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Foot-and-mouth disease epidemiology, vaccines and vaccination: Moving forward

Edited by
Alejandra Victoria Capozzo, Teresa de los Santos, Wilna Vosloo, Mariano Pérez-Filgueira and Andres M. Perez

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Foot-and-mouth disease epidemiology, vaccines and vaccination: Moving forward

Topic editors

Alejandra Victoria Capozzo — National Scientific and Technical Research Council (CONICET), Argentina

Teresa de los Santos — Agricultural Research Service, 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

Andres M. Perez — University of Minnesota Twin Cities, United States

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EDITED AND REVIEWED BY
Timothée Vergne,
Ecole Nationale Vétérinaire de Toulouse (ENVT), France

*CORRESPONDENCE
Alejandra Victoria Capozzo
✉ capozzo.alejandra@inta.gob.ar

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Editorial: Foot-and-mouth disease epidemiology, vaccines and vaccination: moving forward

Alejandra Victoria Capozzo[1]*, Wilna Vosloo[2], Teresa de los Santos[3], Andrés M. Pérez[4] and Mariano Pérez-Filgueira[1]

[1]Institute of Virology and Technical Innovations, CONICET-INTA, Buenos Aires, Argentina, [2]Australian Centre for Disease Preparedness, Transboundary Disease Mitigation, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Health and Biosecurity, Geelong, VIC, Australia, [3]Plum Island Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Greenport, NY, United States, [4]Department of Veterinary Population Medicine, University of Minnesota Twin Cities, St. Paul, MN, United States

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Editorial on the Research Topic
Foot-and-mouth disease epidemiology, vaccines and vaccination: moving forward

Vaccination has played a major role in foot-and-mouth disease (FMD) control. There are different approaches to the design and implementation of vaccination campaigns, and epidemiological information is paramount in influencing the vaccine and vaccination strategy that best suit each geographic location. FMD-endemic regions typically organize vaccination campaigns as a routine preventive control policy or to mitigate the impact of the disease. The majority of currently used vaccines are formulated with chemically inactivated whole-viral particles and suitable adjuvants such as single and double oil emulsions. The most recent strains circulating in a particular region are typically selected as antigens based on the results of vaccine-matching data and *in vitro* experiments, however, predictions based on vaccine-matching approaches are usually uncertain without a live virus challenge in natural hosts combined with reliable field data. Vaccine selection and successful vaccination campaigns rely on a deep knowledge of the epidemiology of the region where these vaccines will be used, as well as access to the appropriate diagnostic tools to underpin these campaigns.

Inactivated vaccines are produced by growing large amounts of live virus, which requires facilities with high biosecurity levels and poses a risk of virus escape that may hinder vaccine production in FMD-free areas. In addition, inadequate inactivation of the antigen used to formulate vaccines could potentially cause outbreaks, so a residual risk may persist if the process does not follow adequate quality standards. New-generation vaccines that can be produced without culturing fully infectious virus could provide a solution to these risks. Ideally, these vaccines should protect the host against a vast number of FMD strains and provide at least the same level of protection compared to current, inactivated vaccines.

The main objective of this Research Topic was to gather studies focussed on aspects of FMD vaccine and vaccination to advance the science supporting the implementation of vaccination campaigns that assist the prevention and control of the disease.

This Research Topic hosted by scientists networking through the Global Foot and Mouth Disease Research Alliance (GFRA—https://www.ars.usda.gov/gfra/) includes 14 manuscripts that cover a variety of studies that investigate and discuss the diverse research gaps in FMD vaccine and vaccination; including the progress of FMD control programs in different parts of the world, control measures design and follow-up, risk-assessments for vaccine use, vaccine strain selection, immune responses to currently used vaccines in different species and tools for novel vaccine design, among other issues.

Progress of FMD control programs

Three studies were aimed at evaluating the progress of FMD control programs in which vaccination strategies were used.

Cabezas et al. introduced a retrospective analysis that described the suspensions and recoveries of 45 FMD-free status in the World Organization for Animal Health (WOAH) Members States and evaluated the impact of several risk factors on the time to recover FMD-free status. Most of the FMD-free status suspensions (>50%) were in the Americas and Africa, and about 70% of these suspensions occurred in previously free without vaccination areas. The study noted that implementing a stamping-out or vaccination and removal policy reduced the time to recover FMD-free status, compared with a vaccination and retain policy due to additional requirements for post-outbreak surveillance. Nevertheless, this study confirms once again that vaccination plays a key role in the control of FMD emergencies.

The success of the Progressive Control Pathway (PCP) strategy in Kazakhstan achieving freedom from FMD by combining zoning, movement control, vaccination, and surveillance was described by Sultanov et al.. The study provides an overview of the key factors leading to the successful control of the disease in the country and offers a discussion that is of interest to other countries in the Central Asia region in which FMD is yet to be controlled.

A review of the progress of South America toward FMD eradication (Rivera et al.) was presented, accounting for more than 70 years of dealing with the disease and 35 years of well-organized public-private partnership that finally is leading the region to eradication by 2025. This productive regional governance was accompanied with high-quality and batch-controlled oil vaccines (most of them locally produced), improved surveillance of post-vaccination immunity that permitted to strengthen of control measures in immunologically resized subregions, and the reinforcement of the capacity of the veterinary service and control of animal movement in most of the countries within a particular region. Currently, many territories have taken the step toward withdrawing the vaccine and being recognized as FMD-free without vaccination by the WOAH. This success demonstrates the importance of regional cooperation to control FMD successfully.

Advances in FMD epidemiology

Shurbe et al. provided evidence for using vaccinations in the field through observational or simulation epidemiology approaches. The authors collected and assessed data in Southern Ethiopia, confirming virus circulation, and analyzing risk factors and the socio-economic impact of the presence of FMDV in the region, which is a prerequisite for the design and application of operative control programs in the field. Also critical to accomplish FMDV epidemiological studies, a new approach for a well-known diagnostic tool was developed. A TaqMan-based real-time reverse transcriptase PCR is presented by Chestley et al. using bioinformatics to specifically identify the Southern African Territories (SAT) 1, 2, and 3 serotype strains, excluding other FMDV strains circulating in the region.

Two reports followed modeling approaches to study specific aspects of the vaccine-based controlled strategies. Yadav et al. investigated the economic and epidemiologic impacts of the vaccination-to-live strategy in FMD-free regions. Different scenarios of disease spread, and control were created using the US livestock population as a model. The authors report that production losses were superior when outbreaks began simultaneously in multiple sites, but smaller when compared to trade and consumer avoidance losses. The model predicted a high percentage of potentially persistently infected animals, arising from infected animals in the vaccinated population and discusses the deployment of appropriate post-outbreak management strategies.

An alternative modeling approach was used by Yang et al. to study the impact of different vaccination parameters in managing the disease and comparing the efficacy of the vaccines vs. the vaccine coverage in the field. The authors conclude that increasing vaccine efficacy has a deeper impact on vaccine-based strategies than increasing vaccine coverage.

Vaccine efficacy

Vaccine antigen selection methods and vaccine efficacy in susceptible species were also examined.

Vaccine dose and vaccination schedules optimization in different target species were analyzed in a study performed in Mongolia (Ulziibat et al.). This field study compared the capacity of a two-dose or a single double-dose vaccination of inducing protective levels of neutralizing antibodies and concluded that a single double dose will provide similar results to the traditionally used scheme while being more cost-effective.

A study by Horsington et al. evaluated the protective ability of a bivalent vaccine of different South Asia lineage serotype A strains against the A/Asia/SEA-97 variants in pigs, instead of using the same strains as monovalent preparations. Improved protection with an increased number of virus strains has been shown before, explaining their success due to the availability of a higher number of conserved epitopes available to the immune system (1).

These challenge studies are paramount to provide information that can be used to feed models that can help select antigens without the need of challenging animals with live virus. Laboratory tools with the optimized capacity to score the adequacy of vaccine-induced immunity were also evaluated based on vaccine performance data from the field (Gubbins et al.). In this regard, Ludi et al. presented "PRAGMATIST," a semi-quantitative FMD strain selection tool that uses information on vaccine efficacy trials, laboratory vaccine matching results and risk scores. The authors

highlighted the variation in the vaccine antigens required for storage in FMD-free regions where vaccination is not applied.

Tools for new generation vaccine development

Virus-antibody interactions are also studied to further optimize vaccines and improve quality control (Harmsen et al.), based on the detection of FMDV capsid integrity. Also in this collection, Summerfield et al. delved into the relationship between opsonizing and neutralizing monoclonal FMDV-specific antibodies and assigned a role for low avidity antibodies, as their interactions are enough to mediate Fcγ receptor-mediated functions that could play a role in the protective immunity against FMDV. Harmsen et al. provided knowledge of the particle specificity of VHHs that can be applied to the production of VLPs with improved immunogenicity.

VLPs and live-attenuated vaccines are new-generation vaccine candidates that can be grown in low biosafety environments. An article by Azzinaro et al. supports the view that manipulation of the Lpro coding region can provide a tool to develop FMDV live attenuated strains of FMDV while ensuring that sufficient replication is achieved to induce a protective and sustained immune response. Moreover, the incorporation of mutations that could stabilize attenuating mutations and prevent recombination with circulating viruses, is an important requirement for the success of such an approach.

Conclusions

Altogether, the articles in this collection bring an overview of the current advances in FMD vaccine development, vaccine selection, vaccination, and epidemiology research to produce tools for FMD control and the pathway for eradication. There remains much to achieve, especially in understanding cross-protection, vaccine strain selection and how to perform accurate risk assessments. These gaps can be closed by promoting collaboration between groups working on FMD globally, supported by international initiatives such as the Global Foot and Mouth Disease Research Alliance.

This Research Topic includes articles that improve our capacity of using vaccination as a key tool to prevent and control FMD, contributing to the sustainability of the livestock industry worldwide.

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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Conflict of interest

The 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.

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EDITED BY
Wilna Vosloo,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

REVIEWED BY
Aman Ullah Khan,
University of Veterinary and Animal Sciences, Pakistan
Aurelio H. Cabezas,
World Organisation for Animal Health, France
Charles K. Nfon,
National Centre for Foreign Animal Disease (NCFAD), Canada

*CORRESPONDENCE
Mesfin Shurbe
messhurbe33@gmail.com

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Seroprevalence and associated risk factors for foot and mouth disease virus seropositivity in cattle in selected districts of Gamo zone, Southern Ethiopia

Mesfin Shurbe[1]*, Bekahegn Simeon[1], Wasihun Seyoum[1], Ayelech Muluneh[2], Ephrem Tora[1] and Edget Abayneh[1]

[1]Department of Animal Science and Health, Livestock and Fishery Research Center, College of Agriculture, Arba Minch University, Arba Minch, Ethiopia, [2]National Animal Health Diagnostic and Investigation Center, Sebeta, Ethiopia

Background: Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, which hampers livestock production and productivity in Ethiopia. This cross-sectional study was conducted from January to December 2021 to estimate the seroprevalence of FMD in cattle and to assess farmers' knowledge about the disease in selected districts of the Gamo zone. Three districts and two kebeles (smallest administrative division) from each district were purposively sampled using a simple random sampling technique to select individual animals from each kebeles. A total of 384 sera samples were collected, and concurrently, 100 farmers were interviewed. The samples were tested for antibodies against nonstructural proteins of the FMD virus using a 3ABC enzyme-linked immunosorbent assay (ELISA). Univariable and multivariable logistic regressions were used to analyze FMD-associated risk factors.

Result: The questionnaire survey result revealed that among the interviewed farmers, 66% of farmers had knowledge about the disease, and 28% of farmers reported having a case of FMD in at least one cattle in their farm in the previous 6 months. The overall seroprevalence of FMD in cattle was 26.8%. The multivariable logistic regression revealed that age, breed, and agroecology had a significant association with seropositivity. Higher seroprevalence (64.57%) was observed in lowland, followed by midland (9.30%) and highland (5.88%). Study animals from lowland areas were 9.26 times more likely to be seropositive (OR = 9.26, CI = 2.22–38.62) for FMD than highland animals. Also, adult animals were 9.01 times (OR = 9.01, CI = 3.18–25.53) more likely to be seropositive for the disease than young animals. The multivariable logistic regression revealed that crossbreeds have an 84.7% (OR = 0.153, CI = 0.028–0.82) lower likelihood to be seropositive to FMD than local breeds.

Conclusion: This study result confirms that FMD is highly prevalent in the study area, and farmers' knowledge regarding disease transmission and

vaccine availability is minimal. Hence the regional concerned bodies should implement FMD vaccination campaigns and create awareness for smallholder farmers regarding the disease transmission, FMD vaccine schedule, and vaccination importance.

