with urea/cOD sample without urea) × 100. AI were compared using one-way analysis of variance (ANOVA) and Benferroni's multiple comparison test (33) with a 95% confidence interval (CI) of difference; p-value <0.5 indicated significance binding. The analysis was performed using GraphPad Prism v5.03 for Windows (GraphPad Software, Inc.).

Statistical Analysis of Gene Sequences and Virus Neutralization Titers

To identify genetic predictors of antigenic variation, amino acid substitutions between reference viruses and test viruses were tested using a model fitted to geometric ($\log_2$) VN titers, while accounting for phylogenetic relationships and non-antigenic variation in VN titers that can be attributed to day-to-day variability in tests performed on different dates. To prevent false support for substitutions that arise due to the evolutionary process, phylogenetic information was included in the model (17). A phylogenetic tree was reconstructed from aligned capsid nucleotide sequences using PhyML v3.0 (34). The general time reversible model with a proportion of invariant sites and a gamma distribution describing among-site rate variation (GTR + I + Γ_4) was identified as the best model of nucleotide substitution using jModelTest v2.1.10 (35). Each combination of reference and test virus is separated by a unique combination of branches of the phylogeny. Phylogeny branches separating reference and test viruses were tested as correlating with antigenic change expressed in lower VN titers. In addition, phylogenetic terms associated with changes in immunogenicity were identified (branches). The optimal combination of amino acid position variables and phylogenetic variable was identified using a sparse hierarchical Bayesian model where each variable is associated with parameter estimate and in addition, a binary indicator variable that determines inclusion (1) or exclusion (0) in the model (36). The posterior mean of each indicator variable provides an estimate for the inclusion probability for each variable. Additionally, the model was used to estimate the proportion of all variables tested that should be included in an optimal model. Conditional effect sizes (coefficient estimated for a variable when present in the model, i.e., when associated indicator variable = 1) were mapped to branches of the phylogeny and visualized alongside a heatmap showing VN titers using the *ggtree* R package (37). Separately,

TABLE 2 | Comparison of the number of variable amino acids in a pairwise alignment of the structural proteins (P1 polypeptide) and r_1-values between reference viruses and test viruses.

Strain	Topotype[a]	SAT3/KNP/10/90		SAT3/SAR/01/06		SAT3/BOT/06/98	
		Variable amino acid[b]	r_1-value[c]	Variable amino acid[b]	r_1-value[c]	Variable amino acid[b]	r_1-value[c]
SAT3/KNP/10/90	I	0	1	38	**1.78**	57	**1.28**
SAT3/SAR/14/01	I	40	0.65	44	**1.46**	67	**1.16**
SAT3/ZIM/6/91	I	36	–	37	–	61	–
SAT3/KNP/2/03	I	33	0.39	33	0.6	56	0.45
SAT3/KNP/8/02	I	28	*0.21*	32	0.41	54	0.38
SAT3/SAR/1/06	I	38	*<0.2*	0	1	63	0.87
SAT3/KNP/1/03	I	38	*<0.2*	50	*<0.2*	64	0.39
SAT3/KNP/1/08	I	39	–	37	–	60	–
SAT3/SAR/57/59	I	42	–	41	–	66	–
SAT3/KNP/14/96	I	40	1.09	43	**1.67**	55	**1.32**
SAT3/KNP/6/08	I	36	0.73	42	1	53	1
SAT3/ZIM/4/81	I	34	–	40	–	61	–
SAT3/ZIM/11/94	II	51	0.48	56	1	46	0.83
SAT3/KEN/11/60	II	65	–	77	–	50	–
SAT3/BEC/20/61	II	66	–	78	–	51	–
SAT3/BEC/1/65	II	57	–	61	–	39	–
SAT3/BOT/6/98	II	57	0.6	63	**2.24**	0	1
SAT3/ZIM/5/91	III	59	*0.27*	64	0.54	69	0.64
SAT3/ZAM/4/96	IV	61	–	74	–	62	–
SAT3/ZAM/5/93	IV	56	0.97	70	0.7	58	0.94
SAT3/UGA/2/97	VI	118	–	119	–	113	–
SAT3/UGA/1/13	VI	122	–	128	–	124	–

[a] *The topotypes classification is based on the VP1 phylogeny proposed by Vosloo et al. (38) and Bastos et al. (12).*
[b] *Pairwise alignment was performed for the complete P1 polypeptide of 741 amino acids.*
[c] *r_1-values higher than 1 are indicated in bold and those values lower than 0.3 in italics. The homologous r_1-values are highlighted in light gray.*
"–" VNTs were not performed and sequences of these viruses were retrieved from GenBank.

a two-dimensional hierarchical clustering of reference and test viruses was performed and visualized as a heatmap.

RESULTS

Antigenic Diversity Among SAT3 Viruses in Southern Africa

We applied one-way antigenic relationships (r_1-values), measured by VNTs, to investigate the antigenic variability of viruses belonging to the SAT3 serotype in southern Africa. SAT3 viruses showed a significant degree of cross-reactivity to the sera of the SAT3 reference viruses (SAT3/SAR/1/06 and SAT3/BOT/6/98) (**Table 2**). At least 92% ($n = 11$) and 100% ($n = 12$) of the SAT3 viruses showed r_1-values $\geq$0.3 to the SAT3/SAR/1/06 (topotype I) and SAT3/BOT/6/98 (topotype II) sera, respectively. However, one of the viruses in topotype I, SAT3/KNP/1/03, had an r_1-value of <0.2 when tested against SAT3/SAR/1/06, but cross-reacted with SAT3/BOT/6/98 antisera with an r_1-value of 0.39. Cross-reactivity to the SAT3/KNP/10/90 (topotype I) reference sera indicated that 67% ($n = 8$) of the viruses were neutralized by the sera with an r_1-value above 0.3. Interestingly, three viruses showed r_1-values >1.0 against SAT3/BOT/6/98 antisera and four viruses had a similar high cross-reactivity to the SAT3/SAR/1/06 antisera, one of which was as high as 2.24. We then investigated whether the higher neutralization titers of these viruses (KNP/10/90, SAR/14/01, KNP/14/96 and BOT/6/98) were as a result of increased avidity of the antisera to the particular viruses.

The avidity index of the SAT3/BOT/6/98 and SAT3/SAR/1/06 bovine antisera against the SAT3 viruses with r_1-values >1.0 (SAT3/KNP/10/90, SAT3/SAR/14/01, SAT3/KNP/14/96), and the homologous viruses is shown in **Figure 1**. The avidity index of the SAT3/KNP/10/90 (AI = 72%) and SAT3/SAR/14/01 (AI = 67%) viruses to the SAT3/SAR/1/06 antisera was higher than the avidity to the homologous virus (AI = 56%), albeit statistically insignificant ($p > 0.05$) (**Figure 1A**). Avidity values of <25% were observed for SAT3/BOT/6/98 and SAT3/KNP/14/96 viruses to the SAT3/SAR/1/06 antisera. In contrast, antibodies in SAT3/BOT/6/98 antisera bound with high avidity to the SAT3/KNP/10/90 (AI = 65%; $p < 0.01$), SAT3/SAR/14/01 (AI = 72%; $p < 0.001$), and SAT3/SAR/1/06 (AI = 47%; $p > 0.05$) viruses, while the avidity against the SAT3/KNP/14/96 (AI = 11%) and the homologous virus, SAT3/BOT/6/98 (AI = 28%), was lower (**Figure 1B**).

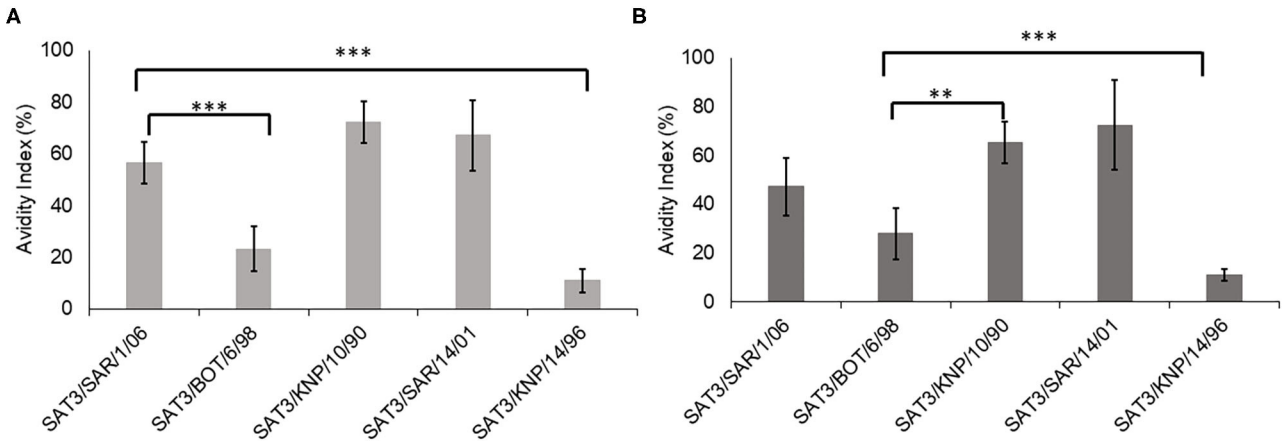

FIGURE 1 | Avidity index of the SDG-purified SAT3 viruses SAT3/SAR/1/06, SAT3/BOT/6/98, SAT3/KNP/10/90, SAT3/SAR/14/01, and SAT3/KNP/14/96, to the bovine antisera raised against SAT3/SAR/1/06 **(A)** and SAT3/BOT/6/98 **(B)** are indicated. The data are means ± SD of quadruplicate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ at 95% CI.

Genetic Variation in the Capsid Proteins of SAT3 Viruses

The intratypic nucleotide variation of the SAT3 P1 region was calculated to be 45.6% ($n = 22$) and is comparable to the intratypic variation reported for SAT1 (47.3%; $n = 20$) and SAT2 (48.9%; $n = 23$) viruses, but higher than types A (42.5%; $n = 50$) and O (38.2%; $n = 41$) (16, 19). The nucleotide and amino acid variation in a complete alignment of the SAT3 capsid proteins and coding region is summarized in **Table 3**. With the exception of SAT3/KNP/10/90, the P1 region of SAT3 viruses was 2,220 nucleotides in length and encodes 740 amino acids representing the four structural proteins. The VP1-coding region of SAT3/KNP/10/90 contains a three-nucleotide-insertion between nucleotides 252 and 253, which translates to an additional amino acid (lysine, K) in the βD-βE loop of the VP1 protein. Overall, in the capsid coding region, a total of 1,015 (45.7%) nucleotide positions were variant. The majority of the mutations in the P1 region (36.5%) were synonymous; however, at least 45% of the nucleotide substitutions in the VP1-coding region resulted in amino acid changes in the complete alignment.

A maximum phylogenetic tree constructed from this alignment with topotypes and the positions of viruses further investigated using virus neutralization assays is shown in **Figure 2**. Phylogenetic resolution of capsid protein sequences of the SAT3 viruses confirmed five of the six topotypes, each with its unique geographic distribution. Topotype I included viruses from South Africa and southern Zimbabwe, topotype II encompassed viruses from Botswana and western Zimbabwe, and topotype IV viruses from Zambia.

A pairwise alignment of the capsid proteins of the SAT3 viruses with the corresponding proteins of each reference virus displayed variation in 28–70 of the amino acid positions, with most variation in the pairwise alignments with SAT3/BOT/6/98 (46–69 variable residues) (**Table 2**). No clear correlation was observed between the number of variable residues and r_1-values to each of the reference viruses (**Table 2**).

In a complete alignment of the structural proteins, three regions of notable variability (amino acid entropy >1) were observed in the VP2 protein at amino acid positions 92–101 (βC-βD loop), 128–138 (βE-βF loop), and 208–217. In the VP3 protein, 23.5% variable amino acid positions were observed and residues with high entropy (>1) were positioned on the surface-exposed βE-βF loop at 131, 135, and 139 and in the C-terminus at residues 219–220. However, several regions with hypervariability were identified throughout the VP1 protein including: (i) N-terminal residues 7–16; (ii) the linear amino acid region that correlates with a T-cell epitope region in serotype O (39), also in the N-terminus (aa 21–26); (iii) a region in the βB-βC loop (aa 44–55) correlating with O1BFS antigenic site 3 (40); (iv) βD-βE loop (aa 79–91); (v) βF-βG loop (aa 109–116); (vi) residues 137–146 and 149–163 of the βG-βH loop; (vii) residues 175–185 and lastly, (viii) the C-terminus (aa 196–206 and aa 207–216).

Predicting Antigenic Substitutions in the Outer Capsid Proteins of SAT3 Viruses

Next, we explored the genetic basis of variation expressed in VN titers. In **Figure 3**, two heatmaps show the same VN titers (log10) for 12 viruses (rows) and reference antisera (columns) raised to three reference viruses organized in two ways: firstly, where test viruses (rows) are sorted according to the phylogeny and secondly where test viruses (rows) are sorted according to a hierarchical clustering of the VN titers. The hierarchical clustering analysis, also expressed in the dendograms to the right of the heatmaps, indicated that viruses of the same topotype did not consistently cluster together on the basis of cross-reactivity data.

To probe the relationship between VN titers and genetic differences in greater detail, a sparse hierarchical Bayesian model was used to test whether substitutions at each non-conserved amino acid residue, within the VP1, VP2, and VP3 proteins, were predictors of reduced antigenic cross-reactivity. Residues would be selected if substitutions between test and reference

TABLE 3 | Variation within the nucleotide and amino acid sequences of the P1 coding region and deduced polyprotein in a complete alignment to each of the SAT3 reference viruses.

Genome region	No. nucleotide positions aligned	No. variant nucleotides	Variant nucleotides (%)	No. amino acid positions aligned	No. variant amino acids	Variant amino acids (%)
VP4 (1A)	258	84	32.5	86	1	1.2
VP2 (1B)	651	276	42.4	217	54	24.8
VP3 (1C)	663	272	41.0	221	52	23.5
VP1 (1D)	651/4	377	57.9	217/8	98	45.2
P1	2220/1	1015	45.6	740/1	205	27.7

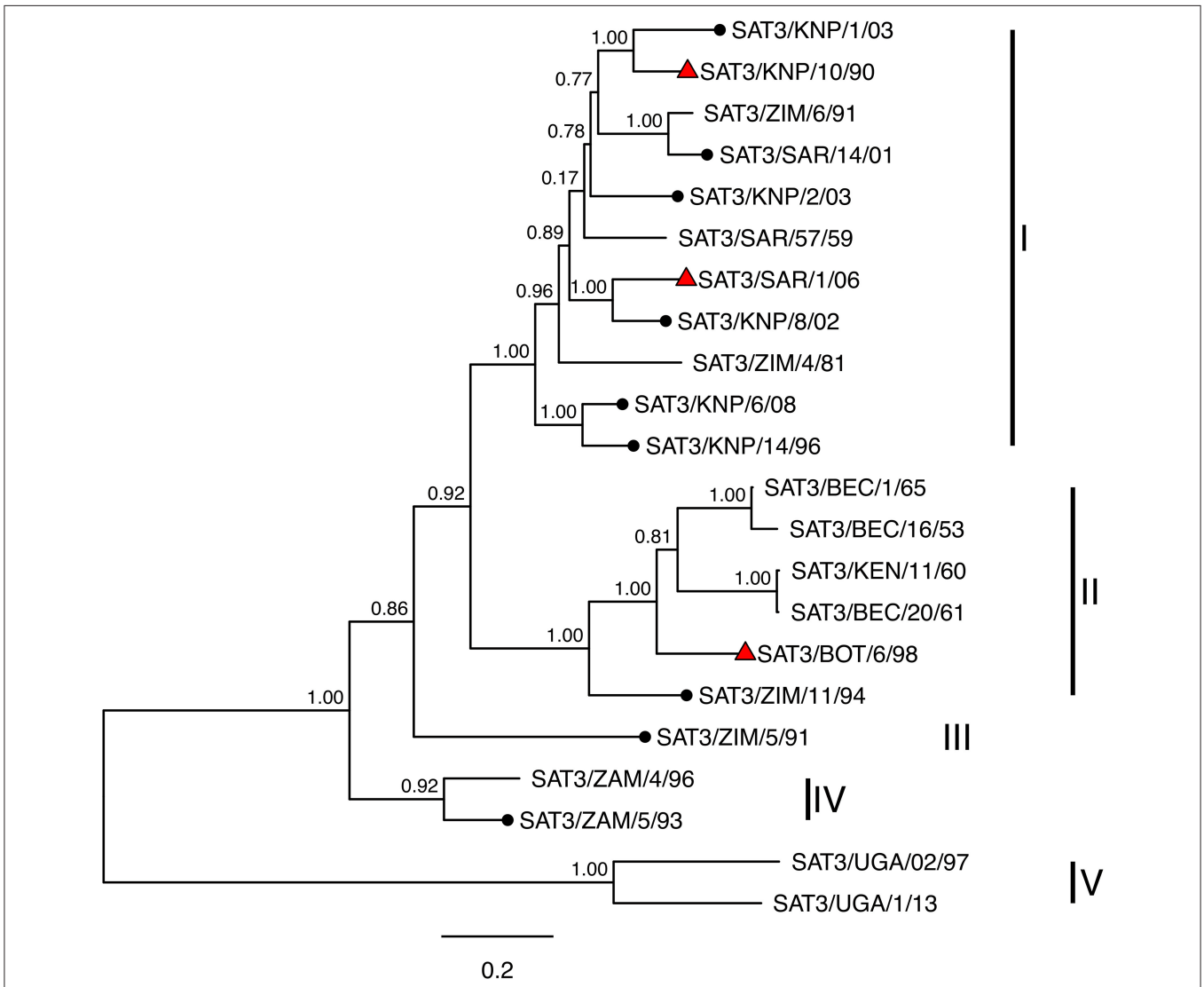

FIGURE 2 | Maximum likelihood phylogenetic tree constructed from aligned capsid (P1) nucleotide sequences, with mid-point root. Clades of the phylogeny corresponding to topotypes are labeled. Viruses tested as antigen in virus neutralization (VN) assays are marked with black circles and reference viruses also used to generate antiserum for VN assays are marked with red triangles. Internal nodes are labeled with bootstrap values and branch lengths indicate the estimated number of nucleotide substitutions per site.

virus tended to correlate with lower VN titers. The model also accounted for other sources of variation in measured VN titers (**Supplementary Figure 1**). When compared with the mean titers recorded for each virus and reference virus combination, the mean difference to these for individual recorded titers was 0.25 $\log_{10}$ titer (maximum 0.89). Some of the variation in recorded

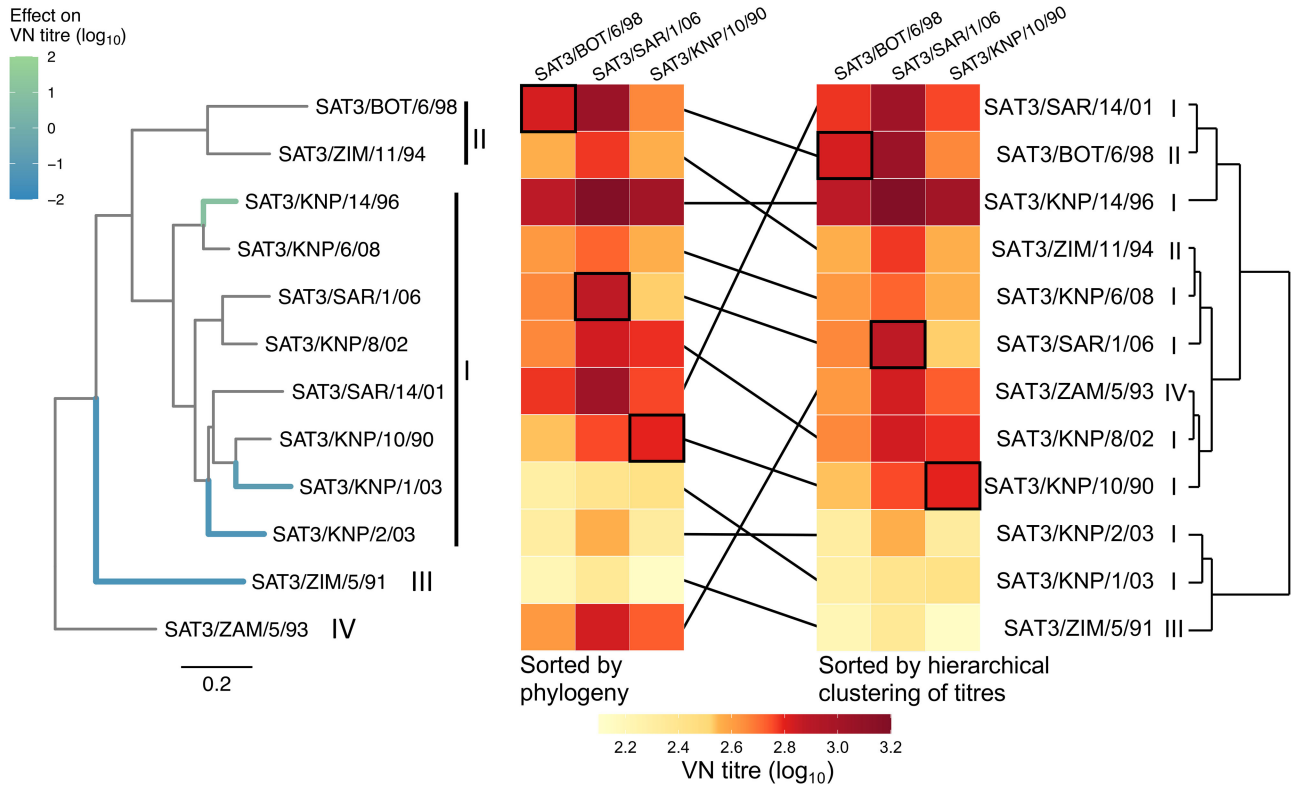

FIGURE 3 | Heatmaps of virus neutralization titers (VN) ordered by phylogeny and by hierarchical clustering. The two heatmaps show the same VN titers (log10) for 12 viruses (rows) and reference antisera (columns) raised to reference viruses SAT3/BOT/6/98, SAT3/SAR/1/06, and SAT3/KNP/10/90. To the left, rows of the heatmap are ordered according to the phylogenetic tree constructed from capsid nucleotide sequences (far left). To the right, rows of the heatmap are ordered according to a hierarchical clustering algorithm applied to VN titers—a dendogram generated by this algorithm is shown (far right). In both heatmaps, black framing is used to highlight homologous titers. Highlighted branches of the phylogeny were associated with variation in VN titers using a sparse hierarchical Bayesian model. Branch color indicates the average effect on titers: green indicates a branch where amino acid substitutions tended to lead to an increase in the VN titer to the three reference sera pools, while blue branches correlated with decreases in VN titers. No internal branches of the phylogeny tended to correlate with variation in VN titers. Topotype nomenclature appears next to clades of the phylogeny and alongside each virus name associated with the hierarchical clustering dendogram.

titers was attributed to day-to-day variability in the assay (**Supplementary Figure 1B**). The average residual difference between measured and fitted titers, after accounting for day-to-day variability was reduced to 0.15 $\log_{10}$ titer (maximum 0.83).

Variable representing amino acid substitutions were tested alongside terms representing branches of the phylogeny that could also identify branches leading to individual viruses or groups of viruses that tended to have higher VN titers, perhaps as a result of differences in avidity for the cellular receptor. Four well-supported branches, to which variation in VN titers mapped, are shown in the phylogenetic tree in **Figure 3**. Each branch effect is caused by the combined effect of one or more residue changes that significantly affect cross-reactivity between reference and test viruses. Each of the four identified branches were terminal branches leading to a single virus, three correlated with low VN titers and one branch with higher VN titers. The terminal branch for SAT3/KNP/14/96 significantly accounts (inclusion probability = 0.97) for an increase in antigenic cross-reactivity to all three reference sera pools. The increase in cross-reactivity reflected as high VN titers regardless of antisera used and was not due to a higher virus titer (4.7 ± 0.2 $\log_{10}$/ml) or increased avidity (AI = 13.95). Three branches in the phylogenetic tree significantly accounted for a reduction in antigenic cross-reactivity against all three reference sera pools. These branches could indicate that the viruses are antigenically distinct, or that they have low VN titers as a result of increased avidity for the cellular receptor. One highlighted branch caused a partitioning of a single topotype (III) from the rest of the tree (SAT3/ZIM/5/91). The virus SAT3/KNP/1/03, although genetically similar to SAT3/KNP/10/90 (38 aa differences in the capsid proteins), is antigenically distinct from the SAT3/KNP/10/90 and SAT3/SAR/1/06 reference viruses (r_1-value <0.3). Similarly, the separation of SAT3/KNP/2/03 from the other topotype I viruses was associated with a decrease in cross-reactivity to the reference sera pools. From our data, the topotype IV virus SAT3/ZAM/5/93 does not seem to be antigenically different from the topotype I and II viruses.

The three branches in the phylogeny in **Figure 3** identified as correlating with reduced VN titers lead to single viruses that

TABLE 4 | Amino acid positions in the SAT3 capsid proteins with substitutions explaining a decrease in the VN titers.

Capsid protein and amino acid position in the SAT3 alignment*	Serotype(s) where residue is antigenic	Antigenically distinct amino acids	Inclusion probability	Impact of substitutions on cross-reactivity (log_{10} VN titer)
VP1 83/VP1 164	None/O^{3}	L-Q, C-R	0.88	−1.2
VP2 134/VP3 168	SAT2, $O^{1,2}$/None	K-Q-T, F-Y	0.42	−0.6
VP1 201		T-V-A-R	0.32	−0.22
VP2 209		Y-F-H	0.30	−0.37

Substitutions at each amino acid position (or combination of positions sharing the same pattern of substitution across viruses in the VN dataset) was tested in a Bayesian model with an indicator variable determining inclusion (1) or exclusion (0) from the model and a coefficient or effect size. The inclusion probability represents the posterior mean value of the indicator variable and the level of support. Model fitting indicated inclusion probabilities above 0.25 have been reasonably well-supported. For each position(s), the conditional effect size is the estimated average impact on VN titers when substitution(s) between test and reference viruses are present.

**Where more than one amino acid appears in a row, this indicates the pattern of substitution at these residues to be identical in the dataset.*

are potentially antigenically distinct due to amino acid residue substitutions in the capsid protein. Amino acid substitutions mapping to each of these branches were identified. The branch leading to the virus SAT3/ZIM/5/91 correlated with 13 residue substitutions in the outer capsid proteins, while the branch leading to SAT3/KNP/1/03 correlated with substitutions at five residue positions, therefore there were several candidate substitutions in these two instances. Only two amino acid substitutions, VP1 L83Q and C164R, mapped to the terminal branch separating SAT3/KNP/2/03 from the rest of the tree; in fact, VP1 83L and 164C are conserved across each of the other 11 viruses in the dataset. Therefore, the substitutions VP1 L83Q and C164R are plausible candidates for causing a reduction in antigenic cross-reactivity. Of these two residues, VP1 164 aligns to a residue that is part of a known epitope in serotype O (17).

Across the phylogeny, three other terms representing amino acid substitutions were identified as correlating with reduced VN titers (**Table 4**) (model selection indicated terms with posterior inclusion probability >0.25 to have a reasonable level of support). The first of these terms with greatest support (inclusion probability = 0.88) represented simultaneous substitutions at VP2 residue 134 [Lys (10), Gln (1), Thr (1)] and VP3 168 [Phe (10), Tyr (2)], which only substituted together in this dataset and therefore could not be distinguished. The positions in the phylogeny where these residues were both substituted were terminal branches leading to viruses SAT3/KNP/1/03 (VP2 K134Q and VP3 F168Y) and SAT3/ZIM/5/91 (VP2 K134Q and VP3 F168Y), both of which had low titers against each of the three antisera used. Of these two residues, the VP2 residue 134 has been identified as being part of an epitope for serotype O and SAT2 viruses (17, 29, 40). Finally, genetic terms associated with VP1 residue 201 [Thr (8), Val (2), Ala (1), Arg (1)], which forms part of the VP1 C-terminus, and VP2 residue 209 [Tyr (9), Phe (2), His (1)] were also identified as potentially antigenically important with substitution, though with reduced support. The location of the latter six residues can be resolved on the predicted structure of serotype SAT3 capsid and is shown in **Figure 4**.

DISCUSSION

The present study confirms the close antigenic relationship between SAT3 viruses in southern Africa using *in vitro* cross-reactivity studies. We then used the antigenic variability data and corresponding genetic and structural data to predict naturally occurring amino acid positions that correlated with antigenic changes. Knowledge of the molecular mechanisms of antigenic evolution are essential to implement systematic approaches to predict protection offered by reference vaccine viruses during prophylactic vaccination in endemic regions or emergency vaccination during an outbreak.

Of the three SAT serotype FMD viruses that occur in southern Africa, SAT3 has the most restricted distribution and outbreaks in livestock are only observed sporadically every 8–15 years (12). A comparison of the genetic diversity within the VP1 coding region of SAT3 viruses, collected between 1965 and 1999 in southern Africa and South-western Uganda, divided the SAT3 viruses into six topotypes (12). Findings from our study, using the complete P1 capsid-coding sequences of SAT3 viruses recovered between 1990 and 2008, substantiated the topotype definitions for SAT3 viruses in southern Africa. Viruses recovered from buffalo in the Kruger National Park, South Africa, and southern Zimbabwe clustered together based on the capsid-coding sequences. Topotype I lineage viruses in South Africa are maintained in buffalo from the Kruger National Park with an incursion every 8–15 years to cattle neighboring this endemic area. Topotype II viruses include viruses from Botswana and western Zimbabwe, while virus isolates from Zambia clustered separately in the phylogenetic tree, defined as topotype IV. The single isolate from northern Zimbabwe was genetically distinct and correlated to topotype III, as described by Bastos et al. (12).

The *in vitro* cross-reactivity analysis of SAT3 viruses was notably high, i.e., 67, 92, and 100% of the SAT3 viruses reacted strongly (r_1-values ≥0.3) to the SAT3/KNP/10/90, SAT3/SAR/1/06, and SAT3/BOT/6/98 reference antisera. The implication is that, in a case of a cattle outbreak, vaccines

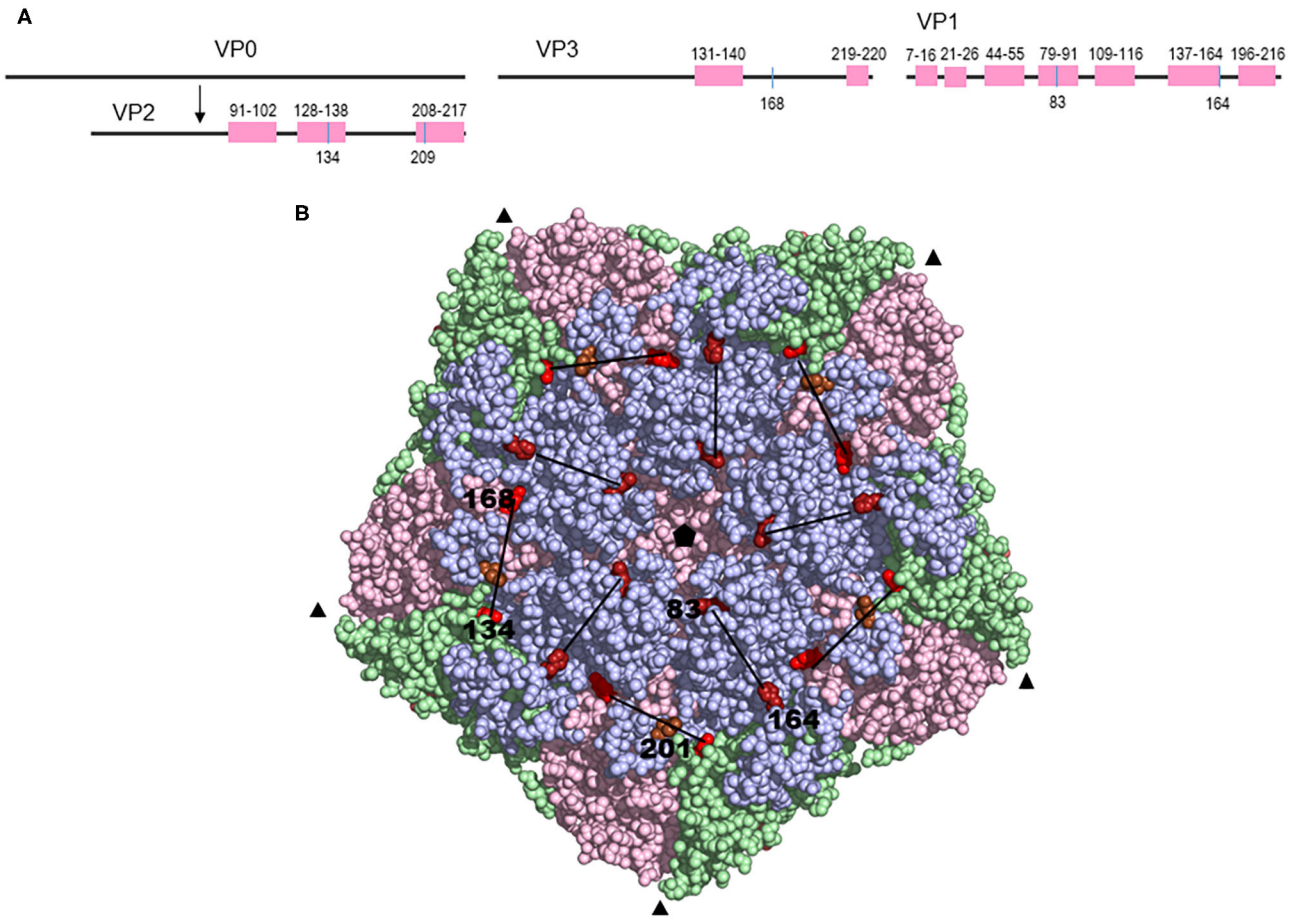

FIGURE 4 | (A) Schematic diagram of the capsid proteins showing the amino acid variation in a complete alignment and the relative positions of amino acid substitutions explaining a decrease in the VN titers. **(B)** A model of the FMDV SAT3 pentamer showing the amino acid residues that correspond to branch formation in a phylogenetic tree and a decline in cross-reactivity in VNTs. The inferred 3-D structure were generated using the SAT1 virus (protein data bank ID: 2WZR) as a template and the structural model rendered by Pymol v 1.8 (DeLano Scientific LLC). The capsid proteins VP1, VP2, and VP3 are shown in blue, green, and pink, respectively, while the exposed amino acid variants are indicated by red spheres. The 3-fold axis is depicted by the black triangles. The pore, located at the 5-fold axis of the capsid (black pentagon), is shown in the middle of the structure. The black lines connect the residues that changed simultaneously.

consisting of any one of the three reference viruses will provide sufficient protection. Moreover, some of the SAT3 viruses reacted stronger to the reference sera than with the homologous virus (r_1-value >1). Particularly, the SAT3/SAR/1/06 and SAT3/BOT/6/98 antisera were highly cross-reactive to the test viruses as indicated by r_1-values >1. Similar results where heterologous cross-reactivity was higher than homologous reactivity have been documented with serotype A FMDV (17, 41). These findings indicate (i) similarities in shared epitopes between the reference and the field viruses, (ii) the reference viruses elicited broadly reactive antibodies in cattle, or (iii) antibodies with high avidity to SAT3 viruses were present. In an attempt to further investigate factors influencing this cross-reactivity, an avidity ELISA was performed to assess and characterize this high heterologous cross-reactivity (32, 42). An increased avidity of SAT3/BOT/6/98 antisera in binding to heterologous viruses (i.e., SAT3/KNP/10/90 and SAT3/SAR/14/01) could explain the higher neutralization titers observed for these viruses. Although higher avidity indexes have been linked to high neutralization titer, this is not always the case. Other factors, such as antibody class or IgG isotype, may also play a role.

The high amino acid variation of the VP1 protein (45% variable residue positions), compared to the other capsid proteins, indicates that VP1 is likely to be under immunologic pressure. Genetic changes and selection of antigenic variants are generally accepted to occur in persistently infected wildlife (8, 43, 44). The majority of variable residues are limited to particular surface-exposed structural loops and changes elsewhere may be under stringent structural and selective constraints (45). The fact that most of the SAT3 capsid amino acid positions with high entropy were identified in the VP1 protein emphasizes that this protein has a major immunogenic role and it also modulates the antigenic variability of the virus. Previous crystallographic studies and structure-based epitope predictions revealed that

VP1 is important to interact with antibodies, especially the βG-βH loop and residues toward the 5-fold axis of the capsid (46, 47). The immunological role of an additional K residue within the βD-βE loop of the VP1 protein of one isolate is unknown. Although less variation was identified in the VP2 and VP3 proteins, these proteins still play an important role in antigenic variation of FMDV. A conformational epitope comprising of residues from the VP2 and VP3 capsid proteins and spanning the 3-fold axis, was also present (47–49). This emphasizes that cross-reactivity is influenced by main, variable capsid amino acid residues and may be affected more by residue interactions rather than residue changes (48, 50–52).

We identified substitutions with a profound effect on antigenic variation that were likely associated with immune evasion. Variation at two residue positions in the VP1 protein, residues 83 and 169, were associated with reduced titers against SAT3/KNP/10/90 and SAT3/SAR/1/06 antisera. The VP1 residues 83 and 169 are located at opposite sides of an elevated plateau on the capsid surface, with residue 83 forming an exposed cluster around the 5-fold axis and residue 169 located at the C-terminal base of the VP1 βG-βH loop. The VP1 residue 83 of SAT2 viruses has been found to be accessible to interact with glycosaminoglycan (18), confirming its accessibility to interact with cellular receptors. Similarly, residues 134 in VP2 and 168 in VP3 together, were associated with an antigenic effect for SAT3/KNP/1/03 and SAT3/ZIM/5/91. It is reasonable to hypothesize that the two residues together function as a conformational epitope, however, the same variation in VN titer is equally well-explained by VP2 K134Q/T substitution. The VP2 residue 134 has been described as an antigenic site for serotypes O and SAT2, and is located on a surface exposed structural loop and is structurally more favorable to contribute to variation in antigenicity (48, 49). Residue 168 in VP3 has not been described to play a role in antigenicity before. Both residues are located in a shallow, structural depression, located at the junction between the three major capsid proteins VP2, VP3, and VP1 (**Figure 4**). Two other residues have also been associated with antigenic variation in SAT3 viruses, one in the C-terminal end of VP2 and the other located on the C-terminus of VP1. Only the VP1 C-terminus residue corresponds to a described antigenic site in serotype O (40, 48).

Amino acids that are important for the antigenicity of SAT1 viruses have been identified at positions 135 or 71 or 76 of VP3; 72 of VP2 and 181 of VP1; and 111 of VP1 using MAb resistant (mar) mutants (49). Similarly, residues 72 or 79 of VP2; 158 of VP1; and 154 or 158 in the βG-βH loop of VP1 of SAT2 viruses have been shown to interact with MAbs or affect the antigenicity of the virus (29, 49). At least five neutralizing antigenic sites, involving the three outer-capsid proteins, have been identified for serotype O viruses. The most prominent surface exposed structure, the βG-βH loop of VP1, and the C-terminus of VP1 have been shown to contribute to antigenic site 1 of serotype O viruses, with critical residues at position 144, 148, 154, and 208 (53–56). Amino acid residues at positions 70–73, 75, 77, and 131 of VP2, 56 and 58 of VP3, and 43 and 44 of VP1 contributes to the remaining antigenic site for serotype O (56). Mar-mutants identified three antigenic sites within the VP1, VP2, and VP3 proteins for serotype A viruses A10, A12, and A22 with residue positions 148, 149, 152, 153, 168, and 205 within VP1 important for antigenicity (57–60). Here, for the first time, we have mapped four unique amino acid regions associated with antigenic changes in SAT3 viruses. In two of these regions two amino acid residues changed together to affect the antigenicity of the virus, i.e., residues 83 and 169 of VP1 and residues 134 in VP2 and 168 in VP3.

We have successfully used phenotypic data, combined with genotypic and structural information in our mathematical models to delineate antigenic sites for SAT3 viruses. The analysis of antigenic differences in outbreak viruses is critical to allow proper vaccine selection for effective control or the design of vaccine antigens tailored for specific geographic localities, using reverse genetics. This work could be further validated using a reverse genetics approach to immune-dampen specific residues to identify its antigenic significance. We anticipate that identifying unique residues associated with a change in cross-reactivity will contribute to improved vaccine development and assessment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Agricultural Research Council, Onderstepoort Veterinary Research, Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

WH, JT, RR, and FM conceived and designed the experiments. LM, WH, LR, and PO performed the experiments. RR and FM contributed the materials/analysis tools. LM, WH, LR, PO, JT, RR, and FM wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00568/full#supplementary-material

Supplementary Figure 1 | Variability in virus neutralization titers (log_{10}) for 12 viruses tested using antisera raised to reference strains SAT3/BOT/6/98, SAT3/SAR/1/06, and SAT3/KNP/10/90 ($N = 198$). **(A)** Histogram of absolute differences in measured titers and the mean log_{10} titer recorded for each virus and reference strain combination. **(B)** Violin plot showing posterior model estimates of the variation in VN titers that can be attributed to variability between experiments carried out on 15 different days. Each violin represents 1,600 values sampled from eight independent MCMC chains. Black horizontal lines represent median values. **(C)** Histogram showing residuals from a model fitted to VN titers—each residual is the absolute difference between a measured titer adjusted for day-to-day variation and the fitted, underlying titer for the particular virus and reference strain combination. **(D)** Scatterplot showing measured VN titers (black circles) and those same VN titers adjusted to account for day-to-day variability (red crosses) plotted against the fitted, underlying titer for a particular virus and reference strain combination.

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A Meta-Population Model of Potential Foot-and-Mouth Disease Transmission, Clinical Manifestation, and Detection Within U.S. Beef Feedlots

Aurelio H. Cabezas[1,2], *Michael W. Sanderson*[1,2]* *and Victoriya V. Volkova*[1,2]*†

[1] Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, United States, [2] Center for Outcomes Research and Epidemiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, United States

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Cyril Gerard Gay,
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United States Department of Agriculture, United States

***Correspondence:**
Victoriya V. Volkova
vv88@vet.k-state.edu
Michael W. Sanderson
sandersn@vet.k-state.edu

†Present address:
Victoriya V. Volkova,
National Institutes of Health, Bethesda, MD, United States

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Foot-and-mouth disease (FMD) has not been reported in the U.S. since 1929. Recent outbreaks in previously FMD-free countries raise concerns about potential FMD introductions in the U.S. Mathematical modeling is the only tool for simulating infectious disease outbreaks in non-endemic territories. In the majority of prior studies, FMD virus (FMDv) transmission on-farm was modeled assuming homogenous animal mixing. This assumption is implausible for U.S. beef feedlots which are divided into multiple home-pens without contact between home-pens except fence line with contiguous home-pens and limited mixing in hospital pens. To project FMDv transmission and clinical manifestation in a feedlot, we developed a meta-population stochastic model reflecting the contact structure. Within a home-pen, the dynamics were represented assuming homogenous animal mixing by a modified SLIR (susceptible-latent-infectious-recovered) model with four additional compartments tracing cattle with subclinical or clinical FMD and infectious status. Virus transmission among home-pens occurred via cattle mixing in hospital-pen(s), cowboy pen rider movements between home-pens, airborne, and for contiguous home-pens fence-line and via shared water-troughs. We modeled feedlots with a one-time capacity of 4,000 (small), 12,000 (medium), and 24,000 (large) cattle. Common cattle demographics, feedlot layout, endemic infectious and non-infectious disease occurrence, and production management were reflected. Projected FMD-outbreak duration on a feedlot ranged from 49 to 82 days. Outbreak peak day (with maximum number of FMD clinical cattle) ranged from 24 (small) to 49 (large feedlot). Detection day was 4–12 post-FMD-introduction with projected 28, 9, or 4% of cattle already infected in a small, medium, or large feedlot, respectively. Depletion of susceptible cattle in a feedlot occurred by day 23–51 post-FMD-introduction. Parameter-value sensitivity analyses were performed for model outputs. Detection occurred sooner if there was a higher initial proportion of latent animals in the index home-pen. Shorter outbreaks were associated with a shorter latent period and higher bovine respiratory disease

morbidity (impacting the in-hospital-pen cattle mixing occurrence). This first model of potential FMD dynamics on U.S. beef feedlots shows the importance of capturing within-feedlot cattle contact structure for projecting infectious disease dynamics. Our model provides a tool for evaluating FMD outbreak control strategies.

Keywords: mathematical modeling, foot-and-mouth disease, transmission dynamics, meta-population, environmental transmission, waterborne transmission, beef feedlot, infectious disease dynamics

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease affecting livestock and over a hundred wildlife species (1). Foot-and-mouth disease virus (FMDv) is of the genus *Apthovirus*, family *Picornaviridae*. There are seven antigenically distinct FMDv serotypes: A, O, C, SAT-1, SAT-2, SAT-3, and ASIA-1. Serotypes O and A are most widely distributed world-wide according to a recent review (2). In the Americas, major FMD outbreaks have not occurred since an outbreak in Paraguay in 2012, in which FMDv strains of serotype O predominated. An on-going program is aimed at eradicating FMD in South America by 2020 (3). The disease has not been reported in the U.S. since 1929 when southern California was affected (4). The last outbreak in North America occurred in 1952 in Saskatchewan, Canada (5). Economic impacts of an FMD outbreak in disease-free countries can be devastating due to export bans for susceptible animal species and their products, disease-associated animal losses, and outbreak control expenses. For example, the FMD outbreak in United Kingdom in 2001 resulted in the estimated overall costs over £8 billion ($15 billion) (6).

The U.S. beef industry is one of the largest in the world with over 30,000 feedlots, primarily concentrated in the Central U.S. (7). Almost 50% of the national fed cattle inventory are in large commercial feedlots, each with the on-time capacity $\geq$24,000 head of cattle. Approximately 1,160 million kilograms of beef are exported by the U.S. producers each year (8). Response by the world animal-health community to an FMD outbreak in the U.S. would likely involve a ban on beef exports. Schroeder et al. (9) estimate that an FMD outbreak in the U.S. could result in $188 billion overall costs without emergency vaccination and $56 billion with high-capacity emergency vaccination in the Midwest. Pendell et al. (10) estimated $16–140 billion costs for an outbreak if FMDv would be released from a high-security laboratory facility in the Midwest. Others estimated a decrease in farm income of $14 billion, ∼6% of the national gross farm income, in the U.S. for an outbreak assuming the outbreak characteristics were similar to the UK 2001 outbreak (11).

For long-term FMD-free countries, such as the U.S., mathematical modeling is the only tool for projecting dynamics of a potential FMD outbreak and evaluating control strategies. Previous modeling studies of FMDv transmission and control in the U.S. focused on projecting the impact on the outbreak of the virus transmission dynamics between farms. In the models, individual farms were considered as FMD positive or negative (12–17). A similar assumption has been made in models of FMD outbreaks in territories other than the U.S. (18–25).

In a U.S. beef cattle feedlot, the cattle are compartmentalized in multiple home-pens (e.g., 200 head per home-pen). The home-pen subpopulations contact via multiple routes conducive to contagious agent transmission, forming the meta-population of cattle in the feedlot. There is a multi-route, complex, and heterogeneous in time and space contact structure among the home-pen subpopulations. The relevant contact routes include mixing of some cattle from different home-pens during short stays in hospital-pens, fence-line contact for contiguous home-pens, waterborne contact for contiguous home-pens sharing water-troughs, environmental due to the care-givers moving between the home-pens located in the same home-pen row (the rows are separated by feed-delivery alleys and drover alleys), and airborne across the feedlot. Thus, an assumption of a contagious virus transmission via an instantaneous and homogeneous mixing of all cattle present on a feedlot is implausible. Projecting the transmission among the home-pen subpopulations necessitates a more explicit model of the contact structure. Reflecting the meta-population contact structure when modeling infectious agent transmission is necessary because the agent temporal dynamics and likelihood of persistence in a meta-population are different from in a homogenously mixing population (26–28). Three teams have modeled within-farm FMDv transmission in cattle (14, 29, 30). However, the animal contact structure, demographics, and production management represented were dissimilar to those in U.S. beef feedlots. One study (31) modeled within-farm FMDv transmission in swine. Models of potential FMDv transmission dynamics in the cattle meta-populations on U.S. beef feedlots have not been reported.

The aim of this study was to develop a mathematical model of potential FMDv transmission, infection, and FMD clinical manifestation dynamics in U.S. beef feedlots, reflecting the animal meta-population contact structure, animal demographics, and contemporary production management. The model was developed as a stochastic meta-population model. In the model, FMDv transmission within a home-pen occurred via homogenous cattle mixing. Relevant contacts among the home-pen subpopulations occurred via cattle mixing in hospital-pen(s), and through fence-lines, shared water-troughs, environment due to care-giver movements between the home-pens in a row, and airborne. The model reflected commercial U.S. beef cattle feedlot demographics and production management, including the incidence and control approaches to endemic infectious diseases and non-infectious diseases. We used the model to project FMDv infection dynamics and clinical manifestation in the absence of control measures on feedlots of several sizes and layouts typical for the U.S. We analyzed the model outputs to describe the projected outbreak characteristics. To our knowledge, this is the first model of potential FMD dynamics on commercial U.S. beef feedlots.

MATERIALS AND METHODS

Host Population and Feedlot Size and Layout Cases Modeled

The model reflected the following assumptions. Beef finishing cattle in an open-air feedlot was the target population. No other FMD-susceptible animal species were included on the feedlot or in the surroundings. The cattle were not vaccinated against FMD. Cattle were housed 200 per home-pen, with 22 m^2 floor space per animal. Cattle morbidity due to production diseases including endemic infectious diseases and non-infectious diseases, e.g., bovine respiratory disease (BRD) and lameness, determined the rate of pulling cattle from the home-pens to hospital-pen(s). Cattle mortality rates due to the production diseases and clinical FMD were incorporated. The model parameter definitions and values are listed in **Table 1**. We simulated the feedlot cattle meta-population as closed, with no cattle introduced or leaving the feedlot after FMD latent animals were introduced in the index home-pen. Five hypothetical feedlot size and layout cases were modeled: a small-size feedlot with 4,000 cattle in 20 home-pens in four rows and one hospital-pen (FS1); a medium-size feedlot with 12,000 cattle in 60 home-pens in eight rows and one hospital-pen (FM1); a medium-size feedlot with 12,000 cattle in 60 home-pens in eight rows and two hospital-pens, (FM2); a large-size feedlot with 24,000 cattle in 120 home-pens and two hospital-pens, the feedlot includes two sections each with eight home-pen rows and one hospital-pen (FL1); and a large-size feedlot with 24,000 cattle in 120 home-pens and four hospital-pens, the feedlot includes two sections each with eight home-pen rows and two hospital-pens (FL2). See **Figure 1** for a schematic diagram of the model. The feedlot layouts are detailed in **Supplementary Figure 1**.

Model Formulation

Two levels of FMDv transmission inside the feedlot cattle meta-population were modeled: within each home-pen (1 route of transmission: direct cattle contact) and between home-pens (5 routes of transmission detailed below).

FMD Infection and Clinical Manifestation Dynamics in a Home-Pen

The FMD infection and clinical disease dynamics in each home-pen were modeled using a modified SLIR (susceptible-latent-infectious-recovered) model. The model was modified to add four compartments for tracing the numbers of cattle that were subclinical infectious 1 (I_1 animals), subclinical infectious 2 (I_2 animals), clinical infectious (I_3 animals), and clinical non-infectious (C). The two subclinical categories were of equal duration and infectiousness but were included to allow for future parameterization of variability in infectiousness between stages. A schematic of the infection and clinical disease progression stages in individual cattle and how those were reflected in the model compartments is provided in **Figure 2**. Cattle started in the susceptible compartment (S) (Equation 1). Susceptible cattle were infected via direct contact with infectious home-pen-mates at a rate reflecting homogenous cattle mixing and density-dependent transmission within the home-pen (the transmission parameter β_{wp}, Equation 1) or due to between-home pen FMDv transmission (detailed below) and moved into the latent compartment (L) (Equations 1, 2). The cattle then moved into a subclinical infectious compartment (I_1) at a rate $1/\delta$ (Equations 2, 3), proceeded into a subclinical infectious compartment (I_2) at a rate $1/\theta$ (Equations 3, 4), then into a clinical infectious compartment (I_3) at a rate $1/\varepsilon$ (Equations 4, 5), and then into a clinical non-infectious compartment (C) at a rate $1/\gamma$ (Equations 5, 6) where they were still manifesting clinical disease but no longer shed the virus. Finally, the cattle proceeded into a non-clinical non-infectious recovered compartment (R) at a rate $1/\tau$ (Equations 6, 7). Cattle mortality (i.e., culling) due to endemic infectious diseases and non-infectious diseases occurred at a rate μ in all the compartments (Equations 1–7). Cattle mortality (i.e., culling) due to clinical FMD in the compartments I_3 and C occurred at a rate ψ (Equations 5, 6). Definitions and values of the model parameters are given in **Table 1**.

The modified SLIR model of FMD infection and clinical manifestation dynamics in cattle in a home-pen on a beef feedlot

The modeled home-pen is denoted *i*. *j* is the contiguous home-pen preceding *i* in the home-pen row. *h* is the contiguous home-pen following *i* in the home-pen row. *k* is any other home-pen than *i*. *n* is the number of home-pens in the feedlot. If the feedlot had more than one hospital-pen, cattle were always pulled to the hospital-pen nearest to their home-pen for either a production disease or clinical FMD treatment. The nearest hospital-pen, or the only hospital-pen if there was one on the feedlot, is denoted *l*. The other parameters are defined in the following sections on the FMDv transmission between home-pens. The time step was 1 day, $dt = 1$ (all the rates in the equations including those with the values sampled from Binomial distributions are daily rates).

Susceptible:

$$\begin{aligned}\frac{dS}{dt} = &-\beta_{wp}S(I_1+I_2+I_3) - \varphi S - Bin\left(\varphi_{(t-1)}S_{(t-1)},\ p\_inf\_hp_{l_{(t-1)}}\right) - \\ &\left\{\begin{matrix}S\beta_{bp}(I_1+I_2+I_3)_j;\ j\text{ present}\\ 0;\text{ otherwise}\end{matrix}\right\} - \left\{\begin{matrix}S\beta_{bp}(I_1+I_2+I_3)_h;\ h\text{ present}\\ 0;\text{ otherwise}\end{matrix}\right\} - \\ &\left\{\begin{matrix}Bin(S,\ 0.5);\ j\text{ present, shares water-trough with }i\text{, and FMDv load in 1 L of the water }\geq ID_{50}\text{ per oral}\\ 0;\text{ otherwise}\end{matrix}\right\} - \\ &\left\{\begin{matrix}Bin(S,\ 0.5);\ h\text{ present, shares water-trough with }i\text{, and FMDv load in 1 L of the water }\geq ID_{50}\text{ per oral}\\ 0;\text{ otherwise}\end{matrix}\right\} - \\ &\left\{\begin{matrix}Bin\left[\left(\frac{FMDv\_floor_j\times\sigma}{ID_{50}\text{ per oral}}\right),\ 0.5\right];\ j\text{ present and }\left(\frac{FMDv\_floor_j\times\sigma}{ID_{50}\text{ per oral}}\right)\leq S\\ 0;\text{ otherwise}\end{matrix}\right\} - \\ &\left\{\begin{matrix}Bin(S,\ p\_air_i);\ \sum_{k=1}^{n} I_3 \geq 0\\ 0;\text{ otherwise}\end{matrix}\right\} - \mu S\end{aligned} \quad (1)$$

TABLE 1 | Definitions and values of parameters used in modeling potential foot-and-mouth disease transmission, infection, and clinical manifestation dynamics on U.S. beef cattle feedlots.