KEYWORDS

foot and mouth disease, Gamo zone, knowledge, perception, seroprevalence

Background

Ethiopia has the largest livestock population in Africa comprising 60.9 million cattle, 31.3 million sheep, and 32.7 million goats. The livestock sector plays a crucial role in the national economy, as well as in the socioeconomic development of millions of rural smallholder farmers; it has considerable prospective opportunities for income generation, employment, and poverty alleviation (1, 2) and sustains livelihoods for 80% of all rural population (3).

However, livestock production in the country is severely affected by several constraints, including the widespread distribution of animal diseases in different agroecological zones, resulting in high annual mortality rates (4). Of the animal diseases hindering productivity, foot-and-mouth disease (FMD) is considered a bottleneck for livestock production and has become the leading cause of blocking the trade of live animals and animal products (5, 6).

Foot and mouth disease is a contagious viral disease caused by the FMD virus (FMDV) of the genus *Aphthovirus*, in the family of Picornaviridae, and it affects cloven-hoofed animals (7). The FMDV genome consists of an 8,400-nucleotide single-stranded ribonucleic acid (ssRNA) that encodes a polypeptide that cleaves into several nonstructural proteins (NSPs) and four structural proteins (SPs) (8). The disease is clinically characterized by fever; loss of appetite; vesicles on the tongue, dental pad, gums, soft palate, nostrils, or muzzle that lead to excess salivation; vesicular eruptions on the feet and teats; and sudden death of young stock (9). There are seven serotypes of FMDVs (i.e., O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3) (10), which have distinct immunologic, antigenic, and genetic properties (11). At present, five serotypes of FMDVs have been reported in Ethiopia (12), which indicates that the disease is endemic in Ethiopia, and varying degrees of their existence were found in different parts of the country, with seroprevalence ranging from 8.18% in South Omo to 44.2% in selected districts of Afar Pastoral Area (13).

The FMD is diagnosed using a combination of history, clinical symptoms, and laboratory investigations. FMDV can be isolated on cell cultures, the viral nonstructural protein can be detected using ELISAs, and the presence of viral genomic material can be detected using PCR assays (14). Anti-NSP antibody testing is commonly utilized to distinguish infected animals from vaccinated animals in both FMD endemic areas (15) and FMD-free countries (16).

The current situation of FMD in Ethiopia is alarming due to its wide distribution with variant strains in different parts of the country. Thus, livestock are at risk from endemic strains as well as from antigenic variants prevailing in neighboring countries (17). Regardless of its wide geographic distribution, broad host range, ability to establish carrier status, and poor cross-immunity, the control of the disease is complicated in FMD endemic areas due to limited disease surveillance together with lack of molecular characterization and lack of proper identification of the origin of the disease (18). Thus, continuous FMD disease surveillance together with serotyping of the virus is a paramount role in undertaking efficient control schemes. Therefore, this study was designed to determine the seroprevalence of antibodies against FMDV and assess the potential risk factors associated with the seroprevalence of the disease in Gamo zone, southern Ethiopia.

Materials and methods

Study area description

This study was conducted from January to December 2021 in three purposely selected districts, namely, Geresse, Arbaminch Zuria, and Chencha of Gamo zone, Southern Ethiopia (Figure 1).

Geresse district is one of the newly established districts of Gamo zone, formerly which is part of Bonke district. Gresse district is located 55 km from the capital of Gamo zone, which is situated between 800 m and 2,700 m above sea level. The district has 23 kebeles, of which 43% kebeles are highland, 32% kebeles are midland, and 25% kebeles are lowland. The total area of the district is estimated to be 66,683.02 hectares with an annual rainfall of 800–1,200 mm. The estimated livestock populations of the district are 137,171 cattle, 189,557 sheep, 65,758 goats, 36,566 equines, and 226,026 poultry (19).

Arbaminch Zuria district has a bimodal rainfall system, short rain season that occurs from January to April and long rain season that occurs from June to September. The altitude of the district ranges from 1,001 to 2,500 m above sea level. The district has two agroecological zones, namely, Woina Dega (midland) and Kola (lowland). The district has

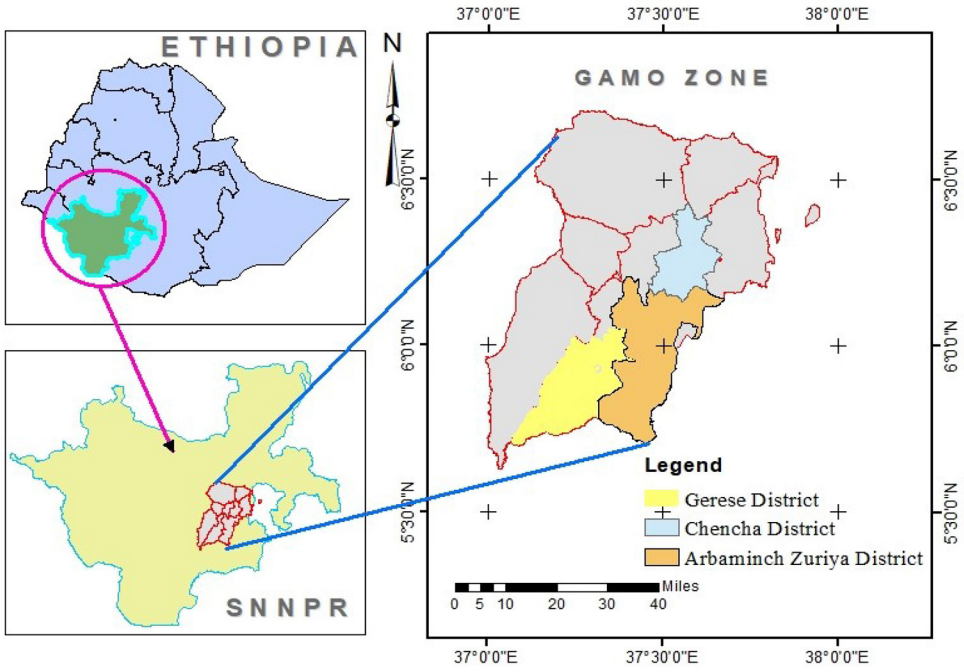

FIGURE 1
Map of the study area.

18 kebeles, of which 8 kebeles were found in midland and the remaining 10 kebeles were found in lowland agroecological zones of the district. Within the district, livestock husbandry is generally characterized by an extensive farming system, in which animals are allowed to graze freely during day time and kept in open enclosures during night time. The livestock populations of the district are 101,628 cattle, 27,339 sheep, 42,662 goats, 3,204 equines, and 140,050 poultry (20).

Chencha District is situated between 1,300 m and 3,250 m above sea level. Astronomical location of Chencha Woreda is between 37 29′ 57″ East to 37 39′ 36″ West and between 6^0 8′ 55″ North and 6^0 25′ 30" South. Due to a high altitudinal range, the area is characterized by diverse agroclimatic distribution. The district is divided into two agroecological zones, namely, Dega and Weyna Dega, which account for about 82 and 18% of the total area, respectively. The rainfall regime in the district is bimodal. The first round of rain occurs between March to April. The second round of rain occurs from June to August. The annual rainfall distribution in the district varies between 900 mm to 1,200 mm. The minimum and maximum temperatures in the district range from 11°C to 13°C and 18°C to 23°C, respectively. The farming system in the district is a mixed farming system where the crop sub-system and the livestock sub-system are practiced. Chencha has 67,269 cattle, 106,594 sheep, 11,870 goats, and 22,554 equines (21).

Study design

A cross-sectional study was conducted from January to December 2021 to estimate the seroprevalence and associated risk factors of FMD. In addition, a survey was used to assess farmers' knowledge of FMD in the study area.

Study population

The study animals were cattle that were kept under different management systems (extensive, intensive, and semi-intensive farming systems). All local breed and crossbreed cattle that were > 6 months of age were included in the study. In the sample collection period, information concerning animal level risk factors such as age, sex, and breed was collected and recorded. The age of each study animal was determined by consulting the owners of the cattle. Accordingly, animals were categorized as calves (<2 years), young (2–4 years), and adults (> 4 years) (22).

Inclusion criteria

All local breed and crossbreed cattle > 6 months of age were included in the study. Also, cattle owners who showed willingness to participate in the survey were included in the study.

Sampling method and sample size determination

Three districts, namely, Geresse, Chencha, and Arbaminch Zuria, and two kebeles from each district were selected purposively based on their agroecology, proximity to livestock market, contact with wildlife, accessibility for transportation and immediate laboratory procedure, and population density. Then, simple random sampling was employed to select each study animal from each kebeles. The sample size required for the study was calculated based on the following formula (23):

$$n = \frac{1.96^2 \times P_{exp} \times (1 - P_{exp})}{d^2}$$

where n = sample size, P_{exp} = expected prevalence, and d = absolute precision.

Considering the expected prevalence of 50% with 95% confidence level and 5% absolute precision, the sample size computed was 384. Then, proportionate numbers of animals were sampled from each of the three districts based on their cattle population size. Consequently, 172, 127, and 85 animals were sampled from Geresse, Arbaminch Zuria, and Chencha districts, respectively.

The survey of farmers' knowledge was carried out in three districts in conjunction with blood sample collection. From cattle owners whose cattle were sampled for serology, a total of 100 farmers were randomly selected from the three districts. Accordingly, 33 individuals from Geresse, 34 individuals from Arbaminch Zuria, and 33 individuals from Chencha districts were interviewed. The sample size was determined using the formula ($n = 0.25/SE^2$) as per Arsham (24) at the standard error (SE) of 0.05 with 95% confidence interval.

Study methodology

Questionnaire survey

A semi-structured questionnaire was used to assess the farmers' knowledge of FMD. The questionnaire was pretested and modified before the final interviews were conducted. The questionnaire was designed to assess whether the informants know FMD, its clinical signs, source of infection, and prevention and control methods.

Blood sample collection

From each animal, 10 ml of blood was collected from the jugular vein using a 21-gauge needle, and serum samples were transported in a cold chain to the National Animal Health Diagnostic and Investigation Center (NAHDIC) and stored at −20°C until further use (25).

Serological diagnostic tests

The collected sera were tested by FMDV 3ABC-Ab ELISA (ID Screen® FMD NSP Competition, ID-VET, Grabels, France) at the NAHDIC according to the manufacturers' recommendation and the procedure provided by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (14). Percentage inhibition equal to or < 50% was considered positive.

Associated risk factors

Age, sex, body condition, and breed of study animals were considered as intrinsic risk factors of FMD during the study period, while management system (extensive, semi-intensive, and intensive), herd composition, herd size, history of movement of animals, contact with wildlife, awareness of farmers, agroecology, and communal grazing and watering practices were considered as extrinsic risk factors for FMD. This information was recorded in the prepared data sheet for each animal. Herd size is classified into *three* categories such as small herd <10 animals, medium herd 10–50 animals, and large herd >50 animals (26).

Data management and analysis

Data generated by laboratory investigations and the questionnaire survey were recorded and coded using a Microsoft Excel spreadsheet (Microsoft Corporation) and was analyzed using STATA version 14.0 for Windows (Stata Corp. College Station, TX, USA). Descriptive statistics were used to present the survey results and to calculate the proportion of FMD-related risk factors. Disease seroprevalence was computed by dividing the number of positive ELISA results by the total number of collected samples. In addition, univariate and multivariate logistic regression analyses were conducted to identify the main FMD risk factors, including sex, age, breed, body condition score, agroecology, herd size, herd composition, management system, communal grazing, communal watering, and cattle owner awareness of the FMD symptoms and risks. After checking the data for collinearity, all variables with $p < 0.25$ in the univariable analysis were subjected to stepwise backward multivariable logistic regression analysis. Hosmer-Lemeshow test was performed to check the goodness of fit of the final model. In the serological study, odds ratios (ORs) were calculated to determine the degree of association between each risk factor and FMD seropositivity. In all analyses, a 95% confidence interval (CI) was calculated, and $p < 0.05$ was considered statistically significant.

Results

Cattle owners' knowledge of FMD

The survey revealed that 66% (66/100) of the surveyed farmers were aware of the FMD and were familiar with its local name "Massa." Those who knew about the disease were instructed to indicate the typical symptoms of the disease. The most commonly cited symptoms were hyper-salivation lesions on the mouth (37.8%, 25/66) and feet (35%, 23/66), lameness (24.2%, 16/66), and inappetence (3.2%, 2/66). In addition, 44% (29/66) of the farmers that were aware of FMD also indicated that they were familiar with the possible causes of the disease. About 30% (20/66) of the farmers believed that contact with infected animals during grazing led to FMD, and 14% (9/66) ascribed the disease outbreaks to the introduction of diseased animals into a herd. However, 98% (98/100) of the interviewed cattle owners did not know about a vaccine that would protect their livestock from FMD.

Foot and mouth disease: 6-month occurrence

The survey results revealed that 28% (28/100) of the surveyed cattle owners reported having a case of FMD in at least one cattle in their farm in the previous 6 months. Those that reported FMD cases were instructed to indicate the disease management strategy they had adopted. While 50% (14/28) of the farmers who had FMD cases in their livestock opted for medical treatment, 32% (9/28), 7% (2/28), 7% (2/28), and 4% (1/28) of this subgroup chose isolation, selling, slaughtering, and doing nothing, respectively. Cattle owners' questionnaire responses are summarized in Table 1.

TABLE 1 Cattle owners' response to knowledge, prevention, and control practices of FMD.

Variables	Number of respondents	Response (%)
FMD knowledge		
Yes	66	66
No	44	44
FMD vaccine information		
Yes	1	1
No	99	99
Symptoms		
Hypersalivation	25	37.8
Lesions on feet and mouth	23	34.8
Lameness	16	24.2
Inappetence	2	3.2
FMD 6 months occurrence		
Yes	28	28
No	72	72
Control method		
Treatment	14	50
Isolation	9	32
Selling	2	7
Slaughtering	2	7
Doing nothing	1	4
Knowledge about causes of FMD		
Contact with wild life	0	0
Contact with infected animal	20	30
Introduction of infected animal	9	14
Do not know	37	56

Overall seroprevalence of foot-and-mouth disease virus

The study revealed that out of 384 samples tested, 103 (26.82%) samples were positive for the presence of antibodies against FMDV NSP (Table 2).

Association of risk factors with seropositivity of FMD

The association between seropositivity and hypothesized risk factors was analyzed using both univariable and multivariable logistic regressions. From a total of 10 hypothesized risk factors that were statistically significant when analyzed by univariable logistic analysis, only 3 risk factors had a statistically significant ($p < 0.05$) association with seroprevalence of FMD in a final model. The result of the two models is summarized and presented in Tables 3, 4.

The age of the study population was categorized into three groups, namely, calves (6 months to 2 years), young (2–4 years), and adult (> 4 years). Higher seroprevalence of FMD was seen in adult animals (40.24%), followed by young (26.55%) and calves (4.90%). The multivariable logistic regression result revealed that adult animals were 9.01 times (CI = 3.18–25.53) more likely to be positive for the disease than young animals.

The other hypothesized intrinsic factor for FMD was breed, which was categorized as local breed and crossbreed. The prevalence of FMD is higher in local breeds (34.24%) than crossbreeds (2.25%). The multivariable logistic regression revealed that the odds of being seropositive is 84.7% (OR = 0.153, CI = 0.028–0.82) less likely in local breeds than crossbreeds.

The association between seropositivity and hypothesized extrinsic risk factor like agroecology was analyzed using multivariable logistic regression and is summarized in Table 4. Study animals that were living in lowland areas were 9.26 times

TABLE 2 Summary of the risk factors of FMD.

Variable	Categories	No. of examined	No. of positive	Prevalence
Age	Calves	102	5	4.90
	Young	113	30	26.55
	Adult	169	68	40.24
Sex	Male	89	25	28.09
	Female	295	78	26.44
Breed	Local	295	101	34.24
	Cross	89	2	2.25
Body Condition	Poor	70	16	22.86
	Medium	169	45	26.63
	Good	145	42	28.97
Agroecology	Lowland	127	5	5.88
	Midland	172	16	9.30
	Highland	85	82	64.57
Management	Intensive	75	5	6.67
system	Semi intensive	221	61	27.60
	Extensive	88	37	42.05
Herd size	Small	242	30	12.4
	Medium	58	17	29.31
	Large	84	56	66.67
Herd composition	Mixed	246	26	18.84
	Not mixed	138	77	31.30
Contact with	Yes	177	19	9.18
wild life	No	207	84	47.46
Communal grazing	Yes	273	8	7.21
	No	111	95	34.80
Movement history	Yes	167	67	40.12
	No	217	36	16.59

TABLE 3 Univariable logistic regression results of risk factor analysis.

Variable	OR	CI	*p*-value
Sex			
Female	-	-	-
Male	1.08	0.64–1.85	0.758
Age			
Calves	-	-	-
Young	7.01	2.60–18.89	0.001
Adult	13.06	5.05–33.77	0.001
Breed			
Local	-	-	-
Cross	0.044	0.011–0.183	0.001
Body condition			
Poor			
Medium	1.22	0.64–2.35	0.543
Good	1.37	0.701–2.67	0.345
Agroecology			
Highland	-	-	-
Midland	1.64	0.58–4.64	0.350
Lowland	29.15	11.01–77.21	0.000
Herd size			
Small	-	-	-
Medium	2.93	1.48–5.79	0.002
Large	14.13	7.81–25.57	0.001
Herd composition			
Not mixed			
Mixed	1.96	1.185–3.250	0.009
Management type			
Intensive			
Semi-intensive	5.34	2.06–13.85	0.001
Extensive	10.15	3.73–27.64	0.001
Communal Grazing			
No			
Yes	6.87	3.20–14.71	0.001
Movement History			
No			
Yes	3.36	2.09–5.41	0.001
Contact with wildlife			
No	-	-	-
Yes	8.93	5.12–15.59	0.000

CI, Confidence interval; OR, Odds ratio.

more likely to be seropositive (OR = 9.26, CI = 2.22–38.62) for FMD than study animals from highland areas. The highest seroprevalence (64.57%) was observed in the lowland district followed by the midland (9.30%) and highland (5.88%) districts.

Discussion

Farmer's knowledge and perception on FMD

This study revealed that 66% of the respondents had knowledge regarding FMD clinical signs with its local name "Massa." This finding goes in line with a study conducted in the Amhara region by Mesfine et al. (27), who reported that 82.4% of respondents knew FMD. Also, Tesfaye et al. (28) reported that pastoralists living at the Borena zone are well aware of the clinical signs of FMD and it was known by the local name Oyale. About 30% (20/66) of the farmers believed that contact with infected animals during grazing led to FMD, and 14% (9/66) ascribed the disease outbreaks to the introduction of diseased animals into a herd. This result strongly agrees with a study conducted in the Amhara region by Mesfine et al. (27), who showed that about 78% of farmers surveyed expect FMD to be transmitted by coming into contact with infected animals during communal grazing and watering activities, and about 22% think that it is primarily by infected animals coming from markets. The survey includes a question about the typical

TABLE 4 Multivariable logistic regression results of risk factor analysis.

Variable	OR	CI	*p*-value
Age			
Calves	-	-	-
Young	4.55	1.50–13.67	0.007
Adult	9.01	3.18–25.53	0.001
Breed			
Local	-	-	-
Cross	0.153	0.028–0.82	0.029
Agroecology			
High land	-	-	-
Mid land	0.725	0.21–2.55	0.617
Low land	9.26	2.22–38.62	0.002

*Result of the Hosmer-Lemeshow goodness-of-fit test $\chi^2 = 9.41$; $p = 0.309$.

symptoms of the disease for those who have knowledge of it. The most commonly mentioned sign was hypersalivation (37%) followed by lesion on feet and mouth (35%), lameness (24%), and inappetence (4%). This finding goes in line with a study conducted in the Amhara region by Mesfine et al. (27) and in the Oromia region by Bayissa et al. (29), who reported that most of the farmers in the study areas were able to describe clinical signs of FMD. Additionally, the study conducted in the Adea Berga district of central Oromia by Urge et al. (30) showed profuse salivation as the most frequently observed clinical sign (39%), followed by oral cavity and interdigital vesicle (22.6%), lameness (7.5%), and inappetence (7.5%).

The survey results revealed that 28% (28/100) of the surveyed cattle owners reported having a case of FMD in at least one cattle in their farm in the previous 6 months. This finding agrees with a study conducted in Kenya by Nyaguthii et al. (31), who reported that out of a total of 220 smallholder farmers, 13 (5.9%) respondents replied having FMD in at least one cattle in their herd in the previous 6 months. Those that reported FMD cases were instructed to indicate the disease management strategy they had adopted. While 50% (14/28) of the farmers that had FMD cases in their livestock opted for medical treatment, and 32% (9/28), 7% (2/28), 7% (2/28), and 4% (1/28) of this subgroup chose isolation, selling, slaughtering, and doing nothing, respectively. This finding agrees with a study conducted in the Amhara region by Mesfine et al. (27), who reported that about 48% of farmers practiced one or more types of FMD control measures following disease occurrence.

Seroprevalence and associated risk factors of FMD

The overall prevalence in this study was 26.82%, in agreement with those of other studies in the country (32) and (33) whose overall prevalence was 24.22 and 26.5%, respectively. In the central Tigray zone (34) and the South Omo zone (35), seroprevalences of 26.6% and 23.9% were recorded, respectively. The highest overall seroprevalence reports were in Adiss Abeba (72.1%) (36), followed by 49.2% in Oromiya (30), 41.5% in Tigray's Eastern zone (34), and 40.4% in West Shewa Zone (37). In Southern Ethiopia, the lowest seroprevalence was reported at 9.5%, and in the Gamo Gofa zone and Sidama zone, the seroprevalence input was 6.9 and 5.9%, respectively (35). Those prevalence differences might have emerged from differences in sampling method, study design, and the presence and absence of extrinsic risk factors like agroecology, contact of animals with wildlife, free movement of animals, communal grazing, and communal watering.

In this study, prevalence varied between age groups in a statistically significant manner. This finding is in line with the findings of Dubie and Negash (38), who found a higher prevalence in adult animals than in young animals in a study conducted at the Afar region. Other scholars who reported the same finding were Awel and Dilba (36) in Addis Ababa, Megerssa et al. (35) in Southern Ethiopia, Sulayeman et al. (32) in central Ethiopia, Gelana (39) in Western Oromiya, and Abunna et al. (40) in Dire Dawa. These statistically significant prevalence differences between different age groups reported might be due to increased exposure to disease risk factors as an animal's age increases. In this study area, calves were kept in barns until they were old enough to graze communally. This habit decreases their exposure to the disease. Additionally, calves < 1 year are protected from the disease due to their passive maternal immunity (28). In contradiction with the above findings, Gelaye et al. (17) in the Benchi Maji zone and Belina et al. (41) in the Eastern Showa zone reported no statistically significant difference in the seroprevalence of FMD in different age groups.

In terms of breed, the current study has shown a statistically significant difference between local breed and crossbreed animals. This finding agrees with Sulayeman et al. (32), Urge et al. (30), and Ahmed et al. (37) who reported statistically significant differences between the local breed and crossbreed prevalence estimates in central Ethiopia, Welmera district of Oromia region, and West Showa zone, respectively. This study finds a higher prevalence in local breeds than crossbreeds as opposed to Sulayeman et al. (32), Urge et al. (30), and Ahmed et al. (37). Possibly, this result variation was caused by non-proportionate sample allocation, and local breeds were more prone to FMD risk factors such as wildlife contact, free movement, semi-intensive/extensive management systems, and communal grazing. Even though the difference was not statistically significant, Awel and Dilba (36) reported a higher prevalence in local breeds than crossbreeds.

In this study, agroecology displayed statistically significant variations in seroprevalence. The magnitude of seroprevalence decreases when agroecology changes from lowland to midland and from midland to highland. The logistic regression result showed that lowland areas were 29.15 times more likely to be seropositive (OR= 29.15, CI =11.01, 77.21) than highland areas. There is strong agreement with the findings of Megerssa et al. (35) and Mesfine et al. (27), who reported that animals found in midlands and highlands were 83% (OR = 0.17, CI = 0.04–0.85) less likely to be seropositive for FMD than lowland animals. Similarly, Tesfaye et al. (42) found significant differences between areas of different altitudes with a prevalence of 53.6% and 10.1% at low and high altitudes, respectively. This prevalence variation arises due to an increase in exposure of animals to the putative risk factors in lowland areas.

Conclusion and recommendations

The study revealed that more than half of the respondents were aware of FMD but had limited knowledge about the presence of the FMD vaccine. Moreover, the survey also revealed that farmers' awareness of the source of FMD infection was minimal. Instead, they followed a strategy of selling, slaughtering, isolating, and doing nothing as a means of preventing it. The serological findings confirmed that the disease is endemic in this study area. An analysis of multivariable logistic regression showed that age, breed, and agroecology are statistically significant risk factors for the disease. The seropositivity of the disease is higher in animals that are living in lowland areas than in midland and highland areas. Therefore, the regional government should give an emphasis on massive vaccination campaigns, especially for animals found in lowland areas, and create awareness through training of smallholder farmers about the disease transmission, FMD vaccination schedule, and vaccine importance.

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 at: https://zenodo.org/record/5834604#.YmuF1tPMJPY.