Parameter	Definition (units)	Mean value and distribution	References[a]
WITHIN A HOME-PEN			
lat_initial	Initial proportion of latent cattle in the index-pen	0.05, Vector (0.005, 0.105, 0.020)	Assumed
β_{wp}	Beta transmission parameter for virus transmission via direct animal contact in a home-pen (animal^{-1} day^{-1})	0.026, Triangular (0.020, 0.026, 0.031)	Derived from Chis Ster et al. (30)
lat	Duration of latent period (days)	3.2, Weibull (α 1.782, β 3.974)	(32)
sub	Duration of subclinical period (days)	2.0, Gamma (α 1.222, β 1.672)	(32)
inf	Duration of infectious period (days)	4.0, Gamma (α 3.969, β 1.107)	(32)
cli	Duration of clinical period (days)	7.5, Fixed	(33)
cliinf	Duration of clinical infectious period (days)	(inf-sub) in each model simulation	
clinon_inf	Duration of clinical non-infectious period (days)	(cli-clininf) in each model simulation	
δ	Rate of progression to subclinical infectious 1 status (day^{-1})	1/lat	
θ	Rate of progression to subclinical infectious 2 status (day^{-1})	1/(sub/2)	
ε	Rate of progression to clinical infectious status (day^{-1})	1/(sub/2)	
γ	Rate of recovery from being infectious (day^{-1})	1/cliinf	
τ	Rate of recovery from clinical disease after recovering from being infectious (day^{-1})	1/clinon_inf	
υ	Proportion of home-pens with cattle just placed in the feedlot (dmnl)	0.20	Feedlot expert opinion
π	Morbidity rate for bovine respiratory disease (BRD) during the first 30 days since cattle placement in the feedlot	0.162, Vector (0.050, 0.300, 0.050)	(34)
ρ	Morbidity rate for other production diseases during the 200 days since cattle placement in the feedlot	0.1280, fixed	(34)
brdtrt	Probability for an animal with BRD to be pulled to a hospital-pen for treatment during the disease course (dmnl)	0.8750, fixed	(34)
endtrt	Probability for an animal with other than BRD production diseases to be pulled to a hospital-pen for treatment during the disease course (dmnl)	0.6908, fixed	(34)
$\varphi_{t=1 \text{ to } 30}$	Per-animal pull rate from a home-pen to hospital-pen due to BRD and other production diseases during the first 30 days since cattle placement in the feedlot (day^{-1})	0.0052	Calculated, $\left(\frac{\pi * brdtrt}{30}\right) + \left(\frac{\rho * endtrt}{200}\right)$
$\varphi_{t=31 \text{ to } 200}$	Per-animal pull rate from a home-pen to hospital-pen due to production diseases between the days 31 and 200 since cattle placement in the feedlot (day^{-1})	0.0004	Calculated, $\frac{\rho * endtrt}{200}$
ς	Per-animal pull rate from a home-pen to hospital-pen due to clinical FMD (day^{-1})	0.02800	FMD expert opinion
μ	Mortality rate for animals with BRD and other production diseases (endemic infectious diseases and noninfectious diseases) (day^{-1})	Triangular (0.01, 0.03, 0.05)	(34)
ψ	Mortality rate for animals with clinical FMD (day^{-1})	Triangular (0, 0.005, 0.010)	FMD expert opinion
BETWEEN HOME-PENS			
In hospital-pen(s)			
β_{hp}	Beta transmission parameter for virus transmission via direct animal contact in a hospital-pen (animal^{-1} day^{-1})	Same as β_{wp}	Derived from Chis Ster et al. (30)
Fence-line			
β_{bp}	Beta transmission parameter for virus transmission via fence-line direct animal contact (animal^{-1} day^{-1})	β_{wp}/4	Assumed [β_{wp} derived from Chis Ster et al. (30)]
Environmental by pen-riders			
uri	Urine volume produced by an animal (L/day)	Uniform (8.8, 22.0)	(35)
sal	Saliva volume produced by an animal (L/day)	Uniform (98, 190)	(35)
fec	Volume of feces produced by an animal (kg/day)	Uniform (14, 45)	(35)
uriv	Virus quantity shed in urine [plaque forming units (PFU)/mL] by an animal in the FMD clinical high infectious status	Uniform ($10^{2.5}$, $10^{5.5}$)	(35)
salv	Virus quantity shed in saliva (PFU/mL) by an animal in the FMD clinical high infectious status	Uniform (10^{6}, 10^{8})	(35)

(Continued)

TABLE 1 | Continued

Parameter	Definition (units)	Mean value and distribution	References[a]
fecv	Virus quantity shed in feces (PFU/mL) by an animal in the FMD clinical high infectious status	Uniform (10^2, $10^{4.1}$)	(35)
fsal_env	Proportion of the cattle daily saliva volume deposited into the home-pen environment (dmnl)	0.3, Vector (0.1, 0.5, 0.1)	Assumed
fsal_env_floor	Proportion of *fsal* that lands on the floor (dmnl)	0.33	Assumed
vir_dec_env	Virus decay rate in the home-pen floor environment (day^{-1})	0.28, Fixed	(36)
σ	Amount of the home-pen floor materials moved daily to the next home-pen in the row by pen-riders (g/day) (300 g per pen-rider round, two rounds per day)	600, Fixed	Assumed plausible amount carried on horse hooves between pens
w_pen	Width of a home-pen (m)	61.0, Fixed	Typical industry value
l_pen	Length of a home-pen (m)	75.2, Fixed	Typical industry value
d_pen	Depth of a home-pen floor top contaminated with the animal fresh secretions and excretions (m)	0.02, Vector (0.02, 0.05, 0.03)	Expert opinion, typical pen surface loosened by hoof action
min_oral	Minimum infective dose of FMDv via oral exposure in cattle (PFU/mL)	10^6, Fixed	(37)
Via shared water-troughs			
fsal_env_w	Proportion of *fsal* that lands in the water-trough (dmnl)	(1-*fsal_env_floor*)	Assumed
vir_dec_w	Virus decay rate in water (day^{-1})	0.12, Fixed	(36)
vol_watert	Volume of the water trough shared between two home-pens (L)	6,000, Fixed	Expert opinion, typical tank size to provide sufficient water reservoir for cattle needs
min_oral	Minimum infective dose of FMDv via oral exposure in cattle (PFU/mL)	10^6, Fixed	(37)
Airborne			
α	Power of the exponential function of decay in the airborne transmission with increasing distance between home-pen centroids (dmnl)	−3.5, Fixed	(24)
	Proportion of clinical infectious cattle in a home-pen *k*	Modeled	
$d_{i,k}$	Scaled distance between centroids of a home-pen *i* and home-pen *k* (*k* is any other home-pen than *i*) (dmnl)	1.0–22.4, Fixed	Euclidean distance between each two home-pen centroids scaled by the shortest Euclidian distance between two home-pen centroids in the feedlot

[a] *In the reference column: "Assumed" refers to parameter values assigned based on our knowledge/judgement. "Derived from [x]" refers to values that we estimated based on data in the cited references. "[x]" is the reference from which the value was adopted directly. "Expert opinion" refers to values obtained via personal communication with experts in the epidemiology of FMD, and in the feedlot industry.*

dmnl, indicates the value does not have a unit of measure.

PFU, plaque forming units.

Latent:

$$\begin{aligned}\frac{\mathrm{dL}}{\mathrm{dt}} = {} & \beta_{wp}\mathrm{S}(\mathrm{I}_1+\mathrm{I}_2+\mathrm{I}_3) - \varphi\mathrm{L} + Bin\left(\varphi_{(t-1)}\mathrm{S}_{(t-1)},\ p\_\mathrm{inf\_hp}_{l_{(t-1)}}\right) + \\ & \left\{\begin{matrix}\mathrm{S}\beta_{bp}(\mathrm{I}_1+\mathrm{I}_2+\mathrm{I}_3)_j;\ j\text{ present} \\ 0;\text{ otherwise}\end{matrix}\right\} + \left\{\begin{matrix}\mathrm{S}\beta_{bp}(\mathrm{I}_1+\mathrm{I}_2+\mathrm{I}_3)_h;\ h\text{ present} \\ 0;\text{ otherwise}\end{matrix}\right\} + \\ & \left\{\begin{matrix}Bin(\mathrm{S},\ 0.5);\ j\text{ present, shares water-trough with } i\text{, and FMDv load in 1 L of the water } \geq ID_{50}\text{ per oral} \\ 0;\text{ otherwise}\end{matrix}\right\} + \\ & \left\{\begin{matrix}Bin(\mathrm{S},\ 0.5);\ h\text{ present, shares water-trough with } i\text{, and FMDv load in 1 L of the water } \geq ID_{50}\text{ per oral} \\ 0;\text{ otherwise}\end{matrix}\right\} + \\ & \left\{\begin{matrix}Bin\left[\left(\frac{FMDv\_floor_j \times \sigma}{ID_{50}\text{ per oral}}\right),\ 0.5\right];\ j\text{ present and } \left(\frac{FMDv\_floor_j \times \sigma}{ID_{50}\text{ per oral}}\right) \leq \mathrm{S} \\ 0;\text{ otherwise}\end{matrix}\right\} + \\ & \left\{\begin{matrix}Bin(\mathrm{S},\ p\_air_i);\ \sum_{k=1}^{n}\mathrm{I}_3 \geq 0 \\ 0;\text{ otherwise}\end{matrix}\right\} - \delta\mathrm{L} - \mu\mathrm{L}\end{aligned} \tag{2}$$

Subclinical infectious 1:

$$\frac{\mathrm{dI}_1}{\mathrm{dt}} = \delta\mathrm{L} - \theta\mathrm{I}_1 - \varphi\mathrm{I}_1 + \varphi_{(t-1)}\mathrm{I}_{1_{(t-1)}} - \mu\mathrm{I}_1 \tag{3}$$

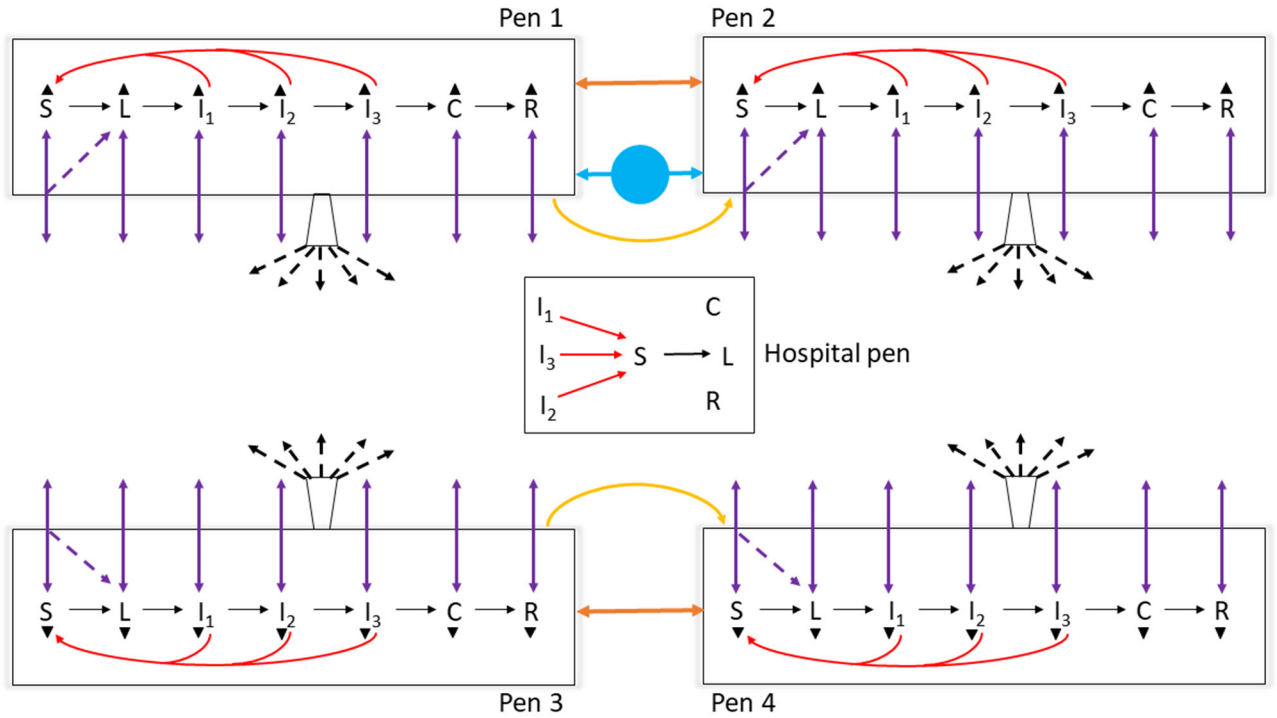

FIGURE 1 | Schematic diagram of the model of foot-and-mouth disease (FMD) virus transmission and FMD clinical manifestation dynamics in cattle within home-pens and among home-pens in a beef cattle feedlot. S, susceptible; L, latent; I_1, subclinical infectious; I_2, subclinical infectious; I_3, clinical infectious; C, clinical but no longer infectious; and R, non-infectious clinically recovered. The black solid arrows show the home-pen subpopulation progression through the infection and disease stages. The red solid arrows show the virus transmission via direct contact between infectious to susceptible cattle in the home-pens and hospital-pen. The purple solid arrows show the animal movements from home-pens to the hospital-pen and back to the home-pens, and the purple dotted arrows show the possibility that susceptible animals moved acquired infection in the hospital-pen and returned as latent to the home-pens. The orange solid arrows show the virus transmission via animal direct contact fence-line. The yellow solid arrows show the virus contaminated material transmitted by pen-riders. The blue circle with solid arrows shows the virus transmission via contaminated water-troughs shared by home-pens. The buckets with black dotted arrows represent the airborne virus transmission. The black triangles represent animal mortality in each of the infection and disease stages.

Subclinical infectious 2:

$$\frac{dI_2}{dt} = \theta I_1 - \varepsilon I_2 - \varphi I_2 + \varphi_{(t-1)} I_{2_{(t-1)}} - \mu I_2 \quad (4)$$

Clinical infectious:

$$\frac{dI_3}{dt} = \varepsilon I_2 - \gamma I_3 - (\varphi + \varsigma) I_3 + (\varphi_{(t-1)} + \varsigma) I_{3_{(t-1)}} - (\mu + \psi) I_3 \quad (5)$$

Clinical non-infectious:

$$\frac{dC}{dt} = \gamma I_3 - \tau C - (\varphi + \varsigma) C + (\varphi_{(t-1)} + \varsigma) C_{(t-1)} - (\mu + \psi) C \quad (6)$$

Recovered:

$$\frac{dR}{dt} = \tau C - \varphi R + \varphi_{(t-1)} R_{(t-1)} - \mu R \quad (7)$$

FMDv Transmission Between Home-Pens

Transmission of FMDv between the home-pen subpopulations occurred via five routes: direct contact of cattle from different home-pens in hospital-pen(s) when they were pulled from the home-pen for treatment in the hospital, fence-line direct contact of cattle from contiguous home-pens, environmental contact through pen-riders moving between home-pens in the same home-pen row (only from a preceding to the next home-pen in the row), waterborne between contiguous home-pens that shared a water-trough, and airborne.

i. *Transmission via direct contact of cattle in hospital-pen(s)*

An S-L (Susceptible-Latent) model was implemented in each hospital-pen *l*. The susceptible and infectious (I_1-I_3) cattle originated from the home-pens when morbid cattle were sent to this hospital-pen. The new latents infected in the hospital-pen and remaining susceptibles (as well as the prior infectious, prior clinical non-infectious, and prior recovered pulled to the hospital-pen) returned to their home-pens the next day (Equations 1–7). Recall that cattle from a home-pen were always pulled to the nearest hospital-pen, except in the FS1 and FM1 feedlots where all cattle were pulled to the single hospital-pen.

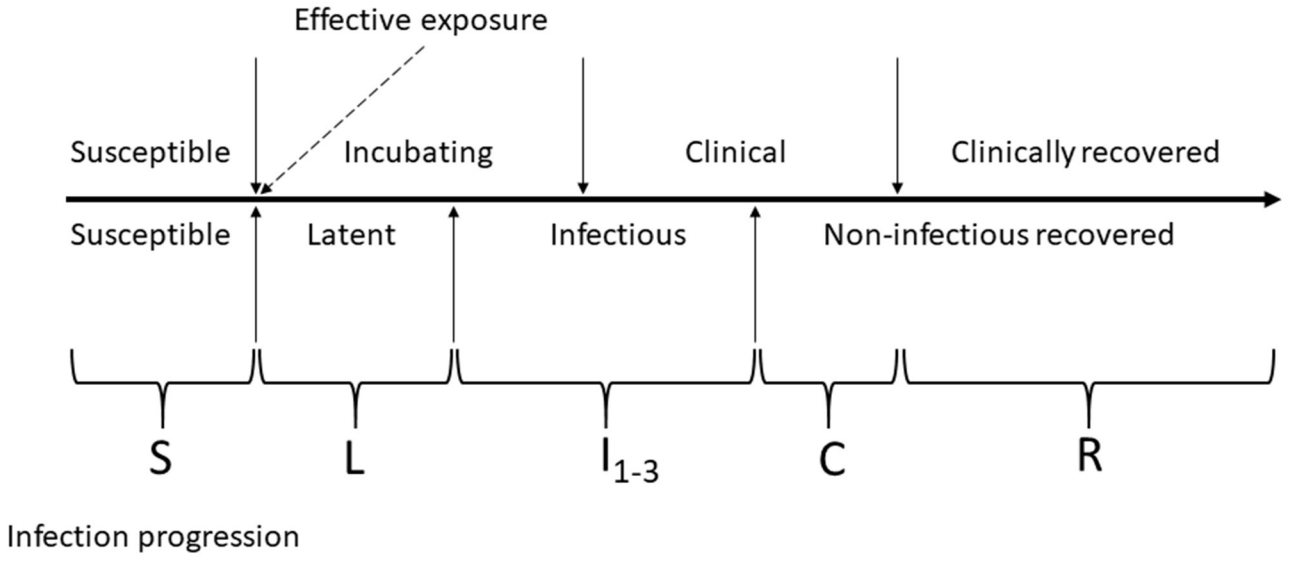

FIGURE 2 | Schematic of foot-and-mouth disease (FMD) infection and clinical disease progression in individual cattle. The compartments of the modified SLIR model of FMD dynamics are indicated by letters: S, susceptible; L, latent; I_1, subclinical infectious; I_2, subclinical–infectious; I_3, clinical infectious; C, clinical but no longer infectious; and R, non-infectious clinically recovered.

From a home-pen, a number of cattle were daily pulled to the hospital-pen due to production diseases—endemic infectious and non-infectious diseases—and returned next day; the per-animal daily pull probability (φ) was equal for all cattle irrespective of their FMD status. This probability was a product of the expected production disease morbidity and the probability to be pulled to the hospital-pen for treatment depending on the disease. For cattle in a home-pen, the expected production disease morbidity was the sum of the bovine respiratory disease (BRD) daily morbidity (π) during the first 30 days after placement onto the feedlot, and the aggregated daily morbidity for all other production diseases (ρ), such as lameness and digestive conditions during the entire 200-days period in the feedlot. The probability of cattle with BRD to be pulled to the hospital-pen for treatment was *brdtrt* and with the other diseases it was *endtrt*. The total per-animal daily probability to be pulled due to the production diseases from a home-pen to the hospital-pen was $\varphi = \pi * brdtrt + \rho * endtrt$. In a feedlot, cattle are placed in individual home-pens, i.e., placed "on-feed," at different times; all cattle are placed in a given home-pen simultaneously. At the start of the model simulations, a fraction (υ) of the home-pens were assumed to just have been placed (day 1 in the feedlot); the home-pens were assigned randomly using a random number generator. The rest of the home-pens were assumed to have been placed >30 days prior. There was also a per-animal daily probability (ς) to be pulled to the hospital-pen for cattle with clinical FMD.

In a hospital-pen, there was homogenous mixing of the cattle pulled from different home-pens that day. The susceptible cattle were infected via direct contact with infectious cattle (I_1-I_3) at a rate reflecting the homogenous mixing and density-dependent transmission (as in the home-pens), and with the same transmission parameter value, β_{hp}in the hospital-pen(s) = β_{wp}.

S-L Susceptible-Latent model of FMD infection dynamics in a hospital-pen l

$$\frac{dS_{hpl}}{dt} = \sum_{i=1}^{m} \varphi S_i - \beta_{hp} \sum_{i=1}^{m} \varphi S_i \left[\sum_{i=1}^{m} \varphi I_{1_i} + \sum_{i=1}^{m} \varphi I_{2_i} + \sum_{i=1}^{m} (\varphi + \varsigma) I_{3_i} \right]$$

$$\frac{dL_{hpl}}{dt} = \beta_{hp} \sum_{i=1}^{m} \varphi S_i \left[\sum_{i=1}^{m} \varphi I_{1_i} + \sum_{i=1}^{m} \varphi I_{2_i} + \sum_{i=1}^{m} (\varphi + \varsigma) I_{3_i} \right]$$

Where i is a home-pen, m is the number of home-pens from which cattle are pulled to the hospital-pen l, and φ and ς are defined in the preceding paragraph. All the parameters are also defined in **Table 1**. $m = n$ if the feedlot had one hospital-pen. The probability for a susceptible animal pulled to the hospital-pen l to be infected by FMD in the hospital-pen that day was:

$$p\_\text{inf\_hp}_l = \frac{\beta_{hp} \sum_{i=1}^{m} \varphi S_i \left[\sum_{i=1}^{m} \varphi I_{1_i} + \sum_{i=1}^{m} \varphi I_{2_i} + \sum_{i=1}^{m} (\varphi + \varsigma) I_{3_i} \right]}{\sum_{i=1}^{m} \varphi S_i}$$

The number of latent cattle returning to a home-pen i that were pulled a day earlier to the hospital-pen l while still susceptible and infected by FMD in the hospital-pen was

$$Bin\left(\varphi_{(t-1)}\mathrm{S}_{i_{(t-1)}},\ p\_\underline{\mathrm{inf}}\_\mathrm{hp}_{l_{(t-1)}}\right).$$

ii *Fence-line transmission via direct contact of cattle from contiguous home-pens*

A fence-line contact between cattle from neighboring home-pens is typical on U.S. feedlots. Home-pens are separated by fences, which do not prevent animal nose-to-nose contact. The fence-line FMDv transmission between each two contiguous home-pens was modeled assuming a homogenous animal mixing and density-dependent transmission along the fence (Equations 1, 2). The effective contact rate fence-line was assumed to be 25% of that within the home-pens, $\beta_{bp} = \beta_{wp} \times 0.25$. Definitions and values of the parameters are given in **Table 1**. The number of cattle infected on a given day by FMD in a home-pen i via the fence-line transmission from a contiguous home-pen j (or home-pen h on the other side of i) was $\mathrm{S}_i\beta_{bp}(\mathrm{I}_1+\mathrm{I}_2+\mathrm{I}_3)_{j\ (\mathrm{or}\ h)}$.

- The viral quantities shed per unit volume of each of the secretions and excretions by an animal in the clinical high infectious FMD stage (I_3),
- The floor size and floor top depth that can be contaminated by the secretions and excretions, and
- The daily viral decay in the floor materials were reflected to model the remaining viral load in the floor materials.

We assumed that only secretions and excretions from the I_3 cattle contributed to this transmission route. Each I_3 animal daily excreted *uri* urine and *fec* fecal volumes, and secreted *sal* saliva volume. We assumed that a fraction *fsal_env* of the daily saliva secreted by an animal was deposited into the home-pen environment and a fraction *fsal_env_floor* of that landed on the floor. The total daily volume of saliva deposited into the home-pen floor by the clinical high infectious cattle was $\mathrm{I}_3 \times sal \times fsal\_env \times fsal\_env\_floor$, of urine it was $\mathrm{I}_3 \times uri$, and of feces it was $\mathrm{I}_3 \times fec$. The virus quantity shed by a highly infectious animal with clinical FMD per unit volume of saliva was *salv*, per unit volume of urine it was *uriv*, and per unit volume of feces it was *fecv*. The deposited secretions and excretions from the I_3 were evenly distributed across the home-pen floor top in j. The viral decay in the resulting mixed floor materials occurred at a daily exponential rate *vir_dec_env*. The width of a home-pen was *w_pen*, the length was *l_pen*, and the contaminated floor top depth was *d_pen*. The remaining viral load in the contaminated floor-top materials in the home-pen j as

$$FMDv\_floor_j = \left(\frac{\mathrm{I}_{3_j} \times sal \times fsal\_env \times fsal\_env\_floor \times salv + \mathrm{I}_{3_j} \times uri \times uriv + \mathrm{I}_{3_j} \times fec \times fecv}{w\_pen \times l\_pen \times d\_pen}\right)^{-vir\_dec\_env}.$$

iii *Environmental transmission due to pen-riders moving between home-pens*

Beef feedlots in the U.S. employ personnel to visually monitor cattle health as an observational disease surveillance method; they are known as pen-riders, pen-checkers, or cowboys and move between the home-pens on foot or on horses. The home-pen floor materials attached to the pen-rider boots or horse hooves could serve as a fomite for FMDv transmission. Such environmental virus transmission between each two contiguous home-pens sequentially visited by a pen-rider in the same home-pen row was modeled (see **Supplementary Figures 1A–E** for the feedlot layouts modeled). A possibility of such environmental transmission between the home-pen rows separated by feed-delivery or drover alleys was not modeled, assuming that majority of the floor materials picked up by a pen-rider in a home-pen are deposited in the next visited home-pen.

In the originating home-pen j we considered:

- The daily volumes of cattle secretions (saliva) and excretions (urine and feces) in which FMDv can be shed,
- The fractions of the secretions deposited into the home-pen environment and then on the floor (the excretions were assumed to be entirely deposited on the floor),

We assumed that an amount σ of the virus-containing floor materials from the originating (visited by pen-riders first) home-pen j was transported on the boots of the pen-riders or hooves of the horses during each pen-rider round to the next—receiving—home-pen i in the same row. The pen-rider rounds through the home-pen row occurred twice per day. In the receiving home-pen i, we assumed that the maximum number of cattle that could be infected due to consumption of the transported contaminated materials was $\frac{FMDv\_floor_j \times \sigma}{ID_{50}\ \mathrm{per\ oral}}$. The infections occurred on the same day when the materials were introduced to i. The daily number of cattle in i infected via this route was modeled as $Bin\left[\left(\frac{FMDv\_floor_j \times \sigma}{ID_{50}\ \mathrm{per\ oral}}\right),\ 0.5\right]$ (Equations 1, 2). Definitions and values of the parameters are given in **Table 1**.

iv *Waterborne transmission*

We assumed that only contiguous home-pens which shared a drinking water-trough were at risk of waterborne FMDv transmission (see **Supplementary Figures 1A–E** for the feedlot layouts modeled). Potential waterborne transmission among home-pens that did not share a drinking water-trough was not modeled. Hospital-pens did not share drinking water-troughs with home-pens in feedlot layouts modeled. We assumed that a fraction (*fsal*) of the daily saliva secreted by an animal (*sal*) was deposited in the home-pen environment, of which a

fraction *fsal_env_w* was deposited in the drinking water-trough. We assumed that all the saliva deposited into the home-pen environment was deposited in the water-trough or the floor, hence,$fsal\_env\_w = 1 - fsal\_env\_floor$. We assumed that only saliva of the clinical high infectious cattle (I_3) contributed to this transmission route. Daily volume of saliva produced by an animal, the fraction of the daily saliva volume deposited into the home-pen environment and what fraction of that deposited in the shared water-trough(s) by the I_3 animals from the two home-pens that shared the water-trough, viral quantity shed per unit volume of saliva by an animal in the clinical high infectious FMD stage, volume of water in the shared water-trough, and viral decay in the water were reflected to model the remaining viral load in the water in the shared trough. A homogenous mixing of the deposited saliva with the water in the trough was assumed. The viral decay in the cattle drinking water occurred at a daily exponential rate *vir_dec_w*. The water volume in a shared trough was *vol_watert*. The home-pen *i* only shared a water-trough with one other home-pen *j* (here, either *j* or *h* could be on either side of *i*). If the home-pen *i* was at the end of the home-pen row in a row with odd number of home-pens, it did not share the water-trough with other home-pens and waterborne transmission was not modeled. The viral load per L of water in the water-trough shared by *i* and *j* was:

$$FMDv\_watert_{i,j} = \left(\frac{(I_{3_i} + I_{3_j}) \times sal \times fsal \times fsal\_env\_w \times salv}{vol\_watert} \right)^{-vir\_dec\_w}$$

We assumed an animal consumed at least 1 L of water every time they visited the water-trough. On each day when$FMDv_watert_{i,j}$was $\geq ID_{50}$ of FMDv for oral exposure, the number of cattle infected by FMD in the home-pen *i* via consumption of contaminated water from that shared trough was modeled as a $Bin(S_i, 0.5)$ (Equations 1, 2). Definitions and values of the parameters are given in **Table 1**.

v *Airborne transmission*

Airborne transmission was modeled using a kernel function that incorporated an exponential decay in the FMDv transmission probability with increasing Euclidian distance between home-pen centroids. Based on the feedlot layout detailed in **Supplementary Figure 1**, we estimated the Euclidean distance between centroids of a home-pen *i* and *k* (where *k* is any other home-pen than *i*) and scaled it by the shortest Euclidean distance between any two home-pen centroids in the feedlot. The scaled distance between two home-pen centroids was $d_{i,k}$. The airborne transmission probability to a home-pen *i* depended on the distances to and proportions of FMD clinical highly infectious cattle (I_3) in the other home-pens. The proportion in a home-pen *k* was $\frac{I_{3_k}}{N_k}$. The probability of FMD infection of a susceptible animal in *i* via the airborne transmission was$p_air_i = 1 - \left[\prod_{k=1}^{n} k \left(1 - \frac{I_{3_k}}{N_k} \times e^{-\alpha \times d_{i,k}} \right) \right]$, and the daily number of cattle infected was $Bin(S_i, p\_air_i)$ (Equations 1, 2). Value of the parameter α reflected the power of the kernel function (**Table 1**).

Outbreak Characteristics Analyzed

We defined the following characteristics of the projected FMD outbreaks, traced these outputs during the model simulations, and analyzed sensitivity of the outputs to the model structure and parameter values. The outbreak characteristics were:

- Outbreak peak day defined as the day with the highest number of clinical cattle (those in the I_3 and C compartments) in the feedlot, counting from the day of introduction of FMD latent cattle into the index home-pen.
- Number of clinical cattle in the feedlot on the outbreak peak day.
- Day of outbreak detection in the feedlot, counting from the day of introduction of FMD latent cattle into the index home-pen. The detection was assumed to occur on the day when the proportion of clinical cattle in the index home-pen reached a detection threshold of 3, 5, or 10% (the lowest detection threshold of 3% was chosen based on data provided via personal communication by veterinarians with experience of FMD investigation on cattle farms during the FMD outbreaks in South America in the 2000s).
- Proportion of latent cattle in the feedlot on the day of outbreak detection.
- Cumulative number of infected home-pens (a home-pen was counted on the day when FMD latent cattle occurred in it for the first time) in the feedlot throughout the outbreak.
- Outbreak duration defined as the day when the last clinical infectious cattle became clinical non-infectious, counting from the day of introduction of FMD latent cattle into the index home-pen.

Model Implementation, Verification, and Validation

The model was implemented in Vensim® PLE Plus Version 6.4a (Ventana Systems Inc., Harvard, MA, USA). The figures were made in R using the ggplot package and in Microsoft Office Power Point® 365 ProPlus (Microsoft, Redmond, WA, USA). The statistical analysis was done in STATA® 13 (StataCorp LP, College Station, TX, USA). Distances between home-pen centroids in each of the feedlot size and layout cases were estimated using Autodesk® Fusion 360 (Autodesk, Inc., San Rafael, CA, USA).

Model verification and validation were performed systematically during the model development and implementation process, i.e., after adding each new component, such as a virus transmission route or a new module, such as a section of the feedlot layout, and following recommended approaches (38, 39). Specifically, at each verification a dynamic approach described by Reeves et al. (39) was used to confirm the model behavior and outputs were logical when giving extreme parameter value inputs. A population balance check was done for the total number of cattle in the feedlot on each day simulated. We conducted a conceptual validation that the model met the intended purpose which was to project FMDv transmission and clinical manifestation dynamics within the feedlot by capturing the effects of the different processes reflected in the model, and a face validation which consisted of an assessment of the system

modeled and model outputs by experts in epidemiological models (38, 39).

Sensitivity Analyses of the Model Outputs to the Model Structure and Parameter Values

i *Sensitivity analysis of the model outputs to the index home-pen location within the feedlot and time spent by individual cattle in the hospital pen per visit*

Cattle with latent FMD were introduced into one home-pen; a proportion of cattle in the index home-pen were FMD-latent at the start of simulations on day 0. Three scenarios of the index home-pen location within the feedlot were modeled: S1—index home-pen was located at the edge of the feedlot and shared a drinking water-trough with one contiguous home-pen; S2—index home-pen was located at the edge of the feedlot and did not share a drinking water-trough with another home-pen; and S3—index home-pen was located centrally within the feedlot and shared a drinking water-trough with one contiguous home-pen. In the base scenario individual cattle pulled to the hospital-pen on one day returned to the home-pen on next day (the beta transmission parameter value for the FMD transmission via direct contact of cattle in the hospital-pen(s) per day was β_{hp}). In a comparative scenario, cattle spent a half day in the hospital-pen, returning to the home-pen same day when pulled (the transmission parameter value was $\beta_{hp}/2$). The model was simulated for each of the feedlot size and layout cases (detailed in **Supplementary Figure 1**) with each of the three scenarios of the index home-pen location within the feedlot, and each of the two scenarios of the time spent by individual cattle in the hospital-pen per pull due to a production disease or FMD. For each case and scenario, 2,000 Monte Carlo simulations were performed and the FMD outbreak characteristics (listed in section Outbreak Characteristics Analyzed) were traced during the simulations. After evaluating the model outputs and if there were no variations in the outputs, a base scenario of the index home-pen location and the time spent by individual cattle in the hospital-pen per visit was chosen based on closest representation to production systems. The base scenario was implemented in the remainder of the sensitivity analyses.

ii *Sensitivity analysis of the model outputs to the parameter values*

The model output sensitivity analysis to values of a set of target parameters was performed. Values from each of the target parameters were sampled for each of 2,000 Monte Carlo simulations of the model. The model was simulated for each specific feedlot size and layout case and the chosen base scenario of the index home-pen location and the time spent by individual cattle in the hospital-pen per visit. The sampled distributions of the target parameters are given in **Table 4**. For each of the remaining model parameters, a single value listed in **Table 1** was used for each of the 2,000 simulations. The target parameters included the FMD latent, infectious, and subclinical periods in individual cattle and cattle infectivity as a change in the value of the beta transmission parameter within the home-pens, fence-line, and in the hospital-pen(s). The infection and disease temporal progression and infectivity could vary with the strain virulence (40). Thus, targeting these parameters in the sensitivity analysis allowed evaluating the model outputs for different FMDv strain virulence scenarios. The target parameter set also included the BRD morbidity in the first 30 days since the cattle placement in the feedlot. The morbidity increases the cattle pull rate to the hospital-pens, but it could vary depending on the feedlot production management and time of year. The target parameter set also included the initial proportion of FMD-latent cattle in the index home-pen, the fraction of daily saliva volume secreted by an animal that is deposited to the home-pen environment, the home-pen floor top depth contaminated by the animal secretions and excretions daily, the water intake per cattle visit to the drinking water-trough, the mortality rate for animals with BRD and other production diseases, the mortality rate for animals with clinical FMD, the urine volume produced by an animal, the saliva volume produced by an animal, the volume of feces produced by an animal, the virus quantity shed in urine by an animal in the FMD clinical high infectious status, the virus quantity shed in saliva by an animal in the FMD clinical high infectious status, the virus quantity shed in feces by an animal in the FMD clinical high infectious status, and the proportion of the cattle daily saliva volume deposited into the home-pen environment (**Table 1**).

Sensitivity to the values of the target parameters was analyzed for outbreak peak day with highest number of clinical cattle and outbreak duration in the feedlot. Using the outputs of the 2,000 model simulations for each of the feedlot size and layout cases, statistical significance of a pair-wise association between the value of each of the target parameters and each the outbreak peak day or outbreak duration was tested with the Spearman rank correlation coefficient. The pair-wise correlation was considered statistically significant if the p-value ≤ 0.05. Also using the simulation outputs, a multivariable linear regression model was built to identify a parameter group most associated with each of outbreak peak day and outbreak duration. A predictor variable was excluded from the model if p-value >0.05 for its association with the outcome variable. The predictor variable selection was performed using the backward stepwise regression and the final model was chosen based on largest adjusted R^2 value. The final multivariable linear regression model's adjusted R^2 statistic was partitioned to obtain the fractional contributions of the target parameters to the projected outcome variance.

An additional parameter-value sensitivity analysis was performed for the power (α) of the function of an exponential decay in the probability of airborne FMDv transmission with increasing distance between home-pens (see the Kernel function definition in the section Model Formulation, subsection Airborne transmission). The model simulations were performed similarly to that described above for the target parameter set; additionally to sampling the value of each of the target parameters, the value of α (the sampled values are given in **Table 3**) was sampled for each of the 2,000 Monte Carlo simulations of the model. The model was simulated for each of the feedlot size and layout cases for each of the three-index home-pen location scenarios and assuming individual cattle spent 1 day in the hospital-pen pen visit. The outbreak duration distribution was summarized over the 2,000 simulated outbreaks

with each value of α. The non-parametric Kruskal-Wallis test was used to test statistical significance of differences in the median outbreak duration with different values of α for a given scenario and for a given feedlot of size and layout case. If p-value ≤ 0.05 for the Kruskall-Wallis test, the Dunn's test with Bonferroni correction was conducted for the multiple comparisons.

iii *Relative impact of the FMDv transmission routes on the outbreak duration*

The model structure sensitivity analysis was focused on the relative impact of the five routes of FMDv transmission between home-pens on the outbreak duration. The five routes were the direct animal contact in the hospital-pen(s), fence-line direct contact, via shared drinking water-troughs, via environment by pen-riders, and airborne. The model was simulated for each of the feedlot size and layout cases for each of the three-index home-pen location scenarios and assuming individual cattle spent 1 day in the hospital-pen pen visit. The value of each of the target parameters (**Table 4**) was sampled for each of the 2,000 Monte Carlo simulations of the model, while setting to zero the parameter values related to one of the transmission routes. The outbreak duration distribution was summarized over the 2,000 simulated outbreaks for the full model and each of the reduced models with one of the routes of transmission excluded, for each feedlot size-layout case and index home-pen location scenario. The non-parametric Kruskal-Wallis test was used to test statistical significance of differences in the median outbreak duration between the full and reduced models and for a given scenario for each of feedlot and layout cases. If p-value ≤ 0.05 for the Kruskall-Wallis test, the Dunn's test with Bonferroni correction was conducted for the multiple comparisons.

RESULTS

Characteristics of Projected FMD Outbreaks in Feedlots of Different Sizes and Layouts

There was no significant variation in the outbreak characteristics among the three scenarios of the index home-pen location within the feedlot, in any of the feedlot size and layout cases modeled (see **Supplementary Figure 1**). There was also no significant variation in the outbreak characteristics when individual cattle were assumed to spend a full day vs. a half of day in the hospital-pen per visit, in any of the feedlot size and layout cases and index home-pen location scenarios. In the light of this, we present results of the FMD latent cattle introduced in an index home-pen that was located centrally within the feedlot, shared a drinking water-trough with one contiguous home-pen (S3 scenario) and cattle spending a full day in the hospital-pen per visit. For each feedlot size and layout scenario, 2,000 Monte Carlo simulations of the model were performed with sampling of the target parameters from the distributions specified in **Table 1**. In short, FS1 was a 4,000 cattle (20 home-pens) feedlot with one hospital-pen; FM1 was a 12,000 cattle (60 home-pens) feedlot with one hospital-pen; FM2 was a 12,000 cattle feedlot with two hospital-pens; FL1 was a 24,000 cattle (120 home-pens) feedlot with two hospital-pens; and FL2 was a 24,000 cattle feedlot with four hospital-pens.

The projected outbreak duration ranged from 49 days in the smallest FS1 to 82 days in the largest FL2 feedlot. The outbreak peak day ranged from 23 in FS1 to 49 days in FL2. The outbreak proceeded slower and lasted longer in a feedlot of a given size if more hospital-pens were operated. The median outbreak duration was 16 days longer in a medium-size feedlot FM2 where 2 hospital-pens were operated compared to FM1 where 1 hospital-pen was operated (**Table 2**). In a large-size feedlot, the median outbreak duration was 9 days longer if two hospital-pens per section of home-pens were operated (four hospital-pens total, FL2), compared to one hospital-pen per section (two hospital-pens total, FL1) (**Table 2**). All home-pens were infected by day 15 following introduction of FMD latent cattle onto the feedlot in FS1, on day 22 in FM1 vs. day 40 in FM2, and on day 37 in FL1 vs. day 46 in FL2 case (**Figure 3**). The number of clinical cattle on the outbreak peak day decreased with a larger number of hospital-pens (**Figure 4**). The median number of clinical cattle on the outbreak peak day was 1,760 (44%) in FS1, 5,520 (46%) in FM1 vs. 2,880 (24%) in FM2, and 6,240 (26%) in FL1 vs. 5,520 (23%) in FL2. Thus, a higher number of hospital-pens had a larger impact on the FMD outbreak dynamics—slowing the outbreak and decreasing the percentage of clinical cattle on the peak day—in a medium-size (12,000 cattle) than in a large-size (24,000 cattle) feedlot, for the layouts modeled.

FMD Outbreak Detection

The outbreak detection was assumed to occur on the day when the proportion of cattle with clinical FMD in the index home-pen reached 3, 5, or 10%. The detection timeline was therefore similar for all the feedlot size and layout cases. The results presented are for the base scenario of FMD latent cattle introduced in an index home-pen that was located centrally within the feedlot and shared a drinking water-trough with one contiguous home-pen, and when the pulled cattle spent a full day in the hospital-pen per visit. The results are summarized over the 2,000 model simulations for each feedlot size-layout case. The day of detection ranged from 4 to 12 days since introduction of FMD latent cattle in the index home-pen for 3 and 5% detection thresholds, and from 6 to 13 days for the 10% threshold (**Table 2**). The median day of detection was 6 for 3 and 5% detection thresholds, while it was 7 for the 10% threshold. Overall, the longer it took to detect the outbreak, the larger was the proportion of latent cattle in the feedlot at detection; however, the relative magnitude of this impact declined with the feedlot size. In ∼50% of the simulations, the outbreak was detected on day 5–9 with any of the three detection thresholds modeled. The median proportion of latent cattle in the smallest FS1 feedlot increased from 4% at detection on day 5–24% on day 9, with 25 and 50% of home-pens infected, respectively. In both FM1 and FM2, the median proportion of latent cattle increased from

TABLE 2 | Characteristics of projected foot-and-mouth disease outbreaks on a U.S. beef cattle feedlot.

Feedlot	Number of home-pens	Total number of cattle	Number of sections of home-pens	Number of hospital-pens	Outbreak duration[a], days (10th, 50th, and 90th percentiles of n = 2,000 simulated outbreaks)	Outbreak peak day with highest number of cattle with clinical FMD (10th, 50th, and 90th percentiles of n = 2,000 simulated outbreaks)	Day of FMD outbreak detection[b] based on detection threshold of 3, 5, and 10% clinical animals in the index home-pen (10th, 50th, and 90th percentiles of n = 2,000 simulated outbreaks)		
							3%	5%	10%
FS1	20	4,000	1	1	39, 49, 59	21, 23, 27	4, 6, 9	5, 6, 10	6, 7, 12
FM1	60	12,000	1	1	46, 58, 69	25, 28, 33	4, 6, 9	5, 6, 10	6, 7, 12
FM2	60	12,000	1	2	61, 74, 89	26, 31, 43	4, 6, 9	5, 6, 10	6, 7, 12
FL1	120	24,000	2	2	60, 73, 86	33, 41, 48	4, 6, 9	5, 6, 10	6, 7, 12
FL2	120	24,000	2	4	68, 82, 95	42, 49, 57	4, 6, 9	5, 6, 10	6, 7, 12

[a] *Outbreak duration was defined as the number of days since introduction of FMD latent cattle in the index home-pen on the feedlot until the last animal infected within the feedlot proceeded from the clinical high infectious stage to the clinical non-infectious stage.*

[b] *Outbreak detection was assumed to occur via routine visual surveillance of cattle health by the pen-riders, on the day when proportion of cattle with clinical FMD in the index home-pen reached 3, 5, and 10%^. This detection threshold was assumed to be independent of the feedlot size (cattle head count).*

1% at detection on day 5 with 7% of home-pens infected to 6% on day 9 with 15% (FM1) and 13% (FM2) of home-pens infected. In both FL1 and FL2, the median proportion of latent cattle increased from 1% on day 5 with 3% of home-pens infected to only 3% on day 9 with 7% of home-pens infected (**Table 3**).

Sensitivity of the Projected Outbreak Characteristics to the Model Parameter Values

The sensitivity analysis was performed for the base scenario detailed above. Of the target parameters, the durations of the FMD infection stages in individual cattle were most influential on the outbreak duration and outbreak peak day in the feedlot (**Table 4** and **Figure 5**). Using the simulation outputs, a multivariable linear regression model was built for each the outbreak duration and peak day of outbreak variables with the target parameters as the predictor variables (**Table 4**). The duration of the FMD latent period was the most influential parameter. The fractional contribution of the latent period duration to the variance in the outbreak duration ranged from 53% in FS1 to 66% in FM1, and to the variance of the outbreak peak day it ranged from 4% in FM2 to 42% in FS1 (**Figure 5**). The duration of the FMD infectious period was the second most influential parameter. Its fractional contribution to the variance in the outbreak duration ranged from 20% in FM1 to 25% in FS1. The infectious period contribution to the outbreak duration variance decreased with a larger feedlot size and for a feedlot of a given size it decreased if more hospital-pens were operated. This contribution was 25% for FS1, 20% for FM1 vs. 5% for FM2, and 13% for FL1 vs. 9% for FL2 (**Figure 5**). The subclinical period was less influential compared to the latent and infectious periods, with a fractional contribution of 5% or less to the variances of both the outcomes in all feedlots modeled.

A larger value of the beta transmission parameter (β_{wp}) reflected a higher cattle infectivity for FMDv transmission via direct contact in the home-pens, fence-line, and in hospital-pen(s). A larger value of this parameter was negatively correlated with each the outbreak duration and outbreak peak day (**Table 4**). This appears straightforward that a higher virus transmission rate via direct animal contact could lead to a faster outbreak progression. However, the relative contribution of β_{wp} to the total variance in either the outbreak duration or outbreak peak was $\leq$5%, being low compared to that of the durations of the FMD infection stages in individual animals (**Figure 5**). The initial proportion of FMD-latent cattle in the index home-pen had smaller fractional contributions to the variances in the outbreak duration and outbreak peak day compared to the durations of the FMD stages and the beta transmission parameter (**Figure 5**). The contribution of the initial FMD-latent proportion in the index home-pen to the outbreak duration decreased with a larger feedlot size and in a medium-size feedlot was lower if more hospital-pens were operated. This contribution was 24% for FS1 and 11% for FM1, but it was <4% for FM2 and both FL1 and FL2 (**Figure 5**). The morbidity rate of BRD during the first 30 days since cattle placement in the feedlot was weakly correlated with

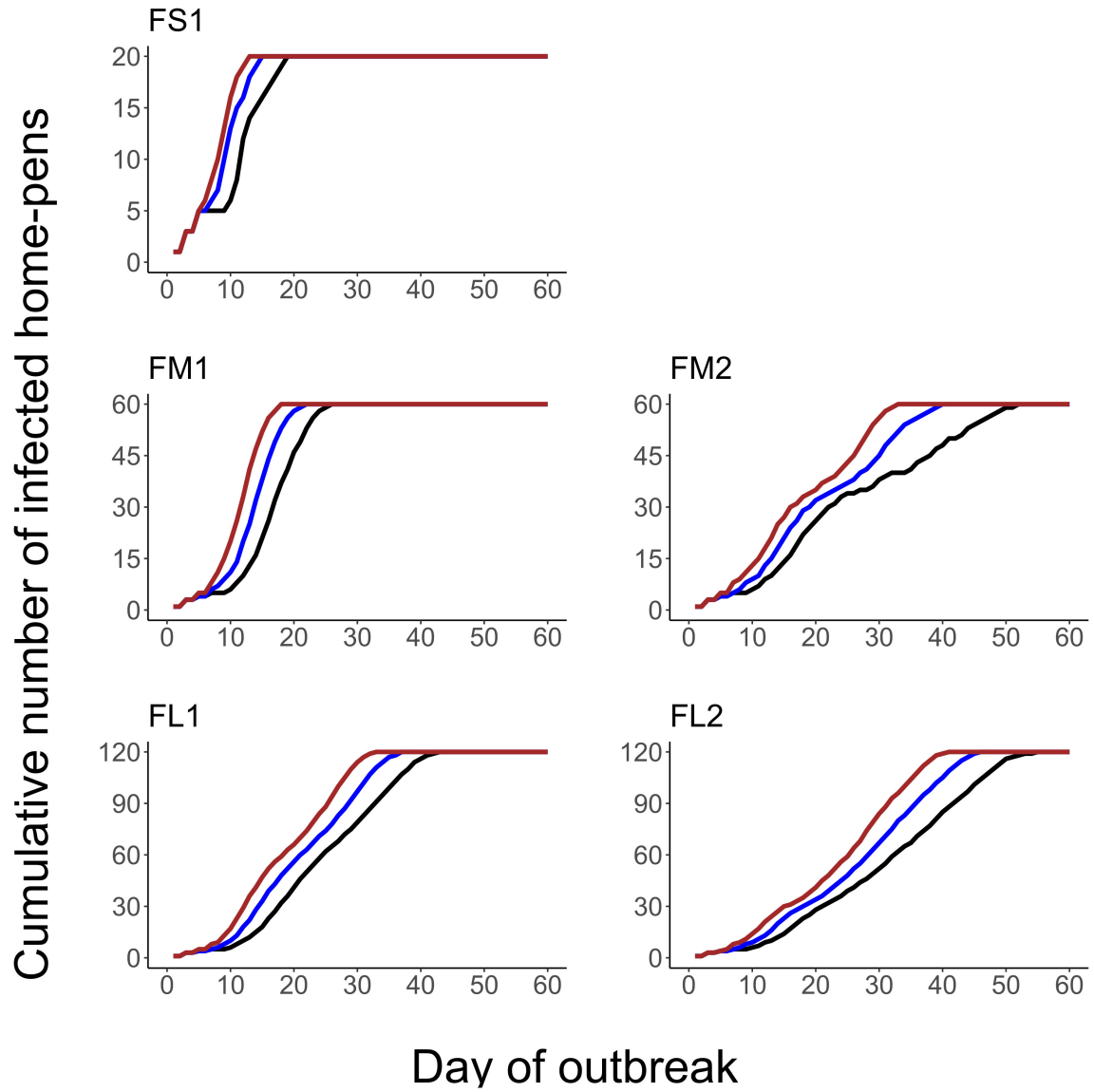

FIGURE 3 | The cumulative number of the home-pens infected with foot-and-mouth disease during a projected outbreak on a U.S. beef cattle feedlot. The lines represent the percentiles (brown lines the 90th percentile, blue lines the 50th percentile, and black lines the 10th percentile) for $n = 2{,}000$ simulated outbreaks in the feedlot of that size and layout sampling the values of the target parameters. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts $n = 200$ cattle per home-pen).

both the outcome variables in each of the feedlot size and layout cases (**Table 4**). The fractional contribution of the BRD morbidity to the variance in the outbreak duration ranged from 1% in FL1 to at most 17% in FM1 (**Figure 5**).

The target parameter set for the sensitivity analysis (**Table 4**) included the parameters values that were initially assigned based on our judgment in the absence of data (**Table 1**). These were the fraction of daily saliva secreted by an animal that is deposited to the home-pen environment; the home-pen floor top depth daily contaminated by the animal secretions and excretions; and the water intake per cattle visit to the drinking water-trough. The values of each of these parameters had low correlations with the outbreak duration and outbreak peak day (**Table 4**), and low fractional contributions to the variances in these outcomes (**Figure 5**) across the feedlot size and layout cases. The remainder of the investigated target parameters (**Table 4**) were not influential for the two outcomes (results not shown) and are not discussed further. These were: the mortality rate for animals with BRD and other production diseases; the mortality rate for animals with clinical FMD; volumes of urine, saliva, and feces produced daily by an animal; proportion of the cattle daily saliva volume deposited into the home-pen environment; and the

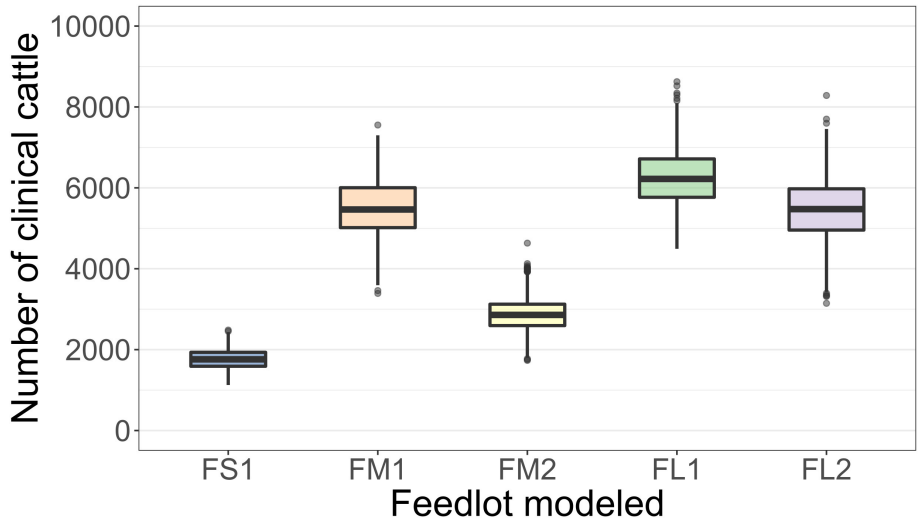

FIGURE 4 | Boxplot of the projected number of cattle with clinical FMD on the outbreak peak day for each of the feedlot size and layout cases modeled. The outbreak peak day was defined as the day with the highest number of clinical cattle (infectious and non-infectious) since the FMD introduction in each of n = 2,000 simulated outbreaks in the feedlot of that size and layout sampling the values of the target parameters. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts n = 200 cattle per home-pen).

TABLE 3 | Estimated percentage of latent cattle and home-pens with latent cattle on a U.S. beef cattle feedlot depending on the outbreak detection day since foot-and-mouth disease introduction.

Feedlot[a]		Percentage (%) of latent cattle and home-pens with latent cattle in the feedlot on the day of FMD outbreak detection (10th, 50th, 90th percentiles of *n* = 2,000 simulated outbreaks)[b]				
		Day 5	Day 6	Day 7	Day 8	Day 9
FS1	Cattle	<1, 4, 7	1, 10, 14	2, 14, 18	6, 18, 24	13, 24, 25
	Home-pens	25, 25, 25	25, 25, 30	25, 30, 41	25, 35, 50	25, 50, 65
FM1	Cattle	<1, 1, 2	0, 3, 4	1, 5, 5	3, 6, 6	4, 6, 7
	Home-pens	7, 7, 8	7, 7, 8	8, 10, 13	8, 12, 18	8, 15, 25
FM2	Cattle	<1, 1, 2	0, 3, 4	1, 5, 5	3, 6, 6	4, 6, 7
	Home-pens	7, 7, 8	7, 7, 8	8, 10, 13	8, 10, 15	8, 13, 18
FL1	Cattle	<1, 1, 1	0, 2, 2	1, 2, 3	1, 3, 3	2, 3, 4
	Home-pens	3, 3, 4	3, 3, 4	4, 4, 7	4, 5, 8	4, 7, 11
FL2	Cattle	<1, 1, 1	0, 2, 2	1, 2, 3	1, 3, 3	2, 3, 4
	Home-pens	3, 3, 3	3, 3, 4	4, 4, 7	4, 5, 8	4, 7, 9

[a] *Feedlot sizes and layouts modeled are detailed in **Table 2** and **Supplementary Figure 1**. Briefly, FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts n = 200 cattle per home-pen).*

[b] *We show results of latent cattle and latent home-pens on days 5–9 (only) of outbreak detection on each feedlot size and layout modeled because those were the most common days of outbreak detection for the three detection thresholds modeled (3, 5, and 10% clinical cattle in the index home-pen).*

virus quantities shed in urine, saliva, and feces by an animal in the FMD clinical high infectious stage.

Relative Impact of Individual Routes of FMDv Transmission Between Home-Pens on the Outbreak Duration

The results presented are for the base scenario detailed above. For each feedlot size-layout case, 2,000 model simulations were performed with sampling the values of the target parameters (**Table 4**), and also setting to zero the parameter values related to one of the between-pen FMDv transmission routes. Exclusion of the transmission via environment by pen-riders or the transmission via contaminated drinking water in the shared water-troughs did not result in a substantially different median outbreak duration or outbreak peak day (each $p > 0.05$ for the *post-hoc* multiple comparisons test) compared to that in the full models across the feedlot size and layout cases (**Figure 6**).

TABLE 4 | Target parameters investigated for associations with the projected outbreak's peak day with highest number of clinical cattle since foot-and-mouth disease introduction and the total outbreak duration on a U.S. beef cattle feedlot.