Ethics statement

The animal study was reviewed and approved by Arba Minch University Animal Ethical Clearance Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MS, BS, and WS participated in the study design, data analysis, write-up of the draft, and final version of the manuscript. AM, ET, and EA participated in the data collection through questionnaire and encoding of raw data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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Wilna Vosloo,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

REVIEWED BY
Nagendrakumar Singanallur Balasubramanian,
Australian Animal Health Laboratory (CSIRO), Australia
Valerie Mioulet,
The Pirbright Institute, United Kingdom

*CORRESPONDENCE
Taeyo Chestley
taeyo.chestley@inspection.gc.ca

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Development of reverse-transcriptase, real-time PCR assays to distinguish the Southern African Territories (SAT) serotypes 1 and 3 and topotype VII of SAT2 of Foot-and-Mouth Disease Virus

Taeyo Chestley[1]*, Patrycja Sroga[1], Michelle Nebroski[1], Kate Hole[1], Hussaini Ularamu[2], Oliver Lung[1] and Charles Nfon[1]

[1]National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada, [2]National Veterinary Research Institute, Vom, Plateau State, Nigeria

Foot-and-Mouth Disease Virus (FMDV), the causative agent of Foot-and-Mouth Disease, is a highly feared, economically devastating transboundary pathogen. This is due to the virus' extremely contagious nature and its ability to utilize multiple transmission routes. As such, rapid and accurate diagnostic testing is imperative to the control of FMD. Identification of the FMDV serotype is necessary as it provides the foundation for appropriate vaccine selection and aids in outbreak source tracing. With the vast genetic diversity, there is a desperate need to be able to characterize FMDV without relying on prior knowledge of viral serotypes. In this study, the Neptune bioinformatics tool was used to identify genetic signatures specific to each Southern African Territories (SAT) 1, 2 and 3 genomes but exclusionary to the other circulating FMDV serotypes (A, O, Asia1, and the heterologous SAT1, SAT2 and/or SAT3). Identification of these unique genomic regions allowed the design of TaqMan-based real-time reverse transcriptase PCR (rRT-PCR) primer/probe sets for SAT1, SAT2 and SAT3 viruses. These assays were optimized using prototypic FMDV cell culture isolates using the same reagents and thermocycling conditions as the FMDV pan-serotype 3D rRT-PCR assay. Cross-reactivity was evaluated in tandem with the FMDV pan-serotype 3D rRT-PCR utilizing representative strains from FMDV serotypes A, O, Asia1, SAT1, SAT2 and SAT3. The SAT1, SAT2, and SAT3 primer/probe sets were specific for the homologous serotype and exclusionary to all others. SAT1 and SAT3 primer/probe sets were able to detect several topotypes, whereas the SAT2 assay was revealed to be specific for topotype VII. The SAT2 topotype VII specificity was possibly due to the use of sequence data deposited post-2011to design the rRT-PCR primers and probes. Each assay was tested against a panel of 99 bovine tissue samples from Nigeria, where SAT2 topotype VII viruses were correctly identified and no cross-reactivity was exhibited

by the SAT1 and 3 assays. These novel SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays have the potential to detect and differentiate circulating FMD SAT viruses.

KEYWORDS

Foot-and-Mouth Disease Virus, FMDV, Southern African Territories, serotyping, detection, real-time reverse transcriptase polymerase chain reaction

Introduction

Foot-and-Mouth Disease (FMD) is a highly contagious viral disease affecting even-toed ungulates. While mortality rates are often low in adult animals (1%−5%) they are inversely correlated with age and have been reported to be up to 94% in lambs, 80% in calves, and 100% in suckling piglets (1). However, the disease is devastating to the animals as they lose their ability to eat, drink, and walk due to extremely painful lesions. These debilitating effects subsequently lead to many direct losses including, lower weight gains, decreased milk production and a loss in draught power (2).

The characteristic clinical manifestation of FMD is the formation of vesicles in the mouth and on the feet of afflicted animals, often accompanied by fever and profuse salivation. Disease signs appear between 1 and 14 days after initial infection depending on infectious dose, transmission route and housing (3). Suspicion of FMDV infection must be confirmed through laboratory diagnosis as signs are nebulous and clinically indistinguishable from other vesicular diseases.

The causative agent of FMD is the Foot-and-Mouth Disease Virus (FMDV), a member of the *Picornaviridae* family in the *Apthovirus* genus. Virions are non-enveloped and utilize capsid proteins to encase a ∼8.3 kilobase single-stranded, positive-sense RNA genome (4). Extensive genetic heterogeneity is a key characteristic of FMDV and is reflected at both the genetic and antigenic levels. Seven immunologically distinct serotypes exist and include A, O, C, Asia1, Southern African Territories (SAT)1, SAT2 and SAT3, however, serotype C has not been detected since 2004 (5). Within these serotypes, there are many subtypes/topotypes and lineages. There is no antigenic cross-reactivity between serotypes, and this is often also extended to subtypes (6).

Preparedness through having established rapid, sensitive, and readily-available diagnostic tests is critical to FMDV control. Accurate tests and quick turnaround times are imperative to cease the spread and manage unnecessary animal culling. Most FMDV diagnostic testing methods that detect viral antigen or genomic RNA are serotype independent and verify FMDV presence. Pan-serotype real-time reverse transcriptase PCR (rRT-PCR) that detects either the 3D or internal ribosome entry site (IRES) portion of the FMDV genome are highly sensitive and accurate first-line diagnostic tests (7–9). These tests are capable of determining the presence of the FMDV genome only, therefore, in order to fully characterize an FMDV incursion, it is essential to identify the virus serotype.

FMDV serotyping provides the necessary first step in establishing a VP1 Sanger-based sequencing approach and identifying an appropriate FMDV vaccine. The FMDV antigen detection ELISA (Ag-ELISA) is the most common methodology for identifying FMDV serotype. The Ag-ELISA consists of seven serotype-specific polyclonal antibodies that capture the FMDV capsid antigen which is then detected *via* a serotype-specific guinea pig antibody. A major pitfall of the Ag-ELISA is the low sensitivity of 80%−90% for positive bovine samples and <80% for porcine samples (10). Sensitivity issues also extend to the sample source. While vesicular fluid and vesicular epithelium are preferred samples utilized in the Ag-ELISA as viral titers are the highest, less-invasive samples such as blood, oropharyngeal fluids, and mucosal swabs may lead to false negatives or require additional passage in cultured cells (3, 11).

Currently, sequencing of the 1D (VP1 protein) region of the FMDV genome is typically accomplished utilizing Sanger termination sequencing methodology. The VP1 capsid protein contains a surface exposed G–H loop formed by residues 140–160 of the βG and βH chains and this exposure results in its constant evolution (12, 13). This lack of genetic conservation provides enough sequence information to differentiate FMDV to the strain level. However, current FMDV Sanger sequencing protocols require prior knowledge of the serotype/subtype sequence, and the lengthy protocol requires two amplification procedures as well as costly reagents and equipment (14). Next-generation sequencing (NGS) methodologies are powerful tools to generate sequence data but some technologies, such as the widely used Illumina short-read sequencing, are time-consuming and require expensive equipment. Generally, NGS requires more specialized technical and bioinformatics expertise and can have longer turnaround times when compared with PCR-based methods.

Real-time conventional PCR methods, in combination with size differentiation based on agarose gel electrophoresis, were the first attempts to utilize PCR technology to identify FMDV serotypes (15–24). However, several of these assays demonstrated serotype cross-reactivity (15, 16). Issues with

strain sensitivity were also noted as several strains within a serotype eluded detection (19, 21). In order to produce assays with higher sensitivity and specificity, the approach to target specific geographic regions and thus specific FMDV pools was adopted. As an example, Giridharan et al. described a method where primer sets were designed based on isolates circulating in India that successfully detected O, A, Asia, and C serotypes (22). In another example, using TaqMan-based rRT-PCR, Reid et al. (25) designed FMDV serotyping assays directed to Middle Eastern O, A, and Asia1 viruses. Jamal and Belsham in 2015 (26) designed primer/probe sets capable of distinguishing FMDV serotypes A, O, and Asia1 circulating in pools present in West Eurasia. Likewise, Bachanek-Bankowska et al. were able to discern FMDV A, O, SAT 1 and 2, restricted to viruses found in East Africa (27). El Bagoury et al. (28) produced rRT-PCR assays capable of detecting and distinguishing O and SAT3 viruses circulating in Egypt. Several other lineage-specific FMDV rRT-PCR assays have been reported (25, 29–32).

With the constant emergence of new FMDV strains and variants contributing to the already vast genetic diversity, there is a need to consistently develop assays capable of identifying FMDV serotypes. In this study, an innovative bioinformatics tool, Neptune, was used to generate genetic signatures that were specific to the FMDV SAT1, SAT2, and SAT3 serotypes. Degenerate Taq-Man-based rRT-PCR assays were designed to both detect and differentiate FMDV SAT1, SAT3, and topotype VII of SAT2. Serotyping assays were optimized to utilize the same reagents and thermocycling conditions as the previously validated pan-serotype 3D rRT-PCR assay.

Materials and methods

FMDV samples

Cell culture isolates

Viruses utilized in this study were obtained from the World Reference Laboratory for FMDV, The Pirbright Institute, UK, and from the National Veterinary Research Institute (NVRI), Vom, Nigeria. FMDV isolates were propagated in either baby hamster kidney 21 (BHK-21), fetal porcine kidney (LFBK-$\alpha_v\beta_6$), porcine kidney (IB-RS-2) or primary lamb kidney (LK) cell lines as previously described (33–35). Viral isolates were stored at −70°C until use.

Clinical field samples

Tissue samples from FMDV – infected cattle in Nigeria were collected by NVRI veterinarians, stored at −70°C, and eventually transported to NCFAD with the cold chain maintained. A 10% tissue suspension was prepared in sterile phosphate-buffered saline (PBS) using the Precellys Lysing Kit (BER-P000918LYSK0A0, ESBE Scientific) and the Precellys 24 dual tissue homogenizer as previously described (36).

Identification of FMDV SAT serotype-specific genetic signatures

Specific FMDV SAT1, SAT2 and SAT3 genetic signatures capable of serotype identification and differentiation were identified by the Neptune bioinformatics tool, version 1.2.5 (37). Neptune analysis was performed utilizing six comparisons representing six of the seven FMDV serotypes [serotype C was excluded as it is extinct (5)]. The input required for the Neptune tool is that two arguments are presented. The first is a list of nucleotide acid sequences that are the inclusion group and the second is a list of sequences defined as the exclusion group. FMDV sequences representing the homologous target serotype populated the inclusion group and sequences from the remaining six heterologous serotypes populated the exclusion group. For SAT1, the inclusion group consisted of 510 sequences and the exclusion group contained 6,986 sequences, SAT2 758 vs. 6,738 and SAT3 115 vs. 7,381. The generated file of interest is a FASTA file called "consolidated.fasta." Each line of the output file contains an identified genetic signature accompanied by the overall Neptune score, the values for which are based on the inclusion and exclusion group scores that are used to calculate the overall score. Also included is the accession number for the reference sequence that the marker is based on and the position in that reference sequence that the marker begins at. Neptune scores measured genetic signature confidence based on a positive value that represents the inclusion group component and a negative value representing the exclusion group component. Scores were then used to rank the produced genetic signatures by sensitivity and specificity. The signature sequences produced by Neptune for FMDV SAT1, SAT2 and SAT3 were all located in the highly variable VP1 region of the genome and are listed in Table 1.

FMDV SAT1, SAT2, and SAT3 serotype-specific primer/probe design

Primers and probes designed to identify and differentiate between FMDV serotypes SAT1, SAT2 and SAT3 were based on the signature sequences with the highest score produced by the Neptune bioinformatics tool. To facilitate serotype inclusive primer/probe design, FMDV VP1 and full-length genome sequences belonging to the SAT1, SAT2, and SAT3 serotypes were retrieved from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Sequences collected were limited to those deposited between 2011 and September 2021. This included 286 SAT1 sequences, 378 SAT2 sequences and 50 SAT3 sequences. Multiple alignments were performed using Geneious software, version 11.1.5, and the MAFFT version 7.450 algorithm (38–40). FMDV SAT1, SAT2, and SAT3 genetic signatures were mapped to the consensus sequences produced by the alignment and serotype-specific primers and probes were designed using the modified

TABLE 1 Top ranking FMDV SAT1, SAT2 and SAT3 serotype-specific genetic signatures generated by Neptune version 1.2.5 software.

FMDV serotype	Gene	Genome location	Sequence (5′→3′)
SAT1	1D (VP1)	3200	CTGAACCAGTCACAACTGATGCCTCACAACATGGTGGTAACGCCCGTCCCACACGGCGATA
			CCACACCAATGTTGAGTTCTTGCTTGACCGTTTCACGCTCATAGGCAAGACACACAACAAC
			AAAATGGTTTTGGACATGCTACGGACCGAGA
SAT2	1D (VP1)	3600	CCGATGTCGTCACGACCGGCCCTGCCACACACGGTGGTGTTGCAAACACTGCGCGACGTG
			CCCACACAGACGTCGCTTTCTTGCTGGATCGCAGCACACACGTGTACACCAACAAAACGTC
			ATTCAGCGTCGATCTCATGGAAACAAAGG
SAT3	1D (VP1)	3549	CAACGGATCCTGTAAATACACCAAAACGCGAAGTGTTGGCCCGCGCCGTGGAGACTTGGC
			NACGCTGGCACAACGCGTAGAAACTGAGCAAGCAAGGTGTATACCCACGACATTCAACTTC
			GGTCGTTTGTTGTGTGATTCAGGTGAGGTGTACTACCGCATGAAGCGA

Genomic numbering corresponds to the consensus sequences generated from MAFFT alignments of 286 FMDV SAT1 sequences, 378 SAT2 sequences and 50 SAT3 sequences.

version of Primer3 2.3.7 available in Geneious (40, 41). Multiple sets of primers and probes were generated and were evaluated *in silico* to determine which primers and probes aligned to the majority of the individual sequences in the alignment. Once an rRT-PCR assay set containing two primers and a probe were identified, the nucleotides present within the sequence were evaluated against the individual sequences to determine the level of conservation across all sequences in the alignment. If a nucleotide was not conserved, a degenerate nucleotide was incorporated into the primer and/or probe to increase FMDV strain inclusivity but restricted to three degenerate nucleotides per oligomer. Primer3 2.3.7 was utilized for the *in silico* evaluation of the primer pair properties. FMDV serotype exclusivity was evaluated for all primers and probes by first utilizing BLASTn to determine that the top identifications were all the homologous serotype, followed by testing the alignment of the primers and probe against the heterologous SAT serotypes using Primer3 2.3.7 (41). The FMDV SAT serotype-specific probes were designed as dual-labeled hydrolysis TaqMan probes with a modified 5′ terminus containing a 6-carboxyfluorescein (FAM) reporter dye and a Black Hole Quencher dye (BHQ1) appended to the 3′ terminus. The SAT1, SAT2, and SAT3 serotype-specific primer and probe set sequences are listed in Table 2.

Total RNA extraction

Total RNA was extracted from both the FMDV cell culture isolates and tissue suspensions using the MagMAX™-96 Viral RNA Isolation Kit (AMB1836-5, Life Technologies) in combination with the MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies) following the manufacturer's protocols. One microliter of VetMAX™ Xeno™ Internal Positive Control RNA (A29761, Life Technologies) was added per sample to serve as an RNA extraction control. Extractions utilized 55 µl of the sample and total RNA was eluted into 50 µl of MagMAX elution buffer (34). All RNA was stored at −70°C until evaluated by PCR or nucleic acid sequencing.

FMDV 3D pan-serotype and SAT-specific rRT-PCR assays

Detection of pan-serotype FMDV viral genomic RNA *via* real-time reverse transcriptase PCR (rRT-PCR) was accomplished utilizing a previously published primer/probe set that detects the conserved, serotype-independent 3D RNA-dependent RNA polymerase gene of FMDV (FMDV 3D rRT-PCR) (7). The forward 1186F (5′-ACT GGGTTTTAYAAACCTGTGATG-3′) and reverse FMDV 1237R (5′-TCAACTTCTCCTKGATGGTCCCA-3′) primers amplify an 88-base-pair fragment. The FMDV dually labeled hydrolysis TaqMan probe was modified so that the 5′ end contains a 6-carboxyfluorescein (FAM) reporter dye and the 3′terminates with a Black Hole Quencher dye (BHQ1; 5′-6FAM-ATC CTC TCC TTT GCA CGC-BHQ1-3′). The Xeno internal positive control RNA was detected utilizing the proprietary VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay (A29767, Applied Biosystems). Detection of pan-serotype FMDV and Xeno reactions were performed in a multiplex reaction comprised of 6.25 µl of TaqMan® Fast Virus 1-Step Master Mix (4444432, Applied Biosystems), 1 µl of 25× FMDV primers/probe mix (0.5 µM each of forward and reverse primers and 0.2 µM FAM-labeled probe), 1 µl of the VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay, 5 µl RNA template topped to a final volume of 25 µl with nuclease-free H_2O. Testing was performed on the QuantStudio™ 7 Pro Real-Time PCR System (A43183, Applied Biosystems) using a standard thermocycling program consisting of a reverse transcriptase step (50°C for 5 min), an inactivation/denaturation step (95° for 20 s) and a 45 cycle amplification step cycling between 95° for 15 s and 60° for 45 s. FMDV positive controls consisting of synthetically prepared

TABLE 2 FMDV SAT1, SAT2 and SAT3 serotype-specific rRT-PCR assay primers and probes.

FMDV serotype reagent	FMDV SAT specific oligo name	Sequence (5′→3′)
SAT1 forward	SAT1_3437_F	AGGCANCACACTGAYGTG
SAT1 reverse	SAT1_3502_R	GCAGRTCCAGTGTCAGTYT
SAT1 probe	SAT1_3474_P	FAM-CCTYGACCGGTTCACHCTDGT-BHQ1
SAT2 forward	SAT2_3765_F	YGTCTACAAYGGYGAGT
SAT2 reverse	SAT2_3934_R	CCKCTTCATCCKGTAGTARA
SAT2 probe	SAT2_3867_P	FAM-CGDACCCGAAGTTGAAGGTBGRCG-BHQ1
SAT3 forward	SAT3_3736_F	GYGTTGAGAMTGAAACCAC
SAT3 reverse	SAT3_ 3834_R	CWGCHCTCTTCATCCGGTA
SAT3 probe	SAT3_probe1.2	FAM-AVAGWCGCCCGAAGTTGAATGTYGTGGG-BHQ1

Characters in sequences represent degenerate bases: Y (C, T), B (C, T, G), H (C, A, T), K (G, T), M (A, C), D (A, G, T), R (A, G), W (A, T), and N (A, C, T, G).

FMDV RNA fragments amplifying at 130 base pairs and a no template negative control (NTC) composed of nuclease-free H_2O were utilized on every run. Quantification cycle (Cq) was determined for every reaction with Cq values $\leq$35.99 considered positive for FMDV genome when accompanied by appropriate amplification curves. The Xeno reaction also adhered to the Cq cut-off of $\leq$35.99. Detection of the FMDV SAT1, SAT2, and SAT3 serotype-specific viral genomic RNA was optimized to utilize the same assay conditions and thermocycling parameters as the pan-serotype FMDV rRT-PCR with the exception that the reaction mixture contained 1.0 μM each of FMDV SAT1, SAT2, and SAT3 forward and reverse primers and 0.5 μM of the serotype-specific FAM labeled probes Table 2.

SAT-specific rRT-PCR assay optimization and standardization

SAT1, SAT2 and SAT3 specific rRT-PCR assay primer/probe concentration optimization

Optimization of the concentrations of the FMDV SAT1, SAT2 and SAT3 assay primers and probes was completed by testing the ability of different reagent dilutions to detect the homologous SAT genomic RNA extracted from prototypic FMDV cell culture isolates. The FMDV cell culture isolates included SAT1/KEN/4/1998, SAT2/SAU/1/2000, and SAT3/ZIM/4/1981. Three assay conditions were examined, primers at a concentration of 2.0, 1.0, and 0.5 μM combined with the probe at a concentration of 1.0, 0.5, and 0.25 μM, respectively. All reagent concentrations were tested at a minimum in duplicate. Optimal assay performance was defined as the lowest Cq value coupled with the lowest discrepancy in Cq values between replicates with no amplification in RNA extraction and no template controls.

Repeatability of the SAT1, SAT2 and SAT3 rRT-PCR assays

SAT-specific rRT-PCR assay repeatability was evaluated for each assay by extracting RNA from two different prototypic FMDV cell culture isolates and performing three replicate tests on three separate days. The isolates utilized were SAT1/KEN/4/1998, SAT1/BOT/12/2006, SAT2/SAU/1/2000, SAT2/EGY/2/2012, SAT3/SAR/1/2006 and SAT3/ZAM/1/2017 as well as a PBS extraction control and a no template control (NTC). SAT1, SAT2, and SAT3 rRT-PCR assays were evaluated against both the homologous FMDV isolates as well as the heterologous SAT isolates to ensure amplification and no signal detection, respectively.

Analytical sensitivity of the SAT1, SAT2 and SAT3 rRT-PCR assays

Prototypic FMDV cell culture isolates representing SAT1, SAT2, and SAT3 viruses of known titer were selected to determine the analytical sensitivity using the limit of detection (LoD) for each assay. The isolates utilized were SAT1/KEN/4/1998, SAT1/KEN/121/2009, SAT2/ SAU/1/2000, SAT2/SEN/27/2009, SAT3/ZIM/4/1981 and SAT3/SAR/1/2006. Duplicate 10-fold serial dilutions of each of the FMDV isolate's cell culture supernatants from 10^0 to at least 10^{-7} were prepared after which RNA was extracted and samples were tested using the homologous serotype SAT-specific rRT-PCR assay as well as the pan-serotype FMDV rRT-PCR assay. A standard curve was prepared from the Cq values. Assay efficiency (E) was calculated utilizing the formula:

$$\mathbf{E} = -\mathbf{1} + \mathbf{10}^{(-\mathbf{1}/\mathbf{slope})}.$$

TABLE 3 Detection of FMDV A, O and Asia1 cell culture isolates with the serotype-specific SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays.

Serotype	Strain	SAT1 Cq	SAT2 Cq	SAT3 Cq	FMDV 3D Cq	Xeno Cq
O	OUKG	No Cq	No Cq	No Cq	13.29	32.30
O	O1 BFS	No Cq	No Cq	No Cq	13.54	33.31
O	O1 Manisa	No Cq	No Cq	No Cq	20.14	33.63
O	O1 Campos	No Cq	No Cq	No Cq	13.54	33.57
O	O/TAN/2009	No Cq	No Cq	No Cq	12.63	33.31
O	O/CAR/2005	No Cq	No Cq	No Cq	13.88	33.29
O	O/VIT/2012	No Cq	No Cq	No Cq	12.31	33.24
O	O/LIB/2012	No Cq	No Cq	No Cq	10.75	32.88
O	O/KEN/2009	No Cq	No Cq	No Cq	13.30	33.12
O	O/NIG/2017	No Cq	No Cq	No Cq	14.78	33.97
A	A/22	No Cq	No Cq	No Cq	10.67	33.17
A	A/MAY/1997	No Cq	No Cq	No Cq	11.35	32.90
A	A/COL/1985	No Cq	No Cq	No Cq	13.52	33.29
A	A/IRN/1/1996	No Cq	No Cq	No Cq	16.69	32.81
A	A/IRN/2005	No Cq	No Cq	No Cq	16.04	33.77
A	GHA/4/1996	No Cq	No Cq	No Cq	11.67	31.41
A	BKF/4/1994	No Cq	No Cq	No Cq	10.62	31.87
A	ERI/2/1998	No Cq	No Cq	No Cq	12.50	31.65
A	ETH/6/2000	No Cq	No Cq	No Cq	11.54	31.98
A	NIG/38/2009	No Cq	No Cq	No Cq	13.81	31.41
A	ETH/12/2009	No Cq	No Cq	No Cq	13.64	32.03
A	EGY/3/2009	No Cq	No Cq	No Cq	17.85	32.00
A	SUD/1/2006	No Cq	No Cq	No Cq	12.03	31.93
A	CAR/10/2013	No Cq	No Cq	No Cq	12.67	31.56
A	NIG/A/6/2019	No Cq	No Cq	No Cq	12.91	33.15
A	NIG/A/7/2019	No Cq	No Cq	No Cq	10.58	31.85
A	NIG/A/12/2020	No Cq	No Cq	No Cq	11.87	31.43
A	NIG/A/1/2019	No Cq	No Cq	No Cq	14.78	31.74
Asia1	Asia1/Shamir/2001	No Cq	No Cq	No Cq	13.99	32.87
Asia1	Asia1/PAK/1994	No Cq	No Cq	No Cq	12.43	33.52

Samples are considered positive when the quantification cycle (Cq) is ≤35.99 for SAT1, SAT2, SAT3, FMDV pan-serotype 3D and Xeno control assays.
The colours used to indicate each of the Foot-and-Mouth Disease virus serotypes. Serotype O is blue, serotype A is red, serotype Asia1 is grey, serotype SAT1 is yellow, serotype SAT2 is purple and serotype SAT3 is orange. When the Cq value cells are highlighted with either yellow, purple or orange, it means that a Cq value was produced for this particular viral isolate or sample. If the yellow, purple or orange is darker and the Cq value is <35.99 then the sample was positive when evaluated by the corresponding assay. If the filled cell is the lighter version of the colour, it indicates that although a Cq value was produced, it is >35.99 and the sample is considered negative.

Serotype specificity of the SAT1, SAT2 and SAT3 rRT-PCR assays

The analytical specificity of the SAT1, SAT2 and SAT3 rRT-PCR assays were determined by evaluating each assay against representative FMDV isolates from the A, O, Asia1 and the heterologous SAT1, SAT2 and SAT3 serotypes.