Target parameter*	Parameter value distribution	Strength of the correlation (Spearman correlation coefficient value) between the model parameter value and outcome variable value for the feedlot of that size and layout									
		Peak day of the outbreak[a]					Duration of the outbreak				
		FS1[b]	FM1	FM2	FL1	FL2	FS1	FM1	FM2	FL1	FL2
Beta transmission parameter in home-pens (β_{wp})	Triangular (0.02, 0.026, 0.031)	**−0.14***	**−0.21***	**−0.09***	**−0.09***	**−0.10***	**−0.05***	**−0.08***	**−0.14***	**−0.09***	**−0.08***
Bovine respiratory disease morbidity during the first 30 days of cattle placement in the feedlot (π)	Vector (0.05, 0.30, 0.05)	−0.01	−0.05	0.03	**−0.10***	**−0.17***	**−0.05***	**−0.13***	**−0.15***	**−0.07***	**−0.05***
Depth of the home-pen floor top contaminated by fresh animal excreta (*d_pen*) (m)	Vector (2, 5, 3)	−0.06	−0.05	−0.01	−0.04	−0.03	−0.05	−0.05	−0.05	−0.06	−0.06
Initial proportion of latent cattle in the index home-pen (*lat_initial*)	Vector (0.005, 0.105, 0.020)	**−0.42***	**−0.29***	**−0.09***	**−0.15***	**−0.17***	**−0.11***	**−0.09***	**−0.08***	**−0.09***	**−0.09***
Fraction of saliva daily produced by the animal that is excreted into the home-pen environment (σ)	Vector (0.1, 0.5, 0.1)	0	−0.05	0.03	−0.05	−0.03	**0.04***	**0.03***	**−0.01***	**−0.01***	**0.01***
Duration of FMD latent period (*lat*) (days)	Weibull (α = 1.782, β = 3.974)	**0.67***	**0.62***	**0.25***	**0.48***	**0.64***	**0.75***	**0.77***	**0.77***	**0.82***	**0.83***
Duration of FMD infectious period (*inf*) (days)	Gamma (α = 3.969, β = 1.107)	0.02	**−0.11***	−0.02	**−0.14***	**−0.12***	**0.48***	**0.42***	**0.23***	**0.35***	**0.29***
Duration of FMD subclinical period (*sub*) (days)	Gamma (α = 1.222, β = 1.672)	**0.19***	**0.25***	**0.07***	**0.18***	**0.22***	**−0.21***	**−0.17***	**−0.03***	**−0.09***	**−0.06***
Water intake by the animal per visit to the water-trough in the home-pen (*wat_int*) (l)	Vector (1, 5, 4)	−0.02	−0.08	0.01	−0.09	−0.10	0.01	−0.01	−0.05	−0.06	−0.06

[a] *Bold coefficients with * indicate $p < 0.05$ for the correlation coefficient between the parameter value and outcome variable value.*

[b] *Feedlot sizes and layouts modeled are detailed in **Table 2** and **Supplementary Figure 1**. Briefly, FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts n = 200 cattle per home-pen).*

** Results of the following target parameters were not included in the table above because were found to be not influential to model outputs: mortality rate for animals with BRD and other production diseases (endemic infectious diseases and noninfectious diseases) (day^{-1}) (μ), Mortality rate for animals with clinical FMD (day^{-1}) (ψ), urine volume produced by an animal (L/day) (uri), saliva volume produced by an animal (L/day) (sal), volume of feces produced by an animal (kg/day) (fec), virus quantity shed in urine [plaque forming units (PFU)/mL] by an animal in the FMD clinical high infectious status (univ), virus quantity shed in saliva (PFU/mL) by an animal in the FMD clinical high infectious status (salv), virus quantity shed in feces (PFU/mL) by an animal in the FMD clinical high infectious status (fecv), and the proportion of the cattle daily saliva volume deposited into the home-pen environment (dmnl) (fsal_env). Their distributions can be found in **Table 1**.*

Exclusion of the FMDv transmission via direct contact of cattle from different home-pens in the hospital-pen(s) resulted in a significantly longer median outbreak duration ($p < 0.001$ for the *post-hoc* multiple comparisons test) in FM1, FM2, and FL1 compared to the full models. The median outbreak duration in FM1 was 27 days longer, in FM2 it was 11 days longer, and in FL1 it was 10 days longer if the β_{hp} was set to 0 (**Figure 6**). Exclusion of the FMDv transmission via fence-line direct contact of cattle from contiguous home-pens resulted in a significantly longer median outbreak duration ($p < 0.001$ for the *post-hoc* multiple comparisons test) in all the feedlot size and layout cases, with largest differences in FM2, FL1, and FL2. Specifically, the median outbreak duration in FM2 was 19 days longer, in FL1 it was 7 days longer, and in FL2 it was 12 days longer (**Figure 6**). Exclusion of the airborne FMDv transmission resulted in a significantly shorter or longer median outbreak duration ($p < 0.001$ for the *post-hoc* multiple comparisons test), depending on the feedlot size and layout. The median outbreak duration in FS1 was 6 days longer, in FM1 it was 3 days shorter, but in FM2 it was 15 days shorter, in FL1 it was 11 days shorter, and in FL2 it was 23 days shorter (**Figure 6**).

Impact of the Power (α) of the Function of an Exponential Decay in the Probability of Airborne FMDv Transmission With Increasing Euclidean Distance Between Home-Pen Centroids on the Outbreak Duration

The results presented are for the base scenario detailed above. For each feedlot size-layout case, 2,000 model simulations were performed with sampling the values of the target parameters (**Table 4**). Additionally, for each simulation a different power [α, modified from Boender et al. (24)] was specified for the Kernel function of an exponential decay in the probability of airborne FMDv transmission with increasing distance between home-pen centroids. The values of α modeled were: −3, −3.5 (baseline), −4,

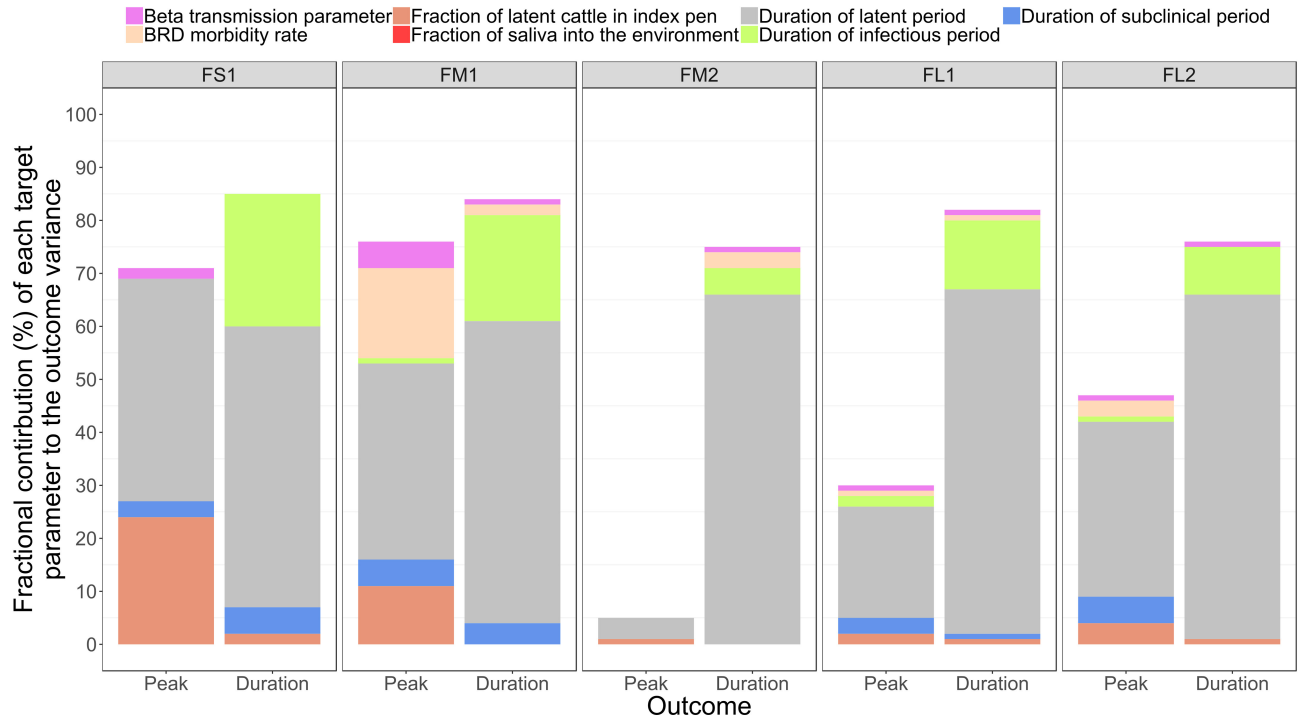

FIGURE 5 | The fractional contributions of select target parameters to the variance in each the outbreak peak day with highest number of clinical cattle and the total outbreak duration in the feedlot since the foot-and-mouth disease introduction, estimated based on $n = 2{,}000$ simulated outbreaks in each of the feedlot size and layout cases modeled. Multivariable linear regression models were developed for each of the outcome variables of the projected outbreak peak day and outbreak duration and the target parameters as the predictor variables. For each outcome, the final regression model adjusted R^2 statistic was partitioned to obtain the fractional contributions of the target parameters to the projected outcome variance. Outcomes: Peak—outbreak peak day, Duration—duration of the outbreak. Target parameters: beta transmission parameter for FMD virus transmission via direct cattle contact [Beta transmission parameter]; morbidity rate of bovine respiratory disease (BRD) during the first 30 days since cattle placement in the feedlot [BRD morbidity rate]; initial proportion of FMD latent cattle in the index home-pen [Proportion of latent cattle in index pen]; fraction of the daily saliva volume produced by an animal that is deposited into the home-pen environment [Fraction of saliva into environment]; and the durations of the FMD latent period [Duration of latent period], infectious period [Duration of infectious period], and subclinical period [Duration of subclinical period] in individual cattle. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts $n = 200$ cattle per home-pen).

-4.5, and -5; a higher value of α represents a higher intensity of the airborne transmission. There was no significant difference in the median outbreak duration ($p > 0.05$ for the *post-hoc* multiple comparisons test) in FS1, FM1, or FM2 with a change in the value of α (**Figure 7**). In each FL1 and FL2, the outbreak duration was shorter with a higher value of α. In FL1, the median outbreak duration was 68 days with the highest α of -3 and 82 days with the lowest α of -5 (**Figure 7**). Similarly, in FL2 the median outbreak duration was 77 days with α of -3 and 92 with α of -5 (**Figure 7**).

DISCUSSION

The initial parameter values were assigned based on available data (**Table 1**). The available data are often for the serotype O that is the most prevalent serotype world-wide (2, 41) and responsible for recent epidemics in non-endemic countries with large livestock populations, such as the UK, France, Netherlands, South Korea, and Japan (42–47). However, the durations of the infection and disease stages in cattle can vary among FMDv strains, e.g., depending on the strain virulence (40). Other strain characteristics can also vary, e.g., transmissibility via direct animal contact (reflected by the β transmission parameter value) or airborne (reflected by the a parameter value in the airborne transmission Kernel function). We analyzed sensitivity of the projected outbreak duration and peak day to potential differences in the FMD strain characteristics associated with different disease period durations. A longer FMD latent period in individual cattle was associated with a later outbreak peak day and longer outbreak duration in all the feedlot size and layout cases modeled (**Table 4**). The transmission can be delayed since it takes longer for the animals to become infectious. A longer infectious period was also moderately correlated with outbreak duration and showed a weak negative correlation with days to peak infection in FM1, FS1, and FS2. A paper published after development of this model used experimental data and application of an Accelerated Failure Time model to estimate FMD disease periods and 95% confidence intervals but not fitted distributions (48). Their estimates are contained within the bounds of our model parameters for disease periods derived from Mardones et al. (32). Notably their point

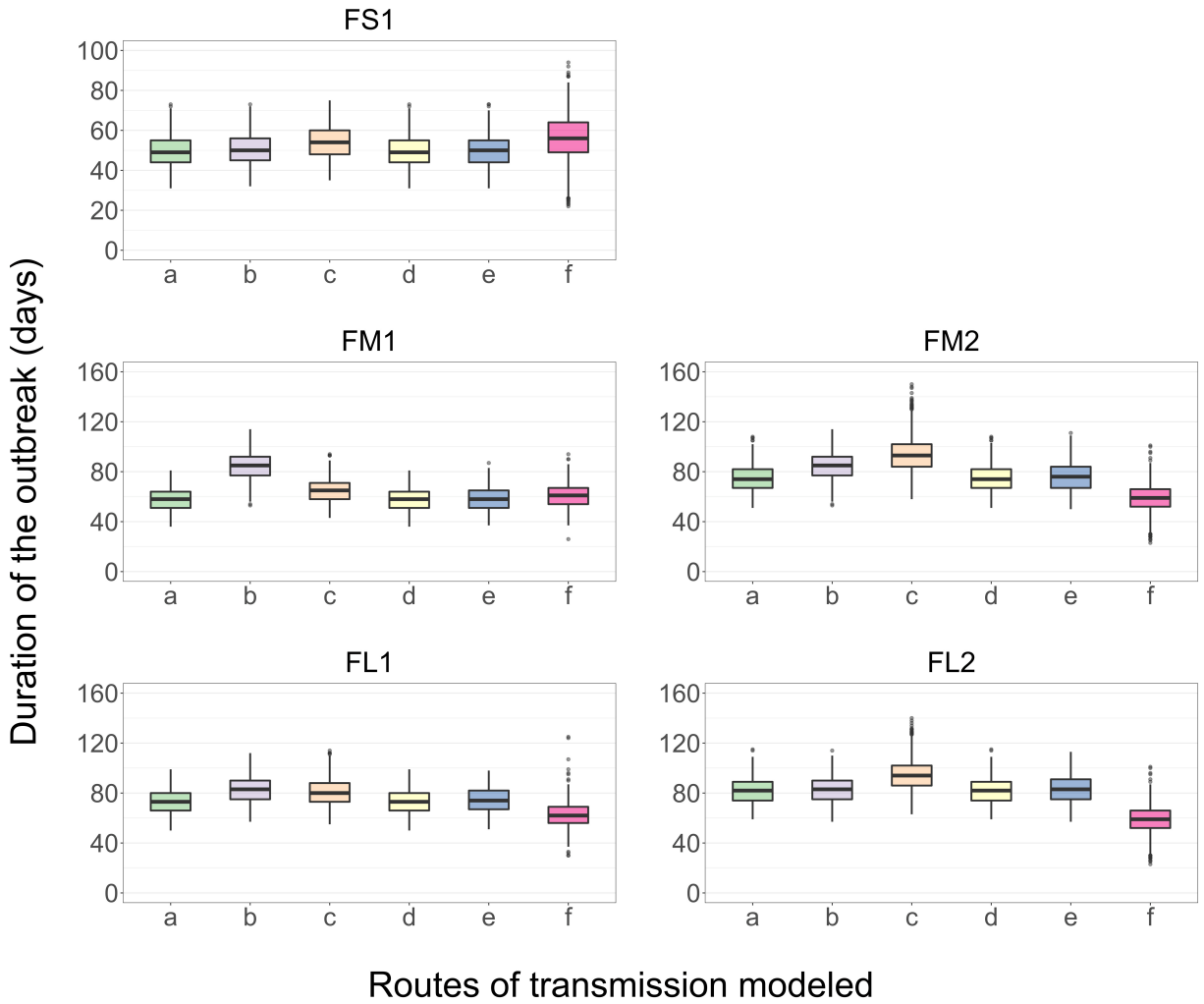

FIGURE 6 | Boxplot of the projected duration of a foot-and-mouth disease outbreak on a U.S. beef cattle feedlot for $n = 2{,}000$ simulated outbreaks in the feedlot of that size and layout sampling the values of the target parameters, when the full model incorporating all the routes of FMD virus transmission among the home-pens or a model with one of the transmission routes excluded was simulated. a—all the routes of FMD virus transmission among home-pens incorporated, b—transmission via direct contact of cattle in the hospital-pens excluded; c—fence-line transmission between cattle in neighboring home-pens excluded; d—transmission of virus contaminated material between home-pens by the pen-riders excluded; e—transmission via contaminated water-troughs excluded; and f—airborne transmission excluded. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts $n = 200$ cattle per home-pen).

estimate of the latent period is shorter and of the infectious period is longer than our point estimates. In each case our distribution includes the estimates from Yadav et al. (48). Our sensitivity analysis suggests that both the latent period and the infectious period may be influential in outbreak dynamics. A shorter latent period may decrease the time to peak outbreak and increase the duration of the outbreak and a longer infectious period my increase the duration of the outbreak. Overall, these results suggest that characteristics of the FMDv strain will likely impact the transmission dynamics within the feedlot and outbreak characteristics. Given that the last documented FMD outbreak in the U.S. was in 1929 (4), an introduction of any FMDv strain would severely impact the U.S. livestock sector due to costs of the associated restrictions on international trade, animal depopulation or other control measures, and production losses (49).

The routes of direct and indirect FMDv transmission between home-pens in the feedlot were explicitly reflected in the model. The virus transmission from cattle with clinical and subclinical FMD via direct contact with susceptible cattle from other home-pens occurred in the hospital-pen(s) and fence-line for contiguous home-pens, along with the indirect waterborne, environmental, and airborne transmission. Of all the direct and indirect between-home-pen transmission routes, the direct transmission in the hospital-pen(s) had the largest impact on the outbreak duration in the median and large size feedlots that

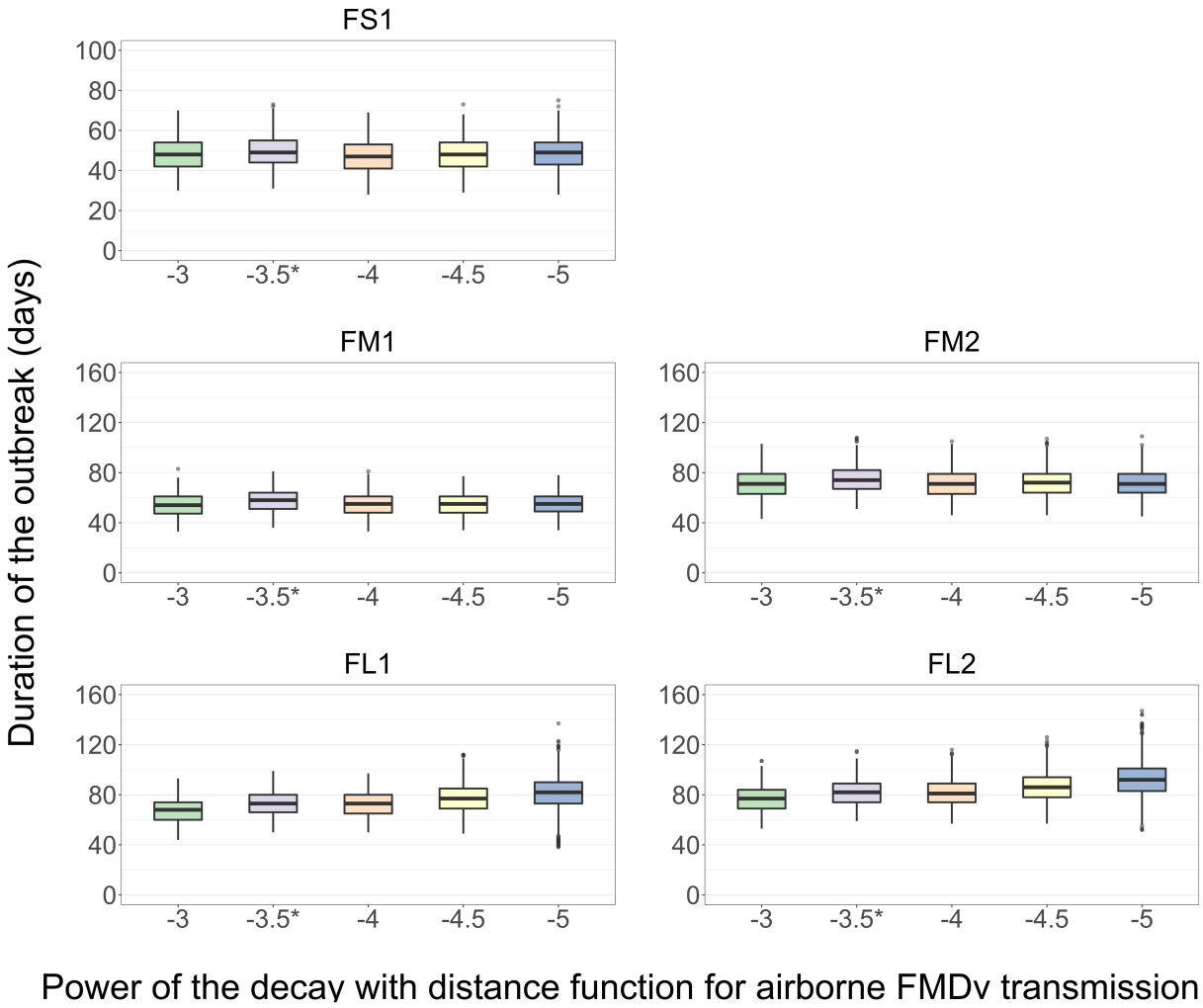

FIGURE 7 | Boxplot of the projected duration of a foot-and-mouth disease outbreak on a U.S. beef cattle feedlot for $n = 2{,}000$ simulated outbreaks in the feedlot of that size and layout sampling the values of the target parameters, depending on the power (α) of the function of an exponential decay in the probability of airborne FMD virus transmission with increasing distance between home-pens. *Baseline value used to simulate the models for the other analyses. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts $n = 200$ cattle per home-pen).

operated one hospital-pen per home-pen section (FM1 and FL1) (**Figure 6**). In the medium and large feedlots that operated two hospital-pens per section (FM2 and FL2), the fence-line direct transmission had the largest impact on the outbreak duration (**Figure 6**). Note that while the FMDv transmissibility via direct contact with infectious subclinical and clinical cattle and the effective contact rates were assumed to be equal in the home-pens and hospital-pens ($\beta_{wp} = \beta_{hp}$), a simplified assumption was made that the fence-line contact rate was ¼ of the within home/hospital-pen rate ($\boldsymbol{\beta_{bp} = \beta_{wp} \times 0.25}$). The detailed role of the fence-line transmission can be explored in future models. We assumed equal FMDv transmissibility via direct contact from infectious subclinical and clinical cattle, because experimental studies show the virus shedding to the environment starts before the clinical signs (50–55). Such parameterization could lead to an overestimation of the within-herd FMD transmission rate as suggested by Kinsley et al. (31). To avoid the overestimation, the subclinical infectiousness and clinical infectiousness durations in our model were limited to the total infectious period reported by experimental and field studies (**Table 1**). The explicit specification of the subclinical and clinical infectious stages can be used in the future to investigate the contribution of animals in each of the stages to the transmission dynamics, if data on the FMDv shedding in excretions and secretions in subclinical and clinical animals become available. Moreover, the developed model structure with the explicit infection/infectiousness vs. clinical disease progression timelines (**Figure 2**) enables investigating the impact of the strain

characteristics (e.g., the sensitivity analysis reported in **Table 4** and **Figure 5**) as well as of specific vaccine formulations and vaccination strategies on the outbreak dynamics.

For the routes of indirect FMDv transmission—waterborne, environmental by pen-riders, and airborne—we only considered the contribution of cattle at the clinical high-infectious stage, because these are known to shed the virus in all the relevant excretions and secretions (55–57). The amount of virus shed by cattle with clinical FMD has been previously reviewed (35, 58–60). Data are extremely scarce on the shedding and other parameters relevant for FMDv indirect transmission in a feedlot; this is also relevant for the risk of transmission to other farms. We made a number of simplifying assumptions to model the indirect transmission in a feedlot. For the environmental transmission, we only considered the transmission of FMDv contaminated home-pen floor materials by pen-riders. We made a simplifying assumption that the FMDv containing animal secretions and excretions were evenly distributed across the home-pen floor, though this is unlikely. We assumed a floor material volume carried by a pen-rider between the home-pens, and the infectivity of the contaminated materials for cattle based on an infectious dose via per oral exposure (**Table 1**). To model FMDv transmission via drinking water in the water-troughs shared by contiguous home-pens, we made a simplifying assumption of an equal water volume consumed per visit to the trough by a healthy animal and an animal with FMD, and assumed the infectivity of the contaminated water for cattle based on an infectious dose via per oral exposure (**Table 1**). We did not model a specific drinking behavior, which is variable among cattle (61), season-dependent, and may change depending on the FMD stage. The drinking and feeding behavior changes during the FMD progression in cattle have not been sufficiently described in literature to enable inclusion in the model; future models could incorporate such data. Within limits of the current model structure and parameterization, the sensitivity analysis showed that neither the environmental FMDv transmission by pen-riders nor the transmission via contaminated drinking water substantially contributed to the projected outbreak duration (**Figure 6**). Other routes of indirect FMDv transmission, e.g., via contaminated fomites or personnel movement other than the pen-riding, may contribute to the transmission dynamics in feedlots but were not reflected in the model due to the lack of data for the parameterization.

The airborne FMDv transmission was influential on the outbreak duration (**Figure 6**). In the small FS1 feedlot, without the airborne transmission the projected outbreak duration varied significantly (**Figure 6**). This suggests the airborne transmission may contribute to a rapid and short outbreak in such feedlots with close spatial proximity of the home-pens. In the feedlots with multiple sections of home-pens (FM2, FL1, and FL2) in which 1–2 hospital-pens were operated for each home-pen sections, the airborne transmission was the only route of FMDv transmission responsible for the virus spread between the sections of home-pens. Without such transmission, only the index home-pen section was affected producing a shorter outbreak while the other home-pens sections remained uninfected. Hagerman et al. (62) showed that weather conditions are permissive of airborne FMDv spread in parts of the U.S. with significant beef cattle populations. However, no data is available on the expected intensity of the spread. To model a decreasing probability of airborne FMDv transmission with an increasing distance between home-pens in a feedlot, we adopted a Kernel function and its parameter values fitted by Boender et al. (24) to data from the UK 2001 FMD epidemic. This was an approximation since the parameter values were for the total probability of FMD spread via all routes among the cattle herds in the UK. To evaluate significance of this approximation, we investigated the impact of varying the key parameter of the function (the power α of the exponential decay in the airborne transmission probability with an increasing distance between home-pens) on the projected outbreak duration. The average outbreak duration was not significantly affected (**Figure 7**). However, in the large feedlots (FL1 and FL2) the outbreak duration was more variable when there was a lower probability of the airborne FMDv transmission via a given distance (a lower a value) (**Figure 7**). This suggests the airborne transmission can contribute to more predictable, shorter outbreaks even in larger feedlots. A simulation study by Donaldson and Alexandersen (63) showed a 100 infected cattle at a source would be enough for the virus to travel up to 1 km and infect a susceptible host which might suggest that within a medium to large beef feedlot, airborne transmission by itself can be responsible to the FMDv spread to the entire population. The airborne transmission might play a large role also for FMD spread between U.S. beef feedlots, because of the concentration of cattle farms within defined geographical areas, such as the Central United States where the majority of cattle is concentrated (62, 64). Environmental conditions however severely impact the airborne FMDv survival and transmission, and in turn depend on factors, such as seasonality and geographical location of the feedlot within the country (62).

The initial proportion of latent cattle in the index home-pen varied between a 0.5 and 10% and the BRD morbidity rate were not influential on the outbreak duration or peak day (**Table 4** and **Figure 5**). We considered the cattle pulled to the hospital-pen(s) due to BRD as the main risk factor for contact of cattle from different home-pens during the first 30 days of the FMD outbreak. In our model, FMD was introduced with cattle arriving on the feedlot; the first 30 days post-arrival is on average the highest risk period to develop BRD in beef feedlots (65–68). Although, that risk period can be affected by several other factors (69–72) that were not further reflected in our model. Beyond designating at the start of the simulations some of the home-pens as just placed and the remainder as placed >30 days prior—to model the post-arrival BRD morbidity—we did not explicitly model the endemic disease incidence dependent on days on feed. Cattle in all the home-pens experienced an equal incidence of common production diseases other than BRD throughout the simulated outbreak. Realistically, cattle arrive on and leave the feedlot on a continuous basis, as a home-pen in/home-pen out.

The feedlot layout and number of hospital-pens operated impacted on the FMD outbreak characteristics. The projected

FMD outbreak duration was shortest for feedlots with one hospital-pen serving one section of home-pens (**Figure 8**), because cattle from the whole feedlot mixed in a single hospital-pen. For medium or large feedlots (12,000 and 24,000 cattle, respectively), operating a lower number of hospital-pens resulted in a shorter outbreak (**Figure 8**). The outbreak peak day occurred earlier in feedlots with one hospital-pen and there was a large burden of the FMD clinical cattle earlier in the outbreak and on the outbreak peak day (FS1 and FM1) (**Figures 4**, **8**). The epidemic curves were bi-modal in feedlots with more than one hospital-pens (FM2, FL1, and FL2); limiting differences in the number of cattle in the clinical stage during the outbreak. Overall, for a feedlot of a given size, the number of clinical cattle at the outbreak peak day(s) was lower with more hospital-pens operated (FM2 vs. FM1, FL2 vs. FL1) (**Figure 4**), which can be a result of the delayed outbreak progression due to the segregation of the hospital-pen catchment sub-populations of cattle. However, all the home-pens were infected during the outbreak in all the feedlots modeled, despite the differences in the cattle population size, number of home-pens sections per

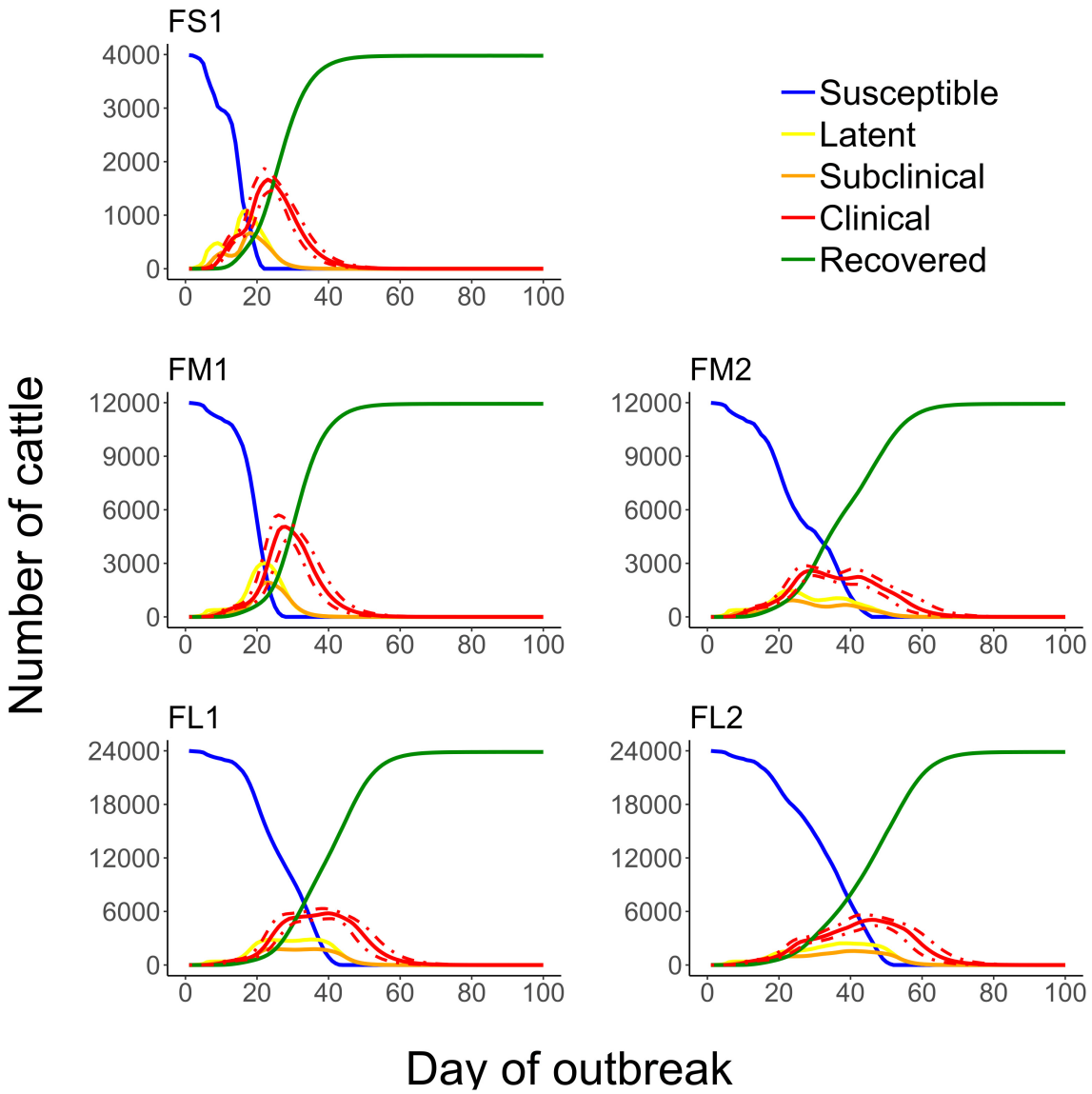

FIGURE 8 | Numbers of cattle in each of the foot-and-mouth disease infection and disease stages during projected outbreaks on U.S. beef cattle feedlots. The solid lines represent the 50th percentiles for the cattle numbers in the infection stages and the red dotted lines represent the 25th and 75th percentiles for the number of cattle with clinical FMD (infectious and non-infectious clinical cattle) of $n = 2{,}000$ simulated outbreaks in the feedlot of that size and layout sampling the values of the target parameters. Feedlot size and layout cases modeled: FS1 is a 4,000 cattle feedlot with one hospital-pen; FM1 is a 12,000 cattle feedlot with one hospital-pen; FM2 is a 12,000 cattle feedlot with two hospital-pens; FL1 is a 24,000 feedlot with two hospital-pens; and FL2 is a 24,000 cattle feedlot with four hospital-pens (in all the layouts $n = 200$ cattle per home-pen).

hospital-pen, or number of hospital-pens. These results suggest that a reduction of cattle contact within the feedlot by operating multiple hospital-pens, each with a defined catchment home-pen sub-population, might slow down the outbreak progression. This would provide time for implementing outbreak control strategies and reduce the FMD clinical cattle burden on individual days. On the other hand, operating a lower number of hospital-pens might lead to a faster and shorter outbreak. If no intervention strategies are implemented, a rapid outbreak progression might be the best scenario, with a lower risk of FMD transmission to other farms.

The outbreak was detected on day 4–12 since introduction of FMD latent cattle on the feedlot, if it was detected at 3% of clinical FMD cattle in the index home-pen (**Table 2**). The time to FMD detection in cattle herds was estimated to be 21 days during the UK 2001 epidemic (42) and 13 days during the 2010/2011 Korean epidemic (73). McLaws and Ribble (74) reviewed the time to detection for FMD outbreaks in livestock in non-endemic areas during 1992 to 2003; it varied from 7 to 24 days and reasons for a delayed detection included misdiagnosis of the disease, mild clinical signs (in small ruminants), delayed laboratory confirmation, and deliberate underreporting by the affected farmers. Prior modeling studies of FMD dynamics in livestock herds suggested the mean time to detection to be 10–11 days (17), 6–7 days (13), and 10–13.5 days (29) since FMD introduction. We modeled the day of detection based on identification of FMD clinical signs by the pen-riders during the routine observational surveillance. Pen-riders represent the first line of surveillance as they monitor cattle for clinical signs of endemic diseases within the feedlots, and are generally experienced in identifying diseased cattle (75). However, it is important to consider the differential diagnosis as there are cattle diseases with similar symptomatology as mentioned by Coetzer and Tustin (56); misdiagnosis can delay the time to detection in the field. The clinical disease severity also depends on the FMDv strain virulence (35, 56). To account for potential delays in the detection, we also considered the detection thresholds of 5 and 10% of FMD clinical cattle in the index home-pen. The outbreak detection was delayed by only 1–2 days for detection at 5 or 10% compared to 3% of FMD clinical cattle (**Table 2**). Nelson et al. (55) suggests the possibility to use qPCR to identify FMDv in cattle during the pre-clinical stage. The use of a surveillance test detecting pre-clinical FMD could potentially decrease the time to detection, however, no such pen-side (practical) test for cattle is currently available. The model simulations suggest that proportion of latent cattle in the feedlot can substantially increase from day 4 to 12 of the outbreak (**Figure 8** and **Table 3**). This had a larger impact in small-size feedlots which in the worst-case scenario of detection on day 12 had up to 28% of the cattle already infected (data not shown). Carpenter et al. (29) modeled FMD transmission within a 1,000-cattle dairy farm; the results suggested 65–97% of the cattle would be infected by the day of detection at a 1 and 5% clinical FMD prevalence, respectively. However, the animal contact structure in dairy farms differs from that in beef feedlots. Studies modeling within-farm FMD dynamics have shown that early detection has a large impact on the scale of the outbreak and the success of intervention strategies (15, 16, 23).

We modeled feedlots as a closed system in which incoming and outgoing animals during the simulations were not considered. While U.S. feedlots generally have continuous turnover of cattle, once FMD was diagnosed quarantine would result in quarantine of the infected feedlot.

To our knowledge, this is the first model of transmission dynamics of FMD in beef feedlots. Kinsley et al. (31) modeled FMD transmission dynamics in swine farms. They estimated an earlier outbreak peak day—with highest number of clinical animals—on a swine farm compared to our estimate for a feedlot. This may be due that swine shed FMDv in larger quantities to the environment compared to cattle (35, 51, 56); this can contribute to the rapid infection transmission across the farm. The within-farm animal contact structure differs between swine farms and beef feedlots, and it can be expected that the FMDv transmissibility via different routes varies due to the different animal contact structure, virus shedding, and potentially virus survival in the farm environment. However, the estimated average time to FMD detection on a swine farm based on observation of the clinical signs was 3–12 days post-introduction (31), which is similar to day 4–12 in our model for beef feedlots (**Table 2**).

CONCLUSIONS

This is the first model projecting FMD transmission, infection, and clinical manifestation dynamics on contemporary U.S. beef cattle feedlots. The model is consistent with data available to date but can be improved with better data on FMDv survival in within-feedlot environments (e.g., in cattle manure and drinking water); FMDv infectious dose depending on the exposure route for cattle that are healthy or experience common production diseases; clinical presentation of FMD in beef feedlot cattle depending on the strain virulence; potential for the virus airborne transmission in areas where the U.S. beef industry is concentrated; and sensitivity of the routine observational surveillance of large cattle populations to detect FMD introduction. Also, the modeling results highlight the importance of understanding the complex contact structure in the cattle meta-populations within feedlots for projecting possible dynamics of FMD and other infectious diseases. The lack of such understanding limits the realism and granularity of current models of within-farm dynamics of foreign animal diseases if (re)introduced to the U.S. The developed model will be used to project and compare impacts of FMD control strategies, such as cattle depopulation, within-feedlot movement restrictions, and vaccination on the outbreak progression. Finally, we emphasize that although mathematical models are powerful tools to understand complex systems, they are simplified representations of real life.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR'S NOTE

This work was adapted from a doctoral dissertation chapter available online at: https://krex.k-state.edu/dspace/handle/2097/40307.

AUTHOR CONTRIBUTIONS

VV conceived the study. MS and VV designed the study. AC implemented the models and performed the sensitivity analyses. All authors contributed to the development, implementation, analysis of the models and the output interpretation, wrote the manuscript, read, and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.527558/full#supplementary-material

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Foot-and-Mouth Disease: Optimization, Reproducibility, and Scalability of High-Yield Production of Virus-Like Particles for a Next-Generation Vaccine

Ana Clara Mignaqui[1], Alejandra Ferella[2], Brian Cass[3], Larissa Mukankurayija[3], Denis L'Abbé[3], Louis Bisson[3], Cintia Sánchez[4], Romina Scian[4], Sabrina Beatriz Cardillo[4], Yves Durocher[3] and Andrés Wigdorovitz[2]*

[1] Instituto de Investigaciones Forestales y Agropecuarias de Bariloche, IFAB, INTA-CONICET, San Carlos de Bariloche, Argentina, [2] Instituto de Virología e Innovaciones Tecnológicas, IVIT, INTA-CONICET, INCUINTA, Buenos Aires, Argentina, [3] Human Health Therapeutics Research Center, National Research Council Canada, Montreal, QC, Canada, [4] Biogénesis-Bagó S.A, Buenos Aires, Argentina

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Cyril Gerard Gay, United States Department of Agriculture (USDA), United States

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Eugene Ryabov, United States Department of Agriculture (USDA), United States
John Gerard Neilan, United States Department of Homeland Security, United States

***Correspondence:**
Ana Clara Mignaqui
mignaqui.anaclara@inta.gob.ar

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Inactivated Foot-and-Mouth Disease (FMD) vaccine has proven to be effective in the control of the disease. However, its production has some disadvantages, including the costly biosafety facilities required for the production of huge amounts of growing live virus, the need of an exhaustive purification process to eliminate non-structural proteins of the virus in the final formulations in order to differentiate infected from vaccinated animals and variable local regulatory restrictions to produce and commercialize the vaccine. Thus, a novel vaccine against FMD that overcome these restrictions is desirable. Although many developments have been made in this regard, most of them failed in terms of efficacy or when considering their transferability to the industry. We have previously reported the use of transient gene expression in mammalian cells to produce FMD virus-like particles (VLPs) as a novel vaccine for FMD and demonstrated the immunogenicity of the recombinant structures in animal models. Here, we report the optimization of the production system by assaying different DNA:polyethylenimine concentrations, cell densities, and direct and indirect protocols of transfection. Also, we evaluated the reproducibility and scalability of the technology to produce high yields of recombinant VLPs in a cost-effective and scalable system compatible with industrial tech-transfer of an effective and safe vaccine.

Keywords: FMDV, VLPs, mammalian cells, transient gene expression, emergency vaccine

INTRODUCTION

Foot-and-Mouth Disease (FMD) is a highly contagious and threatened disease of cloven-hoofed animals, endemic in many parts of the developing world (1). It is one of the most important animal health concerns from an economic point of view and continues to pose a serious threat to farmers, livestock industries and governments. The presence of the disease in developing countries results in severe restrictions to the international trade and an outbreak in FMD-free countries can cause billionaire losses. Vaccination programs in endemic countries or those FMD free with vaccination

consider annual or semi-annual vaccination with conventional, inactivated vaccines. The policy of applying vaccination to respond to incursions in FMD free countries, known as "vaccination to live," gained acceptance as a result of public questioning after the slaughter of millions of animals due to large outbreaks in Europe in 2001 (2, 3). Vaccination is useful in many possible scenarios: for prevention or control of an outbreak in FMD-free areas and for the control of the disease in endemic regions.

The currently marketed inactivated virus vaccine consists of chemically inactivated virus formulated, in most of the cases, in oil-based adjuvant (4). For this type of vaccines, baby hamster kidney-21 (BHK-21) cells are grown in large scale bioreactors and then are infected with a specific Foot and Mouth Disease Virus (FMDV) strain. The virus obtained after this process is inactivated with binary ethyleneimine (BEI) and after purification can be used for vaccine formulation. Although the inactivated vaccines are effective to control the disease, they have many disadvantages that have prompted the development of novel vaccines (5, 6). These disadvantages include the need of costly biosafety facilities that require constant investment in manufacturing plant up-grades and qualified personnel as well as strict controls to eliminate the possibility of viral incomplete inactivation. In addition, rigorous purification steps during production are needed to avoid the presence of FMD viral non-structural proteins in the final formulation and thus later be able to differentiate between infected and vaccinated animals. Another important issue is that related to the policies of regulatory sanitary agencies across many FMD-free regions and countries, which restrict the production of growing live virus in their mainland. Finally, other key obstacles include the high variability of the virus and the lack of cross protection between serotypes or in some cases between members of the same serotype.

FMDV is a non-enveloped positive-sense single-stranded RNA virus, member of the Picornaviridae family and genus Aphthovirus (7). FMDV is classified into seven serotypes: A, O, C, SAT1, SAT2, SAT3, and ASIA1, and into many subtypes within each serotype (8). FMDV structural proteins are encoded by the polyprotein P12A and assembled to form the icosahedral capsid after protease 3C cleavage of P12A into mature VP0, VP3 and VP1. Finally, after RNA encapsidation, VP0 is cleaved into VP2 and VP4 and the infectious viral particle is assembled. One copy of each structural protein forms a protomer (5S), five protomers (12S) form a pentamer and twelve pentamers form the empty capsid (75S). Most of the novel developments in FMD vaccines are based on recombinant empty capsids (also referred as virus-like particles, VLPs). These VLPs are promising alternative antigens for vaccine development because they mimic the viral structure and have the complete repertoire of epitopes in a particulate and repetitive form but lacking the infectious RNA (9). Many expression systems and recombinant strategies, including baculovirus expression in cells and larvae, bacterial expression using SUMO technology, DNA vaccines and viral vector vaccines -especially using adenovirus-, have been developed to produce a novel FMD vaccine based on VLPs (10). Moreover, several researchers have already demonstrated the immunogenicity of this recombinant structures produced in various expression systems and with different strategies (11–18). Among these strategies, the most advanced technologies include the use of a baculovirus expression system in insect cells and adenovirus vector vaccines. Using the baculovirus expression system, a novel mutation in the VP2 sequence of FMDV serotype A has been reported to produce thermostable VLPs which is especially helpful in some developing countries where maintaining the cold chain for vaccine distribution can be complicated (14). Regarding viral vector vaccines using adenovirus technology for serotype A, there is already a license in the USA for emergency use in case of an outbreak (5, 18, 19). We have previously demonstrated that VLPs based on A2001 Argentina strain, produced using transient gene expression (TGE) in suspension-growing cells, were able to elicit an immune response in a mouse model with a 100% protection after viral challenge, a response comparable to the one obtained using a similar amount of inactivated virus (20). We have also recently demonstrated the immunogenicity of FMDV VLPs in cattle (21).

The traditional way of producing recombinant proteins in mammalian cells is the development of stable cell lines (22, 23). However, toxic proteins like protease 3C do not allow the development of stable cell lines. In this context, TGE is a simple and fast technology for the production of recombinant proteins, which was developed to produce mg of proteins for the first steps in clinical trials and thus represents the strategy of choice for mammalian cell expression of toxic proteins (24). In the case of FMD novel vaccines, TGE has some advantages because the process itself is quite similar to the current production of the inactivated virus but the preparation of viral seeds and infection of cells are replaced by plasmid production and transfection, respectively. The possibility of fast cloning the P12A sequence of different serotypes into the expression plasmid makes TGE a promising technology for the development of a novel vaccine against FMD, especially in emergency scenarios where fast responses are required.

Although many of the efforts made to develop a novel VLP-based vaccine for FMD have shown promising results, the challenge is still to have a novel vaccine that is as effective as the currently marketed inactivated one but produced with simple and scalable technology to encourage the tech-transference steps required to move from bench to market. These key aspects of novel strategies are scarcely reported. Considering that it is now generally accepted that VLPs are the best recombinant antigens to produce a novel vaccine against FMD, it is desirable to show the simplicity and scalability of the different recombinant technologies in order to make them attractive to the industry. Thus, the aim of this work was to make an effort in this regard by moving forward to increasing the yield of VLPs from TGE by using codon-optimized plasmids and assaying different DNA:polyethylenimine (PEI) concentrations and cell densities. We also assayed direct and indirect protocols, evaluated the addition of an antiapoptotic gene in the transfection mixture, and focused on the reproducibility and scalability of the already reported technology. Finally, we performed vaccine formulation

assays to study the antigen integrity and stability throughout this process.

MATERIALS AND METHODS

Cells and Viruses

The human embryonic kidney 293 cell line stably expressing a truncated Epstein–Barr virus Nuclear Antigen-1 (293-6E cells) was grown in suspension in serum-free F17 medium (Gibco), as previously described (20). Cells were grown in a humidified incubator at 37°C with 5% CO_2, with agitation at 120 rpm.

Plasmids

FMDV DNA sequences were cloned under the CVM promoter in the pTT5 vector (25). The pTT5-P12A3C plasmid encoding wild type sequences from FMDV A2001 Argentina strain was previously constructed (20). The P12A sequence from FMDV A2001 was codon-optimized for mammalian expression by GenScript (**Supplementary Data**) and the synthetic gene encoding P12A was subcloned in the pTT5 vector with protease 3C in tandem by digesting the pTT5-P12A3C wild type with the restriction enzymes NheI and BstBI. A Kozak sequence was included immediately upstream the start codon. pTT22-hAktDD encoding a constitutively active Akt mutant was co-transfected as described previously (23, 26). *Escherichia coli* (DH5α) grown in Circle Grown medium (MP Biomedicals, Solon, OH, USA), supplemented with 50 μg/mL ampicillin was used for plasmid production. The plasmid was purified using MAXI prep columns (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The A260/A280 ratios were measured and only plasmid preparations with ratios between 1.75 and 2.00 were used.

Production of Recombinant VLPs

293-6E cells were transfected using polyethylenimine (LPEI-MAX) (Polysciences, Warrington, PA, USA), as previously reported (20). Briefly, cells were seeded 2 days before transfection at 0.45–0.5 × 10^6 cells/mL. The day of transfection, viability and cell densities were determined. Only cells with viability >95% and densities between 1.5 and 2 × 10^6 cells/mL were transfected. For the indirect protocol: the plasmid and PEI were diluted in complete medium and PEI was added to the DNA dilution and mixed. After incubation at room temperature for 3 min, the DNA:PEI mixture was added to the culture. For the direct protocol: the plasmid and PEI were diluted in complete medium and added to the cell cultures (27). Shake flasks of different volumes (125, 250, 500 mL, and 1 L) and a 10 L Bioreactor were used for the transfection. Cells were harvested, centrifuged and resuspended in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% Thesit, 0.5% NaDeoxycholate). The use of PEI for transfection may be covered by existing intellectual property rights, including the US Patent 6,013,240, the European Patent 0,770,140, and foreign equivalents, for which further information may be obtained by contacting licensing@polyplus-transfection.com.

Cell Counts

Cell density and cell viability were measured using an automated cell counter, Cedex Analyzer, based on the trypan blue exclusion method (Roche, Laval, Qc).

Recombinant Protein Analyses

For Western Blotting analysis, lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred onto a polyvinylidene difluoride membrane, blocked and then incubated with anti-FMDV guinea pig serum (1/500) produced in house using wild type inactivated FMDV A2001 Argentina strain. After several washes, membranes were incubated with horseradish peroxidase-conjugated anti-guinea pig goat serum (1/1,000) (KPL). The reaction was visualized with an enhanced chemiluminescence method in a GBox (Syngene). The VLPs were quantified by enzyme-linked immunosorbent assay (ELISA). For coating of microtiter plates (Maxisorp), a polyclonal anti-FMDV serum made in rabbit (1/3,000) was diluted in carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C overnight. The washing steps were done with phosphate-buffered saline (PBS) 0.1% Tween-20 and blocking for 30 min at 37°C with 5% normal equine serum in PBS 0.1% Tween-20. Samples were incubated at 37°C for 1 h. For the generation of standard curves, known amounts of inactivated FMDV were serially diluted and added to the wells. Plates were then incubated for 1 h with a polyclonal anti-FMDV serum made in guinea pig (1/3,000), followed by horseradish peroxidase-conjugated anti-guinea pig goat serum (KPL). Then, tetramethylbenzidine was added and, 5 min later, the reaction was stopped with sulfuric acid 12%. Absorbance at 450 nm (A450) was recorded in a microplate reader (Thermo Scientifics MultiskanFC). For gradient assembly, 1 mL of 45, 35, 25, and 15% sucrose solutions (W/V) was added to ultracentrifuge tubes Ultra-Clear tubes 1/2 × 2 in (13 × 51 mm). The most concentrated solution was located at the bottom of the tube and the most diluted at the top. Samples were added on top of the gradient. The tubes were centrifuged in a Beckman Optima-LP X-100 Ultracentrifuge using a SW 55 Ti rotor for 2 h at 45,000 rpm at 4°C, acceleration: 9, deceleration: 9. Once the centrifugation was completed, 0.5 mL aliquots were collected. Aliquots were tested for ELISA-specific FMDV protein. Both the gradient assembly and the sample collection were performed manually.

Vaccine Formulation

Cells were harvested, centrifuged at 4,000 g and the supernatant was discarded. Pellets were resuspended in Tris-salt buffer and subjected to three freeze-thaw cycles at −80/25°C. Finally, the lysate was clarified by centrifugation and VLPs were quantified by ELISA as described above.

For the vaccine formulation, a water-in-oil (W/O) single emulsion was prepared by adding the formulated aqueous phase containing the VLPs to the oily phase containing mineral oil and emulsifier agents. The mixture was then emulsified using an UltraTurrax homogenizer for 8 s at full speed. The particle size distribution of the final emulsion was analyzed by laser diffraction using the Mastersize 2000 (Malvern). To obtain the aqueous phase for analysis, the emulsion was disrupted by adding

1 volume of chloroform in a 15 mL tube. The blend was mixed gently up and down for 1 min and centrifuged for 10 min at 4,000 rpm. The aqueous phase was collected and the procedure of adding chloroform was repeated. The VLPs in the recovered aqueous phase were evaluated by sucrose gradient and quantified by ELISA as described above.

RESULTS

Optimization of FMDV VLPs

First, we compared the VLP yield obtained using wild type viral sequences and codon-optimized synthetic sequences for mammalian cells encoding for FMDV proteins. The expression levels achieved with the optimized sequence were slightly higher than those achieved with the wild type sequence, although the codon adaptation index changed from 0.74 to 0.95 (data not shown). So, after that, all the experiments shown were performed with the codon-optimized sequence.

To further optimize the VLP yield achieved by TGE, we evaluated different DNA:PEI concentrations in both direct and indirect protocols, using the pTT5-P12A3C plasmid. Also, considering the toxic effects of protease 3C on cells, we tested the effect of the addition of an antiapoptotic gene (AKT) in the transfection mixture (**Figure 1A**) (23, 28). Among all

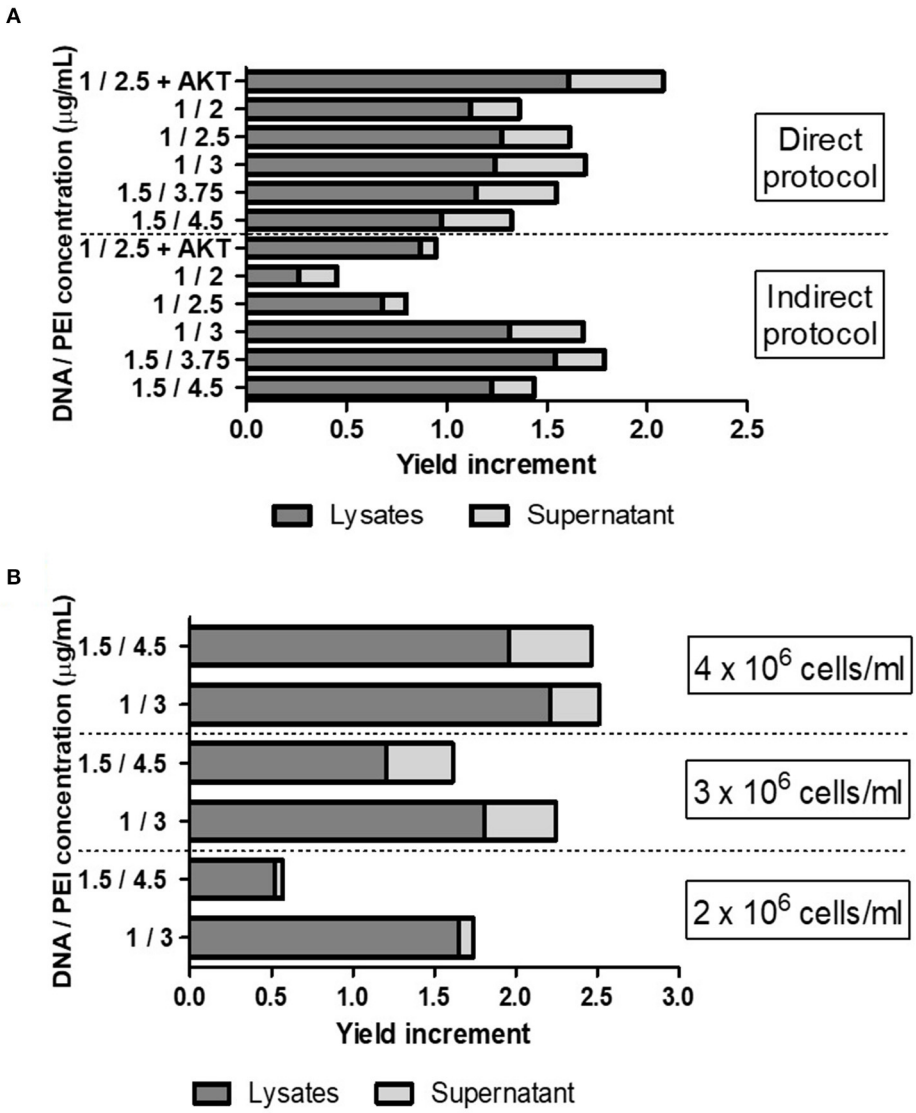

FIGURE 1 | (A) Evaluation of different DNA:PEI concentrations (µg/ml) in both direct and indirect protocols and effect of the addition of an antiapoptotic gene AKT in the transfection mixture. **(B)** Evaluation of different cell densities using the pTT5-P12A3C plasmid plus AKT, using different DNA:PEI concentrations. Cell cultures with 3 and 4 × 10^6 cells/mL were produced using cultures with a cell density of 2 × 10^6 cells/mL and centrifugation steps before transfection. The increase in VLP yield was measured by Western Blotting analysis of the VP0 band intensity, comparing each transfection with the transfection condition previously published in Mignaqui et al. (20).