Analytical specificity of the SAT1, SAT2 and SAT3 rRT-PCR assays

Other vesicular disease-causing viruses were examined and included Vesicular Stomatitis Virus New Jersey Serotype (VSNJV; VS-NJ/92/CIB, VS-NJ/11/84/HBD and VS-NJ/95/COB), Vesicular Stomatitis Indiana Virus (VSIV; VS-IN/97/CRB, VS-IN/94/GUB and VS-IN/85/CLB), Swine Vesicular Disease virus (SVDV; SVD/ITL/2008, SVD/POR/1/2003, SVD/UKG/1972) and Senecavirus A (SVA; SVA prototype strain SVV-001, SVA/CAN/2015, SVA/CAN/2017).

Diagnostic sensitivity of the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays

Diagnostic sensitivity evaluation of the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays was conducted by utilizing SAT1, SAT2 and SAT3 FMDV cell culture isolates as representative true positive samples to test the homologous assay (see Table 3 for the list of FMDV cell culture isolates).

Diagnostic specificity of the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays

Evaluation of the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assay diagnostic specificity was performed using samples that were confirmed to be true negatives by the FMDV 3D pan-serotype rRT-PCR. The samples were remnant negative samples obtained from previous animal experiments conducted in the laboratory (42). The samples included five tissue sample homogenates (porcine lymph node, porcine tongue and three bovine tongue tissues from different animals), five porcine serum samples, four porcine oral fluids samples, four porcine oral fluid samples and BHK-21 cell culture supernatant collected from PBS mock viral infections collected two and three DPI.

Sequencing

VP1 Sanger sequencing

The VP1 gene sequence from FMDV was generated using Sanger nucleic acid sequencing based on the protocol described previously by Knowles et al. (14). Briefly, FMDV RNA was extracted and both cDNA generation and VP1 PCR amplification were accomplished using the qScript XLT One-Step RT-PCR Mastermix (95143-200, Quantabio) with FMDV serotype-specific primers. Reactions consisted of 25 µl of the 2× One-Step Toughmix, 1 µl of the 50× GelTrack Loading Dye, 0.4 mM of both the forward and reverse primer, 2 µl of 25× qScript XLT One-Step RT, 5 µl of extracted FMDV RNA and nuclease-free H_2O to a total volume of 50 µl. VP1 FMDV cDNA amplicons were cleaned of the PCR reaction components using the QIAquick PCR Purification Kit (28104, Qiagen) while sequencing was accomplished using the BigDye® Terminator v3.1 Cycle Sequencing Kit (4337452, Life Technologies) and cleaned with the DyeEx 2.0 Spin Kit (63204, Qiagen) using the manufacturer's specifications. Sequencing primers were chosen according to the recommendations for serotype described in Knowles et al. (14).

Next-generation sequencing

Near-full length FMDV genome sequencing was accomplished using next-generation sequencing (NGS) as previously described (43, 44). All samples were screened with the pan-serotype FMDV rRT-PCR and positives were prepared for NGS using SuperScript™ IV First-Strand Synthesis System (Life Technologies). Libraries were processed for Illumina Nextera XT sequencing and were sequenced on a MiSeq instrument using a V3 cycling kit (Illumina). Sequencing data was evaluated using a previously described workflow (43).

Results

Generation of specific SAT1, SAT2 and SAT3 rRT-PCR assays

The Neptune bioinformatics tool was able to identify genetic signature sequences that were highly specific for each of the SAT1, SAT2 and SAT3 FMDV serotypes (37, 45). The Neptune bioinformatics tool was able to produce these unique genetic signatures based on an inclusion group consisting of sequences from the FMDV serotype of interest and an exclusion group consisting of FMDV sequences from heterologous serotypes. The software applies a reference-based, parallelized exact-matching k-mer strategy for speed while enhancing sensitivity through allowances for inexact matches. Genetic signature identification is based on probabilistic models that derive conclusions based on statistical confidence (37). Each of the FMDV SAT1, SAT2 and SAT3 genetic signatures identified by Neptune were located in the highly variable VP1 coding region of the FMDV genome (Table 1). The signature sequence for SAT1 was predicted to be 100% specific and sensitive to SAT1 with a Neptune score of 1.0. The Neptune scores for the SAT2 and the SAT3 VP1-based signature sequences were 0.8800 and 0.8757, respectively. These genetic signatures were used as the template to discern the genomic location from which to build the SAT serotype-specific rRT-PCR assays. 286 SAT1 sequences, 378 SAT2 sequences and 50 SAT3 sequences deposited into NCBI from 2011-Fall 2021 were utilized to perform multiple alignments in Geneious (40). Primer3 (41) was used to produce SAT-specific primers and probes with non-conserved nucleotides being replaced with a degenerate nucleotide (Table 2).

SAT-specific rRT-PCR assay optimization and standardization

SAT-specific rRT-PCR assay primer/probe concentration optimization

The optimal performance of the SAT1, SAT2 and SAT3 rRT-PCR assay primers and probes were determined by evaluating different concentrations of these reagents against homologous prototypic FMDV cell culture isolates (SAT1/KEN/4/1998, SAT2/SAU/1/2000 and SAT3/ZIM/4/1981). Optimal assay performance was defined as the primer/probe concentration that produced the lowest Cq with the minimal spread between technical replicate Cqs as well as no detectable amplification in the RNA extraction control nor the no template control. All extracted RNA was pre-screened with the FMDV 3D pan-serotype and Xeno rRT-PCR assays to ensure quality RNA templates (data not shown). Interestingly, the SAT1, SAT2 and SAT3 assays each performed optimally with both of the primers

at a concentration of 1.0 μM and the probe at a concentration of 0.5 μM (Supplementary Figure 1).

Repeatability of the SAT1, SAT2 and SAT3 rRT-PCR assays

To assess the repeatability of the SAT1, SAT2 and SAT3 rRT-PCR assays RNA was extracted from FMDV cell culture isolate prototypes (SAT1/KEN/4/1998, SAT1/BOT/12/2006, SAT2/SAU/1/2000, SAT2/EGY/2/2012, SAT3/SAR/1/2006 and SAT3/ZAM/1/2017) on three separate days followed by rRT-PCR performed also on three separate days. Each of the SAT-specific rRT-PCR assays did not amplify any of the heterologous SAT serotype RNA nor the extraction or NTC controls (Figure 1). The SAT1 rRT-PCR assay demonstrated robustness over time as the standard deviations between the three replicates for SAT1/KEN/4/1998 and SAT1/BOT/12/2006, were 0.96 and 1.41 respectively (Figure 1A). Standard deviations for the SAT2 rRT-PCR were different for the two isolates examined with the three replicates producing a standard deviation of 2.15 for SAT2/SAU/1/2000 and 0.48 for SAT2/EGY/2/2012 (Figure 1B). The SAT3 rRT-PCR assay produced standard deviations of 1.08 and 1.27 for SAT3/SAR/1/2006 and SAT3/ZAM/1/2017 (Figure 1C). Each isolate was also analyzed using the FMDV 3D pan-serotype and Xeno rRT-PCR assays to ensure that the extraction was successful and each extraction (except the negative controls) contained FMDV genomic template (Figure 1D).

Analytical sensitivity of the SAT1, SAT2 and SAT3 rRT-PCR assays

The limit of detection (LoD) for the SAT1, SAT2 and SAT3 rRT-PCR assays were evaluated using prototypic FMDV cell culture isolates of a known titer. Ten-fold serial dilutions of cell culture isolated virus had the genomic RNA extracted from each of the serial dilutions and evaluated using the homologous SAT1, SAT2 and SAT3 rRT-PCR assays. There is difficulty with defining a singular LoD and rRT-PCR assay efficiency for FMDV detection as there is no all-encompassing template for a virus as genetically diverse as FMDV. As such, two representative isolates from each of the FMDV SAT serotypes were chosen (SAT1/KEN/4/1998, SAT1/KEN/121/2009, SAT2/SAU/1/2000, SAT2/SEN/27/2009, SAT3/ZIM/4/1981 and SAT3/SAR1/2006). As expected, the LoD and the PCR efficiencies of the SAT-specific rRT-PCR assays differ both with the inter-assay comparison to the 3D pan-serotype FMDV rRT-PCR and intra-assay between the two isolates tested. The LoD for the SAT1-specific rRT-PCR assay defined as the last dilution to produce a positive (Cq ≤35.99) signal was the 10^{-3} dilution corresponding to a detection of $10^{2.92}$ and $10^{2.29}$ $TCID_{50}$ of the SAT1/KEN/4/1998 and SAT1/KEN/121/2009 isolates (Figures 2A,B). The efficiency of the SAT1 rRT-PCR assay was 102.827% for SAT1/KEN/4/1998 and 81.161% for SAT1/KEN/121/2009 (Figures 1A,B). In comparison, the FMDV 3D pan-serotype assay's LoD and efficiency for SAT1/KEN/4/1998 was 8.33 $TCID_{50}$ and 71.689% and for SAT1/KEN/121/2009 it was 1.35 $TCID_{50}$ and 81.161% (Figures 2A,B). The LoD for the SAT2 rRT-PCR assay also varied based on the isolate examined. The two isolates tested were SAT2/SAU/1/2000 and SAT2/SEN/27/2009 where the LoDs and efficiencies were $10^{2.79}$ $TCID_{50}$ and 62.097% and $10^{4.04}$ $TCID_{50}$ and 81.272%, respectively (Figures 2C,D). For both SAT2 isolates, the FMDV 3D pan-serotype PCR was able to detect at least four more 10-fold dilutions of each of the isolates, with estimated efficiencies ranging from 81 to 83% (Figures 2C,D). The SAT3-specific rRT-PCR assay demonstrated greater robustness with the LoDs for each of the SAT3 isolates at 0.11 $TCID_{50}$ and 0.16 $TCID_{50}$ for the SAT3/ZIM/4/1981 and SAT3/SAR1/2006 isolates (Figures 2E,F). The efficiencies were 94.171 and 97.315% (Figures 2E,F). For the SAT3 isolates, the FMDV 3D pan-serotype PCR had similar LoDs but the efficiencies were lower within a range of 72%—81% for the two SAT3 isolates (Figures 2E,F).

Serotype specificity of the SAT1, SAT2 and SAT3 rRT-PCR assays

The serotype specificity of the SAT1, SAT2, and SAT3 rRT-PCR assays were tested using a panel of FMDV isolates of known serotype. All of the isolates were screened using the FMDV 3D pan-serotype and the Xeno RNA extraction control rRT-PCR assays to ensure the RNA present was of good quality by producing Cqs of ≤35.99 on both assays (Tables 3, 4).

The SAT1, SAT2 and SAT3 rRT-PCR assays were then evaluated against FMDV serotypes O, A and Asia1 to determine specificity against non-SAT FMDV serotypes. The serotype O and A FMDV isolate panels contained FMDV isolates originating from Africa so there would be representation of viruses that co-circulate with SAT serotype viruses. All three of the SAT1, SAT2 and SAT3 rRT-PCR assays demonstrated 100% specificity against 10 serotype O isolates, 18 serotype A isolates and two Asia1 isolates (Table 3).

Next, the ability of the SAT-specific serotyping rRT-PCR assays to differentiate detection from the heterologous SAT serotypes (Table 4) was tested. The SAT1 and SAT2 specific rRT-PCR assays demonstrated 100% specificity as no cross-reaction with any of the heterologous SAT1, SAT2 nor SAT3 viruses were observed (Table 4).