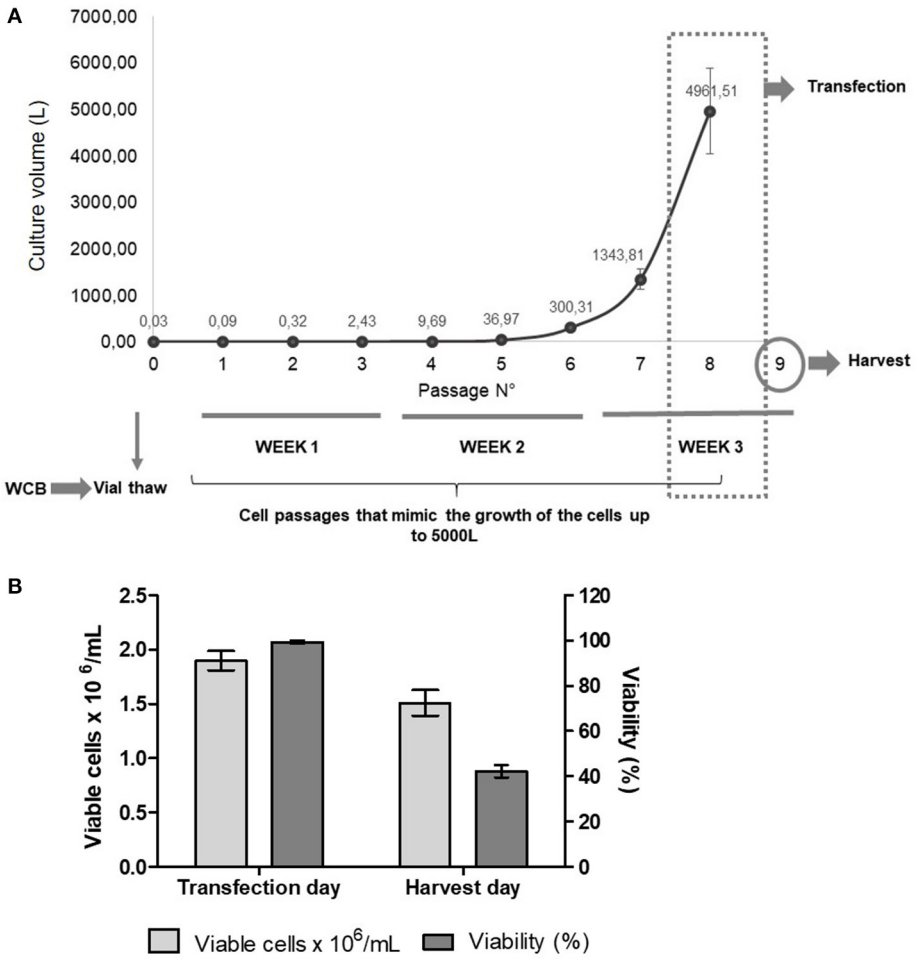

FIGURE 2 | (A) Experimental design of the reproducibility assay. After vials were thawed, cells were grown for 3 weeks, mimicking the passages needed to achieve a volume of around 5,000 L. After 7 passages, cells were transfected and, at 48 hpt, cells were harvested for protein analysis. WCB, Working cell bank. **(B)** Average of viable cells $\times$ 10^6/mL and viability (%) of cell cultures on the day of transfection and on the harvest day (48 hpt).

the conditions evaluated, the highest increase in yield was around 2-fold higher than that obtained by the use of the pTT5-P12A3C plasmid in previously reported conditions and was achieved when the antiapoptotic gene was added in the transfection mixture. Overall, indirect transfection protocols yielded higher recombinant VLPs levels. However, using a direct protocol with a DNA:PEI mixture of 1.5:3.75 µg/ml we obtained 50% of increase in the VLP yield per mL of cell culture. The advantage of the direct protocol is that the DNA:PEI mixture step is avoided because the procedure implies the direct addition of DNA and PEI to the cell culture.

Then, we evaluated different cell densities and different DNA:PEI ratios and used a combination of pTT5-P12A3C plus 15% of a plasmid encoding AKT for the transfection, which allowed us to further increase the recombinant VLP yield (**Figure 1B**). Indeed, the highest yield was achieved when the highest number of cells was used.

Reproducibility and Scalability of FMDV VLPs

To study the reproducibility and scalability of our technology, we prepared a working cell bank with vials with 10 million cells per mL. Then, independent assays were performed by starting cell cultures using four cryovials of the working cell bank. After thawing the cryovials, cells were grown for three weeks at low scale (final volume 50 mL), mimicking the cell passages needed to achieve a 5,000 L culture (**Figure 2A**). Only cells from one vial were also grown to different volumes: 20, 50, 200, 500 mL, and 10 L. Moreover, we performed the transfection by using independent DNA:PEI mixtures. Cell counts and viability were recorded during cell growth, on the day of transfection, and on the harvest day (**Figure 2B**). VLP expression was analyzed by ELISA, Western Blotting and sucrose gradient (**Figures 3A,B**). The growth curves and viability of the different cultures during cell passages during the 3-week period were similar to each other (data not shown). On the transfection day, cell density

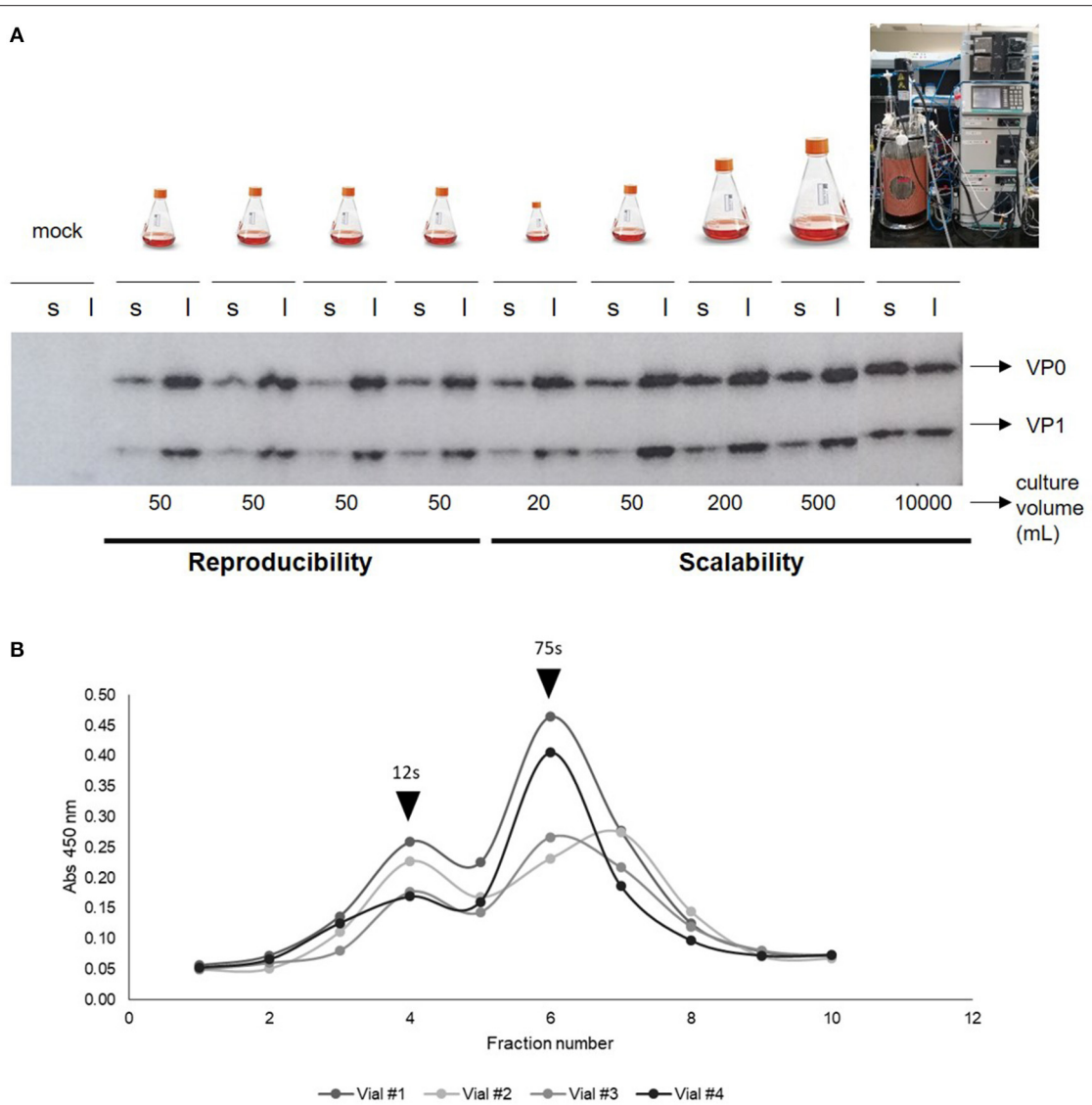

FIGURE 3 | (A) Western Blotting of cell lysates (l) and supernatants (s) after 48 h of independent transfections of different culture volumes (10 L, 500, 200, 20, and 50 mL) with FMDV-encoding plasmids. Primary antibody: anti-FMDV polyclonal serum made in guinea pig (1/500) and secondary antibody: anti-guinea pig peroxidase (1/10,000). **(B)** Cell lysates of cultures of independent transfections of 50 mL at 48 hpt analyzed by sucrose gradient to evaluate VLPs formation. Black arrows indicate 12s or 75s peak, corresponding to structural proteins assembled in pentamers or in complete empty capsids, respectively.

was between 1.5 and 2 $\times$ 10^6 cells/mL and viability >95%, respectively, in all cell cultures. When cells were harvested at 48 h post-transfection (hpt), cell density and viability were similar in all the flasks, with a viability of 42% in average, which is very low and confirms the toxic effects of FMDV proteins, especially protease 3C, on cells. When 20 or 50 mL were transfected, the distribution of VLPs between the supernatant and the lysate was around 20 and 80%, respectively. However, when the transfection volume increased, the specific protein detected in the supernatant also increased, being around 60% in the 10 L bioreactor. Probably, the shear stress in the 10 L bioreactor was stronger than in the 50 mL transfection, thus, if the bioreactor is used, the harvest time should be further optimized. Interestingly, VLP formation was reproducible in the four 50 mL transfections, as determined by sucrose gradient analysis, with more than 92 $\pm$ 8% of the structural proteins assembled in VLPs. Overall, we were able to demonstrate high reproducibility and scalability of the technology for the production of FMDV VLPs. The maximum recombinant protein yield in cell lysates was measured by ELISA being 7 $\pm$ 0.6 mg per liter of cell culture.

Vaccine Formulation and Antigen Stability

To evaluate the stability of the antigen after the process of vaccine formulation, we prepared a W/O emulsion containing 25 μg of VLPs per dose of vaccine. Then, the emulsion was disrupted by the addition of chloroform, and the VLPs present

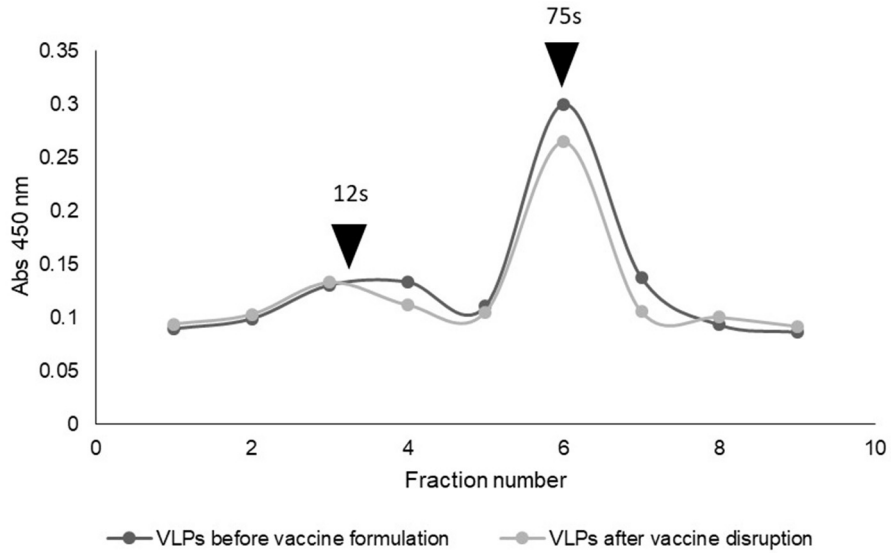

FIGURE 4 | Sucrose gradients showing antigen stability of VLPs within a water-in-oil emulsion vaccine after disruption.

in the recovered aqueous phase were analyzed by sucrose gradient and quantified by ELISA (**Figure 4**). The percentages of complete empty capsids (75s) and pentamers (12s) were calculated. Immediately after the vaccine formulation and the subsequent disruption of the emulsion, 84% of the antigen was present as complete empty capsids. Thus, we were able to demonstrate that a high proportion of the VLP antigen is stable after the emulsification procedure of vaccine formulation.

DISCUSSION

FMD, one of the most devastating diseases of livestock, can cause significant economic losses worldwide, and represents the most important limitation to international trade in live animals and animal products. Although the traditional inactivated vaccine has been proved effective, the development of a novel FMD vaccine that is effective, safer and less expensive than traditional vaccines could be of great value. The feasibility of a tech transfer to occur in the veterinary vaccine technology field after a novel vaccine has proven to be effective relies mostly on the simplicity, scalability and cost-effective properties of the proposed technology. Regarding FMD, many efforts have been made to develop a novel vaccine that overcomes the need of producing huge amounts of growing live virus in costly biosecurity facilities (5, 6, 10). To move from bench to market, the efforts made to develop a novel vaccine against FMD must focus in production aspects related to regulatory concerns, scalability aspects, and cost-effective technology. Here, we focused in the yield of recombinant VLPs, the production procedure, and the reproducibility and scalability of the technology. Although technical details are necessary for an efficient transfer to large-scale production, there are few reports that address them. Most of the efforts have been made to demonstrate that VLPs are the antigens of choice for a novel vaccine for FMD and currently there is relevant evidence that demonstrates that their particulate structure and the complete presence of viral epitopes make them as immunogenic (or almost as immunogenic) as the virus (11–18).

The technology here described could be a great response for emergency vaccines. When an outbreak occurs, the genomic analysis of the viral strain responsible for the outbreak would be enough to start the production of VLPs after a synthetic gene is ordered and cloned into the expression plasmid. After plasmid production by *E. coli* transformation and purification by anion exchange chromatography, cells are transfected, and VLPs are harvested at 48 hpt, The technology allows the optimization of different aspects like the DNA:PEI ratio or the cell density, and the use of direct protocols not only to increase the yield but also to have a more convenient process. Here, we demonstrated that direct transfection protocols can be used, which is a great advantage in case large volumes are transfected because they avoid the step of forming DNA:PEI polyplexes outside the bioreactor (27). Generally, these polyplexes are made in 5–10% of the final volume transfected. So, if the final volume of transfection is 10 L, the polyplex mixture will range between 0.5 and 1 L. Performing the mixture in a flask up to that volume can be possible but doing so with higher volumes would require an extra reactor. In the present study, increasing cell density was effective in increasing the VLP yield. However, the need to concentrate the cell cultures by a centrifugation step is not suitable for large-scale processes. Another key element that should be further studied is the reproducibility of the amount of VLPs produced. In the four independent experiments here performed, we demonstrated that the amount of VLPs obtained was high. There is little information about this key point in other reported technologies.

All novel reported recombinant technologies avoid the need of growing live FMDV with the reduced need of biosecurity

level in the production facilities. However, the issues related to the high variability of the virus have not yet been solved. Interestingly, in the case of TGE, the cloning of P12A sequences of different FMDV into the pTT5 vector can be easily achieved to produce different FMDV serotypes. TGE also allows solving problems related to the adaptation of the virus to the cell culture but further research should be done to demonstrate the recombinant expression of more serotypes. Each technology for VLP production has advantages and disadvantages and thus each could be useful in different situations considering the complex scenario of FMD. Here, we demonstrated the robustness of TGE and the many possibilities that can be used to improve the technology to optimize the yield and make it suitable for technology transfer. To go further, we were able to demonstrate that a high proportion of VLPs produced by this technology was stable after being subjected to the emulsification process of water-in-oil vaccines, which is the type of formulations usually used for traditional inactivated FMD vaccines. Although some decrease in the integrity of VLPs was observed after rupture of the emulsion, it must be considered that this procedure can be quite aggressive, and maybe the decrease in VLPs can be an artifact of the techniques used for the analysis. Further studies in target species must be performed in order to continue with the characterization of the vaccine. Overall, these results suggest the potential use of VLPs produced as here described for the development of a next generation vaccine for FMD control.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AM: experimental design, fulfillment, analysis and interpretation of the results, production, characterization, purification and quantification of FMDV empty capsids, and manuscript drafts. BC, LM, DL'A, and LB: transient gene expression experiments and critical revision of the manuscript. AF, CS, RS, and SC: VLPs characterization and critical revision of the manuscript. YD and AW: conception of the work, experimental design, and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00601/full#supplementary-material

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frontiers in Veterinary Science

ORIGINAL RESEARCH
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Novel Foot-and-Mouth Disease Vaccine Platform: Formulations for Safe and DIVA-Compatible FMD Vaccines With Improved Potency

John M. Hardham[1†], Peter Krug[2], Juan M. Pacheco[2], James Thompson[1], Paul Dominowski[1], Veronique Moulin[1], Cyril G. Gay[3], Luis L. Rodriguez[2] and Elizabeth Rieder[2*†]*

[1] Zoetis Inc, Kalamazoo, MI, United States, [2] United States Department of Agriculture (USDA) Plum Island Animal Disease Center, Agricultural Research Services, USDA, Greenport, NY, United States, [3] United States Department of Agriculture (USDA) Office of National Programs, Agricultural Research Services, Beltsville, MD, United States

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Edited by:
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Animal and Plant Quarantine Agency, South Korea

***Correspondence:**
John M. Hardham
john.m.hardham@zoetis.com
Elizabeth Rieder
elizabeth.rieder@usda.gov

[†] Co-lead authors

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Inactivated, wild-type foot-and-mouth disease virus (FMDV) vaccines are currently used to control FMD around the world. These traditional FMD vaccines are produced using large quantities of infectious, virulent, wild-type FMD viruses, with the associated risk of virus escape from manufacturing facilities or incomplete inactivation during the vaccine formulation process. While higher quality vaccines produced from wild-type FMDV are processed to reduce non-structural antigens, there is still a risk that small amounts of non-structural proteins may be present in the final product. A novel, antigenically marked FMD-LL3B3D vaccine platform under development by Zoetis, Inc. and the USDA-ARS, consists of a highly attenuated virus platform containing negative antigenic markers in the conserved non-structural proteins $3D^{pol}$ and 3B that render resultant vaccines fully DIVA compatible. This vaccine platform allows for the easy exchange of capsid coding sequences to create serotype-specific vaccines. Here we demonstrate the efficacy of the inactivated FMD-LL3B3D-A_{24} Cruzeiro vaccine in cattle against wild-type challenge with A_{24} Cruzerio. A proprietary adjuvant system was used to formulate the vaccines that conferred effective protection at low doses while maintaining the DIVA compatibility. In contrast to wild-type FMDV, the recombinant FMD-LL3B3D mutant viruses have been shown to induce no clinical signs of FMD and no shedding of virus in cattle or pigs when inoculated as a live virus. The FMD-LL3B3D vaccine platform, currently undergoing development in the US, provides opportunities for safer vaccine production with full DIVA compatibility in support of global FMDV control and eradication initiatives.

Keywords: FMD (foot and mouth disease), DIVA, potency, efficacy, diagnostic, vaccine platform, rapid, effective

INTRODUCTION

Foot and Mouth Disease Virus (FMDV) is the causative agent of a highly contagious disease that affects pigs, cattle, sheep, goats, buffalos, and other cloven-hoofed animals. The disease causes severe production losses and disrupts a wide range of agricultural, industrial, and social activities. The FMD status of a country represents the single largest barrier to trade in the agricultural sector. Estimates of the annual economic impact of FMD in endemic countries range from $6.5 to $21

billion, while the economic impact of an FMD incursion in an FMD-free country are >$1.5 billion per year (1–3).

An incursion of FMD in North America represents the single largest risk to the agricultural sector (1, 3, 4). According to the National Pork Producers Council (5), an FMD outbreak in the United States would immediately stop all export markets for U.S. pork and beef. As the export market represents ∼25% of total US pork production, the outcome would be devastating to the US pork industry. Additional follow-on impacts would also result for the corn and soybean markets and have additional negative impacts to related industries (such as food processing plants, food distribution, and restaurants). The impact to the U.S. economy over 10 years is estimated to be over $128 billion for the beef and pork sectors, $44 billion for the corn sector, and $25 billion for the soybean sector with an additional loss of ∼1.5 million jobs (5, 6).

Due to the risk of research on or FMD vaccine production with wild-type, virulent FMD strains, the United States has restricted the presence of the wild-type FMD viruses to only Plum Island Animal Disease Research Center in New York. This restriction has caused the United States to be reliant on overseas production of FMD vaccines. Roth and Spickler (7) stated that the United States should "seek USDA licensure of new technology FMD vaccines that could be safely manufactured in the U.S. and which are based on a platform that allows various capsid serotypes/topotypes to be inserted into the vaccine. These would then be candidates for vendor managed inventory of finished vaccine and of vaccine antigen concentrate (VAC)".

There are currently seven immunologically distinct serotypes of FMDV that contain 60 topotypes. These serotypes, with the exception of serotype C which has not been detected in the field since 2004, circulate in seven recognized "pools" around the globe (8). The genetic and antigenic diversity of FMDV strains results in challenges with vaccine matching for effective FMD control (9). This situation leads to the necessity to maintain specific vaccines for each region. Traditional FMD vaccines (monovalent and multivalent) are comprised of virulent, wild-type viruses chemically inactivated and formulated with adjuvants. These vaccines confer protection from clinical signs of FMD caused by FMDVs closely related to the vaccine strain. However, the traditional FMD vaccines have several challenges and limitations (10–12).

There are four major drawbacks of traditional FMD vaccines that are currently commercially available. First, large quantities of infectious, virulent FMD virus are necessary to produce vaccine antigen, with the associated risk of virus escape from manufacturing facilities or incomplete inactivation during the vaccine formulation process. Therefore, traditional inactivated FMD vaccines must be manufactured in expensive biocontainment facilities utilizing virulent FMD strains. The typical volumes of culture fluids range between 1,000–5,000 liter. The associated risk of escape from the manufacturing facilities is a key reason why many countries restrict FMD vaccine production to only local endemic strains. There have been several examples of the virulent FMD viruses escaping from manufacturing facilities and causing widespread FMD outbreaks (13–16). Second, the vaccine strain must antigenically match to the wildtype FMDV responsible for the outbreak as standard vaccines may provide little or no cross-protection against different strains even within a serotype (17). High potency (emergency use) vaccines may remediate this issue somewhat (18, 19). Third, there are challenges associated with differentiating infected from vaccinated animals (DIVA) when using traditional FMD vaccines in order to serologically discern infected animals and vaccinated animals. Small amounts of residual non-structural proteins may still be present in traditional FMD vaccines, resulting in some animals with false positive results, especially if multiple revaccinations are required due to the inherent short duration of immunity of conventional vaccines (20–26). Fourth, traditional FMD vaccines may not fully protect animals from persistent infection (10, 27–32). In a 2016 study by Stenfeldt *et al.* (30), it was shown that neoteric [new or temporally acute (32)] subclinical infection or persistence resulted following challenge in similar percentage of vaccinated and non-vaccinated animals (62% in vaccinated cattle, 67% in non-vaccinated cattle), indicating that vaccination with traditional vaccines has little impact on the carrier state (30, 33, 34).

In the United States, FMDV is only one of two animal pathogens on the Select Agent List (34) requiring additional security measures. Furthermore, current U.S. law (21 U.S. Code § 113A) states that no live virus of foot-and-mouth disease may be introduced for any purpose into any part of the mainland of the United States. These U.S. regulations and restrictions create challenges for FMDV research along with the discovery, development, and manufacture of FMD vaccines.

For these reasons the search for alternative vaccines has been a focus of extensive research for decades. To address some of the above limitations of traditional FMD vaccines, Zoetis Inc. and the United States Department of Agriculture—Agricultural Research Services have jointly developed a safer next generation marker FMD Vaccine platform that utilizes a proprietary adjuvant system. The vaccine platform consists of an attenuated FMD A_{24} Cruzeiro virus that has been modified in three ways; (1) a 543-bp deletion of the FMDV leader sequence resulting in the complete attenuation of the FMD A24 Cruzeiro virus, (2) insertion of two unique restriction enzyme sites that flank the capsid coding region to accommodate swapping capsid coding cassettes, and (3) negative antigenic markers engineered into the non-structural proteins 3B and $3D^{pol}$ (35). In this study, we describe protective immune responses in cattle and DIVA capabilities after vaccination with the novel FMD-LL3B3D A_{24} Cruzeiro antigen formulated with a proprietary adjuvant. This vaccine platform allows for a rapid response capability by virtue of the easy exchange of capsid coding sequences using the unique restriction sites flanking the capsid coding region (**Figure 1**).

In contrast to the conventional vaccines produced with wild-type FMD viruses, the recombinant FMDV-LL3B3D platform vaccine viruses are fully attenuated as they induce no clinical signs of FMD and no shedding of virus in cattle or pigs when inoculated as a live virus (35,36, Pflaum, in preparation). As a result, this vaccine platform may use existing FMD vaccine manufacturing technology without the concerns associated with current FMD vaccine production where the risk of wild-type virus escape from the manufacturing site may cause an FMD

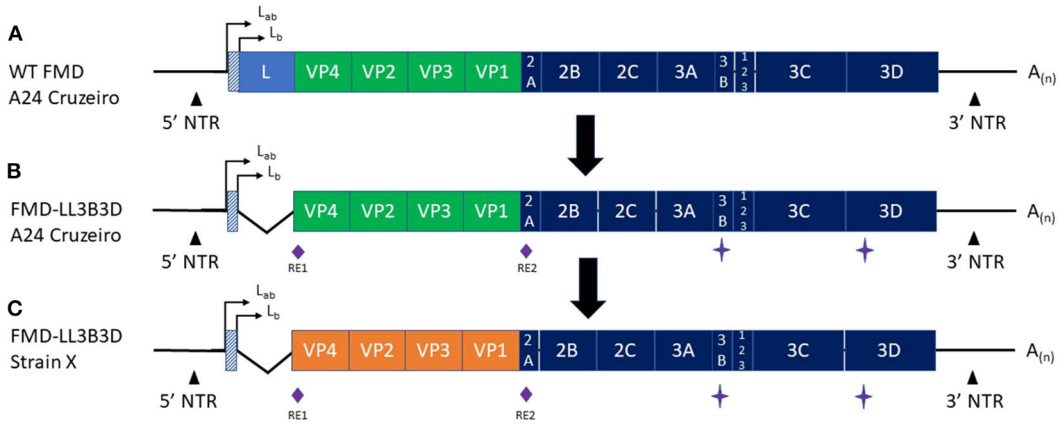

FIGURE 1 | Graphic representation of the **(A)** Wild-type FMD A_{24} Cruzeiro, **(B)** FMD-LL3B3D A_{24} Cruzeiro, and **(C)** FMD-LL3B3D X genomes. The green **(A,B)** and orange **(C)** segments represent the capsid coding regions. RE1 and RE2 represent two unique restriction enzyme sites that were engineered into the genome to facilitate swapping of the capsid coding region cassettes. The star symbol represents mutations introduced into the 3B and 3D genes to negatively mark the virus rendering them fully-DIVA compatible. The angled lines **(B,C)** represent the 543-bp deletion of the leader gene rendering the resultant viruses fully attenuated. The orange region in **(C)** represents that the capsid coding region from any FMD strain may be cloned into the platform to generate a vaccine strain targeting the new strain.

outbreak. The finished vaccine is formulated with a proprietary adjuvant system that induces robust humoral and cellular immune responses. The FMD-LL3B3D A_{24} Cruzeiro vaccine platform strain and a large number of capsid coding cassettes were excluded from the United States Select Agent Program regulations in April 2018 (37, 38). The FMDV-LL3B3D vaccine platform is currently under development with the goal of providing a high potency, fully DIVA compatible FMD vaccines manufactured in the United States. In this manuscript, we will discuss the preliminary vaccine safety and efficacy results and assessment of the DIVA compatibility.

MATERIALS AND METHODS

FMD-LL3B3D Vaccine Virus and Cells

The FMD-LL3B3D A_{24} Cruzeiro (also named $A_{24}LL3B_{PVKV}3D_{YR}$) vaccine virus was derived in baby hamster kidney cells (BHK-21) cell monolayers as previously described (35) following virus adaptation to BHK suspension cells (sBHK). The parental construct pA24Cru cDNA infectious clone, from which the FMD-LL3B3D A_{24} Cruzeiro strain was derived, was described by Rieder *et al.* (39). Suspension BHK (sBHK) cells in Celligen BLU bioreactor vessels (Eppendorf) were expanded in serum free media (Corning) with L-Glutamine and Gentamicin (Invitrogen). Parental A_{24} Cruzeiro FMDV challenge virus (39) was obtained from the Plum Island Animal Disease Center inventory and all uses of this select agent were in compliance with regulations detailing the requirements for possession, use, and transfer for USDA select agents mandated in 9 CFR parts 331 and 121. The cell line BHK-21 was maintained in MEM (Invitrogen) medium supplemented with 10% bovine serum (GE Healthcare), 10% Tryptose Phosphate (Teknova), 2 mM L-Glutamine (Invitrogen) and 1X Antimycotic/Antibiotic (Invitrogen).

The FMD-LL3B3D vaccine platform allows derivation of other relevant strains through the use of unique restriction enzyme sites flanking the capsid coding sequence. Standard molecular biology techniques are used to exchange the capsid coding region of the FMD-LL3B3D A_{24} Cruzeiro infectious plasmid with that of other FMD strains. The resultant infectious plasmids were linearized, transcribed into RNA, and then transfected into BHK-21 cells as previously described (35).

FMD-LL3B3D Antigen Production and Vaccine Formulation

Suspension BHK-21 cells were inoculated with the FMD-LL3B3D A_{24} Cruzeiro virus at a multiplicity of infection of 0.001 in a Celligen BLU bioreactor vessel (Eppendorf/New Brunswick) using optimal growth conditions determined previously and then virus was harvested when values of cell viability reached end points under 20%. Parameters such as pH, temperature, aeration rate and viable cell number were monitored.

The FMD-LL3B3D A_{24} Cruzeiro vaccine antigen was harvested from infected sBHK, clarified by filtering cell debris on successive capsule filters (Pall Corporation), concentrated on Hollow Fiber columns (GE Healthcare) and subjected to two chemical inactivation processes following the standard BEI inactivation protocol for vaccine preparation (40) using a 10 mM solution of 2-bromethylamine hydrobromide (BEA) in 0.7% NaOH. Virus was exposed to BEI at 25°C for up to 24 h per inactivation step. Neutralization of BEI was achieved by addition of 2% Sodium Thiosulfate (W/V). Inactivation kinetics were monitored by standard plaque assay in BHK-21 monolayers (data not shown). Complete inactivation of the bulk antigen was confirmed by a sterility assay in which three blind passages of undiluted material in BHK-21 cell monolayers were monitored for viral growth. For each passage, undiluted and diluted BEI-inactivated virus was used to infect BHK-21 monolayers followed by incubation for at least 72 h at 37°C. Vaccine antigens were stored in aliquots at −70°C until use. Specific concentrations of inactivated vaccine antigen were mixed

with either Zoetis proprietary adjuvant or prepared as a water-in-oil-in-water (WOW) emulsion with Montanide ISA 206 (Seppic Paris) according to the manufacturer's instructions. The integrity of 146S particles and antigen concentration present in the formulated vaccines were determined by using 10–30% sucrose density gradients and 260 nm densitometry as previously described (35, 41, 42).

Preliminary Vaccine Efficacy Study

All cattle experiments were performed in the BSL3Ag FMDV research facility at the Plum Island Animal Disease Center (US Department of Agriculture, Agriculture Research Services). These studies were conducted in compliance with the Animal Welfare Act (AWA), the 2011 Guide for Care and Use of Laboratory Animals, the 2002 PHS Policy for the Humane Care and Use of Laboratory Animals, and U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (43), as well as specific animal protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Animal Disease Center (USDA/APHIS/AC certificate number 21-F-0001).

Holstein steers, weighing between 250 and 300 kg were identified with ear tags and housed for a week of acclimation prior to vaccination. Seven bovines in treatment groups T02 and T03 were vaccinated with the indicated dose of BEI-inactivated FMD-LL3B3D virus formulated with either ISA 206 adjuvant (T02) or Zoetis proprietary adjuvant (T03) intramuscularly (IM) in the neck. Four bovine control animals were vaccinated with sterile phosphate-buffered saline (PBS). Immediately before and at indicated times after vaccination, blood was taken for serum analysis of FMDV-specific neutralizing antibodies. On day 21 post vaccination (dpv), all cattle were challenged with homologous, wild-type FMD A_{24} Cruzeiro intradermolingually (IDL) with 10^4 50% bovine tongue infectious doses ($BTID_{50}$) according to OIE guidance. The animals were monitored at 0, 4, 7, and 10 days post-challenge (dpc) for the appearance of localized and generalized lesions. Sera and temperature were collected daily. Clinical signs were scored as 1 credit for each affected foot, and presence of vesicles in the head was not considered due to lingual inoculation of challenge. FMDV RNA was measured in sera, by rRT-PCR as previously described (35, 44).

Preliminary PD_{50} Study

To further investigate the protective immune responses to formulated FMD-LL3B3D A_{24} Cruzeiro vaccine formulated with the Zoetis proprietary adjuvant system, Holstein steers from 6 to 8 months of age were randomly assigned to one of four treatment groups (with four bovine per group): T01 received phosphate buffered saline (PBS) and served as the negative controls. Three separate vaccine dose volumes of the full dose vaccine formulated with 8 μg of hollow-fiber concentrated inactivated FMD-LL3B3D A_{24} Cruzeiro antigen and the Zoetis proprietary adjuvant system were applied intramuscularly (T02-Full dose = 2.0 ml, T03-1/4 dose = 0.5 ml, and T04-1/16 dose = 0.125 ml). Twenty-one days post-vaccination, all vaccinated and naïve animals were inoculated by the IDL route with 10^4 $BTID_{50}$ of homologous, wild-type FMD A_{24} Cruzeiro. All cattle were followed for 3 weeks to assess development of clinical disease as expressed by fever, nasal secretion, salivation, loss of appetite and/or lameness and to examine the presence of viral RNA in probang samples.

Serology and Assessment of DIVA Compatibility

Serum samples from cattle in the naive (mock vaccinated) and vaccinated groups were tested for the presence of neutralizing antibodies against FMDV using a serum standard micro-neutralization test performed in 96-well plates (in quadruple replicas). End-point neutralizing titers were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of the corresponding FMDV in 50% of the wells (35, 45). The end point titer of the serum against homologous virus was calculated as the reciprocal of the last dilution of serum to neutralize 100 $TCID_{50}$ in 50% of the wells (46). Serum samples at indicated time points were tested for the presence of antibodies against FMDV non-structural proteins (NSPs) using three commercially available competitive 3ABC Enzyme-Linked Imunosorbent Assay (cELISA) kits following the corresponding manufacturer's protocol. The three test kits were the PrioCHECK FMDV NS Antibody ELISA (Thermo Fisher Scientific) (47), VMRD FMDV Antibody Detection Kit (48), and the SERELISA FMDV NSP Antibody Competition ELISA (Zoetis Inc.). Values are cited as means ± Standard Deviations. Cellular immune responses were measured using a cell proliferation assay with PBMCs from both healthy naive and vaccinated steers (49).

Statistical Analysis

Statistical differences of serum neutralization comparing vaccination groups was determined by 2-way ANOVA using the Tukey's multiple comparisons test in GraphPad Prism. Statistical differences of 21-day post infection non-structural protein seroconversion was determined using the unpaired *t*-test in GraphPad Prism.

RESULTS AND DISCUSSION

While traditional FMD vaccine formulations provide adequate safety and efficacy, there are still numerous challenges and gaps. Amongst these are use of virulent FMD strains, need for multiple doses, short duration of immunity, lack of prevention of persistence, and incomplete DIVA compatibility. In addition, timelines for development of traditional vaccines are not compatible with the need for rapid response to new or evolving FMD strains. To address these limitations, the USDA-ARS and Zoetis have developed the FMD-LL3B3D vaccine platform (**Figure 1**) that combines a safe and fully DIVA-compatible platform with rapid development of new inactivated FMD vaccines that are formulated with a proprietary adjuvant system which increases vaccine immunogenicity. The vaccine platform may be adapted to swap out the FMD capsid coding region while maintaining the safety and DIVA-compatibility capabilities.

Figure 1 depicts the generation of the FMD-LL3B3D vaccine platform from the wild-type FMD A_{24} Cruzeiro virus (**Figures 1A,B**) along with the ability to swap the capsid

coding region (**Figure 1C**). The deletion of the leader gene renders the resultant FMD-LL3B3D vaccine viruses completely attenuated (35). Multiple FMD-LL3B3D vaccine viruses have been assessed for live virus safety in both pigs and cattle (Pflaum, in preparation). By utilizing a non-replicating, attenuated vaccine platform, events such as outbreaks associated with manufacturing facility escape (50) may be avoided. Traditional FMD manufacturers are typically limited to producing only those FMD strains that are endemic to the region. The FMD-LL3B3D vaccine platform affords the opportunity to produce global FMD vaccines from a single or a few manufacturing sites, thus improving the economics of vaccine production.

Animals that recover from natural infection are protected longer than traditionally vaccinated animals, and this is likely due to the replication of the virus in the host's cells inducing a more complete TH1 and TH2 immune response against the virus. This includes the presence of the complete repertoire of FMDV non-structural proteins, which are normally depleted in traditional vaccines or not included in recombinant FMDV vaccines. The presence of these NSPs has been shown to contain epitopes for T-cell mediated immunity, which may enhance the response to the viral infection. However, the most common assays to detect the difference between infected and vaccinated animals (DIVA) target the presence or absence of antibodies against the NSPs, making the addition of NSPs to vaccine preparation untenable. The FMD-LL3B3D vaccine platform carries mutations in the 3D polymerase ($3D^{pol}$) and 3B non-structural proteins which act as negative markers to distinguish vaccination with this platform from natural infection, thereby making the exclusion of NSP from vaccine preparations unnecessary.

The FMD-LL3B3D vaccine platform is used to generate vaccine bulk antigen targeting a wide variety of FMD strains. The bulk antigen is blended with a proprietary vaccine adjuvant system that increases the immunogenicity of the finished product. With FMD and other antigens, the adjuvant system has been demonstrated to increase the humoral and cellular immune responses, shorten the onset of immunity, and increase the duration of immunity (unpublished data).

The vaccine efficacy of the FMD-LL3B3D-based vaccine formulations was demonstrated in cattle using a laboratory-based vaccine/challenge study (**Figure 2**). Animals were vaccinated with a single vaccine dose on day 0 and challenged via the intradermal lingual route with virulent, wild-type FMD on day 21. Clinical observations and clinical samples (serum and swabs) were taken over the next 14 days. Probang samples were taken from day 38 through day 52 to assess persistence of the challenge virus.

The serological and cellular immune responses to vaccination with a monovalent FMD-LL3B3D A_{24} Cruzeiro vaccine are shown on **Figure 3**. The serum neutralizing titer generated in response to vaccination with the FMD-LL3B3D A_{24} Cruzeiro vaccine formulated with the proprietary adjuvant system were statistically significantly higher than that generated using traditional commercial adjuvant at days 7, 14, and 21 (**Figure 3A**). In addition, the lymphocyte proliferation index, which represents the cellular immune response, were numerically increased in animals vaccinated using the proprietary adjuvant system vs. commercial-type adjuvant (**Figure 3B**).

Following virulent challenge on day 21, clinical observations (temperature and clinical signs) were taken through day 31.

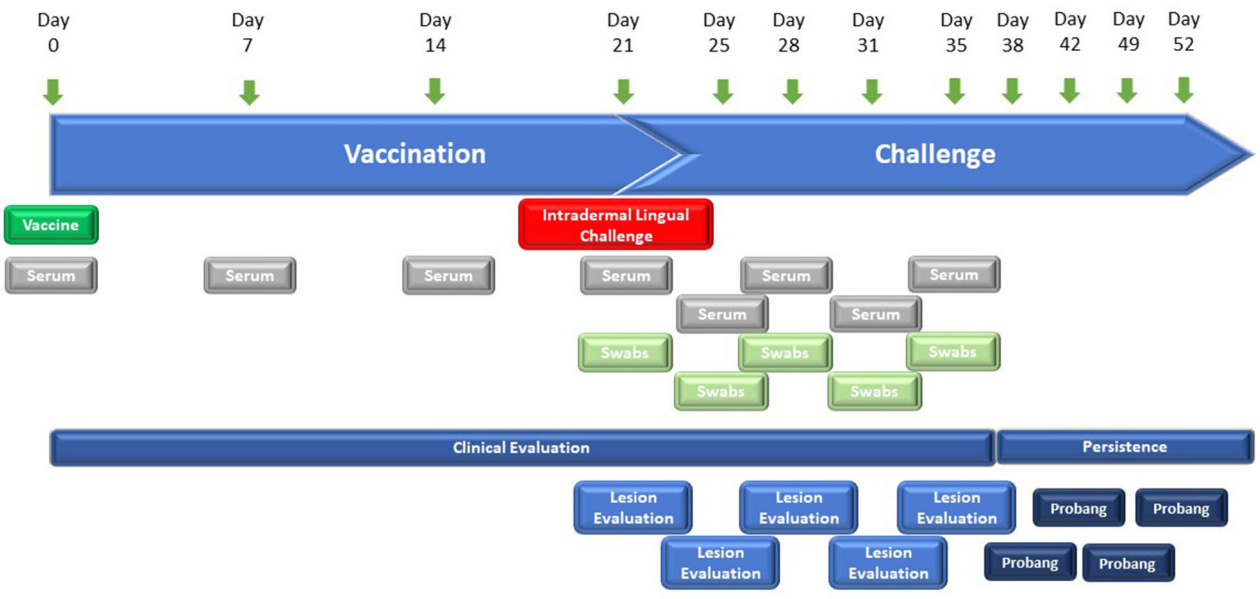

FIGURE 2 | Graphic representation of the study designs for FMD-LL3B3D-based vaccine efficacy studies. Six–twelve-month-old steers were vaccinated on day 0 with the challenge occurring on day 21 where animals are intradermal-lingually challenged with 10,000 $BTID_{50}$ of virulent, wild-type FMD virus. Serum, tonsillar swabs, and clinical observations were taken at the indicated days.

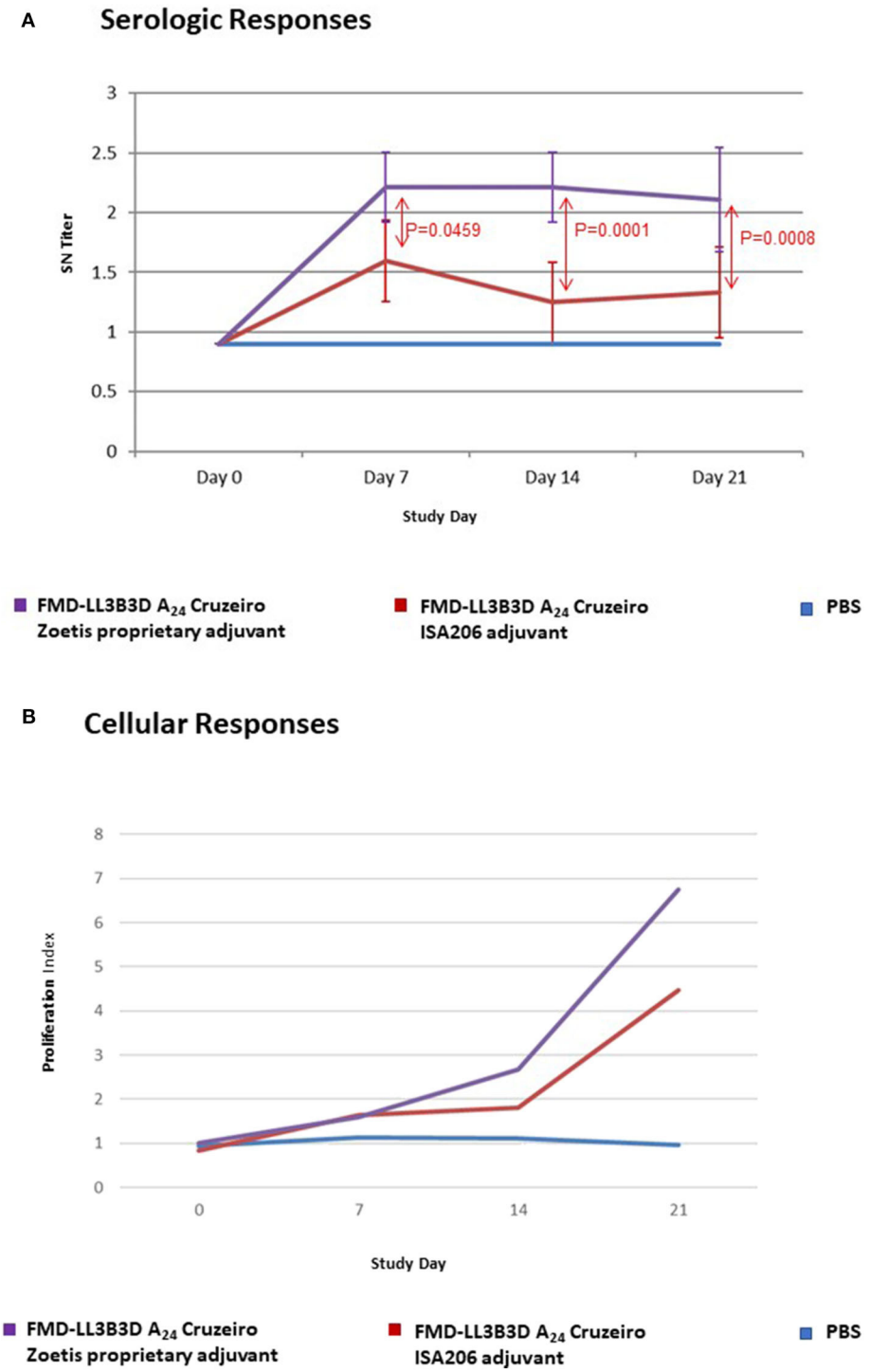

FIGURE 3 | Immune responses of animals vaccinated in a preliminary vaccine efficacy study using FMD-LL3B3D A_{24} Cruzeiro antigen and a proprietary adjuvant system. **(A)** Serum neutralizing titers were measured for each vaccine group pre- and post- vaccination. FMD-LL3B3D-A_{24} vaccination induced dose-dependent levels of neutralizing antibodies. **(B)** Cellular immune responses were measured using cell proliferation assay (on PBMC from both healthy naive and vaccinated steers).

Challenged animals that had been vaccinated with either PBS or commercial-type vaccine had elevated temperatures 1–4 days post challenge while animals vaccinated with the FMD-LL3B3D A_{24} Cruzeiro vaccine demonstrated no temperature elevations (**Figure 4A**). Cattle vaccinated with PBS began to show lesions consistent with FMD on their hooves and mouth starting at

3-days post challenge (**Figure 4B**). By day 10 post-challenge, all control animals had clinical score codes of 4. In contrast, animals vaccinated with either the commercial-type FMD A_{24} Cruzeiro vaccine or with the FMD-LL3B3D A_{24} Cruzeiro vaccine formulation did not show any lesions consistent with FMD.

To determine the potency of the FMD-LL3B3D A_{24} Cruzeiro vaccine, we carried out an experiment in cattle, using full-dose, one-quarter dose, and one-sixteenth dose (2, 0.5, and 0.125 ml, respectively) of FMD-LL3B3D A_{24} Cruzeiro vaccine and challenged at 21 days post vaccination. Cattle in all three vaccinated groups challenged with virulent FMD A_{24} Cruzeiro were fully protected from both viremia (**Figure 5A**) and clinical signs of FMD (**Figure 5B**). In contrast, cattle vaccinated with PBS demonstrated a peak of viremia 3-days post challenge (**Figure 5A**) along with onset of lesions (**Figure 5B**). By day-6 post challenge, all four cattle vaccinated with PBS had clinical scores of 4 indicating that all four hooves had lesions.

Persistent infection of the challenged virus following vaccination was assessed via probang samples taken 17-, 21-, 28-, and 31-days post challenge. Probang samples were tested for the presence of FMD A_{24} Cruzeiro by RT-PCR and virus isolation. With the exception of one RT-PCR positive sample, all probang samples from cattle vaccinated with the full-dose were RT-PCR negative for FMDV and all probang samples were negative for virus isolation (**Figure 5B**). Two of the four cattle vaccinated with the one-quarter dose were negative by both RT-PCR and by virus isolation (**Figure 5B**). Three of the four cattle vaccinated with the one-sixteenth dose were negative by both RT-PCR and by virus isolation (**Figure 5B**). Based on these results, we can only conclude that the full-dose FMD-LL3B3D A_{24} Cruzeiro vaccine contained in excess of 16 PD_{50} per dose.

Because traditional FMD vaccines are produced from wild-type FMDV, the bulk antigen must be purified to deplete the non-structural proteins present in the supernatant. This

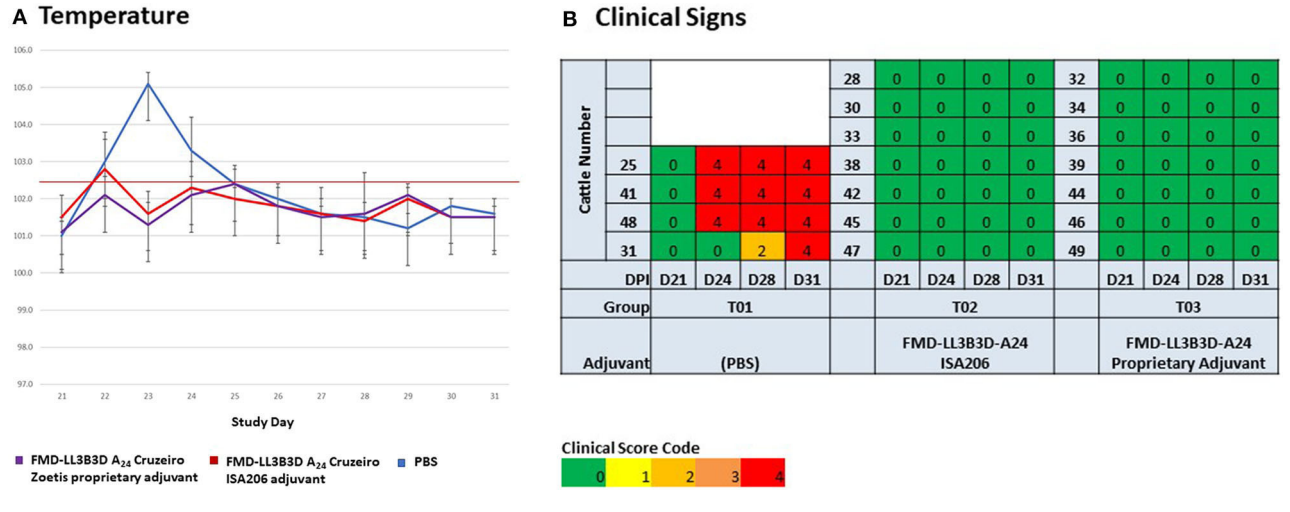

FIGURE 4 | Clinical observations of animals vaccinated and challenged with virulent, wild-type FMD A_{24} Cruzeiro in an adjuvant selection study. All animals were vaccinated on day 0 and challenged on day 21. **(A)** Temperature was assessed on days 21 through 31. **(B)** Clinical observations were taken at days 21, 24, 28, and 31.

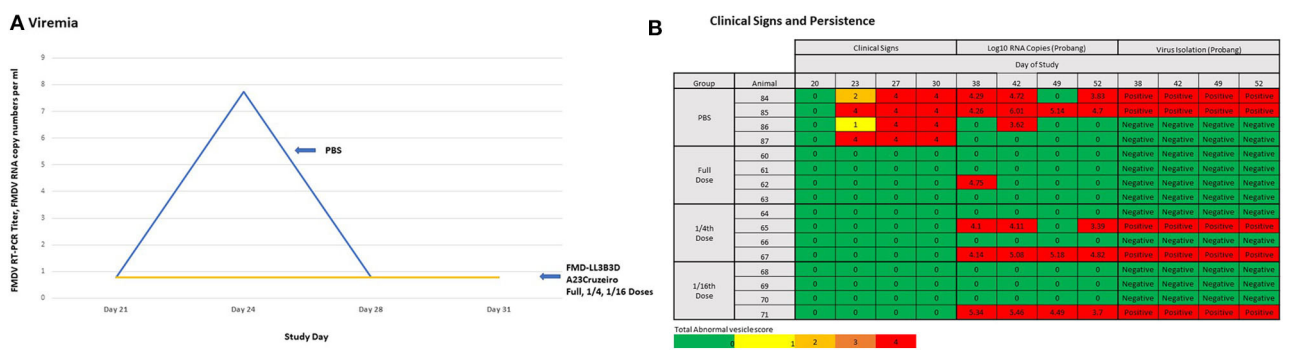

FIGURE 5 | Preliminary Protective Dose 50 (PD_{50}) study for the FMD-LL3B3D A_{24} Cruzeiro vaccine. Animals were vaccinated on day 0 with either a full vaccine dose of FMD-LL3B3D A_{24} Cruzeiro vaccine with a proprietary adjuvant, a quarter of a dose, or a sixteenth of a dose. All animals were then challenged on day 21. **(A)** Viremia was assessed by RT-PCR of serum samples on days 21, 24, 28, and 31. **(B)** Clinical observations were assessed on days 20, 23, 27, and 30 while challenge virus persistence was assessed by either viral isolation or RT-PCR of probang samples on days 38, 42, 49, and 52.

process is not 100% effective as there may still be some non-structural proteins present inside the intact viral particles. As such, vaccinated animals may still react to these trace non-structural proteins and present as positive results when tested by the available DIVA assays (20–26). In contrast, there is no need to deplete the non-structural proteins in the FMD-LL3B3D vaccine platform as the 3B coding region (encoding the non-structural protein) contains mutations which correspond to the epitope of the monoclonal antibodies used in the available commercial FMD DIVA diagnostic assays, thus rendering the FMD-LL3B3D vaccinated animals non-responsive (**Figure 1**) (35). Animals that were vaccinated with the full dose of FMD-LL3B3D A_{24} Cruzeiro vaccine were demonstrated to have reactivities similar to that of animals vaccinated with PBS in three different commercially available FMD DIVA diagnostic tests (**Figure 6**). This DIVA compatibility may facilitate a "vaccinate to live" policy instead of a "vaccinate to cull" as confidence in the immunologic status of the animals may be vastly increased.

In response to an outbreak of a novel FMD strain or incursion of a FMD strain that is not adequately covered by existing FMD vaccine banks, the FMD-LL3B3D vaccine platform is able to be utilized to rapidly generate new vaccine. A shuttle plasmid has been generated in *E. coli* in which the capsid coding region of the FMD-LL3B3D vaccine platform genome has been replace with the G-luc gene (**Figure 7**). This shuttle plasmid is the starting place for the rapid response capability. Upon obtaining a novel FMD strain, the capsid coding region is sequenced, and the capsid coding region is synthesized with the novel restriction sites flanking the capsid coding region. Traditional molecular biological techniques are utilized to clone the capsid coding region into the shuttle plasmid to generate a full-length plasmid construct. Following transcription, the full-length RNA

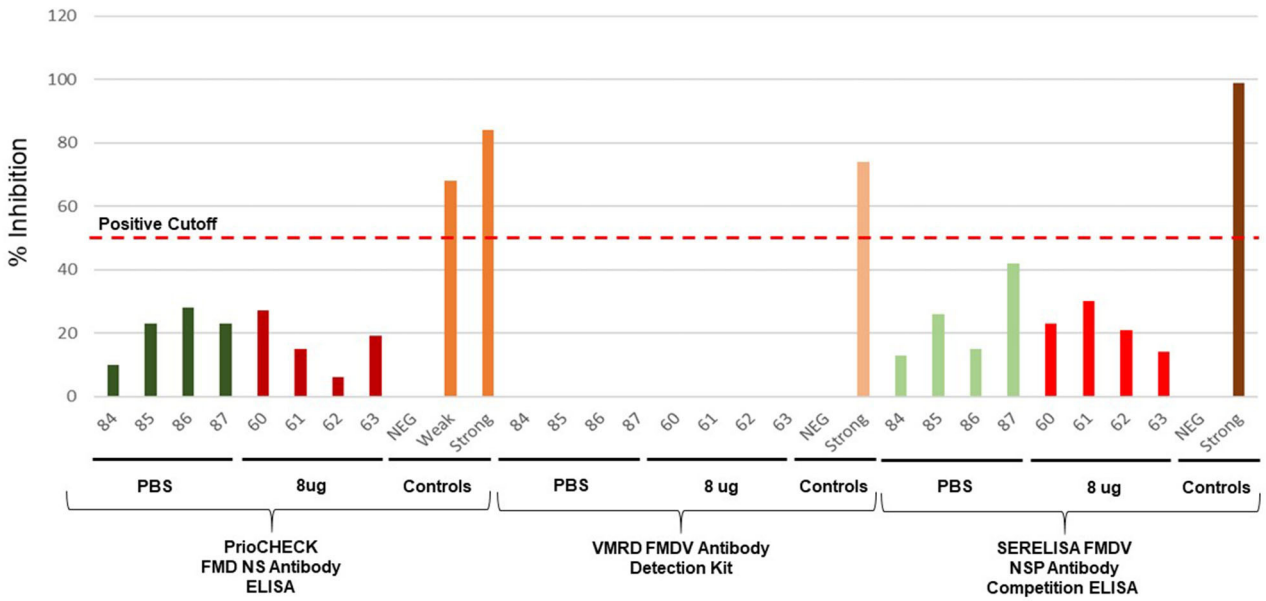

FIGURE 6 | Assessment of DIVA Diagnostic compatibility of animals vaccinated with FMD-LL3B3D A_{24} Cruzeiro vaccine using the PrioCHECK FMDV NS Antibody ELISA (Thermo Fisher Scientific), Foot and Mouth Disease Virus Antibody Detection Kit cELISA (VMRD), or SERELISA® FMDV NSP Ab Competition (Zoetis).

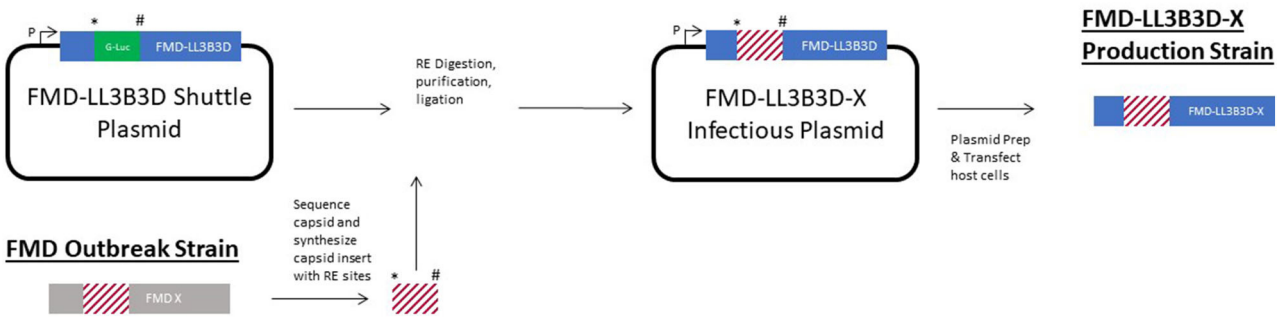

FIGURE 7 | Graphic representation of the rapid response capability of the FMD-LL3B3D vaccine platform. The * and # symbols represent two unique restriction enzyme sites that were engineered into the genome to facilitate swapping of the capsid coding region cassettes.

is transfected into a manufacturing cell line to generate the new vaccine strain from which Premaster and Master Seeds may be derived. In this way, incursion of a new FMD strain into a FMD-free country may be initially addressed with the nearest matching vaccine and followed promptly with the specific FMD strain vaccine.

Cattle immunized with a variety of chemically inactivated FMD-LL3B3D vaccine constructs were protected from challenge with parental virus (**Figures 2**–**5**). Three commercially available FMD DIVA companion assays were shown to be compatible with the negative markers built into the FMD-LL3B3D vaccine platform and facilitate the full DIVA capability (**Figure 6**). Taken together, the vaccine formulations containing FMD-LL3B3D-based antigens represent an improved product profile that addresses the limitations of existing FMD vaccines and create a rapid response capability that may be utilized to promptly address incursions of new FMDV serotypes (**Figure 7**). This new platform technology with high potency, safe antigen production, full DIVA compatibility, and single-dose application may revolutionize the FMD vaccine market and may provide a product profile in line with National efforts to eradicate FMD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Live animals used in these studies were owned by the United States Department of Agriculture—Agricultural Research Service and the animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Plum Island Animal Disease Center.

AUTHOR CONTRIBUTIONS

The studies were designed, directed, and coordinated by JH, ER, and LR. JH, ER, and LR provided conceptual and technical guidance for all aspects of the project. PK adapted virus to sBHK, generated preMaster seeds, determined the antigen content, and with PD formulated the vaccine. All animal experiments were conducted at the PIADC, USDA ARS BSL-3 animal facility. JP participated on the planning and conducted the animal experiment and with PK, VM, JT, ER, LR, and JH collected and analyzed the data. The manuscript was written by JH and ER with contributions and comments of all authors.

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BRIEF RESEARCH REPORT
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In silico Evolutionary Divergence Analysis Suggests the Potentiality of Capsid Protein VP2 in Serotype-Independent Foot-and-Mouth Disease Virus Detection

Israt Dilruba Mishu[1], *Salma Akter*[1,2], *A. S. M. Rubayet Ul Alam*[3], *M. Anwar Hossain*[1†] *and Munawar Sultana*[1*]

[1] Department of Microbiology, University of Dhaka, Dhaka, Bangladesh, [2] Department of Microbiology, Jahangirnagar University, Dhaka, Bangladesh, [3] Department of Microbiology, Jashore University of Science and Technology, Jashore, Bangladesh

Foot-and-mouth disease (FMD) is an economically devastating disease of the livestock worldwide and caused by the FMD virus (FMDV), which has seven immunologically distinct serotypes (O, A, Asia1, C, and SAT1–SAT3). Studies suggest that VP2 is relatively conserved among three surface-exposed capsid proteins (VP1–VP3) of FMDV, but the level of conservation has not yet been reported. Here we analyzed the comparative evolutionary divergence of VP2 and VP1 to determine the level of conservation in VP2 at different hierarchical levels of three FMDV serotypes (O, A, and Asia1) currently circulating in Asia through an in-depth computational analysis of 14 compiled datasets and designed a consensus VP2 protein that can be used for the development of a serotype-independent FMDV detection tool. The phylogenetic analysis clearly represented a significant level of conservation in VP2 over VP1 at each subgroup level. The protein variability analysis and mutational study showed the presence of 67.4% invariant amino acids in VP2, with the N-terminal end being highly conserved. Nine inter-serotypically conserved fragments located on VP2 have been identified, among which four sites showed promising antigenicity value and surface exposure. The designed 130 amino acid long consensus VP2 protein possessed six surface-exposed B cell epitopes, which suggests the possible potentiality of the protein for the development of a serotype-independent FMDV detection tool in Asia. Conclusively, this is the first study to report the comparative evolutionary divergence between VP2 and VP1, along with proposing the possible potentiality of a designed protein candidate in serotype-independent FMDV detection.

Keywords: FMDV, VP2 vs. VP1, evolutionary divergence, serotype-independent, conserved sites, consensus VP2 protein, B cell epitope

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****Correspondence:***
Munawar Sultana
munawar@du.ac.bd

†Present address:
M. Anwar Hossain, Vice-Chancellor, Jashore University of Science and Technology, Jashore, Bangladesh

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, causing a large-scale economic loss for livestock industries worldwide due to the rapid loss of productivity (1–3). The onset of FMD can cause extensive morbidity and mortality, resulting in a disastrous reduction in the yield of animal products. The etiologic agent, FMD virus (FMDV), is a member of the *Picornaviridae* family, possessing a single-stranded positive-sense RNA genome (4–6), which encodes four structural proteins (VP1–VP4) and other non-structural proteins (Lpro, 2A, 2B, 2C, 3A, 3B, 3Cpro, and 3Dpol).

Extensive mutational variations result in the differentiation of the virus into seven immunologically distinct serotypes; O, A, C, Asia1, and Southern African territories (SAT) 1–3 (7). Within serotypes, there are multiple topotypes that are usually related to the geographical region of disease occurrence or subtype and being identified based on a threshold of 15% nucleotide sequence divergence in the VP1 coding region. However, the high genetic diversity of the virus results in the emergence of many distinct lineages within a topotype, which show at least 7.5% VP1 nucleotide divergence (8, 9). Further diversification divides the individual lineages into multiple sub-lineages, although there is no established threshold for VP1 divergence among these sub-lineages.

Due to the relative ease of sequencing and the reliability of virus classification, VP1-based molecular epidemiology has been accepted as the traditional way of evolutionary divergence analysis. Studies suggest that VP1 is the most diversified capsid protein of FMDV at both nucleotide and amino acid (aa) levels (10–12), but there is lack of information regarding the extent of VP1 divergence in comparison with other capsid proteins. Comparative evolutionary divergence analysis can provide us with information regarding the possible chance of mutation and evolutionary stability of two different proteins. This information can be used for selecting the most conserved one for the future development of a more reliable diagnostic tool that will be capable of detecting FMDV regardless of its serotypes. Among the three surface-exposed capsid proteins of FMDV (VP1–3), VP2 is reported to be relatively conserved (10) and to also possess potential immunogenic sites capable of eliciting the development of anti-VP2 antibody upon infection (13). That is why VP2-based evolutionary divergence of FMDV has been investigated in the current study and has been compared with VP1-based divergence at multiple taxonomic hierarchies (serotypes, topotypes, lineages, and sub-lineages).

Overall, the current study aims at analyzing the comparative evolutionary divergence of VP2 and VP1, along with identification of the surface-exposed conserved antigenic sites in VP2 and designing a consensus VP2 protein as a potential candidate to develop a rapid and cost-effective tool for FMD diagnosis in the Asian region, which would be entirely serotype independent. Herein we have analyzed the molecular diversity of VP2 at both genomic and proteomic levels, considering multiple taxonomies (*i. e.*, serotypes, topotypes, lineages, and sub-lineages), and identified the surface-exposed conserved antigenic sites in VP2. Based on these findings, we have designed a consensus VP2 protein sequence of 130 amino acid long and mapped possible B cell epitopes in this protein. To our present knowledge, this study is the first one to report on VP2-based evolutionary divergence cutoff values of FMDV at each subgroup and to design a consensus VP2 protein carrying surface-exposed B cell epitopes to be used in serotype-independent FMD diagnosis.

MATERIALS AND METHODS

Sequencing of VP2 Coding Region

Viral RNA extraction and cDNA preparation of 20 local FMDV isolates were performed from cell culture supernatants of selected isolates using Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, USA) and GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The reverse-transcribed cDNA samples were used for amplification of the VP2 coding regions by the designed primers VP2-F (GACAAGAAAACCGASGAGACCAC) and VP2-R (TCTTTGGAAKGGAACTCACSCG). After purification of the amplified PCR products, sequencing of the desired VP2 coding region was performed using Sanger sequencing method, and the obtained sequences were analyzed with SeqMan (14). The sequences were submitted to NCBI, and the accession IDs are listed in **Supplementary Table 1g**.

Dataset Generation and Compilation

Three major FMDV serotypes circulating in Asian regions (O, A, and Asia1) were focused on in this study to generate 14 datasets containing VP2 nucleotide sequences, which are listed in **Supplementary Tables 1a–h**. The sequences were obtained from the NCBI GenBank (15). The first 10 datasets (**Supplementary Tables 1a–f**) were used for determining the diversity of VP2 protein at multiple taxonomic hierarchies (serotypes, topotypes, and lineages), and the obtained divergence value was compared with the previously established cutoff value for VP1 divergence. Datasets 11–13 (**Supplementary Table 1g**) were prepared with polyprotein sequences of FMDV to analyze the sub-lineage-level divergence of both VP2 and VP1 as there is no previously established cutoff value for the sub-lineage-level divergence of VP1. Twenty local isolates sequenced in the current study were compiled in dataset 11 (**Supplementary Table 1g**). Dataset 14 (**Supplementary Table 1h**) was used for the determination of conserved antigenic sites in VP2 and designing of consensus VP2 protein sequence.

Phylogenetic Analysis Based on VP2 Coding Region

Multiple sequence alignment of each dataset was performed and subsequent phylogenetic analysis was carried out in MEGA7 using ClustalW algorithm (16). Unweighted pair group method with arithmetic mean (UPGMA) trees were constructed which indicated cutoff values of VP2 divergence at multiple subgroup levels of FMDV (serotypes, topotypes, and lineages). The VP2-based cutoff divergence value was then compared with the VP1-based cutoff value of divergence reported elsewhere (17–19). The

sub-lineage-level divergence was compared by creating UPGMA trees based on both VP1 and VP2 nucleotide sequences.

Calculation of Protein Variability Index

For calculating the protein variability index of 279 VP2 sequences (**Supplementary Table 1a**) of FMDV serotypes O, A, and Asia1, Protein Variability Server (PVS) (20) was used. The Wu–Kabat variability coefficient, with a variability threshold of 1.0, was calculated to find out the maximum variable positions and the most conserved regions in the VP2 proteins of all three serotypes.

Visualization of Mutations

MEGA7-based mutation analysis was performed in two levels—one is mutations in the previously reported antigenic sites [**Supplementary Table 2**] of FMDV VP2 protein of serotypes O, A, and Asia1 (**Supplementary Table 1a**). Another one is more precise—mutation in the PVS returned conserved fragments of VP2 protein of FMDV focusing on the Asian region (**Supplementary Table 1h**).

Determination of Conserved Fragments and Validation of Conservancy

Based on the aim of this study, which is the assessment of the credibility of VP2 protein in serotype-independent diagnosis of FMDV, the determination of antigenic sites that are inter-serotypically conserved is a major step. A total of 360 FMDV VP2 sequences (**Supplementary Table 1h**), representing the isolates of Asian territory, were taken into account for finding out the conserved regions in VP2. The PVS was used for returning conserved fragments of seven or more consecutive residues, with a variability threshold of 0.5 using Shannon diversity analysis. The accuracy of the conserved fragments was validated by comparing with the results obtained from the mutation analysis. The mutation score of each position was calculated by the analysis of VP2 protein alignment in MEGA7.

Homology Modeling

Homology modeling of VP2 protein was performed to observe whether there are any structural dissimilarities in the 3D configuration of FMDV serotypes O, A, and Asia1 in the obtained conserved regions. For this purpose, three Bangladeshi isolates were selected as a representatives of three FMDV serotypes: BAN/JA/Ma-180/2013 (accession KJ175183) as representative of serotype O, BAN/CH/Sa-304/2016 (accession MK088171) as representative of serotype A, and BAN/DH/Sa-318/2018 (accession MN722609) as representative of serotype Asia1. SWISS-MODEL server (21) was used to search suitable template protein structures and generate models of protein 3D structure in PDB format using 1qqp as a template. The stereo-chemical quality of the generated 3D structures was validated by Ramachandran plot using PROCHEK (22). The alignment and the superimposition of the 3D structures were performed in PyMOL (23).

Antigenicity Calculation and Structural Assessment of the Conserved Sites

The conserved fragments that fall within the previously reported antigenic sites of VP2 [**Supplementary Table 2**] were identified, followed by the detection of their antigenicity value by vaxijen v2.0 server (24), with a threshold of 0.4. Additionally, the surface accessibility and other structural properties of the conserved antigenic sites were determined using NetSurfP-2.0 server (25), which provides information regarding protein secondary structure, probability of disordered residue, and the relative surface accessibility of individual amino acid residues in the protein structure.

Designing of Consensus VP2 Protein and Mapping of Possible B Cell Epitopes

N-terminal 130 residues of VP2 were targeted for designing a consensus sequence since this region contained all the surface-exposed conserved antigenic sites of VP2. The alignment of 360 VP2 sequences (**Supplementary Table 1h**) was analyzed to find out the most frequently observed residue at each position in the alignment, and by combining these residues, a consensus VP2 protein sequence of 130 amino acids in length was designed. The secondary structure and surface accessibility of the designed protein was studied using NetSurP-2.0 server, and a 3D model was generated using SWISS-MODEL server, followed by visualizing at PyMOL. The possible B cell epitopes were identified using three of the most popular epitope prediction tools [BepiPred 1.0 (26), ABCpred, and Bcepred (27)] to minimize false-positive predictions. The predicted epitopes from each server were compared manually, and the common epitopes were chosen as the most probable B cell epitopes.

RESULTS

Evolutionary Divergence of VP2 in Comparison with VP1

We determined the VP2-based cutoff divergence value of FMDV at serotype, topotype, lineage, and sub-lineage levels using a UPGMA phylogenetic study. With these values, a comparative analysis between VP2- and VP1-based divergence has been shown (**Table 1**).

Thirteen individual datasets (**Supplementary Tables 1a–g**) were used to generate unweighted pair group method with arithmetic mean trees for comparing the VP2- and VP1-based cutoff divergence values of different subgroups under three serotypes of FMDV(O,A, and Asia1).

Serotype-Level Divergence

Serotype-level divergence analysis performed considering the VP2 sequences of 279 FMDV isolates (**Supplementary Table 1a**) representing serotypes O, A, and Asia1 revealed two closest clusters (serotypes O and Asia1) in the tree [**Supplementary Figure 1**], providing a cutoff value of ∼14% divergence among these three FMDV serotypes. In contrast, the serotypes of FMDV possess at least 30% nucleotide sequence diversity in the VP1 coding region (17). These findings

TABLE 1 | Comparative evolutionary divergence of foot-and-mouth disease virus (FMDV) at multiple taxonomic levels based on VP2 and VP1.

Serotype-level divergence							
Serotype	**O**			**A**			**Asia1**
VP2-based nucleotide divergence value	14%						
VP1-based nucleotide divergence value	30–50% (17)						
Topotype-level divergence							
Serotype	**O**			**A**			**Asia1**
Topotype	ME-SA, SEA, Euro-SA, CATHAY, EA-1, EA-2, EA-3, WA			ASIA, AFRICA, Euro-SA			ASIA
VP2-based nucleotide divergence value	5.5%			8%			–
VP1-based nucleotide divergence value	15–20% (18)						
Lineage-level divergence							
Serotype	**O**			**A**			**Asia1**
Topotype	**ME-SA**	**SEA**	**Euro-SA**	**ASIA**	**AFRICA**	**Euro-SA**	**ASIA**
Lineage	Ind2001 PanAsia I PanAsia II	CAM-94 MYA-98	O1 O2	G-VII A-15 A22 Iran-05 Iran-87 Iran-96 Thai-87 Sea-97	G-I G-II G-III G-IV G-V G-VI G-VII	A5 A12 A24 A81	G-I G-II G-III G-IV G-V G-VI G-VII G-VIII G-IX
VP2-based nucleotide divergence value	2.9%	5.5%	4.25%	3.9%	5.4%.	5.1%	4.8%
VP1-based nucleotide divergence value	7.5% (19)						
Sub-lineage-level divergence							
Lineage	**O/ME-SA/Ind2001**			**O/ME-SA/PanAsia II**			**A/ASIA/IRN-05**
Sub-lineage	Ind2001d Ind2001BD1 Ind2001BD2			ANT-10 FAR-09 PUN-10			BAR-08 AFG-07 ARD-07
VP2-based nucleotide divergence value	2.9%			1.8%			2.7%
VP1-based nucleotide divergence value	3.5%			2.7%			2.7%

support that VP2-based nucleotide divergence ensures higher conservancy than VP1 at the serotype level.

Topotype-Level Divergence

UPGMA phylogenetic analysis of eight topotypes of FMDV serotype O and three topotypes of serotype A using datasets 2 and 3 (**Supplementary Tables 1b,c**) showed 5.5 and 8% cutoff divergence values, respectively (**Supplementary Figures 2A,B**). In contrast, the topotypes exhibit ≥15% variation based on the VP1 coding sequence (17, 19).

Lineage-Level Divergence

The topotypes of FMDV are subdivided into different lineages based on at least 7.5% VP1 nucleotide divergence. Here we measured 2.9% inter-lineage VP2 sequence variation between PanAsia-I and PanAsia-II of the most dominant ME-SA topotype in the Indian subcontinent. Topotype South East Asia (SEA) of serotype O showed 5.5% inter-lineage divergence between two lineages (CAM-94 and MYA-98) and O/Euro-SA provided a cutoff value of 4.25% divergence between O1 and O2 lineages (**Supplementary Figures 3A–C**). The lineages of A/Euro-SA topotype (A5, A12, A24, and A81) provided a cutoff value of 5.1% divergence (**Supplementary Figure 4C**). Among eight different lineages of A/ASIA topotype, Thai-87 and Sea-97 were found to be the most closely related and provided a cutoff value of 3.9% divergence. The African topotype of serotype A showed 5.4% inter-lineage divergence (**Supplementary Figures 4A,B**). The only one topotype of serotype Asia1 (topotype ASIA) showed 4.8% inter-lineage divergence between two closest lineages, G-VIII and G-IX [**Supplementary Figure 5**]. Moreover, we observed that topotypes from the Asian region showed less inter-lineage diversity in comparison with the African or the European and South American topotypes.

Sub-lineage-Level Divergence

Sub-lineage-level divergence analysis among three sub-lineages of O/ME-SA/Ind2001 lineage showed VP2 to be less divergent than VP1 (**Supplementary Figures 6A,B**). In VP1-based phylogeny, Ind2001BD1 and Ind2001BD2 were found to be the closest sub-lineages (3.5% nucleotide divergence). In contrast, the VP2-based phylogeny showed Ind2001d and Ind2001BD2 to be the closest relatives (2.9% nucleotide divergence). Similarly, sub-lineages of PanAsia II showed less divergence in VP2 (1.8% nucleotide divergence) than VP1 (2.7% nucleotide divergence). PUN-10 and FAR-09 were found to be the closest sub-lineages in the case of VP1, while these were ANT-10 and FAR-09 in the case of VP2 (**Supplementary Figures 7A,B**). The sub-lineages of IRN-05 showed equal divergence for both VP1 and VP2 (2.7% nucleotide divergence), where BAR-08 and AFG-07 were found to be the closest groups in each case

(**Supplementary Figures 8A,B**). Overall, the phylogenetic analysis ensured a higher conservancy of VP2 over VP1 at each subgroup level.

Protein Variability Index and Mutational Frequency

The protein variability analysis and mutational study using 279 VP2 sequences (**Supplementary Table 1a**) showed that 147 of 218 (67.4%) amino acids were inter-serotypically conserved. In the Wu–Kabat plot (**Figure 1A**), the N-terminal end and the 22–36 motif were found to be highly conserved. A higher Wu–Kabat variability coefficient was found in the B–C and E–F loops and near the C-terminal end.

Mutational study of the previously reported antigenic regions of VP2 delineated that, among 71 varied sites in VP2, only 29 fall within the antigenic regions positioned at three sites each in the N-terminal, T cell epitope I, E–F loop, and T cell epitope III, nine in the B–C loop, and four in T cell epitope II [**Supplementary Table 3**]. In the antigenic regions, only 25 sites were detected to be occupied by more than two amino acids, and as expected, significant variations were observed in the B–C loop and the E–F loop.

The mutational score calculation (**Figure 1B**) of the mutated sites revealed a higher frequency of most common amino acids in comparison to mutated amino acids. Three mutated sites of the N-terminal of VP2 (positions 7, 9, and 10) provided more than 99% frequency of the most common amino acids in comparison with the mutated ones. The only mutated position of the 22–36 motif is position 23, which showed 75.27% frequency of the most common amino acid, threonine, which is satisfactorily high. Among the three T cell epitopes, a high percentage of mutational frequency was observed in T cell epitope II that showed a mutation at four positions, with the mutation score ranging from 32.98% to 56.29%. The highest mutational frequency was observed in the B–C and the E–F loops, with mutation scores ranging from 31.75 to 66.31%. Critical amino acids at positions 100, 172, 188, and 191 showed 98.92, 67.38, 100, and 62% frequency of the most common amino acids, respectively. Overall, the study confirmed a remarkable inter-serotype conservancy of VP2 at the reported antigenic sites.

Inter-serotypically Conserved Regions within VP2 Protein

VP2 sequences representing the isolates of the Asian territory (**Supplementary Table 1h**) revealed nine inter-serotypically conserved fragments (**Table 2**) after Shannon diversity analysis. The accuracy of the conservation of all nine fragments was validated by a mutational study, which also supported the inter-serotypic conservancy of the fragments, except in eight positions with only 0.83–3.33% insignificant mutational frequency (**Figure 1C**). Moreover, homology modeling and superimposition of VP2 proteins from three serotypes displayed no structural dissimilarities in the conserved regions and supported that the 3D configurations of the conserved regions are inter-serotypically invariant (**Supplementary Figure 9A**). Using the modeled 3D structures, the generated Ramachandran plot (**Supplementary Figure 9B**) showed that 89.4–91 and 8.5–9% of residues of the modeled structure are within the most favored (red) and the additional allowed regions (yellow), ensuring the highly reliable stereochemical properties of the generated models.

Antigenicity and Surface Accessibility of the Conserved Fragments

The antigenicity, surface accessibility, and other structural properties of conserved fragments were determined to validate their credibility as suitable candidates for serotype-independent detection of FMDV. Among the nine conserved fragments of VP2, six fell within the previously reported antigenic sites (fragments 1–4, 6, and 9), whereas non-antigenic sites comprised three other fragments (5, 7, and 8). Comparing the PVS returned conserved fragments and the previously reported antigenic sites, we selected six conserved antigenic sites (sites I–VI) in VP2 for calculating the antigenicity value. Among these six sites, four (sites I, II, III, and V) were found to be antigenic, with a sufficient antigenicity value ranging from 0.4189 to 1.1031 (**Table 2**). Among these four conserved antigenic sites, three were found to be entirely surface-exposed (sites I, II, and III), and site V showed partial surface accessibility with a threshold of 25% (**Figure 2A**). In site V (114–124), the first three amino acids (N, Q, and F) were found to be surface-exposed, providing the antigenicity value of 0.7728 (**Table 2**), whereas the remaining amino acids of this site were buried. In contrast, site IV had only two surface-exposed amino acids, and site VI was completely buried. Sites I and III showed a coiled structure, although there is a probability of significant-level disorderliness in site I. The other sites have not shown any significant disordered residue. Sites II, IV, and V showed a co-abundance of stranded and coiled structures, and site VI falls in an entirely stranded structured region (**Figure 2A**).

Designed VP2 Protein and Mapped B Cell Epitopes

Since the four surface-exposed conserved antigenic sites, sites I, II, III, and V (**Figure 2A**), are located within the N-terminal 130 residues of VP2, a consensus VP2 protein was designed by combining the first 130 most common amino acids of VP2 sequentially. The designed protein (**Figure 2B**) showed an abundance of coiled loop structures, which indicate their possible role in the antigenicity of VP2. Five β-strands and two α-helices were also observed. N-terminal 75 residues were found to be exclusively surface-exposed, which have been indicated by underlined residues in **Figure 2B**. Six B cell epitopes have been mapped in the designed protein, which are shown in **Figures 2B,C**. The epitopes are ^{1}DKKTEETT^{8}, ^{16}TTRNGHT^{22}, ^{24}STTQSSVG^{31}, ^{35}GYATAEDFV^{43}, ^{44}SGPNTSG^{50}, and ^{85}TDHKGVYGGLT^{95}. All the epitopes were found to be surface-exposed (**Figure 2B**). A secondary structure analysis of the epitopes revealed that all the epitopes form part of the coiled connecting loops; also, epitopes 1 and 5 entirely fall in the looped region. β-sheeted regions were observed in epitope 2 [first three amino acids (aa)], epitope 3 (first

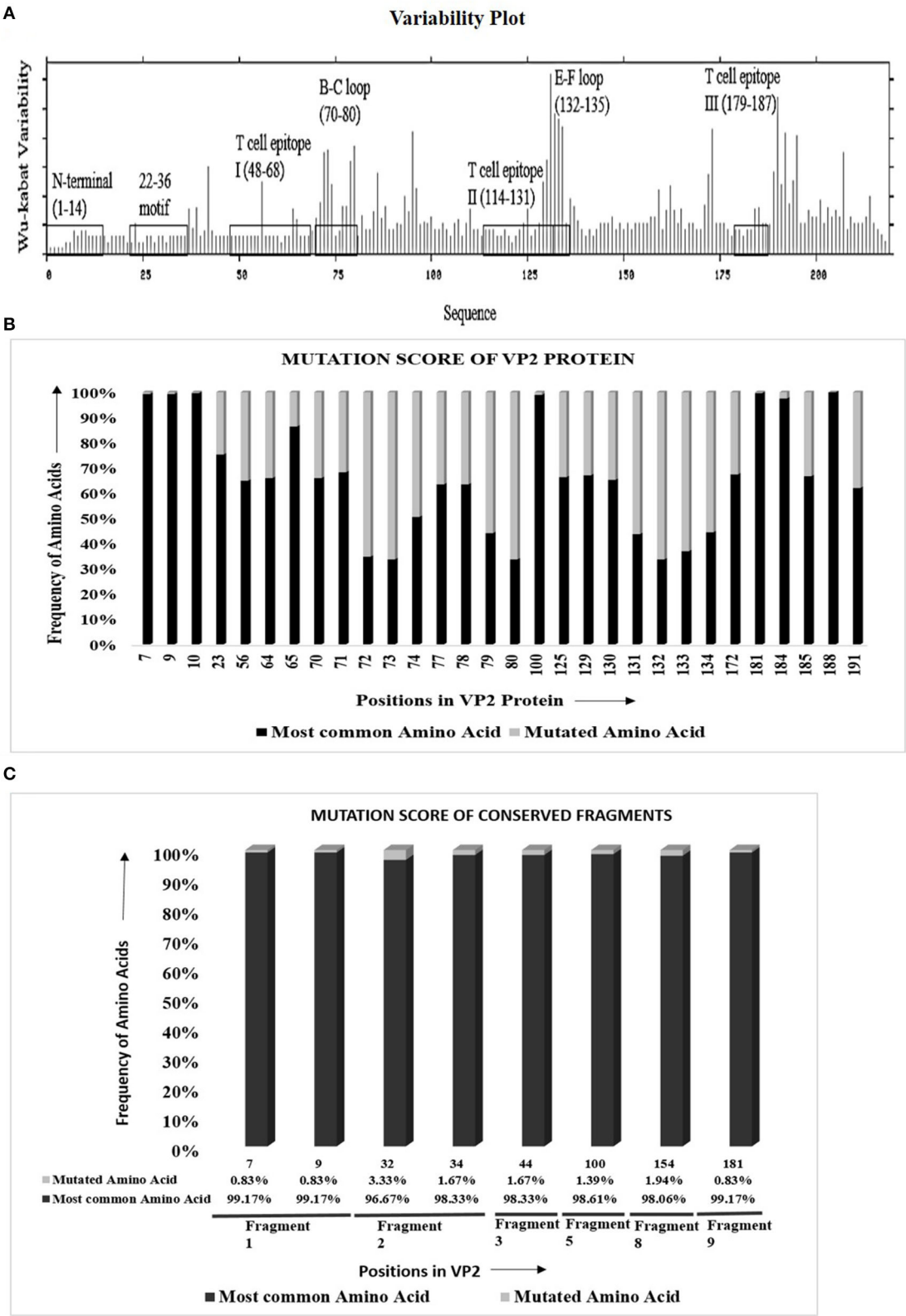

FIGURE 1 | Visualization of amino acid divergence in VP2 protein of foot-and-mouth disease virus serotypes O, A, and Asia1. **(A)** Wu–Kabat protein variability plot for VP2 protein using Protein Variability Server (PVS). The plot was generated using the protein alignment of 279 VP2 sequences. The marked regions indicate the

(Continued)

FIGURE 1 | previously reported antigenic sites of VP2. The X and the Y axis of the plot, respectively, delineates the amino acid positions of VP2 and Wu–Kabat variability coefficient. **(B)** Graphical representation for the overall mutation score of the mutated sites in VP2. The protein alignment of 279 VP2 sequences was used to carry out the mutational study. For each position, the frequency of most common and mutated amino acids is represented with black and gray color, respectively. The X axis shows the amino acid position of VP2, and the Y axis shows the frequency of mutated and most common amino acids. Only the previously reported antigenic sites were taken into consideration for the mutational study. **(C)** Graphical representation for overall mutational score of the mutated positions in the conserved fragments of VP2 protein. The protein alignment of 360 Asian VP2 sequences was used to identify conserved fragments of VP2 using PVS and mutational study of the fragments. The X axis shows the amino acid position of VP2, and the Y axis shows the frequency of mutated and most common amino acids. For each position, the most common amino acids are presented with black color, and the other least common amino acids are presented with gray color. The percentage of frequency of amino acids is attached down to the respective positions. The respective conserved fragments to which the amino acid positions belong are mentioned as fragment numbers (positions 7 and 9 in fragment 1; 32 and 34 in fragment 2; and 44, 100, 154, and 181 in fragments 3, 5, 8, and 9, respectively). Only the previously reported antigenic sites were taken into consideration for the mutational study.

TABLE 2 | Inter-serotypically conserved fragments in VP2 obtained by Protein Variability Server and their antigenicity assessment.

Conserved fragments in VP2	Reported antigenic sites in VP2	Conserved antigenic sites in VP2	Sequence of the conserved antigenic sites	Length	Antigenicity value	Presence of antigenicity
Fragment 1 (1–22)	N-terminal (1–14)	Site I (1–14)	DKKTEETTLLEDRI	14	0.4189	Yes
Fragment 2 (24–36)	22–36 motif	Site II (24–36)	STTQSSVGVTYGY	13	1.1031	Yes
Fragment 3 (43–55)	T cell epitope I (48–68)	Site III (48–55)	TSGLETRV	8	0.7873	Yes
Fragment 4 (57–63)	T cell epitope I (48–68)	Site IV (57–63)	QAERFFK	7	−0.8310	No
Fragment 5 (99–106)	No reported antigenic site	–	–	–	–	–
Fragment 6 (111–124)	T cell epitope II (114–132)	Site V (114–124)	NQFNGGCLLVA	11	0.7728	Yes
Fragment 7 (139–148)	No reported antigenic site	–	–	–	–	–
Fragment 8 (150–158)	No reported antigenic site	–	–	–	–	–
Fragment 9 (174–183)	T cell epitope III (179–187)	Site VI (179–183)	LVVMV	5	Too low to detect	No

Shannon variability index with < 0.5 variability was used as a threshold for finding out conserved fragments of seven or more consecutive residues. The antigenicity value of the fragments that fall in the previously reported antigenic sites of VP2 was calculated with Vaxijen v2.0 server, and fragments with antigenicity values of 0.4 or above were considered to be antigenic.

two aa), and epitope 4 (first four aa). Helices were observed in epitopes 3 and 6. Residues near the C-terminal of the designed protein were found to be buried under the surface structure (**Figure 2B**).

DISCUSSION

Foot-and-mouth disease is a major threat to an economically important livestock population and caused by the foot-and-mouth disease virus (28). Being an RNA virus, FMDV lacks the proofreading mechanism during virus replication resulting in the extensive genetic heterogeneity of the virus. High genetic diversity results in the differentiation of the viruses into different serotypes, topotypes, lineages, and sub-lineages (11, 29). For selecting an appropriate vaccine strain, determination of the infecting FMDV serotype is important during FMD diagnosis. Even so, serotype-independent detection is the most preferred method for the rapid checking of animals during emergency outbreak or international animal trade. Because the detection of individual serotype needs separate diagnostic kits, which eventually increases cost and diagnosis time. Although non-structural protein (NSP)-based diagnostic approaches can offer serotype-independent detection, the generation of anti-NSP antibody requires more time than anti-structural protein (SP) antibodies (30), thus limiting their ability to be used in early diagnosis of the disease. Early diagnosis of the infection will allow the breeder to rapidly separate the infected animals from the uninfected ones, consequently terminating the spread of the disease to the entire farm during an outbreak. Thus, SP-based serotype-independent detection protocols are more beneficial over NSP-based protocols.

Among the four structural proteins of FMDV, VP4 is completely internalized (31) and thus cannot be used for the development of any diagnostic approach. Although VP1-based diagnostic methods are widely used (32–35), serotypic structural diversity due to VP1 sequence variation can be responsible for the false-negative identification of anti-viral antibody and limiting serotype-independent detection of FMD. Considering robust diversity within the VP1 coding sequence, another surface protein having less diversity and potential immunogenicity should be targeted for the serotype-independent detection of

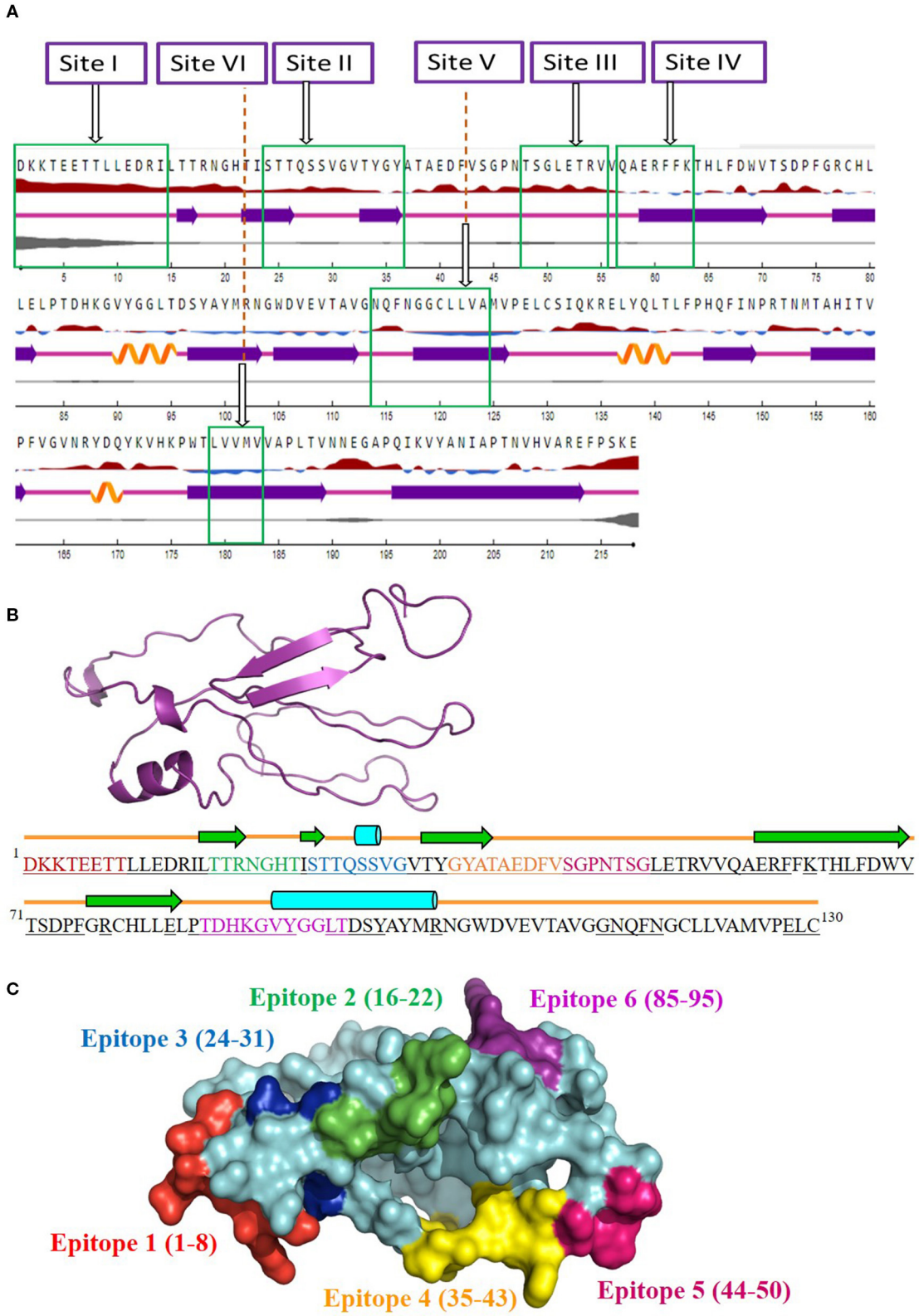

FIGURE 2 | Screening of surface-accessible conserved antigenic regions in VP2 and designing of consensus VP2 sequence along with mapping of possible B cell epitopes. **(A)** Graphical representation of the surface accessibility and the structural properties of VP2 protein. Relative surface accessibility is presented in blue and

(Continued)

FIGURE 2 | red elevation and inclination ( ), where red indicates the exposed surface and blue indicates the buried regions; threshold was kept at 25%. Curved ( ), arrowed ( ), and lined ( ) cartoons depict the secondary structure of different regions of the protein, indicating helical, stranded, and coiled structure, respectively. The disorderliness of residues in the protein is shown by gray-colored curvature, where the thickness of the gray line equals the probability of the disordered residue. **(B)** Designed consensus VP2 protein sequence and its predicted 2D and 3D models. The sequence was designed by combining the most common amino acids at positions 1–130 of VP2 protein. The green arrows highlight the predicted β-strands. Cylinders and lines, shown in cyan and orange colors, highlight the α-helices and connecting loops, respectively. The underlined amino acid residues represent the surface-accessible regions of the designed protein. The 3D structure for the designed protein, in ribbon format, is shown as an insert in the upper-left corner of the figure. Mapped B cell epitopes have been indicated with separate colors. **(C)** Predicted B cell epitopes mapped onto the 3D structure of the designed protein. The whole protein has been colored in cyan, whereas epitopes 1–6 are represented in red, green, blue, orange, pink, and violet, respectively.

anti-FMDV antibody (36). Since VP2 protein is reported to be less divergent than VP1 and VP3 (10–12) and it contains humoral response inducing surface epitopes, using VP2 is more reliable in developing a serotype-independent diagnostic tool. Therefore, we hypothesized that VP2 protein can offer a solution towards serotype-independent diagnosis of FMD. To validate this hypothesis, we at first analyzed the evolutionary divergence of VP2 in comparison with VP1. Besides, we identified the surface-exposed conserved antigenic regions in VP2, and based on the findings, we designed a consensus protein that can be used for the development of a serotype-independent FMD diagnostic kit. Since our prime focus was the Asian region, we used only the most prevalent serotypes (O, A, and Asia1) circulating in this region.

Firstly, VP2-based evolutionary divergence analysis ensured a remarkably higher conservancy in VP2 than VP1 at each taxonomic level (serotypes, topotypes, lineages, and sub-lineages). These findings corroborated with other previous studies stating that VP2 is more conserved than VP1 and VP3 at the serotype level (10–12). However, none of the previous studies showed the extent of VP2-based evolutionary divergence at the other hierarchical levels that we demonstrated here. For instance, Carrillo et al. performed the comparative genomic analysis of 103 FMDV isolates representing all seven serotypes and reported VP2 protein (47% invariant aa) to be more conserved than VP1 (24% invariant aa). Also, transitions *vs*. transversions (Ts/Tv) rate and synonymous *vs*. non-synonymous mutation rate (Syn/non-syn) were higher in VP2 than in VP1 (10). Moreover, Chitray et al. found 33% and 54.2% variant aa in VP2 and VP1, respectively, after a comparative study of 53 sequences of serotype A and O where the Ts/Tv and Syn/non-syn rates were also higher in VP2 (11). After a comparative analysis of 35 sequences of all serotypes, Feng et al. also found VP2 (70–97% aa sequence similarity) to be more conserved than VP1 (45–96% aa sequence similarity) (12). Herein we found 67.4% invariant aa in VP2 after a mutational study of 279 sequences of three FMDV serotypes circulating in Asia (O, A, and Asia1). Although the previous studies relate to our study regarding VP2 conservancy, we extended our analysis by determining the VP2-based cutoff divergence value at each subgroup level of three dominantly circulating Asian serotypes of FMDV by UPGMA phylogenetic analysis using 13 individual datasets (datasets 1–13/**Supplementary Tables 1a–g**), while the previous others showed only the serotype-dependent distinction of clusters by neighbor-joining phylogeny using a rather small dataset. Nevertheless, other studies along with our findings conclude that VP2 is significantly conserved than VP1. Thus, any diagnostic tool designed based on VP2 protein will offer a relatively more stable diagnosis approach.

Several previous studies (37, 38) proposed the N-terminal end of VP2 to be inter-serotypically conserved, which we showed in our study by multiple bioinformatics analysis (protein variability analysis, mutational study, and identification of B cell epitopes). Salem et al. designed and developed an indirect ELISA using the N-terminal conserved regions of VP2, which provided higher sensitivity than VNT and LPBE (37). However, that study used one Egyptian SAT2 isolate (gb|AAZ83686) as model for the development of a VP2-based type-independent indirect ELISA approach and used only the O, A, and SAT2 antisera to evaluate the sensitivity and the specificity of their kit. The authors took 64 sequences for the VP2 invariant site prediction and phylogeny reconstruction (11 O, 10 Asia1, 11 A, and the rest were of SAT1–3 serotypes). We believe that this small dataset does not ensure the conservation of VP2 among the highly diverse O, A and Asia1 serotypes prevailing in the Asian region, and thus using their developed kit should not offer a better output for this region. Freiberg et al. reported another interesting study showing a type-independent detection of foot-and-mouth disease virus by monoclonal antibodies (mAbs) raised against FMDV A22 Iraq/1964, Asia1 Shamir Israel/1989, and SAT1 Zimbabwe/1989 (38). They showed that the monoclonal antibodies bind to the N-terminal of VP2 and were able to recognize 27 representative isolates of six serotypes (O, A, Asia1, C, and SAT1-2). Before concluding the applicability of their recommended mAbs for type-independent detection of FMDVs in the whole Asian region, *in silico* study should be performed to ensure the conservancy of the utilized isolates with other vast majority of isolates circulating in Asia. Oem et al. developed another ELISA-based detection strategy that uses the recombinant pentameric subunit of FMDV as a diagnostic tool, but they used only FMDV type O kit for their study (39). None of those studies offer any solution for the development of diagnostic kits in the Asian region where serotypes O, A, and Asia1 are the most prevalent types. In our study, we determined VP2-based cutoff divergence values (**Table 1**) at multiple taxonomic levels using 13 individual datasets carrying the sequences of almost all subgroups of FMDV circulating in Asia, which will enable the scientific community to visualize the actual picture of VP2 divergence in FMDV strains circulating in the whole Asian region. By establishing the conservation level of VP2 for all Asian subgroups, we demonstrated the possibility of using VP2 for type-independent diagnosis of FMDV in the whole Asian region, which is also a premier report globally.

Interestingly, we observed a difference in divergence among lineages belonging to the same serotype. O/SEA topotype showed the highest inter-lineage variation (with 5.5% divergence) in the VP2 region, while the lowest inter-lineage divergence was observed for O/ME-SA topotype (2.9%). Similarly, inter-lineage divergence in the VP2 region of African (5.4%) and Asian (3.9%) topotypes was the highest and the lowest lineage level divergence for serotype A. This difference in divergence among lineages within the same serotype may possibly be due to the recombination events in FMDVs within the conserved region of VP2 as described by Jamal et al. (40, 41). Importantly, at each hierarchical level, the divergence values for VP2 were lower than those for VP1, except the sub-lineage-level divergence of A/IRN-05 lineage where the values were equal.

Another noteworthy finding of the study is the determination of inter-serotypically conserved fragments in VP2. The highly antigenic, surface-exposed, and conserved fragments can be efficiently used for a serotype-independent diagnosis of FMD. The protein variability data revealed new highly conserved regions in VP2. Nine previously undescribed inter-serotypically invariant fragments within VP2 have been identified in this study (**Table 2**). The antigenicity value calculation determined four sites to have a higher antigenicity value than the preset threshold. The sites are DKKTEETTLLEDRI (1–14), STTQSSVGVTYGY (24–36), TSGLETRV (48–55), and NQFNGGCLLVA (114–124) (**Table 2**). The sites were found to have surface exposure and a functionally active structural configuration (**Figure 2A**), indicating their possible interaction with the immune cells. Thus, these four sites can be a promising focus in designing a serotype-independent diagnostic approach for FMDV detection. Considering this promise, we designed a consensus VP2 protein by combining the first 130 most common amino acids of VP2 sequentially and identified six possible B cell epitopes in this protein. Also, the epitopes were found to be surface-exposed and have a functionally active structural configuration. These findings suggest that the designed protein can be a suitable candidate for the development of a serotype-independent diagnostic tool using ELISA-based approaches. Similar approaches were developed by Salem et al. using one Egyptian SAT2 isolate of FMDV (gb|AAZ83686), which showed the expected level of sensitivity in FMD detection (37). No such strategy is available in the Asian region until now. Yang et al. described a major epitope in VP2 (^{8}TLLEDRILT^{16}) (42), showing that this linear epitope was highly conserved among 21 isolates of all seven serotypes of FMDV and provided proof of concept on an effective serotype-independent test by developing monoclonal antibodies directed to that peptide. Although our study lacks such proof of concept, we performed a robust divergence analysis using a lot more sequences than the previous one. Thus, we can claim our designed protein to be much more reliable at the question of developing a kit for the whole Asian region. Whereas previous studies showed experimental data, we focused on analyzing the actual divergence pattern of VP2 among all Asian subgroups (except a few from which no VP2 sequences were available), and thus proposed a designed protein that will be inter-serotypically highly conserved among all Asian strains. In agreement, our proposed protein will contribute to the scientific world by offering a similar output for all Asian isolates, which was not claimed by previous studies. Though we have lack of experimental data supporting our conclusions, the robustness of our data analysis will widen the window for developing a type-independent detection strategy for FMDV as we are providing necessary information regarding the conserved epitopes of a designed VP2 protein along with their surface accessibility and structural assessment. Finally, the future direction of our study will be the development of a VP2-based indirect ELISA for FMD diagnosis using the designed protein. The first following step in this purpose will be the synthesis of the designed VP2 protein, and then the protein will be used as a coating antigen in an indirect ELISA approach for type-independent detection of anti-FMDV antibody.

CONCLUSIONS

The study has validated that the VP2 protein of FMDV is significantly conserved among three FMDV serotypes (O, A, and Asia1) and other hierarchical subgroups in comparison with VP1. The study also reported four previously undescribed, surface-exposed, inter-serotypically conserved antigenic sites in VP2. Based on these findings, the study reported a designed consensus VP2 protein that carries promise to be used in the development of a serotype-independent diagnostic tool, which will be applicable to the whole Asian region. This is the first report showing the overall diversity of VP2 protein where we validated the credibility of VP2 in serotype-independent detection of FMDV.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MS designed the study. IM performed the experiments, analyzed the results, and wrote the manuscript. SA and AA contributed to the analysis and helped in writing the manuscript. MS and MH provided technical support. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00592/full#supplementary-material

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Bovine Dendritic Cell Activation, T Cell Proliferation and Antibody Responses to Foot-And-Mouth Disease, Is Similar With Inactivated Virus and Virus Like Particles

Valeria Quattrocchi[1], Juan Bidart[1], Ana Clara Mignaqui[2], Vanesa Ruiz[1], Alejandra Ferella[1], Cecilia Langellotti[1], Mariela Gammella[1], Sergio Ferraris[3], Jorge Carrillo[4], Andres Wigdorovitz[1], Yves Durocher[5], Sabrina Beatriz Cardillo[6], Bryan Charleston[7] and Patricia Inés Zamorano[1]*

[1] IVIT, INTA, CONICET, Buenos Aires, Argentina, [2] IFAB, INTA, CONICET, San Carlos de Bariloche, Argentina, [3] Centro de Ciencias Veterinarias, Universidad Maimónides, Buenos Aires, Argentina, [4] CIA - INTA Castelar, Buenos Aires, Argentina, [5] Human Health Therapeutics Research Center, National Research Council Canada, Montreal, QC, Canada, [6] Biogénesis-Bagó S.A., Buenos Aires, Argentina, [7] BBSRC National Virology Centre, The Pirbright Institute, Woking, United Kingdom

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***Correspondence:**
Valeria Quattrocchi
quattrocchi.valeria@inta.gob.ar

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Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals that causes severe economic losses in the livestock industry. Currently available vaccines are based on the inactivated FMD virus (FMDV). Although inactivated vaccines have been effective in controlling the disease, they have some disadvantages. Because of these disadvantages, investigations are being made to produce vaccines in low containment facilities. The use of recombinant empty capsids (also referred as Virus Like Particles, VLPs) has been reported to be a promising candidate as a subunit vaccine because it avoids the use of virus in the vaccine production and conserves the conformational epitopes of the virus. Mignaqui and collaborators have produced recombinant FMDV empty capsids from serotype A/ARG/2001 using a scalable technology in mammalian cells that elicited a protective immunity against viral challenge in a mouse model. However, further evaluation of the immune response elicited by these VLPs in cattle is required. In the present work we compare the effect that VLPs or inactivated FMDV has on bovine dendritic cells and the humoral response elicited in cattle after a single vaccination.

Keywords: FMDV, empty capsids, dendritic cells, bovine, A/ARG/2001

INTRODUCTION

Foot-and-mouth disease (FMD) is an infectious-contagious, acute, and febrile disease of cloven-hoofed animals such as cattle, pigs, sheep, and deer, whose etiologic agent is Foot-and-Mouth Disease Virus (1, 2). The disease is endemic in many parts of the developing countries and is absent in countries from North and Central America, Australia, and Europe. FMD results in, loss of productivity and severe restrictions to international trade and pose a serious and constant threat to livestock industries, since FMD spreads rapidly giving rise to large scale outbreaks (3).

Vaccination with the inactivated virus is still the main strategy for disease control in countries where the disease is endemic (4, 5). Although inactivated vaccine has been effective in controlling the disease, it has some disadvantages. Among them, the need for expensive biosecurity facilities for vaccine production which requires constant investments in manufacturing plant up-grades and personnel qualifications together with the risk of viral escape from the production facilities or live virus contamination of the vaccines. Also, the storage and supply of the inactivated vaccine are cold-chain dependent because of the low vaccine stability at ambient temperatures and the production requires high purification process to remove the non-structural proteins from vaccine formulations to be able to discriminate vaccinated from infected cattle (6, 7). Moreover, many countries have regulatory restrictions that prohibit the production of inactivated FMD vaccines in their mainland. Because of these disadvantages, investigations are being made to produce vaccines as immunogenic as the inactivated viral vaccine in low containment facilities. FMDV recombinant empty capsids (VLPs) seem a promising alternative since they contain all the protein immunogenic sites of the virus, but lack the infectious nucleic acid and natural FMDV empty capsids have been shown to be as immunogenic as virions. Mignaqui et al. (8) have produced recombinant FMDV serotype A/ARG/2001 VLPs with high yield, using Transient Gene Expression (TGE) in mammalian cells. These empty capsids triggered a protective immune response against viral challenge comparable to the response elicited by the same amount of inactivated virus in a mouse model. A key step to move forward through the development of a novel vaccine against FMD based on VLPs is to demonstrate that the immune response elicited by these novel recombinant antigens is similar to the response to inactivated virus in the natural host.

In this regard, dendritic cells (DCs) are key players in initiating immune responses after infection or vaccination since they have unique abilities to stimulate naïve T cells and thus represents an important immune response player to study together with the immune response in the host animal.

In the present work, we compare the effect of FMDV recombinant empty capsids produced by Mignaqui and collaborators, with the response from inactivated virus on bovine DCs and the humoral response they induce in bovines, in order to gain insight about the immunogenicity of these novel recombinant particles in the natural host.

MATERIALS AND METHODS

Experimental Design

Afferent lymph dendritic cells (ALDCs) and peripheral blood mononuclear cells (PBMCs) were obtained from the same prime vaccinated calf as described below. These cells were used to test *in vitro* VLPs immunogenicity by assessing dendritic cell activation and T cells proliferation and to compare it with the response induced by inactivated FMDV contained in the commercial vaccine. Other groups of naïve calves were vaccinated using VLPs or inactivated antigen, both formulated with commercial adjuvant, in order to compare the antibody response of both formulations.

Harvesting ALDCs

Holando-Argentino calves were used to obtain afferent lymph dendritic cells (ALDCs) as described before (9). Briefly: 2 months before cannulation surgery, prescapular lymph nodes were removed. After that time, cannulation surgery was performed on the vessel resulting from anastomosis between afferent and efferent vessels, by inserting a cannula. Lymph was collected in a flask containing heparin and antibiotics. The flasks were replaced daily and lymph cells were concentrated by centrifugation on histopaque-1083 (Sigma) gradient. Cells were cryopreserved with fetal calf serum 10% DMSO under liquid nitrogen until use. Approximately 10–15% of the lymph cells were ALDCs characterized by flow cytometry as DEC205+/FSC^{high}/CD11c+/CD8– as reported previously (9, 10). Calves were vaccinated once with the commercial vaccine regularly used in the national vaccination campaign against FMD, a few months prior to cannulation.

Peripheral Blood Mononuclear Cells (PBMCs) Isolation

Heparinized blood was obtained by jugular vein puncture (from cannulated animal). Blood was centrifuged on Lymphoprep (Ficoll-Paque Plus 1.077 g/ml GE Healthcare) gradient. The white band containing PBMCs was washed and cryopreserved with fetal calf serum 10% DMSO under liquid nitrogen until use.