While no cross-reactivity was observed when the SAT3 assay was evaluated against any of the SAT2 isolates, one of the nine SAT1 isolates was identified as weakly positive. The SAT3 assay produced a Cq value of 35.30 with the SAT1/BOT/12/2006 isolate, a topotype III virus (Table 4). However, the SAT1-specific rRT-PCR assay produced a Cq of 22.60, with that isolate, a Cq

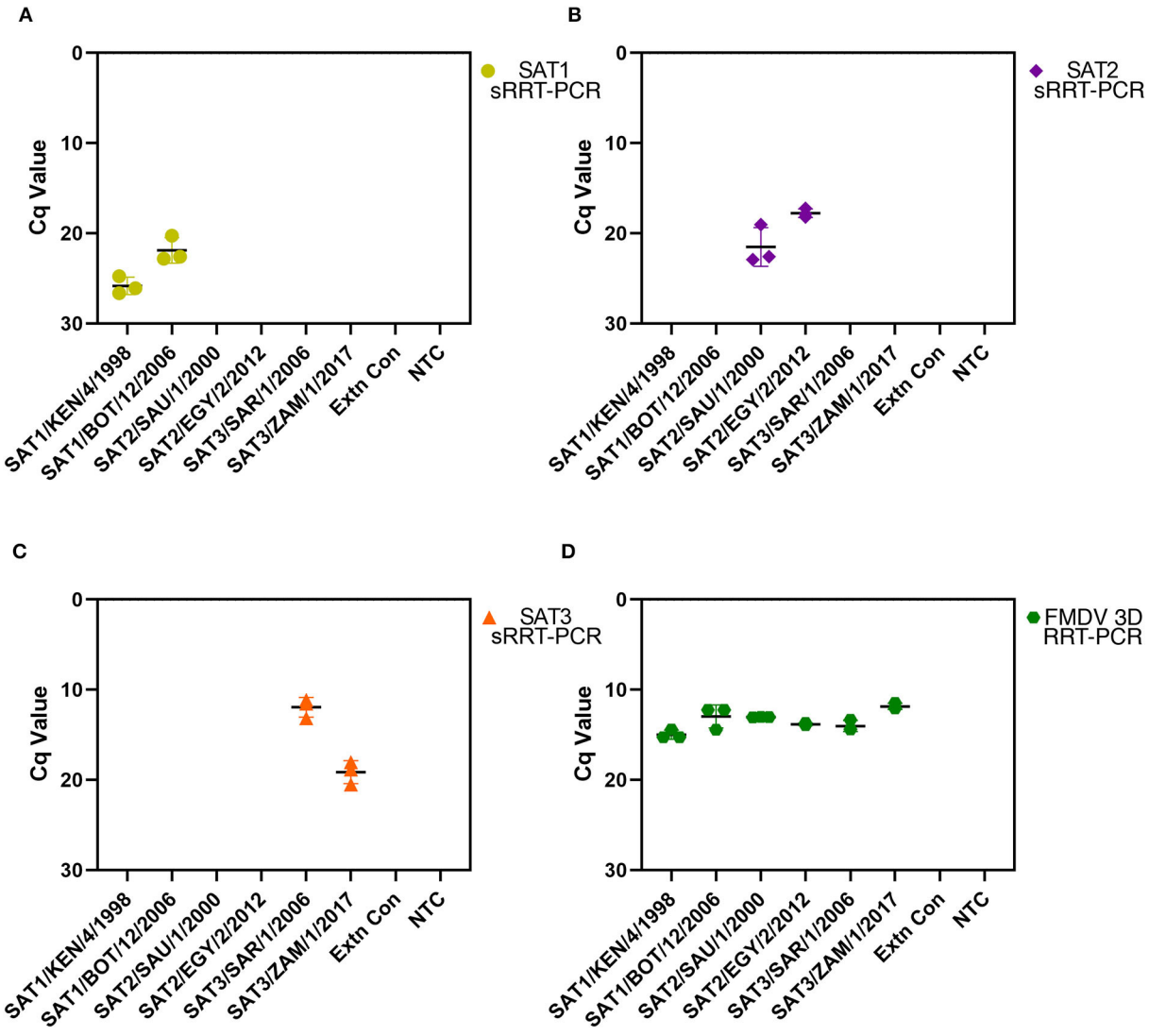

FIGURE 1
Repeatability analysis for the FMDV SAT1, SAT2 and SAT3 assays. FMDV genomic RNA was extracted on three separate days from prototypic FMDV cell culture isolates SAT1/KEN/4/1998, SAT1/BOT/12/2006, SAT2/SAU/1/2000, SAT2/EGY/2/2012, SAT3/SAR/1/2006 and SAT3/ZAM/1/2017 as well as a PBS extraction control (Extn Con). Extracted RNA from each replicate was also analyzed using the SAT1 **(A)**, SAT2 **(B),** and SAT3 **(C)** srRT-PCR assays on three separate days. The FMDV 3D pan-serotype rRT-PCR assay (FMDV) was run in parallel with each replicate to ensure the presence of the FMDV template **(D)**. Replicate Cq values are plotted with a black line representing the mean and error bars with the corresponding FMDV serotype color (SAT1 = yellow, SAT2 = purple, SAT3 = orange and FMDV 3D pan-serotype = green).

value that was 12.70 Cqs lower than that SAT3 assay, correctly identifying the isolate as a SAT1 virus (Table 4). The SAT3-specific assay also demonstrated cross-reactivity with only one of the 22 SAT2 isolates. A Cq of 22.44 was produced when the SAT3 assay was tested against the SAT2/BOT/1/2011 isolate. A nucleic acid sequence for SAT2/BOT/1/2011 was not available on NCBI, so the VP1 coding sequence of the isolate was produced *via* Sanger sequencing. Alignment of the primers and probes to the Sanger-produced SAT2/BOT/1/2011, VP1 sequence revealed that only the reverse primer aligned but, not the forward primer nor the probe.

Analytical specificity of the SAT1, SAT2 and SAT3 rRT-PCR assays

The classic lesions produced by FMDV infection cannot be distinguished clinically from other vesicular disease-causing viruses and a definitive diagnosis is obtained through laboratory diagnostic testing. As such, the analytical specificity of each of the SAT1, SAT2 and SAT3 rRT-PCR assays was evaluated using three cell culture viral isolates representing VSNJV, VS-IV, SVDV and SVA. No detectable amplification was produced by any of the SAT-specific rRT-PCR assays when tested against templates from these vesicular viruses (data not shown).

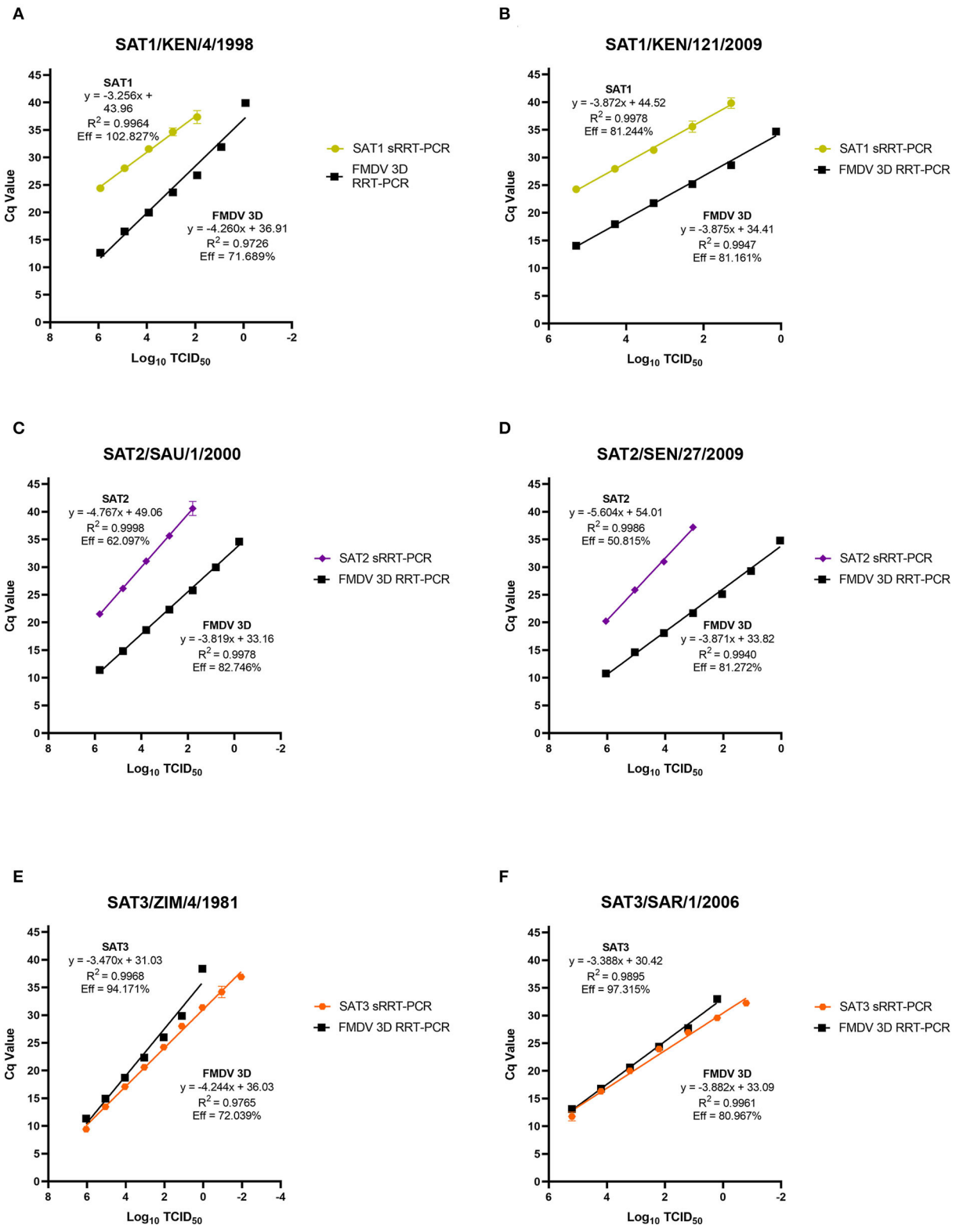

FIGURE 2
Analytical sensitivity limit of detection (LoD) analysis for the FMDV SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays. FMDV genomic RNA was extracted from duplicate 10-fold serial dilutions (10^{0} – 10^{-7} or 10^{-9}) of the FMDV cell culture isolates SAT1/KEN/4/1998 **(A)**, SAT1/KEN/121/2009 **(B)**, SAT2/ SAU/1/2000 **(C)**, SAT2/SEN/27/2009 **(D)**, SAT3/ZIM/4/1981 **(E)**, and SAT3/SAR/1/2006 **(F)** and were tested using the homologous serotype SAT-specific rRT-PCR assay as well as the FMDV pan-serotype 3D rRT-PCR assay. Mean Cq values are plotted with standard deviations.

TABLE 4 Detection of FMDV SAT1, SAT2 and SAT3 cell culture isolates with the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays.

Serotype	Strain	Topotype	SAT1 Cq	SAT2 Cq	SAT3 Cq	FMDV Cq	Xeno Cq
SAT1	KEN/88/2010	I (NWZ)/–	9.37	No Cq	No Cq	13.26	33.16
SAT 1	ZAM/9/2008	I (NWZ)/–	31.95	No Cq	No Cq	14.00	33.52
SAT 1	KEN/21/2004	I (NWZ)/–	19.11	No Cq	No Cq	15.68	33.80
SAT 1	KEN/121/2009	I (NWZ)/–	23.58	No Cq	No Cq	15.38	33.96
SAT 1	KEN/24/2005	I (NWZ)/–	14.32	No Cq	No Cq	15.04	34.06
SAT 1	BOT/12/2006	III (WZ)/unnamed	22.60	No Cq	35.30	14.43	33.72
SAT 1	ETH/3/2007	IX/unnamed	No Cq	No Cq	No Cq	14.10	33.62
SAT 1	KEN/4/1998	I (NWZ)	24.77	No Cq	39.57	14.42	33.49
SAT 1	KEN/BOT/1/1968	III (WZ)	22.20	No Cq	No Cq	11.80	32.86
SAT2	ZIM/10/1991	I	No Cq	No Cq	No Cq	17.18	32.18
SAT2	ZIM/5/1981	II	No Cq	35.90	No Cq	11.21	32.37
SAT2	BOT/1/2011	III/unnamed	No Cq	No Cq	22.44	14.94	33.69
SAT 2	MOZ/1/2010	I/–	No Cq	No Cq	No Cq	13.86	33.56
SAT 2	BOT/1/2010	III/unnamed	No Cq	No Cq	No Cq	14.75	33.56
SAT 2	SUD/1/2008	XIII/–	No Cq	No Cq	No Cq	18.86	33.72
SAT 2	ZAM/8/2008	III/–	No Cq	No Cq	No Cq	14.59	33.83
SAT 2	KEN/13/2004	IV/–	No Cq	No Cq	No Cq	12.40	33.45
SAT 2	BOT/5/2009	III/unnamed	No Cq	No Cq	No Cq	14.68	33.27
SAT 2	SEN/27/2009	VII/unnamed	No Cq	23.88	No Cq	12.37	32.97
SAT 2	TAN/43/2009	IV/–	No Cq	No Cq	No Cq	14.34	33.05
SAT 2	KEN/122/2009	IV/–	No Cq	No Cq	No Cq	14.59	33.50
SAT 2	ETH/2/2007	XIII/unnamed	No Cq	No Cq	No Cq	14.09	33.69
SAT 2	KEN/2/2007	IV/–	No Cq	No Cq	No Cq	13.98	34.11
SAT2	SAU/1/2000	VII	No Cq	22.58	No Cq	13.01	33.09
SAT2	NIG/17/2017	VII	No Cq	12.71	No Cq	11.71	33.09
SAT2	NIG/18/2017	VII	No Cq	12.90	No Cq	11.78	33.87
SAT 2	EGY/2/2012	VII	No Cq	17.87	No Cq	13.70	33.56
SAT 2	NIG/PL/WAS/03/2017	VII	No Cq	15.71	36.80	12.78	34.29
SAT2	NIG/PL/PKN/01/2017	VII	No Cq	13.82	No Cq	11.97	33.86
SAT2	NIG/PL/JS/KA/1/2017	VII	No Cq	14.74	No Cq	12.36	34.27
SAT2	NIG/PL/PKN/02/2017	VII	No Cq	13.41	No Cq	13.01	34.66
SAT3	ZIM/4/1981	I (SEZ)	No Cq	No Cq	13.70	14.19	33.82
SAT 3	UGA/10/1997	V/unnamed	No Cq	No Cq	No Cq	13.25	33.60
SAT 3	SAR/1/2006	I (SEZ)/–	No Cq	37.13	11.54	13.33	34.06
SAT 3	ZIM/4/1981	I (SEZ)	No Cq	No Cq	13.02	14.95	33.47
SAT 3	SAR/1/2006	I	No Cq	No Cq	11.00	12.82	33.54
SAT 3	BEC/1/1965	II	No Cq	No Cq	21.81	14.31	33.35
SAT 3	ZAM/1/2017	II	No Cq	No Cq	18.85	11.46	33.36
SAT3	ZAM/3/2015	II	No Cq	No Cq	15.96	11.19	31.96
SAT3	ZAM/1/2017	II	No Cq	No Cq	14.33	10.07	32.25