Recombinant Empty Capsids Production

VLPs were obtained as described previously (8). Briefly, suspension-growing 293-6E cells were transiently transfected with pTT5-P12A3C plasmid, cells were harvested 48 h post transfection. After centrifugation, cell pellets were lysed by freeze and thawed cycles and analyzed for protein expression. These lysates were used as source of empty capsids and lysates from non-transfected cells were used as mock control.

Inactivated Virus Production

FMDV serotype A/ARG/2001 was grown in BHK-21 cell cultures in Biogénesis-Bagó high biosecurity facilities. Then inactivation was carried out by Binary Ethylenimine (BEI) treatment and inactivated FMDV (iFMDV) was purified by ultrafiltration/diafiltration.

Regulation of Co-stimulatory Molecules on ALDCs

lymph cells were incubated for 16 h with iFMDV serotype A/ARG/2001, VLPs, mock or Poly I:C as positive control, in comparable amounts (1 μg/ml) in IMDM (Gibco) cell culture medium with 10% fetal calf serum (FCS). Regulation of co-stimulatory molecules CD40, CD86, and MHCII, was measured after overnight incubation of lymph cells with

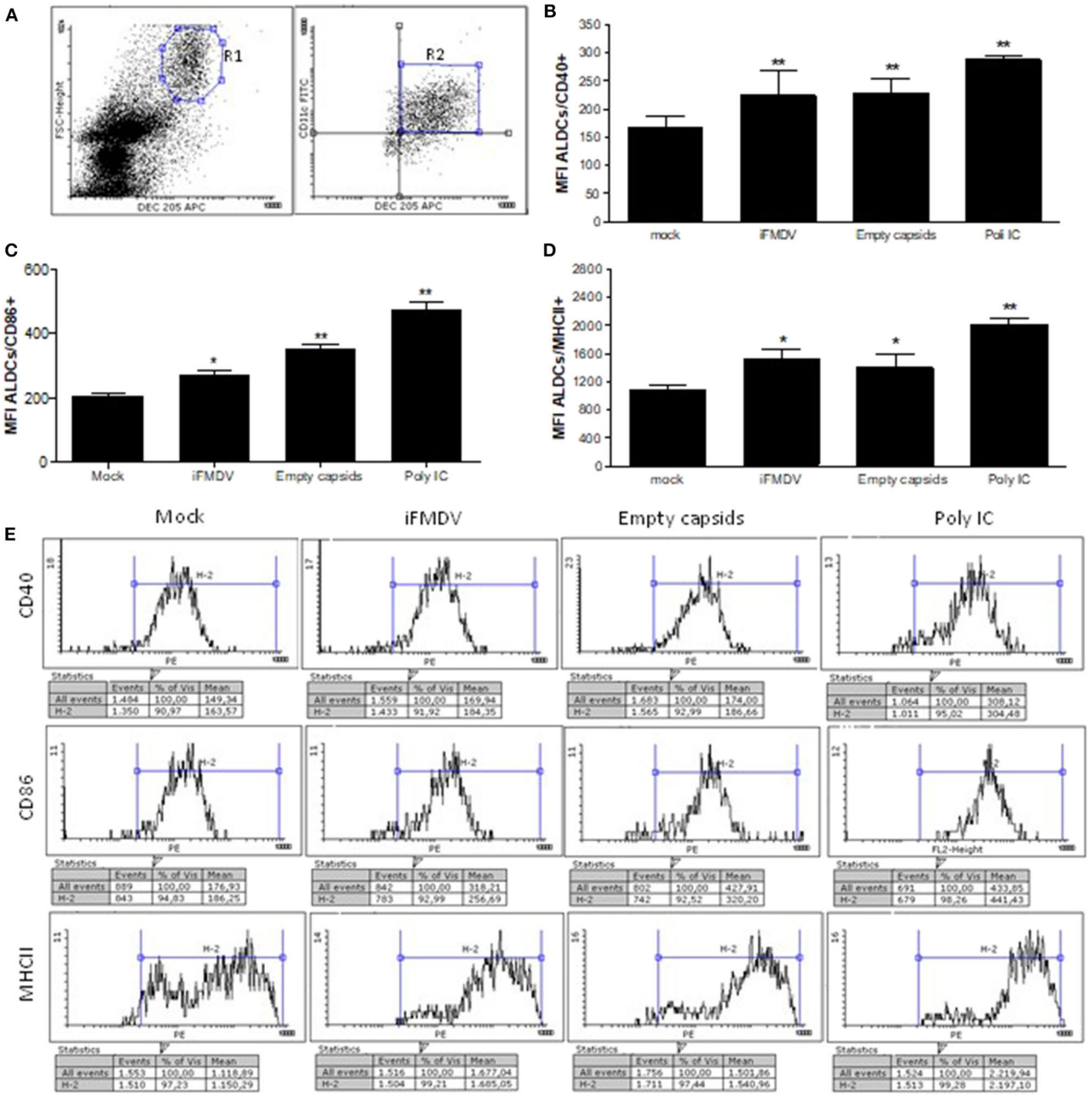

FIGURE 1 | Surface expression of CD40, CD86, and MHCII co-stimulatory molecules on afferent lymph dendritic cells (ALDCs) (FSC^{high}/DEC205+/CD11c+) after overnight incubation with iFMDV or empty capsids, is shown. **(A)** Gating strategy used for cytometric analysis. Co-stimulatory regulation molecules were analyzed in R2 region **(B)** Regulation of CD40 molecule **(C)** Regulation of CD86 molecule. **(D)** Regulation of MHCII molecule. Results in **(B–D)** are expressed as the mean fluorescence intensity plus Standard Deviation (SD) of four replicates in two independent assays. Mock group were lymph cells incubated with supernatant from non-transfected cells. Poly (I:C) was used as a positive control. *Significant difference $p < 0.05$, **significant difference $p < 0.01$. **(E)** One representative histogram of each group is shown.

antigens using an indirect surface staining performed with mouse monoclonal antibodies anti CD40, CD86, and MHCII (SEROTEC), and then anti mouse IgG-Pe conjugated (BD). Finally anti-DEC205 APC-conjugated and anti CD11c FITC-conjugated monoclonal antibodies were added. Cells were fixed with 0.2% paraformaldehyde and were acquired using FACScalibur cytometer and CellQuest software (BD). The analysis of co-stimulatory molecules expression was detected specifically in the FSC^{high}/ DEC205+/CD11c+ cell population. Gating strategy is shown in **Figure 1A**.

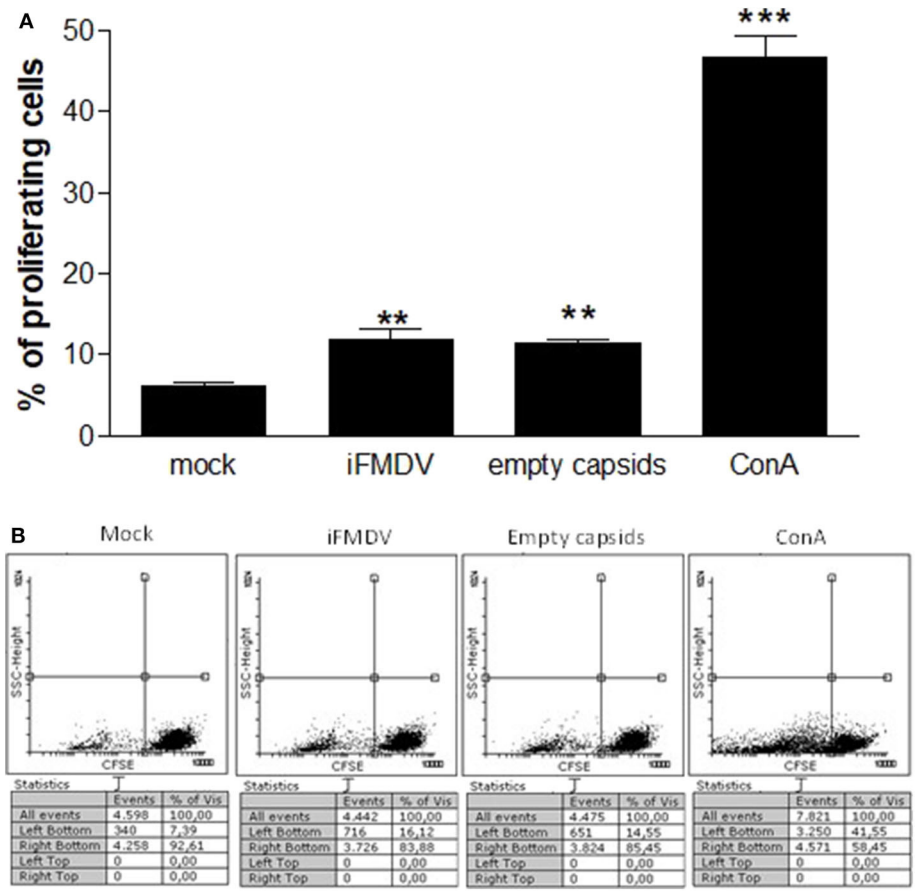

FIGURE 2 | Co-cultures of lymph cells incubated with iFMDV or empty capsids, and CFSE-labeled PBMCs. **(A)** Results are expressed as percentage of proliferating PBMCs. Mock = ALDCs incubated with supernatant from non-transfected cells. ** and *** represent a significant difference ($p < 0.01$ and $p < 0.001$ respectively) regarding mock control. Mean of triplicates + SD of one representative graph of two independents assays, are shown. **(B)** A representative dot plot of CFSE lost for each group is shown.

Proliferation Assessment

iFMDV or VLPs stimulated ALDCs (same procedure as used for regulation of co-stimulatory molecules), were washed with PBS and then with fresh RPMI 1640 (Gibco) 10% FCS medium, and put in contact with Carboxyfluorescein succinimidyl ester (CFSE)-charged PBMCs from the same animal. PBMCs proliferative response was evaluated according to the progressive CFSE staining reduction by flow cytometry, after 5 days incubation at 37°C in a CO_2 incubator. Incubation of PBMCs with Concanavalin A (ConA) was used as positive control.

Humoral Response Assessment

Seronegative calves 8–10 months old, were vaccinated by subcutaneous route with a final volume of 2 ml/dose of formulations containing 25 μg/dose of iFMDV or VLPs ($n = 4$, per group) with a water-in-oil single emulsion adjuvant included in the commercial vaccine currently used in eradication campaigns in Argentine (Biogenesis-Bagó). Calves were bleed at 15, 25, 35, and 45 days post vaccination (dpv) and humoral response were measured by liquid phase ELISA (11). Briefly: Greiner Microlon® plates were coated overnight at 4°C with 1/4,000 dilution of rabbit anti-FMDV serum in carbonate-bicarbonate buffer, pH 9.6. After washing with 0.05% Tween-20/phosphate buffered saline (PBST), plates were blocked with PBST/1% ovalbumin (blocking buffer) for 30 min at 37°C. Sera were serially diluted (first dilution 1:10 and then 1:5 dilutions) in blocking buffer and a fixed amount of iFMDV was added. After 1 h incubation at 37°C with shaking, the virus-antibody mixtures were transferred to the blocked plates, and incubated for 1 h at 37°C. A 1/1,000 dilution of guinea pig anti-FMDV serum in PBS/2% normal bovine serum/2% normal rabbit serum was added for detection, followed by 1 h incubation at 37°C. Plates were washed and peroxidase-conjugated anti-guinea pig IgG (Jackson ImmunoResearch®) serum 1/2,000 diluted in the same buffer was added, followed by 1 h incubation at 37°C. OPD/H_2O_2 was used as peroxidase substrate and A492 was measured in a microplate reader. Positive and negative bovine reference sera were included in each test, for validation. Antibody titers were expressed as the

negative $\log_{10}$ of the highest dilution of serum that causes more than 50% inhibition of color development than in the control samples.

Statistics

ANOVA and Bonferroni post ANOVA tests were used to compare data among groups. $P < 0.05$ was considered as an indicator of significant difference.

Ethics Statement

Experiments involving animals were performed in accordance with protocols approved by the INTA's Ethical Committee of Animal Welfare (CICUAE Permit numbers: 06/2013).

RESULTS

FMDV Empty Capsids Up-Regulates Co-stimulatory Molecules

Lymph cells were incubated with the same concentration of iFMDV or empty capsids. After staining of cells with fluorochrome-conjugated antibodies, anti DEC205, anti CD11c, and MHCII, CD40 or CD86, the analysis of co-stimulatory molecules was made on FSChigh/ DEC205+/CD11c+ cells. As shown in **Figure 1**, iFMDV and empty capsids significantly up-regulates co-stimulatory molecules CD40, CD86, and MHCII. As expected, Poly I: C significantly upregulated the expression of the three molecules.

ALDCs Incubated With FMDV Empty Capsids Are Capable of Stimulating PBMCs Proliferation

ALDCs were incubated with the same concentration of iFMDV or empty capsids, then cells were washed repeatedly in order to eliminate any free inactivated virus or capsids not associated with the ALDCs. The ALDCs were then put in culture with CFSE-labeled PBMCs from the same animal. After 5 days incubation, an anamnestic response was detected. The percentage of proliferating T cells from a vaccinated calf was significantly increased, according to the measurement of CFSE stain loss (**Figure 2**). Concanavalin A was added directly on PBMCs as a positive proliferation control.

FMDV Empty Capsids and IFMDV Are Capable of Stimulating a Similar Humoral Response in Bovines

A dose of 25 μg of iFMDV or empty capsids was used according to previous reports indicating that commercial tetravalent vaccines inducing good levels of protection contain approximately this amount of inactive virus (12). When empty capsids or iFMDV were formulated with commercial adjuvant and inoculated in FMDV seronegative bovines, a specific humoral response was induced in both groups. Antibody titres ($\log_{10}$) ranged between 2.60 and 2.84 for empty capsids vaccinated group, and 2.60–2.9 for iFMDV group. In both groups, these titres of antibodies were maintained up to 45 dpv, without booster immunization (**Figure 3**).

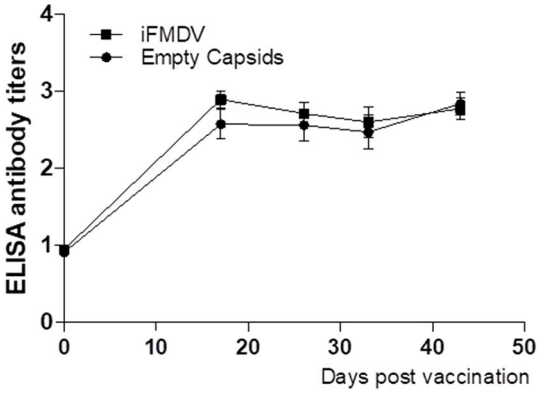

FIGURE 3 | Antibodies titers against FMDV evaluated at 0, 15, 25, 35, and 45 dpv by Liquid phase ELISA in vaccinated cattle. Bovines were inoculated with inactivated FMDV (■) or empty capsids (●) formulated with commercial adjuvant. Results are expressed as mean plus SD error bars of 4 animals.

According with the National Service of Health and Agricultural Quality (SENASA), these antibody titers correspond to a percentage of expected protection between 91.6 and 96.6% (13).

DISCUSSION

In the last years, considerable efforts have been made to develop an effective and safe vaccine against FMD, especially concerning the need to avoid the use of large quantities of live virus because of the risk and the cost of production. Since natural FMDV empty capsids have been shown to be as immunogenic as virions, several approaches have reported the use of empty capsids as an alternative vaccine. In 2013, Mignaqui and collaborators, produced FMDV recombinant empty capsids from serotype A/ARG/2001 in a mammalian expression system, and evaluated them in the mouse model that has been widely used and it has been proved to be a useful tool to predict the immune response against FMDV in bovines (14–17). Humoral responses elicited by FMDV empty capsids in mice were high and empty capsids were fully protective against viral challenge (8). Nevertheless, immune response elicited to these empty capsids has not been assessed in bovines.

This study was undertaken to evaluate the ability of FMDV recombinant empty capsids serotype A/ARG/2001, to stimulate bovine dendritic cells and consequently generate a protective humoral immune response, in order to determine if these VLPs would be a good antigen candidate for vaccine development.

DCs play a main role in the adaptive immune response development, since they are highly specialized in taking, processing and presenting antigens to naive T lymphocytes (18). *In vitro* differentiated DCs from monocytes (MoDCs) are a broadly accepted model, nevertheless, it has been reported

that they functionality could be affected by the treatments used in order to differentiate them (9, 19–21). Moreover, MoDC are different cells from conventional DC (cDCs) (22) that differentiate in peripheral tissues only in inflammatory conditions, and they do not migrate in the lymph or do it weakly (23) while ALDC are mainly cDC. Charleston and collaborators (9) have developed a technique to cannulate bovine afferent lymphatic vessels, allowing collecting of large volumes of lymph containing afferent lymph dendritic cells (ALDCs). This work was carried out using ALDCs which are reported to be a more physiological alternative to *in vitro* differentiated dendritic cells, since they are not subjected to long periods of culture, enzymatic treatment or separation treatments (9, 10).

It has been reported that VLPs contain the complete repertoire of FMDV epitopes with the particulate and repetitive structure of the virus (4, 24). We investigated whether VLPs and inactivated virus would induce similar immune responses. These are important studies to understand if the inactivated RNA genome still present in the inactivated virus provided additional immune stimulation compared to empty capsids. In this sense, we demonstrate that empty capsids are capable of up-regulating co-stimulatory molecules CD40, CD86, and MHCII on the ALDCs surface in the same way as inactive virus. It is known that CD40 signaling induces changes in DCs that make them more effective antigen presenting cells, such as up-regulation of MHC class II and co-stimulatory molecules CD80 and CD86 (25, 26). Accordingly, incubation of ALDCs charged with VLPs or inactive virus, produced a proliferative response when they were incubated with CFSE- labeled PBMCs from the same animal. Taking into account that the donor calf for ALDCs and PBMCs, received one vaccination with the FMD commercial inactivated vaccine previous to the cannulation surgery and the PBMCs period of *in vitro* culture, we hypothesize that the observed proliferation correspond to memory T cells being stimulated by ALDCs. These results indicate that FMDV VLPs are processed and presented to T lymphocytes in a very similar way to iFMDV.

As expected, VLPs were able to produce a high humoral response like the iFMDV when antigens were formulated with a commercial oil adjuvant and antibody response was assessed *in vivo* in FMDV seronegative cattle. Indeed, the humoral response elicited by VLPs lasted the same time post vaccination as the one elicited by iFMDV, in the period analyzed.

Even though the animals were not challenged, the antibody titers achieved by VLPs and iFMDV are above the passmark of approval in potency test of FMD vaccines in Argentina according to statistical correlation previously reported (27).

Our results are in agreement with data from other groups, who reported that recombinant empty capsids of FMDV from different serotypes, produced in different systems, were capable of inducing humoral responses and even fully or partially protecting natural hosts against challenge (3, 28, 29).

Considering the immunogenicity of these VLPs in cattle and the fact that their production is scalable and simple, these empty capsids are a promising antigen to replace the current inactivated vaccine.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by INTA's Ethical Committee of Animal Welfare (CICUAE Permit numbers: 06/2013).

AUTHOR CONTRIBUTIONS

VQ: experimental design, fulfillment, analysis and interpretation of *in vitro* experiments, manuscript drafts, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JB: vaccines formulation for calves, assessment and analysis of antibodies by liquid phase ELISA, and critical revision of manuscript. AM, VR, AF, YD, and AW: production, characterization, purification, and quantification of FMDV empty capsids and critical revision of manuscript. CL and MG: post-surgical cares and daily lymph collect, collaboration in flow cytometry assays, and critical revision of the manuscript. SF and JC: veterinary surgeons, control of anesthetics, cannulation surgeries, and post-surgical cares. SC: inactivated virus production and purification, purification and quantification of FMDV empty capsids used to inoculate bovines, and adjuvant provision. BC: training in cannulation surgery technique and characterization of ALDCs, critical revision of the manuscript, and approval for publication of the content. PZ: conception of the work, participation in experimental design, critical revision of the manuscript, approval for publication of the content, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors: contributed to the article and approved the submitted version.

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Conflict of Interest: SC was employed by the company Biogénesis Bagó.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

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Non-discriminatory Exclusion Testing as a Tool for the Early Detection of Foot-and-Mouth Disease Incursions

Michael Eschbaumer[1], Andrea Vögtlin[2,3], David J. Paton[4], Jamie L. Barnabei[5], Manuel Jose Sanchez-Vazquez[6], Edviges Maristela Pituco[6], Alejandro Mauricio Rivera[6], Dwane O'Brien[7], Charles Nfon[8], Emiliana Brocchi[9], Labib Bakkali Kassimi[10], David J. Lefebvre[11], Roberto Navarro López[12], Eduardo Maradei[13], Sergio J. Duffy[13], Angelika Loitsch[14], Kris De Clercq[11], Donald P. King[4], Stéphan Zientara[10], Christian Griot[2,3] and Martin Beer[1]*

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, [2] Institute of Virology and Immunology, Sensemattstrasse, Mittelhäusern, Switzerland, [3] Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland, [4] The Pirbright Institute, Ash Road, Woking, Surrey, United Kingdom, [5] National Animal Vaccine and Veterinary Countermeasures Bank, Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, Greenport, NY, United States, [6] Centro Panamericano de Fiebre Aftosa y Salud Pública Veterinaria—PANAFTOSA, Rio de Janeiro, Brazil, [7] Diagnostic Surveillance and Response, Australian Animal Health Laboratory, CSIRO, Australian Center for Disease Preparedness, East Geelong, VIC, Australia, [8] National Center for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada, [9] Istituto Zooprofilattico Sperimentale Della Lombardia e Dell'Emilia Romagna, Brescia, Italy, [10] Animal Health Laboratory, UMR1161 Virology, INRAE, Anses, ENVA, Paris-Est Créteil University, Paris, France, [11] Sciensano, Scientific Direction of Infectious Diseases in Animals, Service for Exotic Viruses and Particular Diseases, Brussels, Belgium, [12] Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), Ciudad de México, Mexico, [13] Private Consultants for Animal Health and Epidemiology, Buenos Aires, Argentina, [14] Austrian Agency for Health and Food Safety, Vienna, Austria*

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***Correspondence:**
Michael Eschbaumer
michael.eschbaumer@fli.de

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Endemic circulation of foot-and-mouth disease (FMD) in Africa and Asia poses a continuous risk to countries in Europe, North America, and Oceania which are free from the disease. Introductions of the disease into a free region have dramatic economic impacts, especially if they are not detected at an early stage and controlled rapidly. However, farmers and veterinarians have an obvious disincentive to report clinical signs that are consistent with FMD, due to the severe consequences of raising an official suspicion, such as farm-level quarantine. One way that the risk of late detection can be mitigated is offering non-discriminatory exclusion testing schemes for differential diagnostics, wherein veterinarians can submit samples without the involvement of the competent authority and without sanctions or costs for the farmer. This review considers the benefits and limitations of this approach to improve the early detection of FMD in free countries and gives an overview of the FMD testing schemes currently in use in selected countries in Europe and the Americas as well as in Australia.

Keywords: FMD, early detection, transboundary disease, exclusion testing, surveillance

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals caused by an aphthovirus in the family *Picornaviridae*. The main clinical signs of FMD are lesions on the tongue, oral mucosa and nasal planum, on the teats and in the interdigital spaces and coronary bands of the feet. Except in very young stock, mortality is generally low, but the reduced

productivity and the loss of draft power cause significant economic hardship and food insecurity in endemic areas, which are exacerbated by the costs of control measures, and the forfeiture of trade revenue (1).

FMDV has not occurred in Europe, North America and Oceania for almost 10 years; the last FMD outbreak in any of these regions was in Bulgaria during 2011 (2), while North America and Oceania have been free for much longer. FMD was eradicated in Australia, the United States, Canada and Mexico in 1872, 1929, 1952, and 1953, respectively (3), and has never been reported from New Zealand. However, it is still endemic in Africa and Asia (see also the map in **Figure 1**) (4), and there is always a serious risk of the virus being reintroduced, particularly through the illegal import of animal products. Introductions of FMD into free regions have dramatic social and economic impacts, especially if they are not detected and controlled rapidly (1).

THE CASE FOR NON-DISCRIMINATORY FMD EXCLUSION TESTING

It has often been argued by proponents of pen-side testing that the time from sample submission to the return of results from the diagnostic laboratory is a significant obstacle to the rapid detection of an FMD incursion (5, 6). While this may be the case in areas with insufficient transportation and laboratory infrastructure, the turnaround time in countries with highly industrialized agriculture and sophisticated veterinary services is typically short. For "hot" initial suspicions, samples are sent to official laboratories by courier or government vehicle and tested immediately upon arrival, with results usually returned within 24 h (7–9).

By contrast, much longer periods of time can pass between infection, the first occurrence of clinical signs, their recognition by the farmer, and finally the submission of samples for laboratory testing. The first obstacle here is the recognition of clinical signs and the realization that they may be an indication of a larger issue that requires veterinary attention (see **Figure 2A**). The severity and within-herd prevalence as well as the risk of onward transmission are related to the time that has elapsed since the introduction of FMDV into the herd. Therefore, it is essential that reporting occurs as early as possible, even at a mild incipient suspicion of disease, rather than waiting for a high level of morbidity. These factors are critical elements for limiting the size of outbreaks after a disease incursion into an FMD-free country. Laboratory testing will not occur unless a problem is reported to an official or private veterinarian in the first place. Accordingly, efforts to improve reporting by livestock owners (e.g., awareness programs, streamlined reporting procedures, or the availability of telephone "hotlines") should be considered. But, even if farmers come to realize that there is a problem, some may decide not to consult a veterinarian because of cost implications, a lack of trust in animal health authorities, or the fear of consequences for themselves or their animals (10–13).

An example of this is the large series of FMD outbreaks that originated in the United Kingdom (UK) in 2001 and ultimately affected four countries in Europe, resulting in the culling of over 6 million animals as well as economic losses of 8 billion Euros (1). After the country had been free of FMD for 34 years, a serotype O strain of ultimately unknown, but most likely East Asian origin

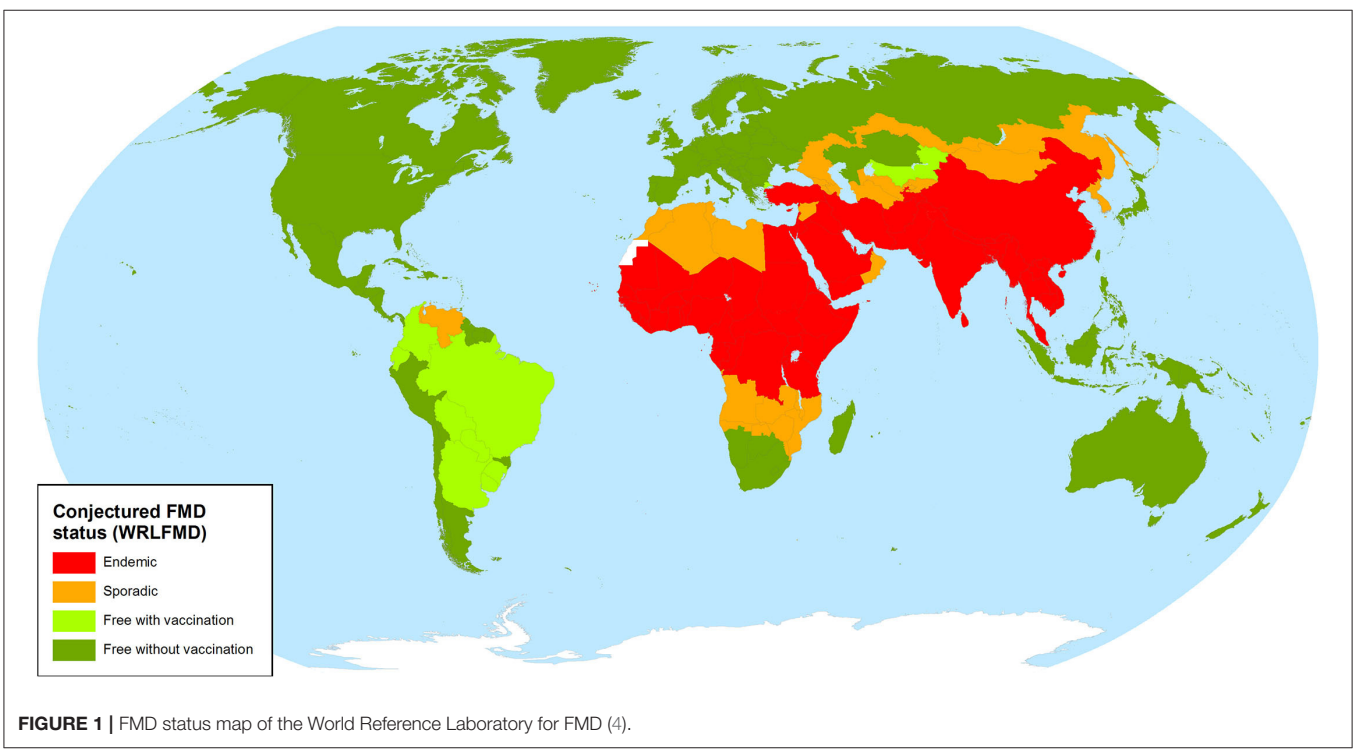

FIGURE 1 | FMD status map of the World Reference Laboratory for FMD (4).

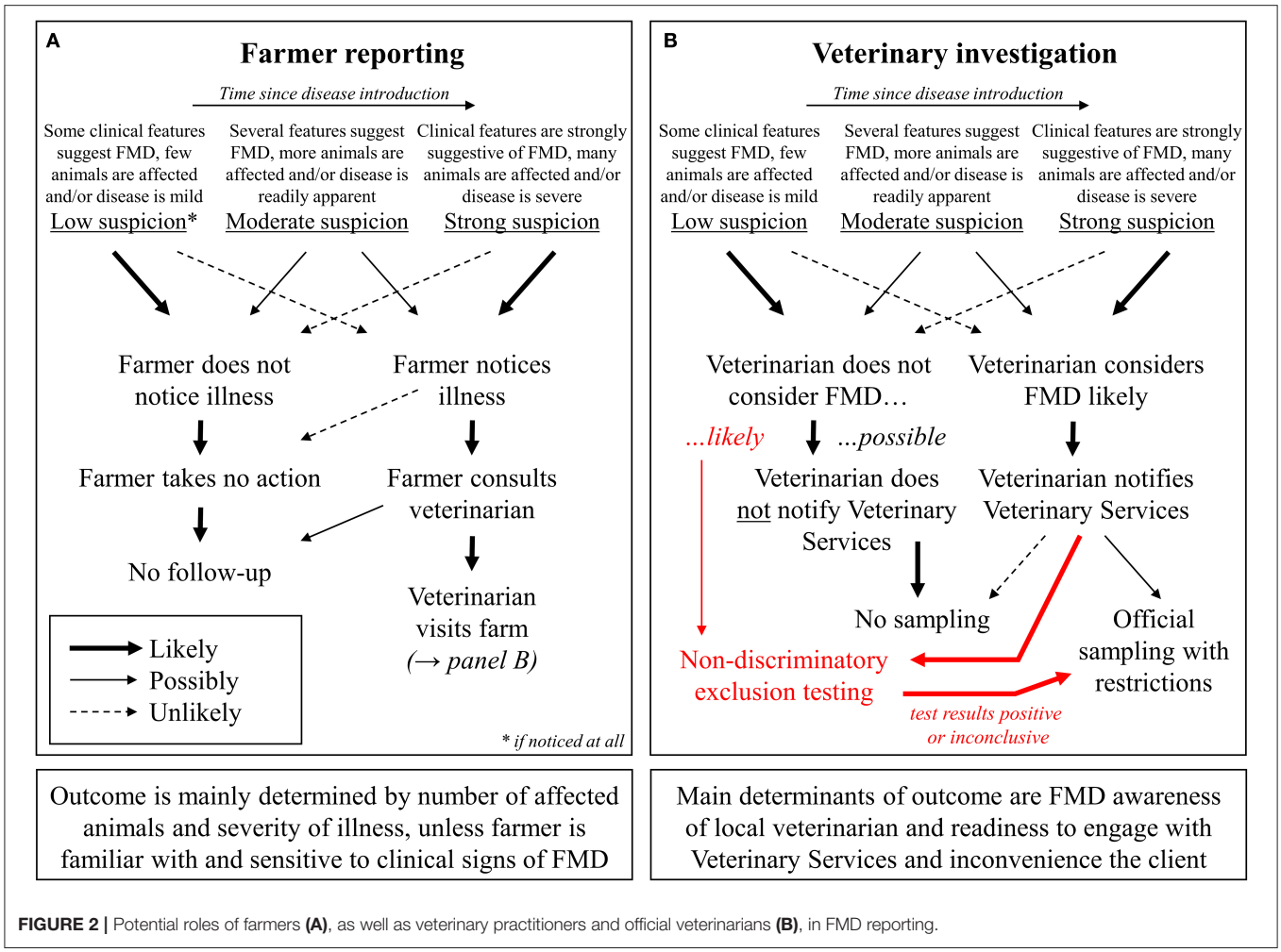

FIGURE 2 | Potential roles of farmers **(A)**, as well as veterinary practitioners and official veterinarians **(B)**, in FMD reporting.

was introduced into a pig fattening unit in northern England by illegal feeding of untreated catering waste (14). This was not realized until weeks later, when clinical signs were detected in pigs that became infected at an abattoir that had received animals from the index herd. At this point, the disease had already spread widely, and it took over 6 months to bring the outbreak under control (15). Sheep played a large role in the early dissemination of the virus, and it was later determined that sheep farmers had also noticed lameness in their animals but did not seek veterinary advice (15). While the pig farmer was found guilty of deliberately hiding the disease in his animals (16), it appears that the sheep farmers were genuinely unaware of the implications of their observations.

In areas where a particular disease has not occurred in decades, most farmers and practitioners are unfamiliar with its clinical manifestations (11). There are a wide variety of conditions (both infectious and non-infectious) that can cause clinical signs that are similar to those of FMD (e.g., oral and/or pedal lesions or lameness) (17), and particularly in countries with routine outbreaks of clinically indistinguishable vesicular diseases, there can be a tendency to think that observed signs are caused by anything but FMD (11). For example, the only outbreak of FMD in Canada in 1951 was initially misdiagnosed as vesicular stomatitis, and samples for laboratory diagnosis were first submitted almost 3 months after the first observation of clinical signs (18). Increased incidence of porcine dermatitis and nephropathy syndrome (PDNS) in the UK likely contributed to the delayed detection of classical swine fever (CSF) in 2000. After a false diagnosis of PDNS had been made in one of the holdings first affected, CSF was not suspected for a further 2 weeks, until losses had accelerated steeply (19). In a more recent example from Germany, it took more than 3 months until a highly virulent strain of bovine viral diarrhea virus was identified as the cause of severe disease in several cattle holdings (20). From the beginning of the outbreak, clinical signs noted by farmers—and veterinarians—on the affected premises had included fever and tongue lesions, but at no point were these animals tested for FMD.

In cases where FMD cannot be ruled out based on the clinical picture alone, samples must be submitted for laboratory examination. However, if the collection and examination of these samples is contingent on the declaration of a formal suspicion of FMD, this can set the bar for a submission very high. Except in the most obvious cases, some veterinary practitioners

TABLE 1 | Benefits, limitations, and risks associated with non-discriminatory exclusion testing schemes for FMD.

Benefits	Limitations and Challenges	Risks
−Allows veterinary practitioners to consider FMD as a differential diagnosis without fear of negative consequences for the farmer or themselves −Testing of low-risk exclusion samples at regional laboratories creates and maintains surge capacity for outbreak response	−Requires outreach to farmers and veterinarians to promote participation −Farmers still need to notice clinical signs and seek veterinary attention first −Requires clear definition of exclusion cases vs. notifiable suspicions −Veterinary services must be kept informed and quickly consulted if problems arise during exclusion testing	−If exclusion testing is used instead of immediate notification in serious cases, an appropriate outbreak response may be delayed −False-negative results can delay the recognition of an FMD incursion −May not be acceptable to trading partners

may be reluctant to raise an FMD suspicion because of the consequences that are precipitated by its formal declaration, regardless of whether the suspicion is eventually confirmed by laboratory diagnosis (see also **Table 1**). In the European Union (EU), national animal health laws reflect the provisions of articles 4–9 of the "FMD Directive" 2003/85/EC, requiring a quarantine of the suspect holding and other holdings around it, a movement ban for all animals in a large area around the suspect holding, and even the preemptive killing of animals of susceptible species if this is deemed necessary by the competent authority (21). Similar regulations are in place in other FMD-free countries (9, 22, 23).

Unsurprisingly, official veterinarians may be viewed by practitioners and farmers in an adversarial role. Even though they are required to do so by law in most jurisdictions, practitioners can be reluctant to report diseases that could result in regulatory action and may be afraid of alienating their clients if they raise suspicions that turn out to be false alarms (24) (see also **Table 1**). This is not entirely unreasonable, because the prior probability of FMD being the cause of suspicious lesions is very low in areas that are free of the disease (15). Nevertheless, if the reporting of suspected foreign animal diseases is only the very last resort, it creates a serious obstacle for their timely detection and control (25).

Notwithstanding the critical importance of implementing rapid control measures in the event of a credible disease suspicion, a possible approach to increase FMD surveillance is to allow veterinary practitioners to submit samples for an exclusion of FMD without restrictions for the farm (with or without the direct involvement of the competent authority; see **Figure 2B**). The laboratory costs for such non-discriminatory exclusion testing should be covered by communal animal health funds or the government, because increased testing helps to protect the entire agricultural sector from disaster, and there should not be any financial barriers to participation for individual farmers (11, 24). In addition to excluding FMDV infection, possible differential diagnoses (both endemic and exotic) can be covered by the laboratory investigation, ideally at no additional charge. This added value will further encourage farmers and their veterinarians to participate in the exclusion testing system (see also **Table 1**). Official testing for notifiable diseases such as FMD is often unsatisfactory for farmers because it does not return a useful diagnostic result once the target diseases have been ruled out.

Where non-discriminatory exclusion testing schemes for FMD are already available (e.g., Switzerland and Germany) (8, 26), educating and encouraging practitioners to make use of these programs is an important part of community outreach activities of the veterinary services (see also **Table 1**). In addition to the classical signs of FMD, exclusion testing can also be warranted for non-specific health problems in a herd. For example, German animal health law has recently been revised to require cattle farmers who observe an increased incidence of febrile illness, a significant reduction in milk yield or increased mortality in young stock to consult a veterinarian to rule out FMDV infection (cf. Section 2a of the *Ordinance on Protection against FMD*[1]). Similar rules for swine holdings have been in place since 1999. At the same time, it must be made very clear to practitioners that the exclusion testing option is not to be used if they actually *suspect* FMD! If critical samples are submitted for exclusion testing only, this can lead to delays in disease confirmation and containment because they are not considered a high priority for delivery to and processing at the diagnostic laboratory.

In the EU, while samples from suspect cases must always be sent to the designated national reference laboratory (NRL), exclusion testing by real-time RT-PCR (RT-qPCR) can be done in any laboratory designated by the competent authority (cf. Annex XV No. 13 of the "FMD Directive") (21). Suitable samples for RT-qPCR tests are lesion material, if available, as well as saliva and serum. In FMD-free countries, laboratories accepting these samples do not need to operate under high-containment conditions, but they are obliged to follow procedures that ensure that the spread of possible FMDV present in the sample material is effectively prevented (27). This includes, among other things, the spatial and organizational separation of areas in which FMD exclusion testing is carried out, the use of Class II microbiological safety cabinets for the processing of samples that have not yet been chemically or physically inactivated, and strict hygiene management for work surfaces, equipment, laboratory waste, and personal protective equipment. All laboratories that do exclusion testing must operate to the highest diagnostic standards. In addition to ISO accreditation, this should include successful participation in FMD diagnostic proficiency tests periodically administered by the NRL. The NRL should be kept informed of any exclusion testing performed by these other laboratories.

[1]MKS-Verordnung in der Fassung der Bekanntmachung vom 18. Juli 2017 (BGBl. I S. 2666, 3245, 3526).

If the results of an exclusion test at another laboratory are not clearly negative, the samples must be immediately forwarded to the NRL for clarification (see **Figure 2B**). Under this scenario, fresh sampling of the animals may be required to ensure the provenance of samples and to eliminate any possibility of accidental cross-contamination at the first laboratory. When sending these samples, the transport regulations for category B biological substances (UN3373) must be observed. At the same time, the veterinary authority responsible for the holding where the samples have been taken must be notified of the situation and it is likely that the farm would be placed under formal suspicion until results from the NRL are known. As long as the first-line tests employed have high specificity, at this point, it is no longer a question of exclusion testing or elimination of differential diagnoses but may already rise to the level of a formal suspicion of FMD. If a formal suspicion of FMD is declared, testing to confirm or clear that suspicion can only be carried out at the designated NRL (cf. Annex XV No. 5 and 13 of the "FMD Directive") where it will be treated with high priority.

Overall, when a non-discriminatory exclusion testing scheme is implemented in a country, it is advisable for the veterinary authorities to create guidelines for practitioners. This should include a decision tree to determine whether or not a case is likely to be FMD (based on the clinico-pathological presentation and the epidemiological context), standard procedures for sample collection and submission, and any follow-up actions or reporting requirements. The veterinary authorities need to devise a strategy for the ongoing training of practitioners to make sure they know how to recognize FMD and the conditions for applying non-discriminatory exclusion testing. Moreover, an active communication channel from the veterinary authorities to practitioners needs to be established to be able to timely disseminate relevant information. For example, this can be used to update stakeholders about the regional risk of FMD according to its presence in neighboring countries, as this information will influence the decision to consider FMD as a likely or unlikely differential in a given case.

FMD EXCLUSION TESTING IN PRACTICE

Europe

Switzerland introduced non-discriminatory exclusion testing in 2011. These examinations are carried out at two central government laboratories and cover not only FMD but also African swine fever (ASF), CSF, avian influenza (AI), and Newcastle disease. Samples are submitted by veterinary practitioners and pathologists and include mostly sera, lesions or swabs; in addition, EDTA blood is requested to test for bluetongue (BT) virus as a potential differential diagnosis. Since the costs are covered by the government through the Federal Food Safety and Veterinary Office, the exclusion examinations are free of charge for the senders. The program is considered a success both by the veterinary services and by practitioners, and it has led to a marked increase in the number of samples tested for foreign animal diseases (23). From January 2012 to February 2020, no "hot" suspicions were declared, but 101 FMD exclusion tests were performed. Nevertheless, considering the number of cattle, sheep, goats and pigs in Switzerland (1.5 million, 340 thousand, 80 thousand and 1.3 million, respectively), it would be desirable to receive even more samples for exclusion testing. In a large population of animals, there inevitably is some "background noise" of oral lesions or lameness, due to endemic infectious diseases such as contagious pustular dermatitis (orf) or non-infectious causes such as chemical burns. For example, a recent field study in sheep (28) found oral lesions in 1% of animals. Even if most of these can be determined not to be FMD by other means, a large number of cases will remain that warrant laboratory diagnosis.

Belgium is using a risk-based "increased vigilance" scheme. Among others, this increased vigilance is currently applied to BT, ASF, and AI. The criteria that trigger exclusion diagnostics in the absence of a clinical suspicion are very different for each disease: for BT, it is importation of ruminants from risk areas with serotypes other than serotype 8; for ASF, it is 2 or more pigs on a farm with symptoms of general disease; for AI, it is abnormal production parameters (e.g., increased mortality). Within the "increased vigilance" scheme, the costs for sampling (or necropsy) are borne by the owner of the animals, whereas laboratory analysis is paid for by the Federal Agency for the Safety of the Food Chain (FASFC). As a result of the aforementioned risk analysis, there currently is no organized system for exclusion testing for FMD and other vesicular diseases, even though these tests are available. Every decision for exclusion testing for vesicular diseases is made on an *ad-hoc* basis. Reasons for FMD exclusion testing can be. e.g., a herd problem of unknown etiology, a (presumably false) positive result in an antibody test for export certification, a lesion at the mouth or foot observed at necropsy or irregularities with animal identification or documentation of origin. In the latter case, the exclusion testing must be paid for by the owner, otherwise it is paid for by the FASFC since laboratory analysis is then done in the context of a—perhaps not (yet) formally expressed—suspicion. On average, there are $\leq$ 2 clinical suspicions and $\leq$10 exclusion diagnostics for vesicular diseases per year in Belgium.

Austria introduced a non-discriminatory exclusion testing scheme in 2014, which was revised in 2019[2]. It consists of five stages (see **Table 2**), whereby stage II (exclusion testing) is divided into two sub-stages A and B. An earlier contingency plan established in 2000 had only included provisions for suspected holdings and holdings with an outbreak (29), similar to stages III, and IV of the new scheme.

In routine cases (stage I), any diagnostic laboratory can test for FMDV as a differential diagnosis, but all exclusion testing (stage II), and investigations of FMD suspicions or outbreaks take place at the Austrian NRL. Any veterinarian can submit samples for FMD exclusion testing directly to the NRL or can notify veterinary services. This notification is mandatory if the veterinarian actually suspects FMD! Upon notification, the official veterinarian visits the farm and determines the level of suspicion. If there is only a weak indication of the disease, samples will be submitted for exclusion testing (stage II.B) and

[2]Untersuchungen zum Zwecke des Ausschlusses von Tierseuchen. Erlass BMASGK-74730/0002-IX/B/10/2019.

TABLE 2 | Stages of the Austrian FMD testing scheme.

Stage	I	II A	II B	III	IV
Type of investigation	**Differential diagnosis**	**Exclusion testing**		**Suspicion**	**Outbreak**
Respondent	Any veterinarian		Official veterinarian		
Laboratory	Any	NRL	NRL	NRL	NRL
Costs covered by ministry	No	Yes	Yes	Yes	Yes
Quarantine of the farm	No	No	No	Yes	Yes

TABLE 3 | FMD exclusion investigations done in Australia between 2017 and 2019 as reported in the Australian Animal Health Surveillance Quarterly (AHSQ).

Reported in AHSQ issue	Cattle	Sheep	Camel	Alpaca	Goat	Pig	Buffalo	FMD exclusion reports
2017–Vol 22/1	8	2	0	0	0	0	0	
2017–Vol 22/2	9	6	0	0	0	1	0	p. 40
2017–Vol 22/3	8	5	0	0	0	2	0	p. 20, 33
2017–Vol 22/4	9	8	0	0	0	1	1	p. 18–19. 25
2018–Vol 23/1	10	5	0	0	0	3	0	p. 44
2018–Vol 23/2	20	6	1	0	2	0	0	p. 42
2018–Vol 23/3	10	3	0	0	0	1	0	p. 38
2018–Vol 23/4	9	2	0	0	0	2	0	
2019–Vol 24/1	13	2	0	1	1	0	0	p. 20
2019–Vol 24/2	13	4	0	0	1	1	0	p. 19
2019–Vol 24/3	19	7	0	1	0	0	0	p. 17–18, 19
2019–Vol 24/4	13	4	1	0	0	0	0	
Total (2017–2019)	141	54	2	2	4	11	1	

no restrictions are imposed on the farm. If the suspicion of FMD (or any other notifiable disease) is confirmed by the official veterinarian, stage III (or IV, depending on severity) will be declared and the holding will be quarantined.

Exclusion testing in Austria is free of charge for the farmer, since the central competent authority covers the costs of the testing. However, if the farmer wants a test for a non-notifiable disease from the same sample material (in addition to the exclusion testing), they have to cover the costs of this additional testing. Although non-discriminatory exclusion testing has been offered in Austria since 2014, not many samples have been submitted to the NRL. There are < 5 cases of exclusion testing for vesicular diseases per year in Austria, for a susceptible population of 1.9 million cattle, 0.5 million sheep, 0.1 million goats, and 3 million pigs.

In Germany, the regional veterinary diagnostic laboratories of all federal states (with the exception of the city states, which only have negligible numbers of livestock), have offered exclusion testing for FMD since 2014, following the earlier implementation of distributed non-discriminatory testing for CSF, AI, and ASF. Private laboratories do not test for FMDV. The participating regional laboratories are enrolled in proficiency tests conducted by the NRLs (30, 31) and will forward positive or inconclusive samples to the NRL for confirmation. Due to the sovereignty of the individual states in matters of animal health, the terms and conditions under which the program is conducted are variable, as is the acceptance among practitioners and the number of samples submitted (32). From 2014 to 2016, the number of samples tested for FMDV RNA across all laboratories increased from 281 to 729 (32) without the concurrent emergence of another vesicular disease such as Senecavirus A (SVA) infection (33), as has happened in the United States (see below). This shows that the concept of FMD exclusion testing is gaining acceptance. But, similar to the situation in Switzerland, the number of tests is still far too low compared to the population of susceptible animals in Germany (2016: 12.3 million heads of cattle, 2 million small ruminants and 28 million pigs), and has to be further increased.

Australia

Similar to the situation in Germany, each of the states and territories that make up the Australian federation operate under different animal health legislation and the Chief Veterinary Officer (CVO) of the relevant state determines if any legal restrictions should apply. Legally binding on-farm restrictions are not mandated whenever an FMD laboratory test is conducted. This allows for exclusion testing to be carried out where FMD is not thought to be a probable differential diagnosis however clinical signs such as lameness or salivation are present. This approach applies across all nationally notifiable diseases and aims to encourage exclusion testing for all livestock species. In the 3 years between January 2017 and December 2019, 215 exclusions for vesicular diseases were carried out in Australia (**Table 3**). All exclusion testing for nationally notifiable livestock diseases is funded by the commonwealth

government and testing is carried out by the Australian Center for Disease Preparedness in Geelong. Detailed case reports on FMD exclusions are frequently published in the Australian Animal Health Surveillance Quarterly (34) (see examples in **Table 3**).

In addition to the laboratory testing being funded by the national government, Australia also has in place a National Significant Disease Investigation Program (SDI) (35). This program provides subsidized veterinary services and diagnostic testing up to the value of AU$1100 to investigate disease events (36). This program aims to encourage non-government veterinarians to investigate the cause of a significant disease event even when the commercial value of the animal is less than the veterinary services required. Although not specifically targeted at vesicular diseases, the SDI program often results in government and non-government veterinarians working together to solve animal health problems.

SYSTEMS IN USE IN COUNTRIES THAT DO NOT ALLOW NON-DISCRIMINATORY EXCLUSION TESTING

Europe

The UK has adopted a binary perspective for initial report cases of FMD in the country—i.e., either (i) there is credible suspicion of disease and on-farm restrictions are adopted until laboratory results are generated or evidence for disease freedom can be provided from other sources, or (ii) based on the clinical and epidemiological context, the animals are not considered to be infected with FMDV and no laboratory testing is necessary or even desirable. This is achieved by a two-tiered alert system (available 24 h a day with veterinary epidemiology support): in stage 1, where restrictions are immediately placed on the premises (by law), clinical suspicion of FMD leads to a visit of an official veterinarian, who undertakes clinical investigation and assesses whether samples are to be taken. If the veterinary visit cannot rule out FMD, stage 2 involves sample collection, laboratory testing and implementation of full restrictions on the farm as well as the need for area restrictions (considered by the competent authority) until a negative test result is returned.

Similar to the UK, France does not employ non-discriminatory exclusion testing for FMD, but its national reference laboratory also has veterinarians and epidemiologists available by phone 24 h a day. Veterinary practitioners who encounter alarming clinical signs can call in and describe and electronically transmit their observations with photos. Taking into account the risk profile of the holding, the epidemiological situation and the clinical signs, the central team will decide if the situation requires laboratory testing or if FMD can be ruled out without it. This relieves the veterinary practitioner of any responsibility for that decision and lowers the bar for reporting possibly suspect cases. If testing is deemed necessary (usually 1–2 cases per year for a susceptible population of 20 million cattle, 9 million sheep and 7 million pigs), the holding will be quarantined, and the samples will be forwarded to the NRL with high priority and tested immediately at any time.

Italy does not offer exclusion testing for FMD. In case of an FMD suspicion, private veterinarians must inform the veterinary services, which will then visit the suspected herd. The cost of laboratory testing for suspect cases is covered by national funds from the Ministry of Health. Formal FMD suspicions were last declared in 2015 and 2016 (2 each), for a susceptible population of 5.6 million cattle, 0.4 million buffalo, 6.2 million sheep, 1 million goats and 8.7 million pigs. Currently, virological testing for FMD in Italy is only done at the NRL, which is located at one of the ten *Istituti zooprofilattici sperimentali* (IZS) of the national animal health and food safety network. However, the IZS network is prepared to carry out post-vaccination FMD serology, and it is planned to extend the existing proficiency testing for the serological assays to include FMDV RT-qPCR. This will build up distributed diagnostic capability that will be very useful if there is an FMD outbreak. If low-risk submissions (e.g., from holdings without clinical signs) can be tested at regional laboratories, this frees up testing capacity for critical samples at the NRL during an outbreak and can decrease sample turnaround time in large countries.

North America

In Canada, based on the experience of the 1951 outbreak (18), all suspicions of vesicular disease in Canada are considered to be FMD until proven otherwise and any suspicion must be reported to the Canadian Food Inspection Agency (CFIA) (37). Following that notification, the local CFIA veterinarian visits the premises. Based on the clinical signs and in consultation with CFIA foreign animal disease specialists, the level of risk is determined, commensurate restrictions are placed on the premises and samples are collected for laboratory testing at the National Center for Foreign Animal Disease. Samples from animals with clinical signs, morbidity, epidemiological data or other factors that indicate a high likelihood of FMD are submitted as "high risk." For cases where the risk of FMD is low but still warrants laboratory testing, the samples are submitted as "confirmatory negative." Even when the risk is negligible, samples can be sent for laboratory testing under the category of "disease investigation."

In the United States, the Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) is the competent authority for the detection of a foreign/transboundary animal disease (FAD/TAD). Samples for suspect vesicular diseases in livestock are sent to an approved laboratory within the National Animal Health Laboratory Network (NAHLN), a network of more than 60 state, university-associated, and federal laboratories across the country that provide both active and passive surveillance, as well as surge capacity support during an outbreak (38). Duplicate samples are also sent to the National Veterinary Services Laboratories (NVSL) Foreign Animal Disease Diagnostic Laboratory (FADDL) on Plum Island for confirmatory testing to rule out FMD, as handling live FMDV is currently prohibited on the U.S. mainland[3]. All suspect FAD

[3]United States Code, 2006 Edition, Supplement 4, Title 21, Chapter 4, Subchapter III – Prevention of Introduction and Spread of Contagion, Sec. 113a – Establishment of research laboratories for foot-and-mouth disease and other animal diseases. Pub. L.116-141; 2020-06-03.

investigations (FADIs) require formal notification within the National Response Framework. All FADIs begin with notification to the state animal health official (SAHO) and the federal Area Veterinarian in Charge (AVIC), at which time a veterinarian trained in FAD sample collection by FADDL, known as a Foreign Animal Disease Diagnostician (FADD), is dispatched to the location. The FADD collects duplicate samples to send to NVSL and a NAHLN laboratory proficiency tested to run the same FMDV PCR assays as FADDL.

Testing results and reporting will be expedited based on the assigned priority level. The FADD, SAHO, and AVIC will agree on a priority level based on risk and on-site epidemiology of the suspect vesicular case. Four priority levels exist: (i) Priority 3, low suspicion for/unlikely to be an FAD, but cannot be distinguished from an endemic condition. (ii) Priority 2, indication this is possibly an FAD, and cannot be distinguished from an endemic condition. Rapid laboratory confirmation is required; (iii) Priority 1, prompt laboratory confirmation is required because there is a high suspicion for an FAD; and finally (iv) Priority A, which also requires prompt laboratory confirmation and is used in situations where animals in commerce are held pending results for FAD testing (39). Testing is conducted at no cost to the customer.

In some situations, testing for endemic diseases that are clinically indistinguishable from FMD, such as SVA infection (33), may also be performed by NVSL FADDL or the NAHLN. The recent emergence of SVA in North America has led to a dramatic increase in costly official disease notifications in Canada and the USA (40). Prior to 2016, FADDL saw an average of 150 FADIs annually, but in 2017, more than 1,300 accessions were received. Through a shared testing program, 343 of 1,314 total cases in 2018 were tested by the NAHLN only and not by FADDL. In 2019, ∼1,542 accessions were received; however, 687 of those were tested only at a NAHLN laboratory.

In Mexico, a specific surveillance program for vesicular diseases of animals—the binational Mexico-United States Commission for the Prevention of FMD and Other Exotic Diseases of Animals (CPA)—has been in place since 1954. Any suspect vesicular disease must be immediately reported. There is an entire program dedicated to promoting such notifications through courses, newsletters, and social networks. All suspect cases are attended to in < 24 h, on any day of the year, and are handled exclusively by CPA personnel, who have the necessary equipment and means to respond to any notification of a suspect foreign animal disease. Diagnosis occurs at a single high-security laboratory and all investigations are paid for by the CPA. In 2019, 2,621 samples from 103 cases were tested for FMDV, 702/41 of which were cattle, 1,029/35 goats, 685/20 sheep, 85/4 swine, and 120/3 wildlife.

South America

In South America, the epidemiological context and the risk perception is different than in Europe and North America. After its introduction to South America in the 19th century, FMD quickly became endemic in the large cattle population. Only through a tremendous and sustained collaborative effort over several decades has the continent now come close to eradication of the disease (41). While all of South America, apart from Venezuela and a small zone in north-eastern Colombia, is recognized as FMD-free (with or without vaccination) by the OIE (42), the fear of this disease and its economic consequences is still very present among the governments and producers in the region. Thus, there is a strong pressure to deal with any suspicion of vesicular disease as if it were FMD. Notably, vesicular stomatitis virus (VSV) is present in the region, particularly in Colombia and Ecuador (43–45). It has a pathological presentation in ruminants and pigs that is indistinguishable from FMD and laboratory testing is required to rule it out. Accordingly, in the presence of suspected vesicular disease, FMD should always be ruled out together with VSV and for swine, it is advisable to include SVA as well.

An informal opinion poll of current and former CVOs and NRL directors in South America conducted for this review revealed strong disapproval of making non-discriminatory exclusion testing available as an option for private veterinarians. It was seen as too difficult to define what constitutes "clear" or "less clear" suspicions of FMD, and even more difficult to explain such a difference to farmers and private veterinarians. There also was concern that exclusion testing may allow farmers or practitioners to intentionally delay the notification of exotic animal diseases, ultimately resulting in a failure to implement appropriate control measures in a timely manner. In addition, an epidemiological investigation by the veterinary services is seen as essential for vesicular disease suspicions because negative laboratory results may be obtained from infected herds due to inappropriate sample collection, handling or analysis.

In 2018, the 13 countries that are part of the South American Commission for the Fight Against FMD (COSALFA) reviewed the way FMD suspicions are addressed (45). It was agreed that any suspicion of vesicular disease needs to be responded to by the official veterinary services which should proceed, in the first place, with an official visit to the farm. On site, the official veterinarian will decide, based on the epidemiological investigation and the clinico-pathological presentation, whether it is a so-called "well-founded suspicion." Well-founded suspicions should always lead to laboratory testing at the NRL and until FMD is ruled out, the farm is quarantined, and movement restrictions are applied. On the contrary, if the official veterinarian rules out FMD during the site visit the case may be closed without laboratory testing, similar to the approach taken by the UK and France.

There are, however, exceptions to this procedure. For example, when vesicular lesions are found in pigs at an abattoir in Brazil, slaughter may proceed normally if the batch is accompanied by official documentation from the veterinary services indicating that the farm of origin has been investigated for FMD with a negative result within the last 30 days (46). This is used by commercial pig farms with high biosecurity standards that are located in recognized FMD-free areas where SVA is known to be present.

In 2018, the last year for which collated data are available from the Pan American Foot-and-Mouth Disease Center (PANAFTOSA) (43), a total of 1,976 suspicions of vesicular

TABLE 4 | Vesicular disease notifications, laboratory diagnoses, and number of cattle and buffalo in South America in 2018.

Country	Notifications of vesicular disease[a]	"Well-founded"	Positive diagnosis*			Negative laboratory diagnosis			Number of cattle and buffalo	
			FMDV	VSV	SVA	FMDV	VSV	SVA	Heads[b]	Farms[c]
Argentina	5	0	–	–	–	5	–	–	55,546,342	205,655
Bolivia	23	0	–	–	–	23	–	–	9,092,286	183,702
Brazil	775	334	–	4	21	775	771	602	218,004,131	2,454,550
Chile	15	15	–	–	–	15	15	–	3,719,507	125,402
Colombia	428	428	7 (1)	241	18	161	–	–	27,590,935	627,239
Ecuador	575	575	–	308 (5)	–	570	114	–	4,313,264	271,590
Guyana	23	0	–	–	–	–	–	–	260,673	4,024
Panama	24	0	–	8 (16)	–	16	2	–	1,521,500	43,948
Paraguay	10	10	–	–	–	10	10	–	13,500,965	145,025
Peru	45	45	–	36 (32)	–	45	13	–	5,156,044	881,920
Uruguay	10	0	–	–	–	10	–	–	11,435,655	40,576
Venezuela	43	43	–	9 (11)	–	2	2	–	15,454,847	108,211

[a]A farm with one or more susceptible animals with pertinent clinical signs. [b]More than 95% of these are cattle. [c]More than 98% of farms have only cattle. [*]Either by laboratory testing or by clinico-epidemiological investigation (numbers in parentheses).

disease were reported in 12 countries, together representing over 365 million heads of cattle and buffalo. In only 7 cases was FMDV infection confirmed by laboratory diagnosis (see **Table 4**).

Argentina

Like other South American countries, Argentina does not offer exclusion testing. It is free of FMD without vaccination in Patagonia, the southern region of the country, and with vaccination in the rest of the country, where only cattle are vaccinated. The susceptible population includes 55 million cattle, 12 million sheep, 4 million goats, and 5 million pigs. The last FMD outbreak occurred in April 2006. Notification of the disease, including suspicious cases, is mandatory. In the event of a FMD incident, or even when it is only a suspicion, farmers and private veterinarians must immediately make a formal report to the local veterinary services. The latter are solely responsible for collecting and sending the samples to the NRL, which is the only laboratory authorized and accredited to perform the diagnostic tests with the appropriate biosafety and issue the final diagnosis. The costs are entirely covered by the national veterinary services.

Although private veterinarians or farmers should not collect and send samples of suspect cases, their participation and contribution to FMD surveillance is very significant. In fact, they receive training from the national veterinary services to recognize an FMD suspicion. Furthermore, farmers are associated to a local animal health association supervised by the National Food Safety and Quality Service SENASA. This association performs activities such as animal surveys, vaccinations, and surveillance. Apart from the concerns listed above, it is important to mention that the countries importing meat from Argentina (including the EU, USA, and China) regularly review the records of all suspect cases of FMD and would probably not accept an exclusion testing scheme instead of the current system.

DISCUSSION

Non-discriminatory FMD exclusion testing can help to quickly detect an introduction of this devastating disease into a previously free area, which is essential for its effective control. In countries where they are available, exclusion testing schemes are gaining acceptance among veterinary practitioners, but more needs to be done to promote the programs and increase awareness about foreign animal diseases in general.

At the same time, the possible pitfalls of exclusion testing should be kept in mind. Concerns about delays in the implementation of control measures are a major reason that many countries do not allow practitioners to submit samples for FMD exclusion. In these countries, any suspicion of FMD must be handled by the veterinary authorities, who will then take measures (quarantines, laboratory testing etc.) based on a risk analysis. The response to suspicions of FMD or other exotic animal diseases is often seen as an inalienable state activity to be carried out by the national animal health service, leading to strong resistance to the concept of non-discriminatory exclusion testing in the hands of private practitioners and laboratories other than the central NRL.

In this context it is important to emphasize that exclusion testing may complement, but cannot replace formally declared suspicions! A lot of the disagreement about non-discriminatory exclusion testing comes from the fact that in many countries, as soon as FMD is even *considered* in the differential diagnosis of a clinical case, it must be reported to the authorities. By contrast, other countries only require the notification of an actual *suspicion*. Where the lines between "no suspicion," "weak suspicion" (consideration as a differential diagnosis) and "well-founded suspicion" are to be drawn is critical but often left to the individual private practitioner or government official.

It is clear, however, that a key step for the success of non-discriminatory FMD exclusion testing is the decision

made by the private veterinarian during the farm visit; i.e., whether they consider FMD likely, unlikely or even rule it out completely (as depicted in **Figure 2B**). Therefore, any country considering the implementation of an exclusion testing scheme should establish guidelines to align the criteria of the private veterinarians with the officially desired benchmark. If the clinical and epidemiological picture clearly warrants directly raising a suspicion with the veterinary authorities, this must be done immediately in order to quarantine the infected farm and ensure prompt laboratory diagnosis.

Where decentralized FMD testing is available, it is critical that all laboratories involved operate to the highest diagnostic standards, particularly when negative results obtained in a regional laboratory are not sent to the NRL for confirmation. Moreover, the official veterinary system should be aware in real time of any ongoing non-discriminatory exclusion testing to keep records and to be alert in case a positive result appears. In addition to high sensitivity, diagnostic pipelines used for exclusion testing must have very high specificity to avoid false-positive results. False-negative results can obviously be catastrophic, but also the potential for false positives reduces the enthusiasm of farmers, veterinarians and government officials to endorse exclusion testing schemes. Due to the infrequent nature of FMD incursions into free countries and the multiplicity of factors that can affect FMD recognition, it is difficult to obtain empirical evidence for the effectiveness of any particular detection measure, including the relatively new exclusion testing schemes.

Either way, in order to maintain the freedom from FMD in any country, active and informed participation at all levels—professional farmers and hobbyists, practitioners, educators and veterinary services—is essential. The longer a disease has not occurred in a country, the more important it is to make sure that all stakeholders are aware of the risk of reintroduction, are well-equipped to identify its clinical signs and know what steps to take should the occasion arise. Online resources (such as webinars or phone apps with visual references) can be of great utility in the implementation of non-discriminatory exclusion testing schemes. In addition, farmers and practitioners must have confidence in the official animal health authorities, and must be assured that any notification is immediately responded to, a quick diagnosis is obtained, and preventive regulatory measures are applied for as short as possible. Non-discriminatory exclusion testing is one of a range of measures that can be considered to improve the detection of FMD incursions.

AUTHOR CONTRIBUTIONS

MB had the idea for this article. ME wrote the original draft. All authors revised and expanded the original draft and approved the submitted manuscript.

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METHODS
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Mathematical Quantification of Transmission in Experiments: FMDV Transmission in Pigs Can Be Blocked by Vaccination and Separation

Aldo Dekker[1], Herman J. W. van Roermund[1], Thomas J. Hagenaars[1], Phaedra L. Eblé[1] and Mart C. M. de Jong[2]**

[1] Wageningen Bioveterinary Research, Lelystad, Netherlands, [2] Department of Quantitative Veterinary Epidemiology, Wageningen University, Wageningen, Netherlands

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Andres M. Perez, University of Minnesota Twin Cities, United States

***Correspondence:**
Aldo Dekker
aldo.dekker@wur.nl
Mart C. M. de Jong
mart.dejong@wur.nl

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Quantitative understanding of transmission with and without control measures is important for the control of infectious diseases because it helps to determine which of these measures (or combinations thereof) will be effective to reduce transmission. In this paper, the statistical methods used to estimate transmission parameters are explained. To show how these methods can be used we reviewed literature for papers describing foot-and-mouth disease virus (FMDV) transmission in pigs and we used the data to estimate transmission parameters. The analysis showed that FMDV transmits very well when pigs have direct contact. Transmission, however, is reduced when a physical barrier separates infected and susceptible non-vaccinated pigs. Vaccination of pigs can prevent infection when virus is administered by a single intradermal virus injection in the bulb of the heel, but it cannot prevent infection when pigs are directly exposed to either non-vaccinated or vaccinated FMDV infected pigs. Physical separation combined with vaccination is observed to block transmission. Vaccination and separation can make a significant difference in the estimated number of new infections per day. Experimental transmission studies show that the combined effect of vaccination and physical separation can significantly reduce transmission ($R < 1$), which is a very relevant result for the control of between-farm transmission.

Keywords: foot-and-mouth, vaccine, transmission, reproduction ratio, pig, separation, disease control, epidemiology

INTRODUCTION

Foot-and-mouth disease (FMD) is a contagious disease affecting cloven-hoofed animals and outbreaks can have major economic consequences. Due to the impacts of FMD, the German government decided in 1896 to finance FMD research which was led by Loeffler and Frosch (1). In the same year they started their research, they described FMD virus (FMDV) as an agent that passes bacterial filters (1), making FMDV the first animal virus ever described. In dairy cattle FMDV infection causes loss of milk production, in meat producing cattle and pigs, it reduces the feed conversion and in draft animals it reduces their availability for plowing and harvesting of crops. Furthermore, it can contribute to fertility problems, due to abortions and reduced conception rates, which will lead to a higher need of breeding animals (2). Control of FMDV has, in many countries,

not only led to better economic results in livestock production but also opened new export markets resulting in increased sales of livestock products. Export of animals and animal products without limitations has, therefore, become very important for FMD free countries. An outbreak of FMD in an FMD free country will consequently not only have an impact on livestock production, but it will also have huge economic consequences due to closure of export markets. The economic losses caused by the 2001 FMD outbreaks in Europe and the repeated introduction of FMDV in South-Korea were enormous (3–6).

Since the presence of FMDV infection limits trade of animals and because of the success of national and regional campaigns in the past to control FMDV, the OIE (World Animal Health Organization) and FAO (Food and Agricultural Organization of the United Nations) have proposed to target FMDV for the next world-wide eradication after rinderpest (7). To improve the prospects for FMDV eradication and to be able to optimize control measures, it is necessary to have knowledge about and understand FMDV transmission. Outbreaks and the applied (different) control measures have been described in the past (8), but these studies did not quantify the effect of different control measures. A quantitative understanding of transmission and the contribution of different control measures in reduction of transmission is needed as input in mathematical models. Although quantitative data can be obtained during outbreaks (8–11), the accuracy of data obtained is limited and the effect of a single intervention cannot be studied. For this reason, experimental transmission studies for disease control are essential.

In studying (FMDV) infections, microbiologists have often tried to quantify certain parts of the transmission chain, e.g., the concentration of infectious particles in secretions and excretions that can contaminate the environment, and the probability of infection for various infection doses [the dose-response relationship (12)]. In principle, the full transmission chain can be simulated by combining these experimental quantifications by modeling the dissemination and dilution of the infectivity in the environment. However, this detailed type of modeling is subject to substantial uncertainties, and historical attempts to model FMD transmission in that way have overestimated the infection risks (13).

In contrast, using experimental observations to quantify the transmission rate parameter, which relates the fraction of susceptible and infectious individuals in a population to the hazard rate of a new infection occurring (14), seems to be an accurate way to estimate transmission and extrapolate to different situations (15, 16). The transmission rate parameter can also be expressed using the reproduction ratio (R) which is the average number of new infections caused by a typical (i.e., average) infected individual, during its whole infectious period in a fully susceptible population (i.e., a population only containing non-infected individuals. Please note that the population can also be non-infected vaccinated individuals if transmission in a vaccinated group is quantified). If R is below 1 only minor outbreaks can occur and the infection will eventually die off; when R is above 1 both minor and major outbreaks can occur (17, 18). The parameter R is determined not only by the average level of susceptibility in the population, but also by the infectivity of a typical infected animal, i.e., the average infectivity of the infectious animals in that population. It is, however, possible to quantify R without quantifying susceptibility and infectivity precisely.

FMD vaccine evaluation in cattle, sheep and pigs is extensively reviewed in Cox and Barnett (19). In pigs many experimental studies have been performed in the past (20–37). Most of these vaccine studies, however, were performed to demonstrate protection against clinical disease, protection against sub-clinical infection, to measure reduction of virus titres in excretions and secretions and/or measure the effect on immune responses. In some studies pigs were infected by injection. In several other experiments contact exposure to non-vaccinated seeder pigs was used, and clinical protection against challenge was studied early and late after vaccination (22–25, 32). In these experiments a short exposure period of 1, 2, and 4 h was used; in one experiment, where the aim was to infect several vaccinated pigs, a 9 h exposure period was used (32). But only a limited number of these experiments were designed to quantify how vaccination can reduce FMDV transmission.

To quantify the transmission rate parameter β (i.e., the average number of new infections caused by a typical infected individual, per unit of time in which the individual is infectious in a fully susceptible population) or reproduction ratio R (definition see above), an experimental design fitting those objectives is necessary (for estimation of β and/or R). In these experiments, infected "seeder" animals are brought in contact with susceptible animals, not for a short period, but for a period similar to what happens in the field. The contact duration should ideally cover the entire infectious period of the seeder animals to make sure that all possible transmission can occur. In pigs, several such transmission experiments have been performed (**Table 1**). For mathematical animal disease models, information on the transmission rate parameter β and reproduction ratio R is very important. Up to now the methodology of determining these parameters has been used on a very limited scale, and therefore it is important to describe the methodology.

In this paper the statistical methods to quantify transmission in an experimental setting are presented. The application of methodology is shown by presenting and discussing articles previously published by the authors and some additional selected contributions where pigs were sampled on a daily basis.

MATERIALS

To show how statistical methods to quantify transmission parameters can be used, we conveniently selected papers where FMDV transmission in pigs was studied. Many of the studies were from our own group in which mostly the reproduction ratio, transmission rate and infectious period had already been analyzed, but we also identified three additional papers that presented data that could be used for analysis.

The raw data from all papers were extracted. Information on author, interval of the observations, distance between

TABLE 1 | Summary of within-pen and between-pen transmission experiments with pigs.

Type of transmission	Type of study (number of replicates)	Virus strain	Vaccine	Infection of source first infected pigs	Vaccination moment	β (95% CI)	R (95% CI)	Method	References
Within-pen	5I 5S (1)[a]	O/TAW/97	not used	ID	-		∞ (0.67–∞)	FS	(26)
Within-pen	5I 5S (4)	O/TAW/97	not used	ID	-	6.1 (3.8–10)		GLM	(27)
Within-pen	5I 5S (4)	O/TAW/97	not used	ID	-		∞ (2.4–∞)	FS	(30)[b]
						6.1 (3.7–10)	40 (21–74)	GLM	
Within-pen	1I 1S (5)	O/NET/2001	not used	ID	-		∞ (1.2–∞)	FS	(31)
Within-pen	5I 5S (2)	O/NET/2001	not used	CE	-		∞ (1.3–∞)	FS	(31)
						6.8 (3.2–14.8)		GLM	(31)
						4.4 (2.1–8.4)	23 (11–47)	GLM	(33)[c]
Within-pen	1I 5S (1)	O/SKR/2002	Not used	ID	-	2.1 (0.70–6.1)	7.4 (1.8–30)	GLM	(38)
Within-pen	2I 4S (1)	O/JPN/2010	not used	ID	-	1.3 (0.46–3.5)	3.6 (1.0–13)	GLM	(34)[d]
Within-pen (Feral swine to domestic or feral swine)	2I 4S and 2I 5S	A_{24}Cruzeiro	Not used	ID	-	73 (0-∞)	470 (0-∞)	GLM	(39)
Within-pen (Domestic swine to feral swine)	2I 4S (1)	A_{24}Cruzeiro	Not used	ID	-	2.3 (0.84–6.2)	15 (4.9–44)	GLM	(39)
Within-pen	5I 5S (1)	O/TAW/97	O/TAW/97	ID	−7 dpi		∞ (0.67–∞)	FS	(26)
Within-pen	5I 5S (2)	O/TAW/97	O/TAW/97	ID	−7 dpi		∞ (1.5-∞)	FS	(30)[b]
						2.0 (1.0–4.0)	11 (4.9–24)	GLM	
Within-pen	5I 5S (1)	O/TAW/97	O/TAW/97[e]	ID	−7 dpi		1.2 (0.2–5.4)	FS	(30)[b]
						0.4 (0.1–1.4)	1.0 (0.1–7.8)	GLM	
Within-pen	5I 5S (2)	O/NET/2001	O Manisa	CE	−14 dpi		2.4 (0.9–6.9)	FS	(31)
						0.66 (0.24–1.8)		GLM	(31)
						0.81 (0.39–1.5)	4.4 (2.1–8.2)	GLM	(33)[d]
Between-pen 0 cm	5I 5S (1)	O/TAW/97	not used	ID	-	0.59 (0.083–4.2)		GLM	(27)
Between-pen 0 cm	5I 4S (2)	O/NET/2001	not used	CE	-	0.14 (0.044–0.33)	1.1 (0.34–2.6)	GLM	(33)
Between-pen 40–70 cm	5I 4S (2)	O/NET/2001	not used	CE	-	0.0 (0.0–0.039)	0.0 (0.0–0.08)	GLM	(33)
Between-pen 0 cm	5I 4S (2)	O/NET/2001	O Manisa	CE	−14 dpi	0.0 (0.0–0.075)	0.0 (0.0–0.35)	GLM	(33)

β is the transmission rate parameter, i.e., the average number new infections per infectious animal per day, R is the transmission ratio, i.e., the average number of new infections per infectious animal during its entire infectious period. ID, Intra-dermal (injection); CE, contact-exposed; dpi, days post-inoculation or infection; FS, Final Size; GLM, General Linear Model. Only experiments with homogeneous groups of pigs are included [so excluding one pair-wise experiment of Orsel et al. (31) with non-vaccinated source pigs + vaccinated contact pigs) and experiments which lasted long enough to reach the end of the infection chain [so excluding experiments with short exposure times, such as Alexandersen and Donaldson (40)]. From the included experiments, β and R can be estimated.

[a] 5I 5S (2) indicates five infected pigs, five susceptible pigs and two replicates.

[b] Meta-analysis of experiments from (26–29).

[c] Re-calculated from Orsel et al. (31) by GLM method with changes in the model assumptions.

[d] Calculated by GLM method.

[e] Pigs were vaccinated with four times the normal dose.

pigs, vaccination status, type of pig as source of infection as well as the recipient, FMDV strain, number of infectious, susceptible and cases as well as the total number of animals were recorded (see **Supplementary Material**: art transmission in pigs Supplementary File 2.csv). These data were used in a meta-analysis using the GLM method (see below) to calculate the transmission rate parameter β. For the analysis of the infectious period information on author, inoculation route, vaccination status, type of pig strain, pig identification duration of excretion and whether or not the data were censored was recorded (see **Supplementary Material**: art transmission in pigs time supplementary file 3.csv). The duration of the virus excretion was calculated using exponential survival analysis (41). The analysis was performed in R (42) (see **Supplementary Material**: art transmission in pigs supplementary file 1.r).

METHODS

Biology of Transmission of FMDV in Pigs

The infection process may be described as a sequence of events as depicted in **Figure 1**. At the very start, susceptible animals become exposed to the presence of one or more seeder animals (Exposure). An animal remains susceptible (S) as long as it has not yet become infected. After exposure to virus it takes time before the virus has sufficiently replicated and is excreted

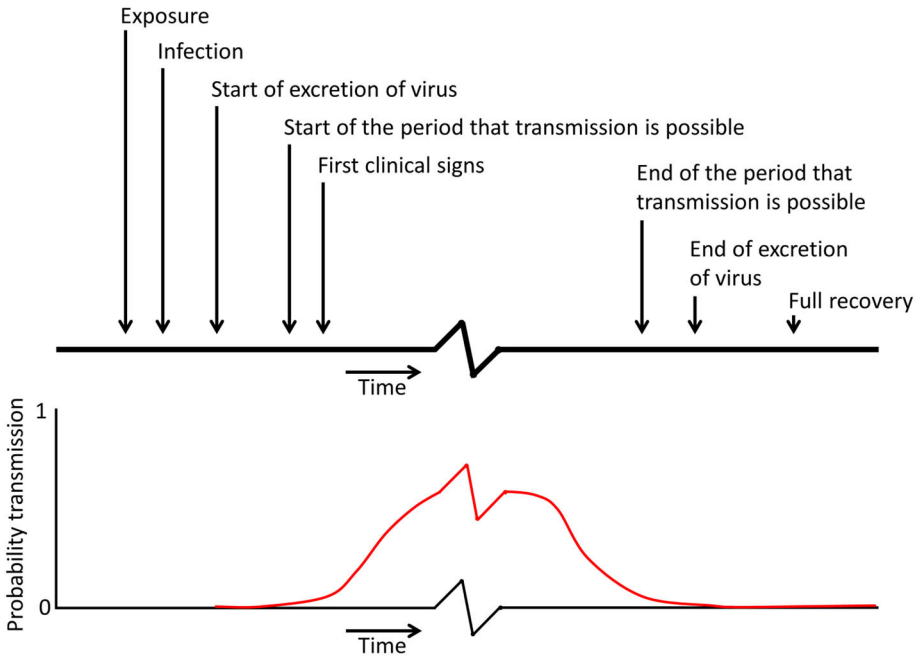

FIGURE 1 | Schematic representation of the different moments in time during infection, which can be used in models to describe the infection process (the x axis and the red curve showing the probability of transmission contain a break as the period in which transmission can occur is probably relatively longer than the other periods indicated on the top of the figure).

and the animal becomes infectious, and during this latent period an animal is often referred to as exposed (E) or latently infected. During the subsequent infectious period the animal is labeled infectious (I). In infection experiments the time of exposure is often well-established, but the exact time that virus replication and the start of secretion is difficult to measure as there are often only one or two observations per day. Orsel et al. (43) estimated the contribution of transmission before clinical signs were observed. In contrast to cattle the contribution to transmission during the incubation period was very high in pigs; the point estimate for the number of new infections per infectious individual in a completely susceptible population during the incubation period was 13 for non-vaccinated pigs and 1.3 for vaccinated pigs (43). These findings were confirmed by Stenfeldt et al. (44) who observed that the transmission period started ∼1 day before clinical signs were observed; even when pigs were only exposed to infectious pigs during a limited 8 h period. In cattle it is assumed that aerosolised FMDV is responsible for infection of cattle, because cattle are highly susceptible for infection via the airborne route (45, 46). Pigs, however, are relatively resistant to infection by natural aerosols (47). The oral route in pigs is also unlikely as the virus will not survive the low pH in the stomach, although infection can occur through exposure of the oropharyngeal tonsil (48). In swine vesicular disease, a disease caused by a different picornavirus but producing clinical disease similar to FMD, infection through the skin is considered the most important route for infection and fighting between pigs can enhance transmission (49). It is not unlikely that in the case of FMDV the skin is also an important port of entry for the virus, but pathogenesis studies after exposure to an FMDV contaminated environment to verify this hypothesis have not been performed.

In pigs the interval between exposure and infection is very short. In cattle newly formed virus is observed within a few hours; the growth rate within the individual host depended on the initial infection dose (50). In the analysis below it is assumed that it is known which pigs are susceptible (S that is not infected), exposed (E), infectious (I), or recovered (R) during the experiment. In many other infections the moment of infection is difficult to estimate, as the time between infection and start of virus shedding is often long and variable. In the FMDV transmission studies discussed in this paper the latent period of infected pigs was ignored. This was based on the observation that virus isolation from oropharyngeal swabs was positive within 24 h in more than 20% of the pigs inoculated by injection in the bulb of the heel. In pigs that were exposed to infectious pigs, a higher proportion was positive in oropharyngeal swabs within 24 h. Therefore, in most experiments, we can conclude that a latent period of <1 day was observed; with only daily observations, inclusion of such a short latent period in the model is not useful. In the studies pigs were classified as being susceptible (S), infectious (I) based on virus isolation from oropharyngeal swabs, not based on clinical signs. All pigs were free of FMD virus and antibodies to the virus before the experiment started.

Statistical Methods to Estimate Transmission of FMDV in Pigs

To estimate transmission repeated observations on all individuals in an isolated group are needed. At each observation all individuals are classified, based on samples taken at the observation moment and analyzed later, as either susceptible (S), exposed (E), infectious (I), or recovered/removed (R). Furthermore, each of these individuals may have multiple other characteristics that may or may not influence transmission. These characteristics can for example be: vaccinated or not vaccinated, being in the same pen or in a neighboring pen, being in the same pen at an earlier moment (1, 2, 3, …, days ago) etc.

The reproduction ratio R can also be estimated when only the number of susceptible, exposed, infectious and recovered animals at the start and at the end of the experiment are known. This is possible, provided that the experiment lasted long enough to reach the end of the outbreak and is called the estimation of R based on the final size of the outbreak (FS). It is based on the fact that for small numbers of individuals per group the likelihood of the observed outcome of the experiment can be (exactly) calculated for each possible value of the reproduction ratio R. The reproduction ratio that yields the maximum likelihood is then selected (51). The FS estimation of R is independent of the presence of a latent period (52) and can be carried out for any assumed distribution of the infectious period [see Formula 1 in (52) based on (53)].

Final Size (FS) Method: Homogeneous Groups

Given any number of susceptible (S > 0) and infectious (I > 0) individuals at the start of the experiment, two events can happen: (i) either an infection occurs and thus the number of susceptible individuals decreases by 1 and the number of infectious individuals (or exposed) increases by 1, or (ii) an infectious individual recovers and the number of infectious individuals decreases by 1 and the number of recovered individuals increases by 1. When an exposed individual does not become infectious immediately, but after a certain lag time (latent period), it is considered E. For sake of simplicity we are assuming no latent period in the formulas used here, so we assume a SIR model; however for SEIR models the same formulas apply (53). In the case of FMDV infection in pigs, virus secretion often starts within the observation period (sampling interval 1 day), so the assumption of no latent period is then valid (see section on Biology above). The alternative event is a recovery of the infectious animal and thus the number of infectious individuals decreases by 1. In formulas:

$$(S, I) \rightarrow (S-1, I+1) \text{ with rate } \beta \frac{\mathrm{SI}}{\mathrm{N}} \quad (1)$$

$$(S, I) \rightarrow (S, I-1) \text{ with rate } \alpha I \quad (2)$$

with N being the total number of individuals, β is the transmission rate parameter (see definition above) and α is the recovery rate parameter of the infectious individuals, i.e., the average number of infectious individuals that recover (or die) per unit of time, and do not contribute to transmission anymore. This recovery rate parameter is equal to 1/infectious period. Clearly there is a third possibility and that is that no infection and no recovery occurs; i.e., the status stays the same. However, conditional on the fact that something has happened, only these two events can have occurred and from the rates above, it follows that the probability of the first event being an infection event (p) is:

$$p = \frac{\beta \frac{SI}{N}}{\beta \frac{SI}{N} + \alpha I} \quad (3)$$

Which simplifies by $R = \frac{\beta}{\alpha}$ and provided I≠ 0 to:

$$p = \frac{R \frac{S}{N}}{R \frac{S}{N} + 1} = \frac{RS}{RS + N} \quad (4)$$

For example, in an experiment with pairs, i.e., with only one infectious individual and one susceptible individual (i.e., $S = 1$ and $N = 2$), the probability (p) that the contact individual becomes infected before the infectious animal recovers is:

$$p = \frac{R}{R+2} \quad (5)$$

and thus, from observing n pairs with k infections in the contacts, the maximum likelihood estimator (MLE) for R is:

$$\hat{R} = \frac{2k}{n-k} \quad (6)$$

For example, when observing 15 contact infections in 30 pairs, $\hat{R} = \frac{30}{15} = 2$. For any experiment with more individuals it is necessary to calculate the final size distribution with a computer algorithm (51) which can also accommodate the case where the infectious period has a different distribution than the exponential distribution (52, 53).

Generalized Linear Model (GLM) Method: Homogeneous Groups

The GLM method can be used when individuals are sampled at regular time intervals during the experiment (yielding interval data). For the ease of explanation, it is first assumed that all individuals are identical (homogeneous group) in all characteristics except for being susceptible or infectious. The SIR model will be used in which each individual moves from Susceptible to Infectious and then to Recovered. Thus, exposed individuals are ignored. Also ignored are infectious individuals which can recover after some time and become susceptible again (they are included in SIS or SIRS models).

To estimate the transmission rate parameter, the number of susceptible individuals (S) and the number of infectious individuals (I) are counted both at the start and at the end of every time interval, and the length of the interval is determined as Δt. With that information for subsequent intervals during the experiment the transmission rate parameter β can be estimated. Those individuals that are S at the start of the interval and I at the end of the interval are the cases (i.e., new infections), counted as C.

The following relationship between the observed counts and the transmission parameter can be considered as a good starting point for analysis (16):

$$c_t = \beta \frac{S_t I_t}{N_t} \Delta t \tag{7}$$

where C_t is the number of observed cases in the interval [t, t+Δt], β the transmission rate parameter (see definition above), S_t, I_t and N_t respectively the number of susceptible, infectious and the total number of individuals at the start of the interval [t, t+Δt] and Δt is the length of the interval. The total number of new cases in interval [t, t+Δt] will be 0, 1, 2, …, with maximum value St (all available susceptibles). In an approximation in which the contribution of new cases to the infectivity occurring during the interval [t, t+Δt] is neglected, the fate of each susceptible individual is independent and identically distributed according to a Bernoulli distribution with infection probability p_t:

$$p_t = 1 - e^{-\beta \frac{I_t}{N_t} \Delta t} \tag{8}$$

Thus, each susceptible individuals' infection status is independent and identically distributed with values (1) for individuals infected and (0) for individuals not infected at the end of the interval. For all susceptible individuals together there are C_t (with value 0, 1, 2, …S_t) new cases observed; thus binomially distributed with parameters S_t and p_t as given by the equation above. This binomial distribution follows from the assumption of independent and identically distributed Bernoulli variables.

From the observed C_t and the observed I_t, S_t and N_t, the transmission rate parameter β can now be estimated. For that, a statistical technique called Generalized Linear Models (GLM) is used (54). In that method a function has to be specified that links the expected value of our observed result variable (here C_t/S_t) to a linear function of the explanatory variables (here I_t and N_t), and a distribution has to be specified around this expected value. That distribution is needed to minimize the variance around the expected values based on estimated coefficients. The distribution that is used here is the binomial distribution, with S_t as the binomial total. As a link function the complementary-log-log function is used.

The expected number of cases per susceptible $\varepsilon \frac{C_t}{S_t}$ is p_t, (the expectation is indicated by ε) which then gives:

$$\begin{aligned} \text{cloglog}(\varepsilon \frac{C_t}{S_t}) &= \log\left(-\log\left(1 - \left(1 - e^{-\beta \frac{I_t}{N_t} \Delta t}\right)\right)\right) \\ &= \log(\beta) + \log(\frac{I_t}{N_t} \Delta t) \end{aligned} \tag{9}$$

Where $\log(\frac{I_t}{N_t} \Delta t)$ is the offset and log(β) is the intercept (regression coefficient) estimated in the statistical analysis. The offset is a value that is subtracted from the transformed result variable before fitting the model. In this homogeneous case the model has only one unknown regression coefficient that has to be estimated [log(β)], as there are no differences in susceptibility or infectivity between the individuals. Note that differences in the infectious period distribution (see above in the FS model) do not play a role in the GLM analysis as we observe whether or not the animal is infectious at the beginning (and at the end) of each interval. We assume that the effect that some animals stop virus excretion during the interval can be ignored.

Generalized Linear Model (GLM) Method: Heterogeneous Groups

In the studies reviewed, heterogeneity was observed because of vaccination (26, 31) and/or because of spatial separation of animals (33). In the example given in this paper, groups containing vaccinated and non-vaccinated individuals (mixed) are used, but the same formulas can be applied to other settings with heterogeneous groups. Vaccination can have two effects on transmission, and thus on the transmission parameter, i.e., it can affect the susceptibility and the infectivity. The susceptibility effect is that vaccinated individuals may under the same circumstances have a lower (or at least different) probability of becoming infected than non-vaccinated individuals. The infectivity effect of vaccination is that the amount of virus and/or the duration of viral shedding is reduced, and thereby reducing the probability of causing infection (32). Note that any individual that is in a group where there are more infectious individuals present than in a comparison group, will get infected more often compared to the individuals in the comparison group. This is the indirect effect of heterogeneity (55). In groups with both vaccinated and non-vaccinated animals both effects of vaccination on susceptibility and infectivity can be estimated.

The observations in homogeneous groups consisted of I_t, S_t, and C_t. This now changes to $I_{u,t}$, $S_{u,t}$, $C_{u,t}$, $I_{v,t}$, $S_{v,t}$, and $C_{v,t}$ where u stands for non-vaccinated and v stands for vaccinated individuals. The result variable in the analysis is now either $\frac{C_{u,t}}{S_{u,t}}$ or $\frac{C_{v,t}}{S_{v,t}}$ corresponding to infections occurring in non-vaccinated and vaccinated individuals respectively. An indicator for vaccination, the dummy variable IndV with value 1 for vaccinated and 0 for non-vaccinated susceptibles, is introduced as explanatory variable to distinguish the estimate of transmission rate parameter for these two situations with the different recipients. The regression coefficient before the explanatory variable IndV, in this case, represents the effect of vaccination on susceptibility [i.e., the relative susceptibility of a vaccinated susceptible compared to that of a non-vaccinated susceptible (set to 1)].

The offset in the statistical model for the heterogeneous situation uses the total number of infectious individuals, i.e., the offset is now: $\log\left(\frac{I_{u,t}+I_{v,t}}{N_t} \Delta t\right)$. The explanatory variable used to estimate the effect of vaccination on infectivity is the fraction of vaccinated infectious individuals: $FrI_v = \frac{I_{v,t}}{I_{u,t}+I_{v,t}}$ and thus the regression coefficient before this explanatory variable represents the effect of vaccination on infectivity.

To illustrate this, the transmission rate parameter (β) can be written as the product of the overall contact rate (c), the relative susceptibility of vaccinated individuals (γ_v) and the relative infectivity of vaccinated individuals (φ_v). Then the transmission rate parameter for the transmission between

vaccinated individuals is $\beta_{vv} = c\ \gamma_v\ \varphi_v$ and between non-vaccinated individuals is $\beta_{uu} = c\ \gamma_u\ \varphi_u = c$ (as the relative susceptibility γ_u and infectivity φ_u for non-vaccinated individuals is set to 1, being only interested in the relative effects). The transmission rate parameter from vaccinated to non-vaccinated is $\beta_{vu} = c\ \gamma_u\ \varphi_v = c\ \varphi_v$.

Now one can write the link function equation and identify the regression coefficients that have to be estimated:

$$\text{cloglog}(\varepsilon \frac{C_{u\text{ or }v,t}}{S_{u\text{ or }v,t}}) = \log\left(-\log\left(1-\left(1-e^{-\beta\frac{I_t}{N_t}\Delta t}\right)\right)\right)$$
$$= \log(c) + \log(\gamma_v)\cdot IndV + \log(\varphi_v)\cdot FrI_v + \log(\frac{I_{u,t}+I_{v,t}}{N_t}\Delta t)$$

The regression coefficients from the analysis are identified by their explanatory variables, thus, these coefficients C0, C1 and C2 are:

C0 (intercept) $= \log(c)$

C1 $= \log(\gamma_v)$

C2 $= \log(\varphi_v)$

Offset $= \log(\frac{I_{u,t}+I_{v,t}}{N_T}\Delta t)$

$\beta = c\cdot\gamma_v^{\ IndV}\cdot\varphi_v^{FrI_v}$

And thus, the four possible values of the transmission rate parameter are:

From non-vaccinated to non-vaccinated: $FrI_v = 0$ and $IndV = 0$ then $\beta_{uu} = c = e^{C0}$

From non-vaccinated to vaccinated: $FrI_v = 0$ and $IndV = 1$ then $\beta_{uu} = c\cdot\gamma_v = e^{C0+C1}$

From vaccinated to non-vaccinated: $FrI_v = 1$ and $IndV = 0$ then $\beta_{uu} = c\cdot\varphi_v = e^{C0+C2}$

From vaccinated to vaccinated: $FrI_v = 1$ and $IndV = 1$ then $\beta_{uu} = c\cdot\gamma_v\cdot\varphi_v = e^{C0+C1+C2}$

Note 1: The link function in the heterogeneous case contains an approximation because in the SIR model the average infectivity is measured by an arithmetic average and in the statistical analysis a geometric average is assumed as a linear model on the log scale was used (56). This approximation causes a small error that can be corrected. As both approximations lead to underestimation of the effects on infectivity and susceptibility it can be chosen to just ignore this small error. For a discussion about these errors and the methods to correct them, see (57).

Note 2: In case where the vaccinated and non-vaccinated individuals do not mix, the effect of vaccination on susceptibility and infectivity is completely confounded as both dummy explanatory variables (FrI_v and IndV) have value 1 for the vaccinated group and value 0 for the non-vaccinated group. Fitting will give us the effect of vaccination on the transmission rate parameter but whether the effect is on susceptibility or infectivity cannot be inferred.

Results of Transmission Studies in Pigs

Of the nine papers used in the analysis six papers were from our own group, the three additional papers described an FMDV transmission study in pigs in sufficient detail to calculate the transmission rate parameter and reproduction ratio; we used the GLM method. In a few cases the results were analyzed by both the GLM as well as the final size method.

In total 14 experiments were identified in which pigs were infected with one of five different FMDV strains (O/TAW/97 $n = 7$, O/JPN/2017 $n = 1$, O/NET/2001 $n = 4$, O/SKR/2002 $n = 1$ and A_{24}Cruzeiro $n = 1$). In two out of 14 experiments there was physical separation between the pigs and in three experiments the pigs were vaccinated. **Table 1** gives an overview of the experimental transmission studies in pigs included in this review. First, results will be reported of the studies in which transmission was studied within a pen, without vaccination as well with homologous and heterologous vaccination. Next, results will be reported of studies on transmission between pens. These latter results are also relevant for transmission between farms, which itself cannot be studied in an experimental setting.

Within-Pen Transmission

The first FMDV transmission experiment, discussed here, was performed with three homogeneous groups of pigs (i.e., two groups of pigs that were vaccinated and one group of pigs that was not vaccinated) (26). Each group was housed in a different stable. This experiment was performed using FMDV strain O/TAW/97 for infection, where the effect of vaccination with homologous FMDV O/TAW/97 vaccine 7 and 14 days prior to exposure was tested (−7 dpi and −14 dpi; dpi = days post-inoculation). As control a group of non-vaccinated pigs was included (29). In each group of ten pigs five were inoculated with FMD virus by intradermal injection in the bulb of the heel and mixed with the remaining five contact pigs. The estimated R for the non-vaccinated controls, based on the FS method, was ∞, with 95% confidence interval 0.67-∞ (see **Table 1**). The wide confidence interval is due to the low number of replicates (only five contact animals in one replicate) and due to the relatively crude estimation method, based on FS only. In a subsequent meta-analysis R was also estimated using the GLM method (30). No significant effect was found for the −7 dpi vaccination compared to the control treatment (also the amount of virus shedding was similar; data not shown here). Vaccination 14 days prior to infection, however, did not result in infectious (vaccinated) pigs, due to complete protection to the injected challenge virus.

In another experiment Eblé et al. (27) studied within-pen and between-pen transmission of FMDV strain O/TAW/97 among non-vaccinated pigs. The within-pen transmission experiment was performed in four replicates of 5S + 5I pigs. Pigs were inoculated by intradermal injection in the bulb of the heel. The estimated transmission rate parameter β was 6.14 per day (see **Table 1**) (27). Although a value for R was not given in the paper, it can be estimated using β × T, where T is the duration of the infectious period of the inoculated pigs (I pigs). With T roughly estimated as 5 days (from data in the paper), R is ∼30 for the within-pen transmission of non-vaccinated pigs according to this study.

In a meta-analysis, in which the above experiments were re-analyzed together with unpublished transmission data (26–30),

the GLM method was used. In the GLM method daily data on virus detection in oropharyngeal swabs are used as indicator for infectivity (number of infectious pigs) and infection (number of cases). Based on the combination of the results the total number of non-vaccinated controls now consisted of four replicates of 5S + 5I pigs, the −7dpi vaccination treatment consisted of two replicates of 5S + 5I, a new −7dpi vaccination treatment with a four-fold vaccine doses consisted of one replicate of 5S + 5I, the −14 dpi treatment with a homologous vaccine (O/TAW/97) consisted of two replicates of 5S + 5I, and a new −14 dpi treatment with a heterologous vaccine (O Manisa) consisted of one replicate of 5S + 5I. In the experiments included in the meta-analysis the pigs were always inoculated by intradermal injection in the bulb of the heel. The corresponding β and R values can be found in **Table 1**. This resulted in a point estimate of R of 40 for non-vaccinated pigs and of a significantly lower value of 11 for pigs vaccinated at−7 dpi. A four-fold higher vaccine dose, also at −7 dpi, reduced the *R*-value (from 11) to a significantly lower value of 1.0, but not significantly below 1 (30).

Orsel et al. (31) studied the effect of vaccination on susceptibility of contact pigs in pair-wise transmission experiments using FMD virus strain O/NET/2001. In the pair-wise transmission experiments non-vaccinated and vaccinated contact pigs (S) were exposed to non-vaccinated seeder pigs (I) inoculated by intradermal injection in the bulb of the heel. The experiment was performed with six replicates of a pair-wise experiment with 1S + 1I pig for both the vaccinated and the non-vaccination contacts. Some of the inoculated pigs did not become infectious. The FS R estimate for transmission between non-vaccinated pigs was ∞ (1.2-∞) (see **Table 1**), not different from the value between 30 and 40 found earlier (28, 30). In the pair-wise experiment using vaccinated contact pigs four inoculated pigs became infectious and transmitted FMDV to all vaccinated contacts (31); this indicated that vaccination did not reduce susceptibility.

Since no significant differences were observed between susceptibility in vaccinated or non-vaccinated pigs, Orsel et al. (31) performed a second experiment in which the effect of vaccination on both susceptibility and infectivity was studied. Due to the fact no infection was seen in the inoculated pigs (26, 28) that had been vaccinated 14 days prior to infection, Orsel et al. (31) changed the needle infection to challenge by contact to non-vaccinated needle infected pigs. The experiments were performed with two homogenous groups of vaccinated or non-vaccinated pigs. Vaccinated pigs (−14 dpi) were exposed to FMDV by housing them together with non-vaccinated seeder pigs. These vaccinated pigs became infectious by this route of infection and shed virus in oropharyngeal fluid for several days. After infection the non-vaccinated and (−14 dpi) vaccinated pigs that were infected by contact exposure were brought into contact with respectively non-vaccinated and (−14 dpi) vaccinated contact pigs. As the objective of that study was the estimation of R within a group of vaccinated pigs, the data on transmission from non-vaccinated seeders to vaccinated contact pigs were not included in the original publication. But based on the original data and the GLM analysis explained earlier we now also calculated an R of 23 (95% CI 11–47) for non-vaccinated pigs and an R of 4.4 (95%CI 2.1–8.2) for vaccinated pigs. This indicates that vaccination reduces transmission significantly, but the estimate for within-pen transmission for vaccinated pigs was not below 1 (**Table 1**).

We identified three studies where transmission between pigs was studied (**Table 1**), but that did not analyse the transmission rate parameter or R. The first study was performed with O/SKR/2002 (38), and yielded an R of 7.4 (95%CI 1.8–30). The second one was performed with O/JPN/2010 (34), this yielded an R of 3.6 (95%CI 1.0–13). The third study (39) was interesting as different types of pigs, feral and domestic, were used as infectious and contact pigs. The GLM model using type of source pig as additional explanatory variable fitted significantly better to the results, i.e., lower Akaikes Information Criterion (14 for the null-model and nine for the model with source as explanatory variable) (58, 59). Analysis of this study alone indicates that feral pigs are more infectious than domestic pigs.

Between-Pen Transmission

Pen-to-adjacent-pen transmission experiments of FMDV in non-vaccinated pigs have been described (27, 40, 46). In the first experiments (27, 40) the total number of contact pens in the studies was four, which is very limited for quantification of between-pen transmission. van Roermund et al. (33) therefore performed three pen-to-pen transmission experiments, where the number of contact pens (cumulated over all replicates) was eight per experiment.

Alexandersen and Donaldson (40) did not observe pen-to-adjacent-pen transmission from non-vaccinated donor pigs to non-vaccinated receiver pigs in any of the four replicates where the exposure time was 24–48 h (47). The only possible transmission route in this experiment was the airborne route. Eblé et al. (27) studied within-pen and between-pen transmission of FMDV strain O/TAW/97 among non-vaccinated pigs. The between-pen transmission experiment was performed in one replicate of 5S + 5I pigs, in which the S and I pigs were separated by a single wall. The I pigs were inoculated by intradermal injection in the bulb of the heel. The estimated between-pen transmission rate parameter β was 0.59 per day, which was significantly lower than the within-pen transmission rate parameter of 6.14 per day (see **Table 1**) (27). The expected time to infection of the first pig in the adjacent pen was estimated at 16 h, much longer than that of the first contact pig within the pen, which was estimated 1.6 h (derived from the estimated β; experimental observations were done on a daily basis, not more frequent). van Roermund et al. (33) performed three pen-to-pen transmission experiments with two replicates. Each replicate consisted of five seeder pigs housed in a central pen surrounded by four separate pens, each containing one contact pig. The FMD virus strain used was O/NET/2001. The seeder pigs in the central pen were infected by contact exposure to needle inoculated seeders. The exposure of the pigs in the adjacent pens was thus to already contact-infected pigs, a more natural infection route than needle infection, this method had been used in a previous direct transmission experiment (31). All pen walls in the experiments consisted of solid barriers ∼1.2 m high that were not glued or cemented to each other or to the floor. In the first experiments,

all non-vaccinated contact pigs were housed in pens which were separated by a walkway of 40–70 cm from the pen with the seeder pigs (so two solid barriers between contact and seeder pigs). In the second experiment, all non-vaccinated pigs were housed in pens that were adjacent, only separated by one solid barrier from the pen with the seeder pigs. In the third experiment, the set-up of the second experiment was repeated but with all pigs vaccinated (−14 dpi). The between-pen transmission events per experiment were analyzed as eight replicates (2 × 4 pens) of 1S + 5I pigs. In non-vaccinated pigs, no transmission occurred when the central pen with five seeder pigs and the pens with contact pigs were separated by a 40–70 cm wide walkway (so two solid barriers). The seeder pigs in the central pen, however, were excreting FMD virus in the oropharyngeal fluid for 8 days and virus could be isolated from air above the pen. The estimated between-separated-pen R was 0 (0–0.08). This observed R was significantly below 1 (see **Table 1**).

In the second experiment using non-vaccinated pigs in adjacent pens, transmission was observed from seeder pigs to contact pigs. In four out of eight individually housed contact pigs FMDV infection was detected, in all cases at 3 days post-exposure. The corresponding between-adjacent-pen R was estimated 1.1 (0.34–2.56) which is not significantly above or below 1 (**Table 1**). When the second experiment was repeated with pigs that were vaccinated 14 days prior to exposure, no transmission was observed to the adjacent pens. The estimated between-adjacent-pen R for vaccinated pigs was 0 (0–0.35), which is significantly below 1 and thus pen-to-adjacent-pen transmission was stopped after vaccination of pigs. Vaccination alone or separation alone did not reduce R significantly below 1, so the combined effect of separation and vaccination is effective to reduce transmission.

Meta-Analysis

The data are given in **Supplementary Material** "art transmission in pigs supplementary file 2.csv" and "art transmission in pigs time supplementary file 3.csv." The analysis is given in **Supplementary Material** "art transmission in pigs supplementary file 1.r." Univariate analysis of the GLM models with distance, vaccination, type of source pig, type of recipient pig and strain as possible explanatory variables yielded the lowest AIC (273) with the model including distance, which is a huge difference with the AIC of the null model (without explanatory variable) which had an AIC of 493. Using forward regression analysis vaccination was the explanatory variable with the lowest AIC in the models with two explanatory factors. Most models using three explanatory variables did not converge, we therefore stopped the analysis. The estimated transmission rate parameter β for within-pen transmission of non-vaccinated pigs was 5.2 day^{-1} (95%CI 4.0–6.8), that for vaccinated pigs (14 days prior to infection with heterologous vaccine) was 0.60 day^{-1} (95%CI 0.31–1.1) whilst the estimated β for between-pen transmission (distance 0 cm) of vaccinated pigs was 0.032 day^{-1} (95%CI 0.013–0.082).

The duration of virus excretion (infectious period) was analyzed by exponential survival analysis. The univariate analysis showed a significant effect of vaccination, but contribution was found for inoculation, type of pig or virus strain. The average duration of virus excretion was 7.5 days (95%CI 5.6–10) for non-vaccinated pigs and 6.3 days (95%CI 5.2–7.5) for vaccinated pigs.

Based on the calculated β and duration of excretion we can calculate the R (the confidence interval is calculated under the assumption that β and the duration of excretion are independent). The R for within-pen transmission of non-vaccinated pig is 39 (95%CI 29–59), and 3.7 (95%CI 1.9–7.3) for vaccinated pigs. The R for between-pen transmission of vaccinated pigs is 0.20 (95%CI 0.079–0.52) which is significantly below 1.

DISCUSSION

The objective of this paper was to give an overview of statistical methods to experimentally quantify transmission; to give an example how these methods can be used, we reviewed papers on experimental FMDV transmission in pigs. We have focussed on the whole transmission chain in pigs. Whilst we are aware that others have analyzed part of the transmission chain (35, 37), although valuable, it does not provide all the information on transmission and was therefore not included in the review. We have limited the review to pig to pig transmission and not included transmission from pigs to other species or vice versa (36).

The results presented show that due to the inherently small scale of the experiments, replications are typically needed to obtain estimates of sufficient quality, i.e., unbiased and with sufficiently small confidence intervals. By combining multiple experiments in one meta-analysis the precision can be improved. For an experiment of a given scale and a given number of replicates, sampling animals at regular time intervals during the experiment, and analyzing the results using the GLM method, allows for a smaller variance in R estimates than if only final size (FS) information is used. In non-vaccinated pigs the within-pen transmission is extremely efficient. The point estimates for R range from 10 to 40 (27, 30). In further transmission studies in pigs two control measures were evaluated: vaccination and physical separation, both separately and in combination.

The reviewed studies show that vaccination 7 days prior to infection with a four-fold vaccine dose resulted in an estimated R close to 1, but not significantly below 1. Thus, even though transmission is reduced significantly by vaccination, within a pen the infection will still spread among vaccinated pigs of −7 dpi (26, 30). Other studies (23, 24) claim full protection in C_1 Oberbayern and O_1 Lausanne exposed pigs when vaccinated at −7 dpi, but in these studies the time of exposure to seeder pigs was limited to only 1–4 h. For vaccinated pigs at −14 dpi, in the first studies R could not be estimated as needle inoculation did not result in infected vaccinated animals (26, 28). The study, however, indicated that susceptibility of the vaccinated pigs for needle challenge was absent, which will in many cases prevent introduction on the farm and reduce between-farm transmission. In the pair-wise study by Orsel et al. (31) no reduced susceptibility was observed when pigs were vaccinated 14 prior to contact with non-vaccinated seeder pigs. Because no infected vaccinated pigs

were observed in the first experiments using needle challenge, in the consecutive experiments the vaccinated pigs were exposed to non-vaccinated seeder pigs. By using this method, it was possible to obtain infected vaccinated pigs (−14 dpi). Although transmission in the vaccinated pigs was reduced compared to the non-vaccinated pigs, R was still above 1 (31).

In our meta-analysis separation is the strongest single factor that influences the transmission rate parameter. The meta-analysis confirms the previous between-pen transmission studies that separation can significantly reduce both β and R. However, R is not reduced to values below 1, when only one solid barrier is present between pigs. Separation of pigs with two solid barriers and a walkway of 40 to 70 cm in between, or vaccination (−14 dpi) in combination with one solid barrier reduced R significantly to values below 1. Thus, these studies show that vaccination helps to block between-pen transmission. Why within-pen transmission of FMD is so efficient compared to between-pen transmission is not clear. The fact that physical separation of pigs reduces transmission so efficiently suggests that airborne infection in pigs does not play an important role. The fact that pigs are relatively resistant to airborne infection has been confirmed in other studies (35, 47, 60). So direct contact with virus excreted in the environment, as has been shown for cattle (61), may play a more important role in pigs as may infection due to fighting (e.g., for food), where virus from pig or environment can directly be transmitted into small wounds of contact pigs. The study by Mohamed et al. (39) give a similar indication, as feral pigs seem to be more infectious than domestic pigs; they are reported to fight more. The role of animal behavior is also shown by the fact that transmission from cattle to pigs is limited compared to transmission from cattle to cattle (36).

Although the hygiene measures taken in the experiments were probably stricter than the current practice on commercial farms, handling of pigs was more intensive as daily samples were collected, so transmission by handling itself can never be excluded. However, the between-pen transmission experiment with non-vaccinated pigs showed no transmission at all when pens were separated by a 40 to 70 cm wide walkway (so two solid barriers), provide proof that hygienic measures can reduce transmission.

The between-pen transmission studies show that transmission of FMDV between farms will be blocked. Still it has been shown to occur even when animal movement is prohibited during an epidemic (62). The major transmission routes left in case of a stand-still are people and inanimate objects moving between farms, in such a case emergency vaccination will be effective as the studies show that vaccination can help even when separation of pigs is limited.

CONCLUSIONS

In many FMD epidemics, vaccination contributed significantly to the control of FMDV as one of the components of the national control and eradication program (4, 63, 64). Transmission experiments as reviewed in this paper can be used to support the FMD control policy. The methods described in this paper can be used to analyse experimental transmission studies. The experimental transmission studies show that vaccination in combination with physical separation can reduce transmission of FMDV in pigs significantly. A combination of a physical and immune barrier is essential for the control FMDV with respect to between-farm transmission and also for reduction of transmission within a pig farm.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AD, HR, TH, PE, and MJ contributed to the ideas represented in this paper. AD was responsible for the analysis of the data. HR and AD were responsible for the reporting and MJ, TH, and PE for the critical reading. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.540433/full#supplementary-material

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Functional and *in silico* Characterization of Neutralizing Interactions Between Antibodies and the Foot-and-Mouth Disease Virus Immunodominant Antigenic Site

Ruben Marrero Diaz de Villegas[1]*, *Cristina Seki*[2], *Nora M. Mattion*[2] and *Guido A. König*[1]*

[1] *Instituto de Agrobiotecnología y Biología Molecular, Instituto Nacional de Tecnología Agropecuaria, Consejo Nacional de Investigaciones Científicas y Tecnológicas, Buenos Aires, Argentina,* [2] *Centro de Virología Animal, Consejo Nacional de Investigaciones Científicas y Tecnológicas, Universidad Abierta Interamericana, Buenos Aires, Argentina*

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***Correspondence:**
Ruben Marrero Diaz de Villegas
marrerodiaz.ruben@inta.gob.ar
Guido A. König
konig.guidoalberto@inta.gob.ar

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Molecular knowledge of virus–antibody interactions is essential for the development of better vaccines and for a timely assessment of the spread and severity of epidemics. For foot-and-mouth disease virus (FMDV) research, in particular, computational methods for antigen–antibody (Ag–Ab) interaction, and cross-antigenicity characterization and prediction are critical to design engineered vaccines with robust, long-lasting, and wider response against different strains. We integrated existing structural modeling and prediction algorithms to study the surface properties of FMDV Ags and Abs and their interaction. First, we explored four modeling and two Ag–Ab docking methods and implemented a computational pipeline based on a reference Ag–Ab structure for FMDV of serotype C, to be used as a source protocol for the study of unknown interaction pairs of Ag–Ab. Next, we obtained the variable region sequence of two monoclonal IgM and IgG antibodies that recognize and neutralize antigenic site A (AgSA) epitopes from South America serotype A FMDV and developed two peptide ELISAs for their fine epitope mapping. Then, we applied the previous Ag–Ab molecular structure modeling and docking protocol further scored by functional peptide ELISA data. This work highlights a possible different behavior in the immune response of IgG and IgM Ab isotypes. The present method yielded reliable Ab models with differential paratopes and Ag interaction topologies in concordance with their isotype classes. Moreover, it demonstrates the applicability of computational prediction techniques to the interaction phenomena between the FMDV immunodominant AgSA and Abs, and points out their potential utility as a metric for virus-related, massive Ab repertoire analysis or as a starting point for recombinant vaccine design.

Keywords: FMDV, neutralizing antibody, peptide ELISA, functional epitope, structure modeling, docking

INTRODUCTION

FMD is a highly infectious animal disease affecting over 70 wild and domestic cloven-hoofed species such as cattle and swine (1). The etiological agent is FMDV, an Aphthovirus from the Picornaviridae family (single-stranded positive-sense RNA genome of ∼8 kb) (2). Direct production losses and international trade restrictions in endemic countries, as well as the high cost of FMD control, are a major problem for governments and producers (1).

The humoral immune response has a fundamental role in the protection against FMDV. Neutralizing antibodies (nAbs) recognize functional regions at the viral capsid (antigenic sites, AgS) (3). The FMDV antigenic site A (AgSA) includes the key virus recognition motif (an RGD amino acid triad) for its major host cellular receptor (αVβ6 integrin). AgSA is located on capsid protein 1 (VP1) and triggers the main humoral neutralizing response (4).

Abs are also valuable biotechnology reagents and key molecules for FMDV diagnosis and surveillance (5). Recent FMDV publications have reported basic and applied Ab studies (6) by harnessing a wider sampling tool like next-generation sequencing (NGS), although a few explored the *in vivo* antibody (or immune receptor) repertoire (7–9). Using this approach, a broad neutralizing antibody was successfully obtained through single B-cell isolation from peripheral blood mononuclear cells of sequentially immunized bovines (10) to improve FMDV diagnostic tests. Other studies have focused on the molecular structure of Ag–Ab interactions of FMDV of serotype C. For example, Verdaguer et al. resolved the structure of a nAb interacting with a peptide equivalent to the AgSA of FMDV by X-ray diffraction analyses and characterization at quasi-atomic level (2.3 Å) (11, 12). A second serotype C Ab-FMDV structure encompassed a Cryo-EM reconstruction of Fab-capsid interactions at 30-Å resolution (13). Recently, another group reported the sequence and molecular modeling of a serotype O mAb (6).

The future for Ab knowledge and veterinary vaccinology applications is undoubtedly promising at the convergence of novel sequencing platforms and bioinformatic tools to explore the cattle and swine immunoglobulin repertoire and Ab structural information (14). Therefore, the Ag–Ab complex modeling is crucial. Herein, we obtained the Fab (antigen-binding fragment) sequence of two site A-specific mAbs of serotype A and characterized their functional epitopes through a peptide ELISA. Finally, we implemented a protocol for modeling the Ab molecular structure as well as their specific interaction with viral Ag at AgSA, based on homolog (X-ray diffraction) structures available for serotype C. Our research demonstrated that it is feasible to model the molecular structure of FMDV antibodies and the topology of Ag–Ab interactions as well as to predict the influence of mutations on those interactions.

MATERIALS AND METHODS

Antibodies and Foot-and-Mouth Disease Virus (FMDV) Peptide Antigen

We developed and supported the modeling protocol by reviewing the mAb 4C4 complex and applying it to novel anti-serotype A mAbs. Published data using mAbs to study FMDV Ag–Ab interaction for their different capsid antigenic sites, including high atomic resolution structures, had only been obtained for the AgSA of serotype C virus (mAb 4C4). Sequence and atomic coordinate data for experiments including the mAb 4C4 (anti-FMDV of serotype C) were obtained from the protein data bank accession, pdb 1EJO (11, 12). Pdb 1EJO describes the interaction complex between mAb 4c4 and AgSA peptide from FMDV strain C-S8c1 (a biological clone derived from FMDV isolate C1 Sta Pau Sp/70) (15).

mAbs 1E12 and 4A2 (IgM and IgG, respectively) against a serotype A FMDV strain (A24Cruzeiro) were obtained from a previous study (16, 17). Both mAbs recognize epitopes at the AgSA of VP1 protein (17).

Fifteen peptides were designed to be used in ELISA (see the *Immunoassays* section) with point mutations designed according to the aa variability found at different positions in several FMDV strains (**Supplementary Table 1**) serologically sampled at the Centro de Virología Animal–CEVAN (16, 17). The 20-aa long, N-terminal biotinylated, peptides comprised the RGD region of the GH loop at VP1. The negative control peptide (ID 15), included the RGD motif, but with multiple mutations relative to the wt peptide (ID 14). The peptides were prepared and used according to the manufacturer's instructions (JPT Peptide Technologies).

Immunoassays

The relative binding affinity between mAbs 1E12 or 4A2 and 15 single mutated antigens (single aa-substituted synthetic peptides) was estimated by two different immunoassays (ELISA) (18, 19).

A competitive peptide ELISA was implemented for mAb 4A2 (monomeric IgG) since this mAb's paratopes were effectively peptide inhibited. A similar peptide inhibition could not be achieved for mAb 1E12, probably due to its pentameric IgM form. Therefore, a sandwich ELISA was developed to assess the peptide interactions instead. ELISA results were normalized by the wt peptide values at each ELISA; however, a rigorous quantitative comparison cannot be established.

For the mAb 4A2 competitive ELISA, 96-well plates (Maxisorb NUNC Fisher) were coated with 15 ng of wt peptide and incubated overnight at 4°C (P1 plates). In a second set of control plates (P2), all peptides (wt and mutated versions) were tested simultaneously by three independent reactions containing a fixed non-saturating dilution of mAb 4A2 and three different amounts of peptide (1,350, 450, and 150 ng). After incubation (2 h at 37°C), peptide–Ab reactions were transferred from P2 to P1 plates and incubated for 1 h at 37°C. Next, plates were washed and further incubated with an anti-mouse IgG-HRP-conjugated mAb (HRPO Sigma) for 1 h at 37°C. After extensive washing with 0.05% Tween 20–0.1% bovine serum albumin in PBS,

TMB substrate (3,3′, 5,5′-tetramethylbenzidine; BD OptEIA) was added to the plate and the resulting OD (optical density) was measured at 450 nm (Thermo Scientific Multiskan® Spectrum) to analyze Ag–Ab interactions. The background values, obtained with no mAb and synthetic peptide, were subtracted for the analysis, and a relative inhibitory concentration (IC50) was determined as the mass of each mutated peptide that causes 50% of binding inhibition relative to the homologous wt peptide (11).

A sandwich ELISA was implemented for the mAb 1E12. In this immunoassay, 96-well ELISA plates were pre-coated (16 h at 4°C) with a fixed 2.5 μg of avidin (NeutrAvidin Invitrogen®), which assured a homogenous capture of the 15 sampled biotinylated peptides (including wt). The procedure consisted of four peptide masses (4,000, 1,333, 444, and 148 ng) for each peptide and incubations for 2 h at 37°C, all in triplicate. Next, mAb 1E12 was added at a fixed dilution per well, followed by the addition of an anti-IgM-HRPO secondary mAb (Axell) and further incubation for 1 h at 37°C. Absorbance was read as described above, and the interaction was quantified as half-maximal effective concentration (EC50), referred to as the concentration of peptide that induces a response halfway between baseline and maximum after a given exposure time.

A qualitative comparison of both ELISA data was performed by defining a relative interaction index 50 (IIR50) according to a settled equivalence between IC50 and the inverse of EC50, relative to the same wt peptide.

Cloning Foot-and-Mouth Disease Virus mAbs Coding Sequence

The molecular cloning of the FMDV neutralizing coding sequences of the evaluated antibodies (4A2 and 1E12) was carried out from mAb-producing hybridoma cell lines (16), without any prior information on their nucleotide or aa sequence (20). This procedure uses the polymerase chain reaction (PCR) to amplify the sequence of variable Ab regions based on the conservation of its flanking regions (21) and a canonical set of degenerate primers capable of amplifying most of the V regions of murine Abs (22).

Briefly, ∼1–2 million mAb-secreting hybridoma cells were harvested and homogenized. Subsequently, total RNA from these cells was isolated using the RNeasy Mini Spin kit according to the manufacturer's instructions (QIAGEN). The cDNA was produced by reverse transcription (RT-PCR) using 10 μg of the RNA template, a 15-base oligo-dT primer, and SuperScript II reverse transcriptase enzyme (Invitrogen). The reactions were incubated at 42°C for 1 h, followed by inactivation of reverse transcriptase at 70°C for 15 min. The resulting cDNA was used as a template for PCR amplification using Taq polymerase (Invitrogen) and various 5′ and 3′ primers specific for VH and VL genes specified in the manual "Current Protocols in Immunology" (20). The PCR reactions were incubated at 94°C for 2 min, followed by 30 cycles of 96°C for 15 s, 56°C for 30 s, and 72°C for 2 min and a final extension at 72°C for 10 min. PCR products of the appropriate size (350–500 base pairs, purified from agarose gels) were cloned into the Invitrogen vector pCR2.1-TOPO-TA™ and transformed into *Escherichia coli* BL21 cells.

Finally, plasmid DNA from five bacterial clones (for each H and L chain of each mAb) was purified using the QIAprep Spin Miniprep DNA isolation kit, according to the manufacturer's instructions (QIAGEN). The presence of appropriate size inserts was verified by restriction enzyme digestions. Up to 10 putative positive clones (by region V cloning event) were sequenced with primers T7 and M13Rev, by using the Big Dye Terminator v3.1 (Applied Biosystems), in Genetic Analyzer 3500xl and 3130xl automatic capillary sequencers (Applied Biosystems) according to the Sanger method (23). The consensus nucleotide sequences were generated using the ContigExpress program within the Vector NTI Advance package (Invitrogen) and submitted to GenBank [accession numbers: 4A2_H (MW435573), 4A2_L (MW435574), 1E12_H (MW435575), 1E12_L (MW435576)].

Sequence and Molecular Structure Data Management and Mining

The general manipulation, analysis, and image production for the aa sequence and Ab molecular structures were conducted with Chimera software (24). Molecular conditioning (e.g., atom protonation and atomic clashes fixes), as a preparatory stage for a mutation task, was implemented with FoldX (25, 26) and MOE (27), which also provides specific modules to perform the alignment of molecular structures and RMSD (root-mean-square deviation of atomic positions) calculus.

The specific calculation and analysis of diverse molecular features of the mAbs, e.g., CDR definition and probability calculations for various kinds of interactions at paratope's residues, were performed with proABC (28) and Paratome (29). The quality of the obtained mAb molecular models was assessed with Molprobity (30).

Ab Modeling and Ag–Ab Docking Protocol

The overall process for Ag–Ab modeling and docking was conducted as outlined in **Supplementary Figure 1**. This process involved three main areas: FMDV Ag, mAbs modeling, and an integrative docking and functional scoring stage. First, the molecular structure of the FMDV A24 Cruzeiro ASA was developed with the FoldX software (25) based on the FMDV AgSA at pdb 1EJO (11). In addition, an ensemble of 30 molecules of the Ag (Ag-M1) was obtained with the Pertmint-MOE software (27, 31).

The nucleotide and the deduced aa sequences for the H and L coding regions of the 1E12 and 4A2 mAbs Fab were first obtained as detailed in the *Cloning Foot-and-Mouth Disease Virus mAbs Coding Sequence* section. Next, multiple molecular mAb models were created by four Ab-dedicated modeling software [KOTAI (32), MOE (27), Rosetta-Antibody (33), and ABodyBuilder (34)]. The quality of the emerging molecular models was evaluated by the MolProbity tool (http://molprobity.biochem.duke.edu/) (30), as suggested by the antibody modeling assessments (AMA) initiative (35). The general denomination for these Ab molecules was mAb-M2. Third, *de novo* Ag–Ab candidate interaction topologies were obtained by two-round computational Ag–Ab docking:

dA: An initial general docking stage (dA) was conducted with the Haddock software (high ambiguity-driven protein–protein

DOCKing) (36), which is an online information-driven flexible docking approach for the modeling of biomolecular complexes. The protocol for dA was implemented as suggested by Trellet et al. with the "guru interface" webserver's client and using the modeled molecules defined above, mAb-M2 and Ag-M1, as input (37).

dB: The emerging top two candidate solutions of every (dA) general docking round became the molecular input for the focused docking stage (dB) implemented using the RosettaDock web server (38, 39). The RosettaDock Server performs a local docking search. That is, the algorithm will search a set of conformations near the given starting conformation for the optimal fit between the two Ag–Ab partners. The resulting Ag–Ab interaction topologies became the input for the last scoring stage.

General modeling and mutations at the FMDV AgSA were computationally accomplished by a single-residue replacement task. Specifically, we employed the FoldX software (academic licensed, http://foldxsuite.crg.eu/) through routine scripts extensively described in the software manual. The scripts for the complex interface repairing (pre-process of the pdb files), mutant building, and energy determination were RepairPDB, BuildModel, and AnalyseComplex, respectively (25, 26). The software yields a mutant complex and a mutant-specific wt complex. These complexes were useful to obtain both, the absolute energetic values of an Ag–Ab interaction, as well as the variation between the mutant and the wt-free energy of unfolding at the interaction interface (ΔG).

Functional Scoring of Ag–Ab Candidate Interaction Complexes

In addition to the software-specific docking scores, the final candidate solutions for near-native Ag–Ab interaction topologies were selected through a functional score. Specifically, we selected the better-scored topologies from a correlation between the two data sets. The antigenic profile A was built with peptide ELISA data for the 13 AgSA peptides (see section Immunoassays). Second, the antigenic profile B was built from *in silico* interaction energy dataset for the same FMDV variant peptides. The profile B for every candidate topology was calculated using the molecular complex as a template. Every scored candidate solution was pre-treated by a multistate modeling (MSM) approach, which implied the generation of a preparative ensemble of 30 molecular structures for every Ag–Ab interaction with PertMin-MOE software (27, 40). Subsequently, every candidate ensemble became the input for the 13 single point mutant modeling. The variation of the interaction energy associated with each mutation (profile B) was calculated using the FoldX program, according to the routines described above. Last, the estimation of the coefficient of determination (correlation) between the *in silico* mutational profile B and the ELISA mutational profile A became the functional score to point out candidate solutions of near-native Ag–Ab interaction complex, the last macromolecular output of our structural modeling pipeline.

RESULTS

Molecular Characterization of Novel Ag–Ab Complexes for Foot-and-Mouth Disease Virus of Serotype A

Antibodies are heterodimeric proteins composed of two heavy (H) and two light (L) chains. The entire macromolecule contains 12 immunoglobulin domain repeats and their variable region, VH and VL domains, form the antigen-binding region (ABR) or paratope. The ABR includes six variable loops termed complementarity-determining regions (CDRs) that are spaced by framework regions (FR) (41).

In a previous work, Seki et al. (16) has described several anti-serotype A FMDV mAbs. Here two AgSA-specific mAbs (1E12 and 4A2) (17) were selected for further characterization. First, we amplified the coding sequence of their VH and VL domains (Fv region) by nested RT-PCR, cloned the resulting amplifications, and their nucleotide and their deduced aa sequences were analyzed for (i) the residues and CDRs that shape the paratopes (**Figure 1** and **Table 1**) and (ii) calculating the interaction probability of residues to its cognate epitope (**Supplementary Figure 2**).

Comparison revealed that 4A2 and 1E12 mAbs displayed smaller paratopes than 4C4 (serotype C mAb reference) and that a lower proportion of the molecular surface of these mAbs was potentially involved in the interaction with the Ag, compared with the reference mAb. Specifically, mAb 4C4 presented a longer H-CDR3 (11 aa) than H-CDR3 of mAb 4A2 (4 aa) and 1E12 (5 aa). The mAb 1E12 also displayed the smallest paratope of the three mAbs because of its shorter L-CDR1 (10 aa) in comparison with 4C4 (15 aa) and 4A2 (16 aa).

The interaction probability data for three different types of molecular contacts of H and L chain paratope residues were consistent with these features. In fact, the prediction of the six loci of greatest probability agreed with the predicted location of the six CDRs (**Supplementary Figures 2**, **3** for H and L chain, respectively). For L-CDR1, the probability of interactions was higher for mAb 4A2 than for 1E12 (potentially because of a shorter L-CDR1 for the latter).

We also elaborated a fine map for 1E12- and 4A2-specific epitopes based on previous basic mapping that allocated the recognition site at the AgSA (16). **Figure 2** summarizes the findings of several relevant residues (for both epitopes) that overlap at the RGD motif of AgSA with 1E12 epitopes involving more RGD distal residues than mAb 4A2. In detail, the AgSA epitopes were defined through two different peptide-ELISA. To that end, we designed a set of 13 peptides with single point mutations based on the aa usage in several FMDV serotype A strains (**Supplementary Table 1**).

For 4A2 mAb, we used a peptide-competitive ELISA and observed that mutations downstream and close to the RGD (peptides 4, 5, 6, 7, 8, and 1) were less tolerated (a lower interaction affinity means a poorer competitor peptide). For the same peptides with the 1E12 mAb (sandwich ELISA), we obtained a more RGD-peripheral effect; where peptides 2 and 3 entailed disruptive mutations, however, peptides 8 and 1,

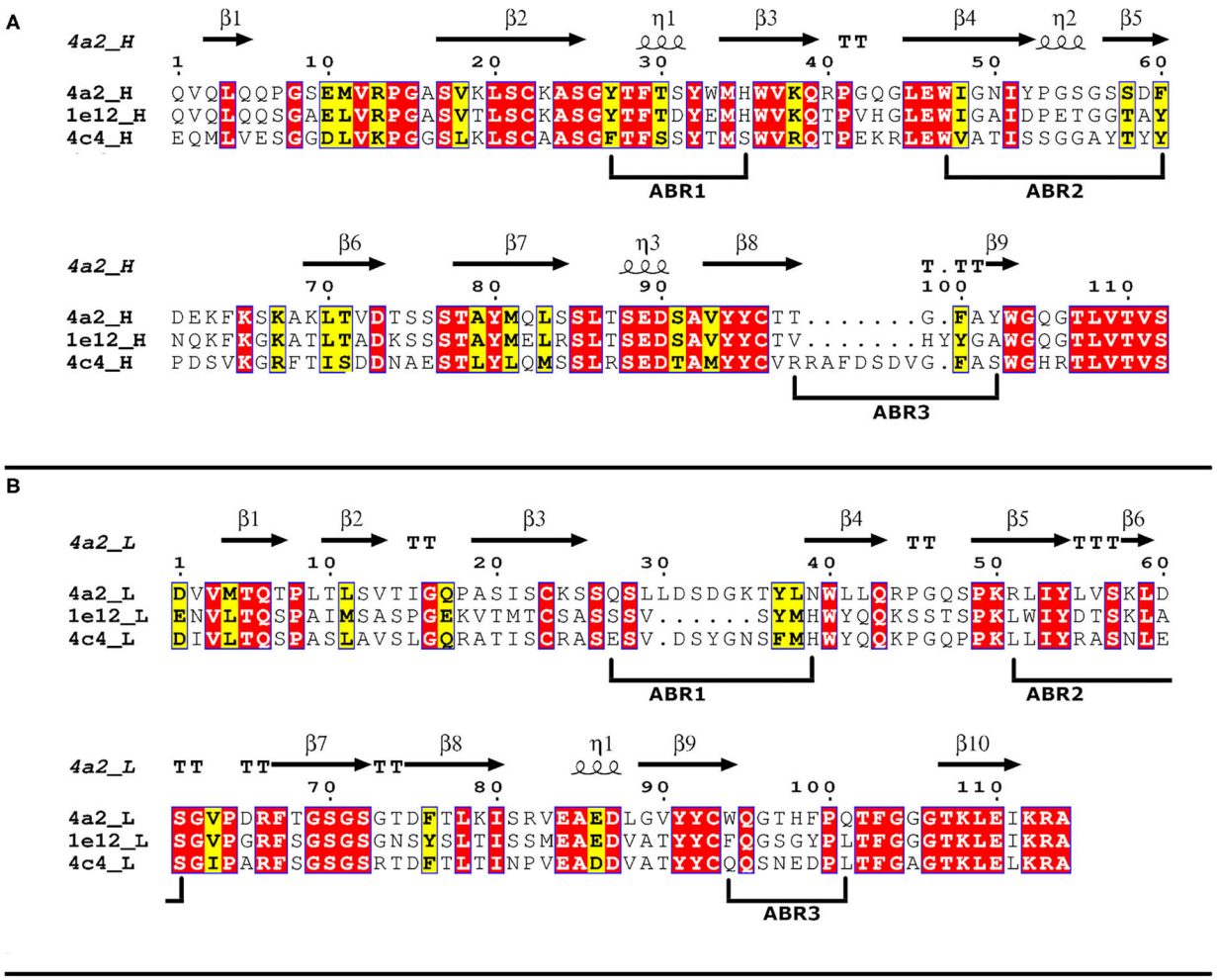

FIGURE 1 | Multiple-sequence alignments of anti-foot-and-mouth disease virus (FMDV) mAb Fv sequences. Amino acid Fv sequences of 4A2, 1E12, and reference 4C4 mAbs. Invariant and conserved residues are highlighted in red and yellow, respectively. Residues are numbered according to the Kabat numbering system and secondary structure elements for variable sequences are indicated above the sequence [spirals, α, and 310(η)-helices; arrows, β-strands; T, turns]. Antigen-binding regions (ABR) are underlined for H **(A)** and L **(B)** chains.

TABLE 1 | Classification and number of aa for six ABRs of mAbs, H, and L chains.

	mAb								
	4C4			1E12			4A2		
H chain									
ABR #	1	2	3	1	2	3	1	2	3
Clasification	1	O	B	1	2	NB	1	2	short
aa num.	5	17	11	5	17	5	5	17	4
L chain									
ABR #	1	2	3	1	2	3	1	2	3
Clasification	k-5	k-1	k-1	k-1	k-1	k-1	k-4	k-1	k-1
aa num.	15	7	9	10	7	9	16	7	9

The table displays features for novel, 1E12 and 4A2, and reference 4C4 mAbs. Classification was according to the Kabat–Chothia canonical structures, where "O" (other) refers to H-ABR not belonging to any canonical structure class. For H3 ABRs, the only two canonical structure classes used were B (bulged) and N (non-bulged). For L chains, "k" stands for kappa. The green and yellow boxes correspond to the more relevant CDR in H and L chains, respectively.

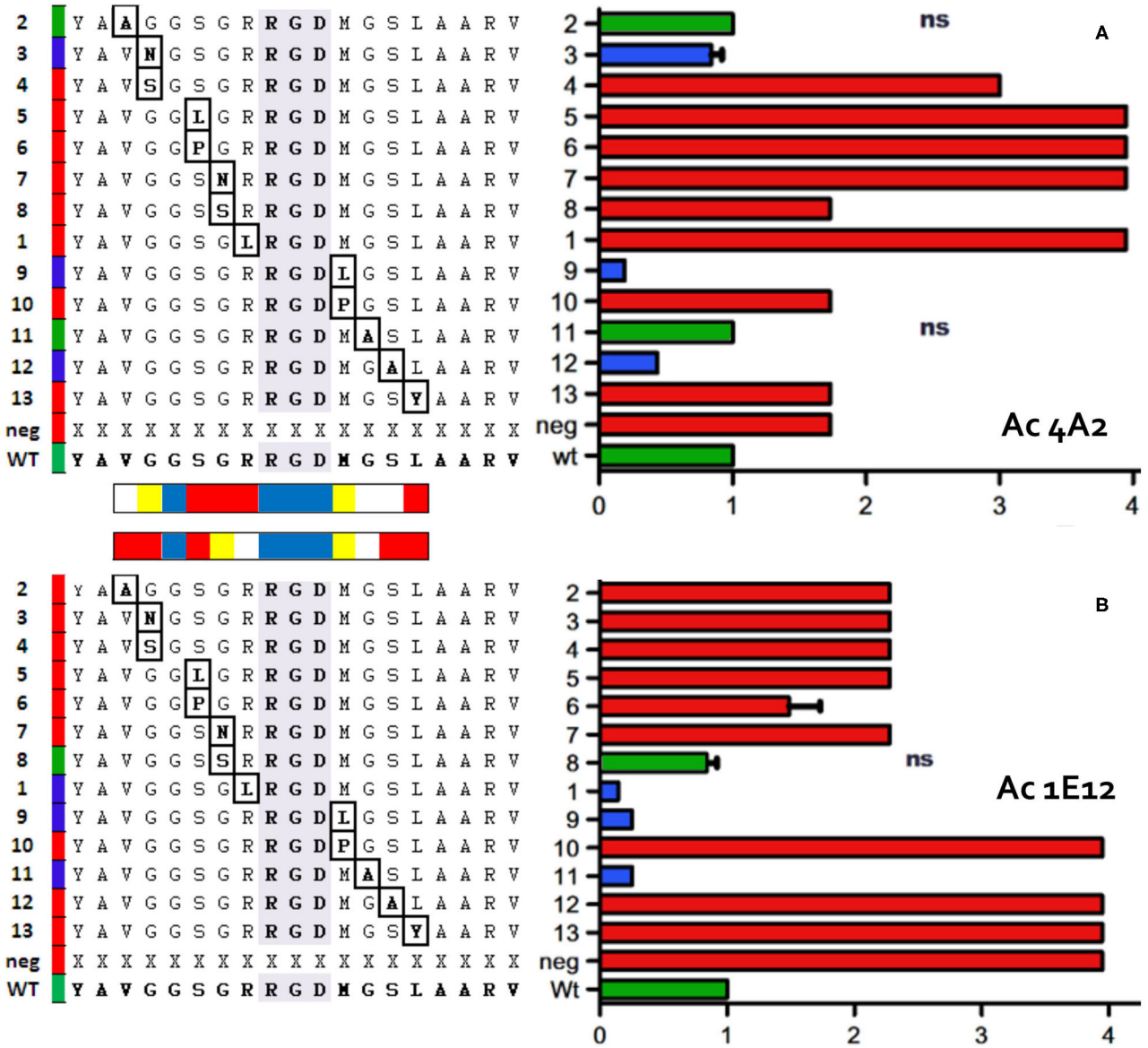

FIGURE 2 | Peptide ELISA mapping of functional epitopes for two anti-FMDV Ab. ELISA results between 15 peptides and mAbs 4A2 or 1E12 are presented in horizontal panels **(A,B)**, respectively. Each panel: Left, the panel displays the specific aa sequences of each peptide; the point mutation is indicated with a box. Right, the panel shows the influence of the point mutations according to the ELISA data; the colors (in the columns) represent IIR50 relative to the wt peptide as follows: in green, the wt peptide and peptides with mutations of IIR50 (relative interaction index 50) equal to wt; in blue, peptides with IIR50 mutations lower than wt (improve interaction) and in red, peptides with IIR50 mutations greater than wt (worsen interaction). The horizontal bar on each peptide matrix summarizes the positions most affected by the mutations in the functional epitope for each mAb, in red and yellow; positions of low and medium tolerance to mutations, respectively, in blue; non-mutated positions and near-native mutations, in white.

with changes closer to the RGD, displayed more tolerated ones. Concerning upstream RGD mutations, both RGD+1 leucine and proline substitutions displayed a similar pattern in both interactions. A proline (peptide 10) was non-tolerated at that short helical fold in the loop GH, although the opposite could be observed with leucine that is more chemically related to the wt aa, methionine (peptide 9). C-terminal mutations demonstrated a similar distal effect, where RGD+4 (peptide 13) was equally disruptive on both interactions, whereas only the 1E12 mAb was sensitive to RGD+3 (peptide 12).

Development of a Foot-and-Mouth Disease Virus Ag–Ab Tuned Docking Protocol

For further characterization of the sequence and function of mAb-Ag (1E12 and 4A2/FMDV AgSA) interaction

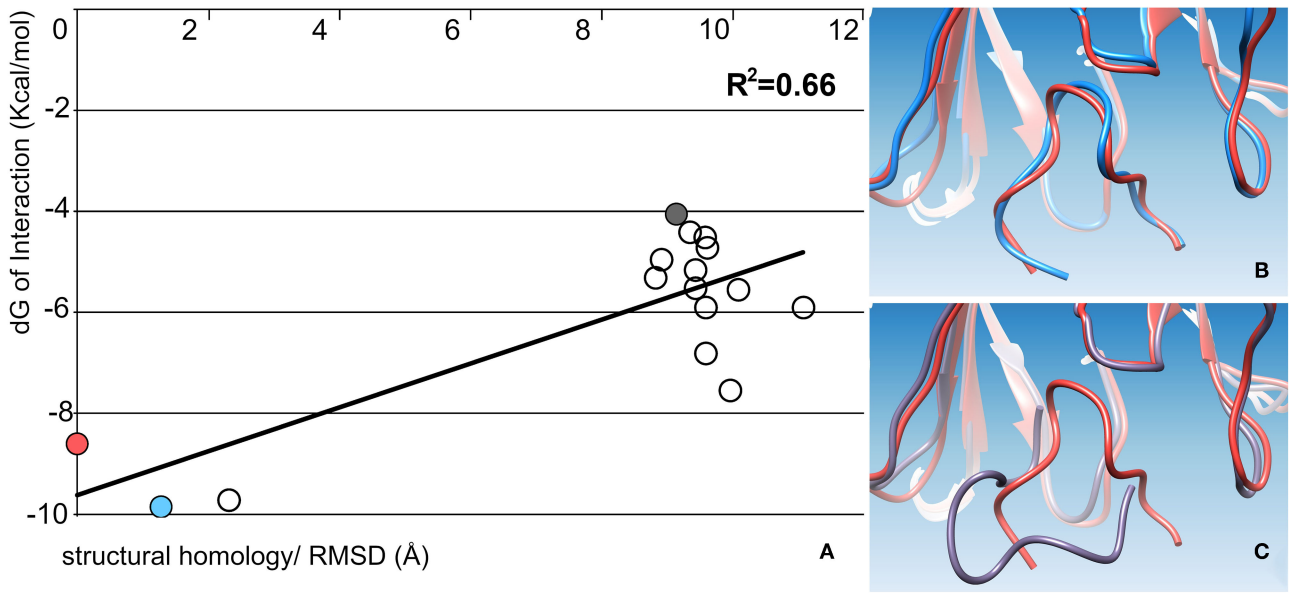

FIGURE 3 | Ag–Ab focused-docking recapitulation for an FMDV interaction complex of reference. The figure displays data for 16 Ag–Ab candidate interaction complexes. **(A)** Regression plot between interface energy score (dG) and RMSD for each Ag–Ab candidate solutions. **(B,C)** exemplifies the structural alignment (of the main chains) between mAbs, revealing the structural distance of FMDV peptide in the wt complex (orange), compared with a top (pale blue), and to a poor (gray) dG scored solution.

complexes, we studied the Ag–Ab molecular structure through a computational modeling approach. However, we first had to define and consolidate an Ag–Ab docking protocol.

To that end, we employed the FMDV Ag–Ab molecular structure of reference [pdb 1EJO (11)] as input. In detail, the exploration of the Ag–Ab interaction topologies was implemented through a two-stage pipeline of docking experiments. First, we evaluated and adjusted a protocol for a general docking stage [software Haddock; (42)] by using individualized Ag and Ab molecules of pdb 1EJO as input (**Supplementary Figure 4**). The Haddock algorithm can sample diverse docking scenarios, including flexible peptide–protein docking supported by well-documented protocols (37, 43). Then, we obtained solution clusters with acceptable i-rmsd and l-rmsd parameters (<1Å), as defined by the Haddock developers and according to the international initiative for the evaluation of docking protocols [Critical Assessment of Predicted Interactions (CAPRI)] (44).

Second, we used the emergent top Haddock solutions as an unbiased input for a local docking stage (dB) implemented at the RosettaDock web server (22, 23). That was a focused docking search near the given starting (input) conformation of the optimal fit between the Ag–Ab interaction partners. Some of the best-scored solutions displayed a remarkable structural homology with the native Ag–Ab topology (R^2 of 0.66) for the wt (pdb 1EJO) Ag–Ab complex (**Figure 3**).

Molecular Structural Characterization of Novel Ag–Ab Interaction Complexes

After defining the docking protocol (section Development of a foot-and-mouth disease virus Ag–Ab tuned docking protocol), we assessed the Ag–Ab interaction partners (section Molecular characterization of novel Ag–Ab complexes for foot-and-mouth disease virus of serotype A). First, the molecular structure of mAbs 4A2 and 1E12 modeled from its aa sequence by using four different programs (see the section Materials and methods) yielded five and 13 models, respectively. Several of those models displayed molecular quality features comparable with the mAb 4C4 [pdb 1EJO (11); **Supplementary Figure 5**]. The AgSA of FMDVA24 Cruzeiro was modeled using FoldX protocols (45) and serotype C FMDV AgSA model (at pdb 1EJO) as a template. Then, the Ag was expanded from a single to multiple molecular state representation, which should bring a more dynamic definition of the Ag input for the next "flexible docking" stage (27, 31).

The sampling of the interaction space for both FMDV AgSA and mAb models using the previous tuned docking protocol (**Supplementary Figure 1**) retrieved five and eight candidate Ag–Ab docking solutions for mAb 4A2 and 1E12 complexes, respectively. All those topologies displayed the characteristic "energy funnel" pattern in a dG score vs. an RMSD plot (**Figure 4**), a feature associated with candidate solutions sampling near the native interaction state (39, 46). Furthermore, the implementation of a functional score biased the results in favor of those candidate Ag–Ab complexes with best mutational fitness between its mutational antigenic profile and a peptide ELISA profile (see the section Molecular characterization of novel Ag–Ab complexes for foot-and-mouth disease virus of serotype A).

For further characterization of top Ag–Ab solutions (**Figure 5**), we modeled and calculated the effect of every single point mutation per each AgSA residue. That computational

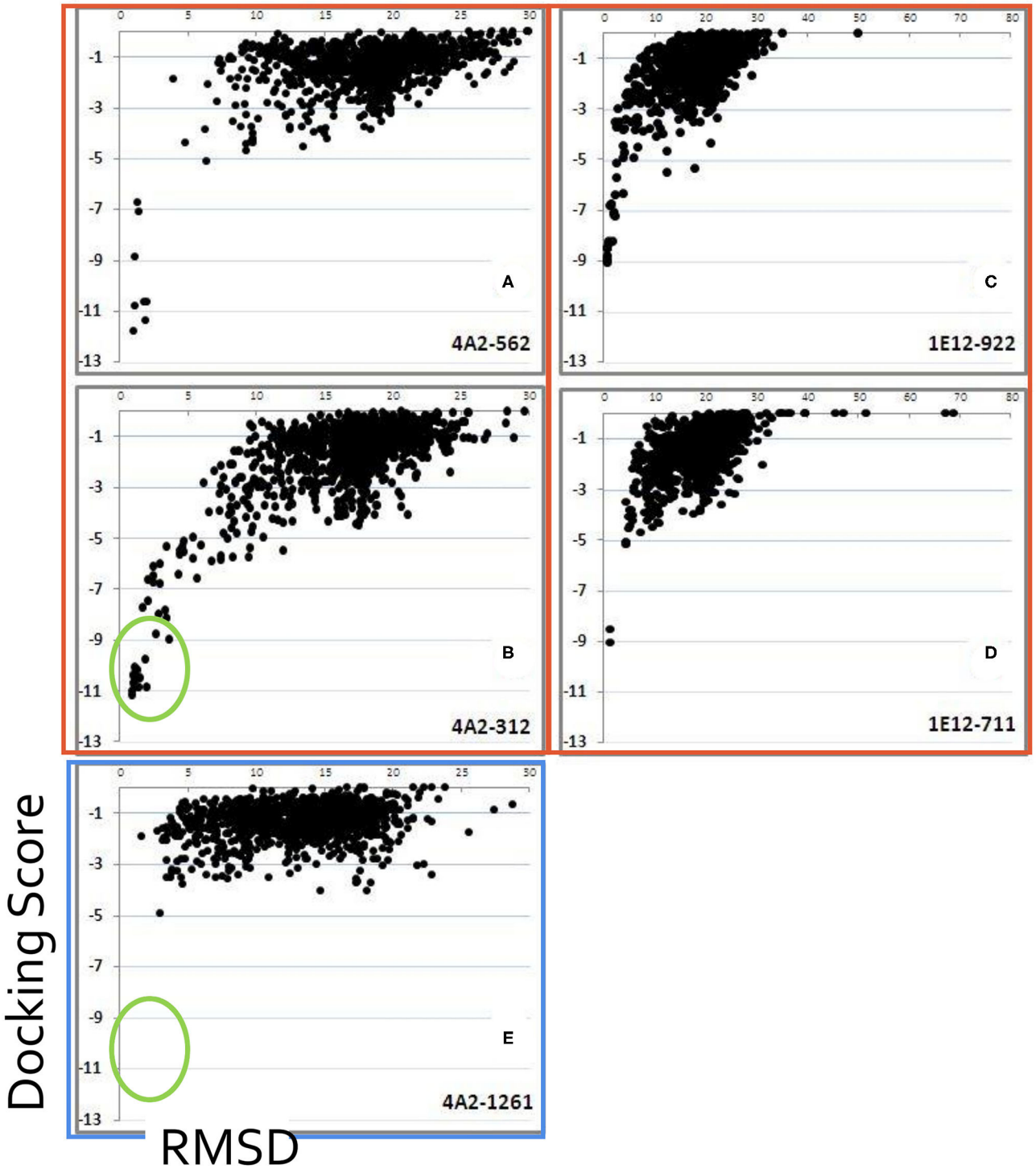

FIGURE 4 | Focused-docking experiments for candidate Ag–Ab complexes. Plots of five different docking experiments using mAb 1E12 or 4A2; dots composed by dG values (Docking score) vs. structural homology values (RMSD, on the horizontal axis). A characteristic pattern called energy funnel **(A–D)**, except for **(E)**, which was a negative control) emerges at near-native docking solutions. Green circles at **(B,E)** denotes the presence or absence of the energy funnel pattern.

exploration of the Ag Tolerated Sequence Space (TSS) was previously demonstrated as feasible by our group (45). Here we obtained three TSS, one for the AgSA-4c4 mAb complex (serotype C reference) and the other two for the novel anti-serotype A mAbs complexes. **Figure 6** illustrates the existence of similar TSS patterns between the "IgG interactions," in

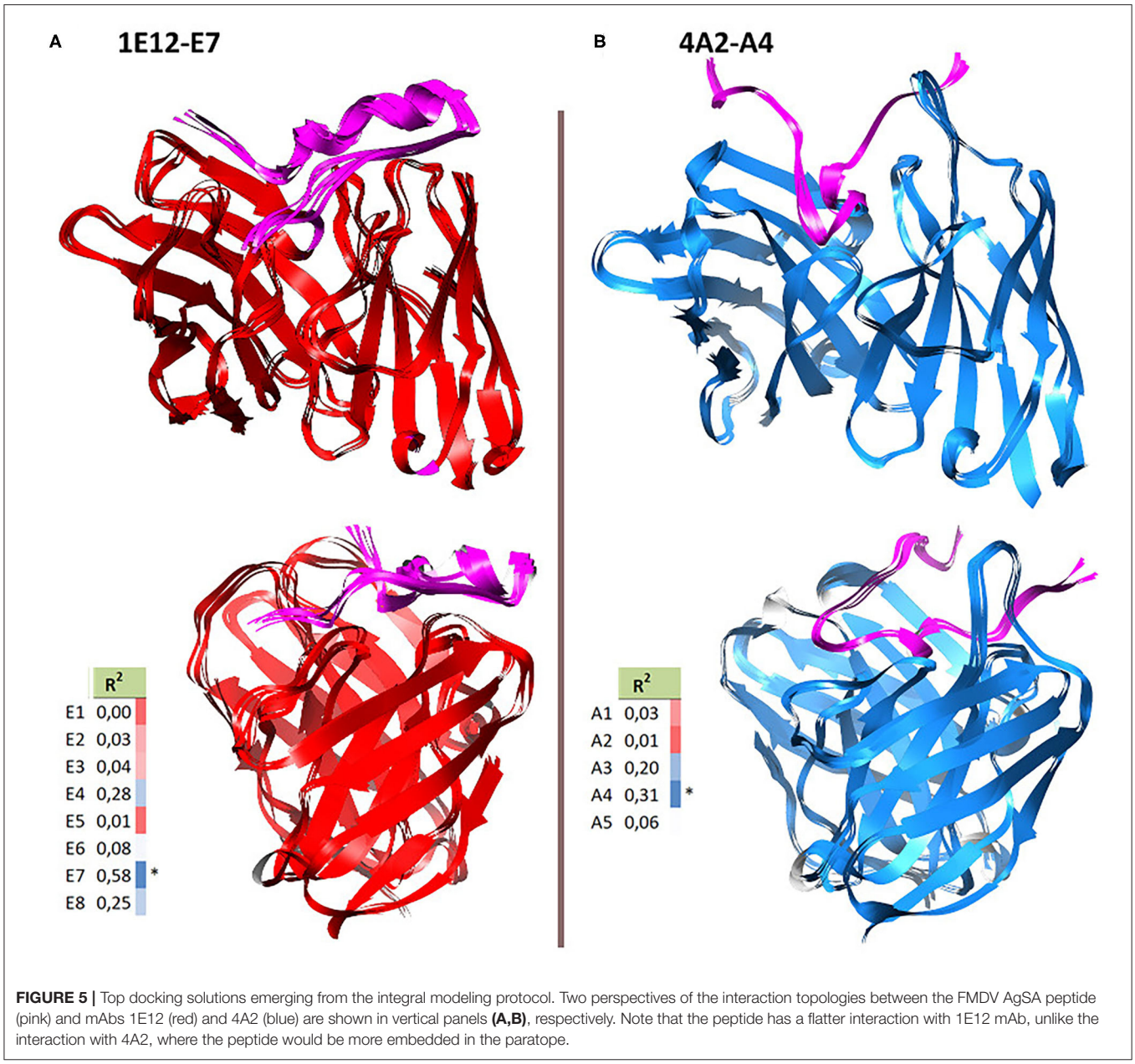

FIGURE 5 | Top docking solutions emerging from the integral modeling protocol. Two perspectives of the interaction topologies between the FMDV AgSA peptide (pink) and mAbs 1E12 (red) and 4A2 (blue) are shown in vertical panels **(A,B)**, respectively. Note that the peptide has a flatter interaction with 1E12 mAb, unlike the interaction with 4A2, where the peptide would be more embedded in the paratope.

opposition to the IgM 1E12-AgSA complex that displayed a more mutation-permissive pattern.

DISCUSSION

Antibody-mediated neutralization is one of the major host mechanisms to decrease and resolve the FMDV infection (47, 48); hence, understanding the Ag–Ab molecular interaction is an essential condition for the development of better FMDV biotechnology tools for disease control (49, 50).

Besides, recent SARS-CoV-2 research has demonstrated a feasible synergy between Ag–Ab molecular structure knowledge and "big data" information from full Ab repertoire analysis (51–53). For FMD, the immunogenomics and polyclonal antibody response have been initially dissected in cattle and African buffaloes (8, 9, 54). With a different approach, we explored and computationally interrogated the sequence space of Ag–Ab interactions at FMDV AgSA of serotype C, and we completed successfully an *in silico* study between two mAbs and more than 200 single point AgSA mutants (45).

Here, we relied on structural data for reference FMDV Ag–Ab complex (pdb 1EJO) to implement a full *in silico* molecular modeling pipeline for the FMDV AgSA–Ab interactions. The adjusted protocol was applied to two novel Ag–Ab interactions for another relevant South American FMDV of serotype A

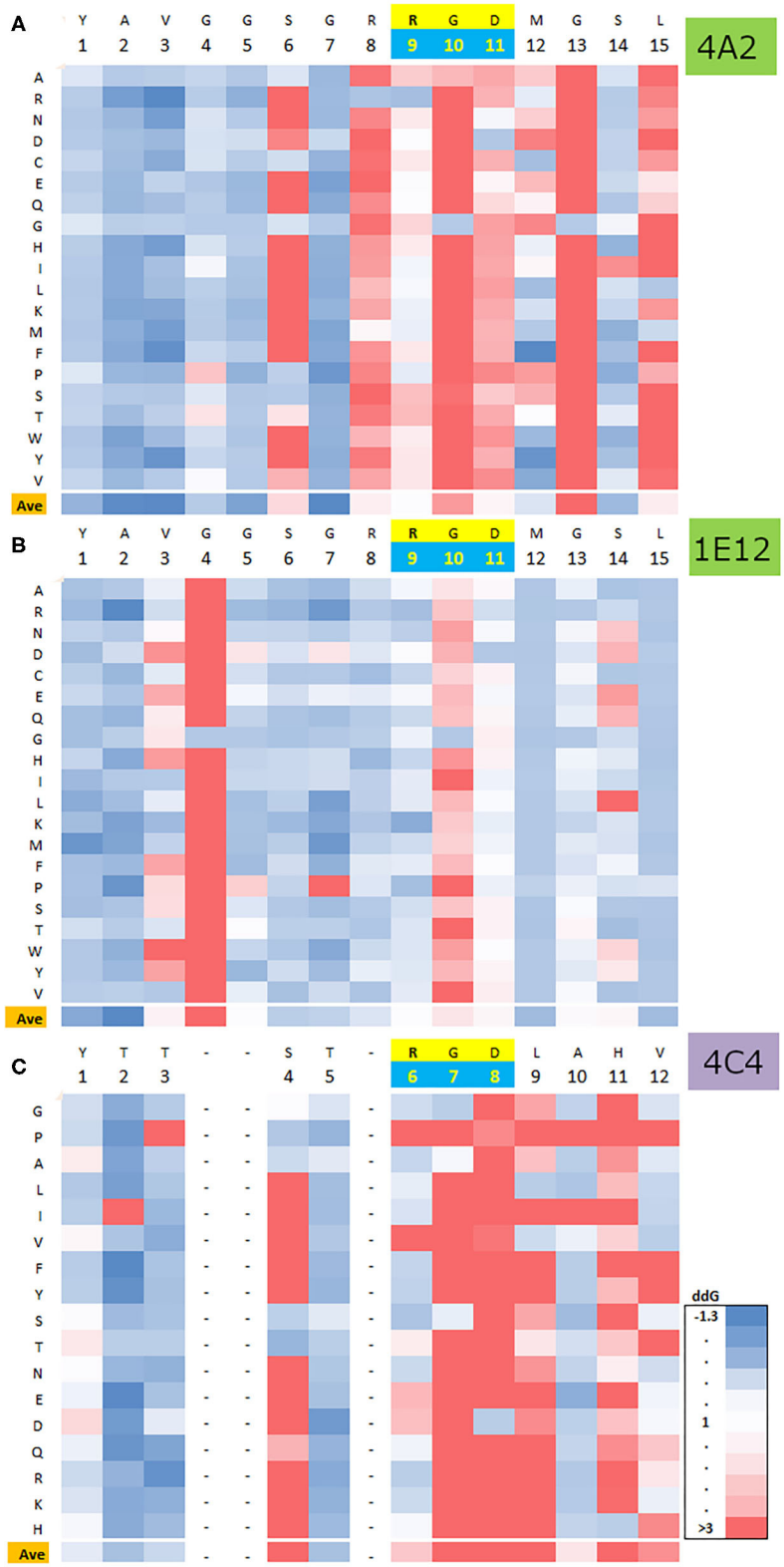

FIGURE 6 | AgSA mutational profile for three FMDV Ag–Ab complexes. Computational mining and comparison of the influence of single point mutations at the FMDV Ag partner for two Ag–Ab complexes and one Ag–Ab complex of reference. Horizontally, each panel contains the native FMDV AgSA sequence (see the conserved RGD motif in yellow) and, vertically, the energetic effect (dG) of single point aa mutations. **(A,B)** correspond to mAbs 4A2 and 1E12 interaction complexes, respectively, and **(C)** displays reference 4C4 mAb (pdb 1EJO) interaction data. Color variation from blue to red implies a worsening of the interaction energy and a less tolerated mutation at a given Ag–Ab. See the similarities between **(A,C)** involving IgG Abs and a different mutational profile for the IgM Ab **(B)**.

(A24 Cruzeiro). In addition, we added a functional-based score to assist the computational exploration of near-native Ag–Ab candidate solutions. A similar data-assisted docking approach has been defined as applicable for docking-algorithm-dedicated research groups (43, 55). Although molecular docking techniques are prone to diverse errors in non-guided experiments (56, 57), here we detected a better predictive performance at the FMDV AgSA scenario that could be associated with the intrinsic simple folding at the RGD turn of the GH loop (58), which means more computational-tractable linear epitopes.

Novel FMDV mAbs Fab sequences were cloned, and their epitopes were characterized by two different ELISAs. The results revealed that the 1E12-specific epitope involved more RGD-distal residues than the mAb 4A2-specific epitope. In addition, both 1E12 and 4A2 mAbs presented smaller paratopes than the reference FMDV 4C4 mAb. However, 4A2 displayed a slight increase in charged residue usage, which could be associated with its characteristic IgG isotype form. Indeed, some researchers have previously reported a net gain of positive charges in IgG in comparison with IgM paratopes (59). Also, mAb 4A2, which is more similar to mAbs 4C4 and SD6 (all of them IgG), featured a long CDR-L1 associated with Abs with greater affinity for protuberant antigens such as the GH loop of FMDV (5). The relevance of the aa positions near the RGD that shapes a linear epitope is undeniable (58). However, in this work, we demonstrated that different residues could have a very distinctive influence on the interaction of the studied Ab–Ag complexes.

Another distinguishing characteristic between the IgM and the IgG Ab is that the molecular modeled structure for mAbs 1E12 and 4A2 displayed a different topology at their paratopes. Specifically, top solutions for mAb 4A2 showed a concave paratope similar to IgG Ab models described for FMDV of serotype C [pdb 1EJO; (11)]. This feature differed from the 1E12 paratope (an Ab of IgM isotype), whose top-ranked molecular model presented a flatter topology of interaction for the same Ag, supporting the differential interaction data obtained by Seki et al. (16). In their work, both were neutralizing Abs, although 1E12 displayed a greater "poly-specificity," as it was positively recognized by more AgSA mutations in FDMV isolates (16). In recent years, there has been a growing interest regarding the relevance of IgM to the FMDV host humoral response (60, 61). Experimental evidence indicates that virus neutralization in vaccinated or infected cattle could be greatly mediated by IgM subtype antibodies by means of thymus-independent humoral immune responses (62). In addition, we obtained more similar TSS patterns between the "IgG interactions" (**Figures 6A,C**) than the IgM 1E12–AgSA complex, which displayed a more mutation-permissive pattern. This contrasting feature could be associated with the above-referred differences at the paratope topology and the AgSA peptide orientation and insertion, concerning the Ab's grooves (**Figure 5**). This could be observed at the TTS differences for the four residues downstream of the RGD motif that build a short helical fold.

Our results suggest the existence of a typical structural diversity of the Ag–Ab modes of interaction at the FMDV AgSA, in contrast with a previous report for mAbs 4C4 and SD6 that showed a greater structural and functional homology at their interaction with a homolog AgSA ligand (FMDV of serotype C). Verdaguer et al. (11) argued the putative existence of a structural bias at the Ab repertoire for the interactions. An impediment in the host humoral response is associated with the presence of the viral RGD motif, which would be acting as a self-antigen (a motif found at several host processes and proteins). However, this contrasting finding could be also attributable to the different isotype nature of the Abs used in our study. For example, a strikingly high aa homology was found between mAb 4A2 with two IgG mAbs (F24G3 and F24G1), also active against A24 Cruzeiro AgSA, produced by another research group (63). In a multiple sequence alignment, the mAbs not only presented a great H chain homology but also contained identical aa sequences at their CDR3-H (**Supplementary Figure 6**) (64). These speculations coming from few examples should be further explored by detailed *in vivo* characterization.

In summary, we generated novel information related to the structural diversity of interactions between epitopes of the FMDV antigenic site and two FMDV Abs of IgM and IgG isotypes. The present work provides evidence about the different characteristics of both isotypes but also points out the similarities among the induced IgG ab structures. This information could be of great biotechnological relevance for the development of new antigens and vaccines with improved cross-protection features. These predictive models, combined with NGS repertoire antibody information, offer a promising tool for the FMDV epidemiology and vaccinology applications (64).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RM and GK designed the study. RM, CS, and GK collected the data and performed the subsequent analyses. RM prepared the manuscript. RM, CS, NM, and GK edited the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.554383/full#supplementary-material

Supplementary Figure 1 | Ag-Ab modeling pipeline. The black outlined rectangles define input points of sequences to the protocol whereas, red, green, blue, and yellow colors refer to modeling stages for the Ag, mAbs, Ag-Ab complexes, and candidate mutational scoring, respectively.

Supplementary Figure 2 | H chain-specific mapping of Ag-Ab contact probability. Contact probability at H chain residues of different Abs and FMDV ASA. Data are displayed as a probability contact over the H chains residues of the reference mAb 4C4 (green), and mAbs 4A2 (blue) and 1E12 (red). The contacts are presented from left to right as follows: A, non-bonded interactions; B, Hydrogen bridge interactions (polar) and C, hydrophobic interactions (apolar).

Supplementary Figure 3 | L chain-specific mapping of Ag-Ab interaction probability. Non-bonded (NB) interaction probability data per aa residues of the L chains are depicted. The reference 4C4 mAb (green) plot is superposed to 4A2 mAb (blue; A) and 1E12 mAb (red; B) plots.

Supplementary Figure 4 | Graphic output from the result page of the HADDOCK web server relative to three different molecular docking experiments. The panels display variable docking clusters obtained from 3 different docking inputs of the same FMDV peptide Ag. Single-state Ag input (A) and two different multiple-state Ag input molecular ensembles obtained after 10 (B) or 50 (C) x,y,z coordinates subtle perturbations cycles. The graphics are based on water-refined models generated by HADDOCK. The clusters (indicated in color in the graphs) are calculated based on the interface-ligand RMSDs assessed by HADDOCK, with the interface defined automatically according to all observed contacts. The various FCC, i-RMSD, and l-RMSD structural analyses are made with the best HADDOCK model (the one with the lowest HADDOCK score). Interface-RMSD (i-RMSD) calculated on the backbone (CA, C, N, O, P) atoms of all residues involved in intermolecular contact using a 10Å cutoff. Ligand-RMSD (l-RMSD) calculated on the backbone atoms (CA, C, N, O, P) of all ($N > 1$) molecules after fitting on the backbone atoms of the first ($N = 1$) molecule. The average values are calculated on the best 4 structures of each cluster (based on the HADDOCK score). Cluster averages and standard deviations are indicated by colored dots with associated error bars.

Supplementary Figure 5 | Quality scores for Ab molecular models. The scoring, according to MolProbity, includes three metrics (cells in blue) with values that improve according to a scale of colors from red to green. The tables reflect data from 3 models for each of the mAbs 4A2 and 1E12, as well as data for the reference mAb 4C4.

Supplementary Figure 6 | Multiple sequence alignment for anti-A24 cruzeiro FMDV mAbs. H-chain aa. sequence alignment between 4A2 mAb (seq. A41-HL) and 2 mAbs developed (F24G3 and G1) against the same FMDV strain. The red boxes indicate CDR1 regions.

Supplementary Table 1 | List of mutant peptides. Mutations and RGD motifs are bold-highlighted. (∗)Leu aa usage to contrast the Arg aa prevalence. (∗∗)Pro and Tyr aa usage as chemical structure modifiers for the RGD +1 +4 short helix span.

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