Samples are considered positive when the Quantification cycle (Cq) is ≤ 35.99 for SAT1, SAT2, SAT3, FMDV pan-serotype FMDV 3D and Xeno control assays.
The colours used to indicate each of the Foot-and-Mouth Disease virus serotypes. Serotype O is blue, serotype A is red, serotype Asia1 is grey, serotype SAT1 is yellow, serotype SAT2 is purple and serotype SAT3 is orange. When the Cq value cells are highlighted with either yellow, purple or orange, it means that a Cq value was produced for this particular viral isolate or sample. If the yellow, purple or orange is darker and the Cq value is <35.99 then the sample was positive when evaluated by the corresponding assay. If the filled cell is the lighter version of the colour, it indicates that although a Cq value was produced, it is >35.99 and the sample is considered negative.

Diagnostic sensitivity of SAT1, SAT3 and SAT2, topotype VII rRT-PCR assays

Next, the diagnostic sensitivity of the SAT-specific assays was evaluated by testing the ability of the SAT-specific serotyping rRT-PCR assays to detect the homologous SAT viral cell culture isolates. The nine SAT1 isolates included topotype I, III and IX viruses, 22 SAT2 isolates that included topotypes I–IV, VII and XIII and nine SAT3 isolates that included topotypes I, II and V (Table 4).

The SAT1 assay was able to detect eight out of the nine FMDV SAT1 isolates with Cqs ranging from 9.37 to 31.95 (Table 4). As such, the diagnostic sensitivity of the assay was 88.89% (Supplementary Table 1). The SAT1 assay failed to detect the SAT1/ETH/3/2007 isolate, a topotype IX virus. An alignment between the SAT1 assay primers and probe and the SAT1/ETH/3/2007 VP1 nucleic acid sequence (accession number: FJ798154.1) was performed in Geneious (40). This *in silico* analysis revealed that neither the forward nor reverse primer were able to bind the sequence and that there were two mismatches in the probe alignment (data not shown).

Testing of the SAT2-specific assay against 22 different SAT2 isolates revealed that the assay had topotype VII specificity (Table 4). No detectable fluorescence was produced from any of the SAT2, topotype I, III, IV nor XIII isolates. One SAT2, topotype II virus, SAT2/ZIM/5/1981, was identified as a very weak positive with a Cq of 35.90 (Table 4). As the SAT2-specific rRT-PCR assay demonstrated specificity for only the topotype VII isolates, the assay was redefined as the SAT2, topotype VII-specific rRT-PCR assay. The diagnostic sensitivity calculated from the nine FMDV SAT2 topotype VII isolates was 100% (Supplementary Table 1).

The SAT3-specific rRT-PCR assay was able to detect eight out of the nine SAT3 isolates with Cqs ranging from 11.00 to 21.80 (Table 4). From these nine FMDV SAT3 isolates the diagnostic sensitivity was 88.89% (Supplementary Table 1). SAT3/UGA/10/1997 was the only SAT3 isolate that the assay failed to detect, and was the only topotype V virus tested. An alignment of the SAT3-specific primers and probe with SAT3/UGA/10/97 (accession number: KY091308.1) revealed that the SAT3 probe was capable of binding to the target nucleic acid sequence, but neither the forward nor reverse primers were (data not shown).

Diagnostic specificity of SAT1, SAT3 and SAT2, topotype VII rRT-PCR assays

Diagnostic specificity of the SAT1, SAT3 and SAT2, topotype VII rRT-PCR assays was investigated utilizing FMDV negative samples. All negative samples were defined as such by no amplification produced when tested by the FMDV 3D pan-serotype rRT-PCR but also the presence of a quality template by producing a Cq $\leq$35.99 on the Xeno rRT-PCR assay. Eighteen remnant clinical samples (42) and two mock viral infections were evaluated. These samples included five tissue sample homogenates (porcine lymph node, porcine tongue and three bovine tongue tissues from different animals), five porcine serum samples, four porcine oral swab samples, four porcine oral fluid samples and BHK-21 cell culture supernatant collected from PBS mock viral infections collected two and three DPI. Each of the SAT1, SAT3 and SAT2, topotype VII rRT-PCR assays did not produce any detectable fluorescent signal and thus the diagnostic specificity of these assays was 100% (Supplementary Table 1).

Detection and serotyping of FMDV samples from Nigeria

The detection and differentiation abilities of the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays were evaluated using bovine tissue samples collected from Nigeria. The 99 tissue samples were collected from various states in Nigeria in 2020. Serotyping of the samples was accomplished utilizing Illumina-Nextera NGS sequencing. Produced sequences had to contain the VP1 coding sequence and the produced contigs were searched against the BLASTn database to obtain the closest serotype match. Of the 99 samples, NGS identified 63 as serotype O/EA3, 12 as A/WAG/IV and 24 as SAT2/VII (Table 5). Samples were organized based on the area collected. All samples were tested using the FMDV pan-serotype 3D rRT-PCR and Xeno RNA extraction control RTT-PCR assays and found to be positive on both. The SAT1-specific rRT-PCR assay demonstrated 100% specificity as the assay produced no Cqs for any of the 99 samples (Table 5). The SAT3-specific rRT-PCR only incorrectly identified one of the 99 samples as positive for SAT3. However, this sample, SAT2/NIG/PL/JA/2/2020, was also identified correctly by the SAT2, topotype VII specific rRT-PCR with a Cq of 18.87 vs. the Cq of 35.56 produced by the SAT3 assay that was also just below the positive cutoff (Table 5). The SAT2, topotype VII specific rRT-PCR demonstrated the assay sensitivity to be 100% in that it correctly identified all 24 SAT2 samples (Table 5). This assay demonstrated no cross-reactivity with any of the samples identified as A/WAG/IV viruses. However, some cross-reactivity was demonstrated by the SAT2, topotype VII specific rRT-PCR assay where five of the 63 samples identified as O/EA3 viruses were positive on the SAT2, topotype VII assay (Table 5). Interestingly, since the tissue samples were collected in the same year and close in proximity, it can't be ruled out that these samples may contain SAT2/VII genomic material.

Discussion

FMDV is one of the most economically devastating pathogens worldwide leading afflicted areas to suffer both direct and indirect losses. For many countries elimination of FMDV is through a strict stamp-out policy leading to mass animal culling. Other nations have controlled the disease through the implementation of a vaccine policy. Due to the virus' highly contagious nature, incursions are feared by all nations. The paramount method to combat FMDV spread is preparedness. This is accomplished at the laboratory level by the establishment of rapid, sensitive, and specific diagnostic tools.

TABLE 5 Detection of FMDV from bovine tissue samples collected from Nigeria using the SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays.

Isolate	Serotype/Topotype/Lineage	SAT1 Cq	SAT2 Cq	SAT3 Cq	FMDV 3D Cq	Xeno Cq
O/NIG/BAU/BAU/1/2020	O/EA3	No Cq	No Cq	No Cq	17.96	31.36
O/NIG/BAU/BAU/5/2020	O/EA3	No Cq	No Cq	No Cq	23.12	31.59
O/NIG/BAU/BAU/13/2020	O/EA3	No Cq	No Cq	No Cq	24.04	31.11
O/NIG/BAU/BAU/14/2020	O/EA3	No Cq	No Cq	No Cq	21.33	31.44
O/NIG/BAU/BAU/17/2020	O/EA3	No Cq	No Cq	No Cq	25.77	31.83
O/NIG/BAU/BAU/21/2020	O/EA3	No Cq	No Cq	37.81	16.22	31.53
O/NIG/BAU/BAU/22/2020	O/EA3	No Cq	No Cq	No Cq	15.73	32.14
SAT2/NIG/PL/BLD/1/2020	SAT2/VII	No Cq	19.57	No Cq	13.73	31.82
SAT2/NIG/PL/BLD/2/2020	SAT2/VII	No Cq	21.01	No Cq	15.32	31.63
SAT2/NIG/PL/BLD/3/2020	SAT2/VII	No Cq	14.84	No Cq	9.82	31.41
SAT2/NIG/PL/BLD/5/2020	SAT2/VII	No Cq	16.63	No Cq	11.80	31.04
SAT2/NIG/PL/BLD/6/2020	SAT2/VII	No Cq	22.26	No Cq	15.79	31.20
SAT2/NIG/PL/BLD/7/2020	SAT2/VII	No Cq	21.25	No Cq	15.22	30.99
SAT2/NIG/PL/BLD/8/2020	SAT2/VII	No Cq	14.69	No Cq	10.34	31.37
O/NIG/PL/BLD/9/2020	O/EA3	No Cq	No Cq	No Cq	26.64	31.72
O/NIG/KN/BMF/1/2020	O/EA3	No Cq	No Cq	No Cq	19.92	31.46
O/NIG/KN/BMF/2/2020	O/EA3	No Cq	No Cq	No Cq	15.76	32.18
O/NIG/KN/BMF/3/2020	O/EA3	No Cq	No Cq	No Cq	17.78	31.60
O/NIG/KN/BMF/4/2020	O/EA3	No Cq	No Cq	36.13	18.17	31.50
O/NIG/KN/BMF/5/2020	O/EA3	No Cq	No Cq	No Cq	27.26	31.94
O/NIG/KN/RMG/1/2020	O/EA3	No Cq	No Cq	No Cq	18.34	31.46
O/NIG/PL/BK/1/2020	O/EA3	No Cq	No Cq	No Cq	16.77	31.29
SAT2/NIG/PL/BK/2/	SAT2/VII	No Cq	28.90	No Cq	22.17	30.84
O/NIG/PL/BK/3/2020	O/EA3	No Cq	No Cq	No Cq	18.08	31.00
O/NIG/PL/BK/4/2020	O/EA3	No Cq	No Cq	No Cq	20.78	31.37
A/NIG/PL/BK/5/2020	A/WAG/IV	No Cq	No Cq	No Cq	13.85	32.71
O/NIG/PL/BK/6/2020	O/EA3	No Cq	No Cq	No Cq	23.23	31.77
A/NIG/PL/BK/7/2020	A/WAG/IV	No Cq	No Cq	No Cq	17.21	32.21
SAT2/NIG/PL/BK/8/2020	SAT2/VII	No Cq	28.62	No Cq	21.72	30.60
O/NIG/PL/BK/31/2020	O/EA3	No Cq	No Cq	No Cq	25.14	32.42
O/NIG/PL/BK/32/2020	O/EA3	No Cq	No Cq	No Cq	25.43	32.05
O/NIG/PL/BK/33/2020	O/EA3	No Cq	No Cq	No Cq	23.00	32.31
O/NIG/KD/ZA/1/2020	O/EA3	No Cq	No Cq	No Cq	19.77	30.67
O/NIG/KD/ZA/2/2020	O/EA3	No Cq	43.16	No Cq	15.72	30.70
O/NIG/KD/ZA/4/2020	O/EA3	No Cq	No Cq	No Cq	24.98	30.63
O/NIG/KD/ZA/5/2020	O/EA3	No Cq	No Cq	No Cq	14.16	31.73
O/NIG/KN/KN/1/2020	O/EA3	No Cq	No Cq	No Cq	18.37	30.94
O/NIG/KN/KN/2/2020	O/EA3	No Cq	No Cq	No Cq	17.47	30.81
SAT2/NIG/PL/JS/1/2020	SAT2/VII	No Cq	19.75	No Cq	14.17	31.26
SAT2/NIG/PL/JS/2/2020	SAT2/VII	No Cq	18.87	35.56	13.69	31.32
O/NIG/KT/KT/1/2020	O/EA3	No Cq	No Cq	No Cq	17.02	30.70
O/NIG/KT/KT/2/2020	O/EA3	No Cq	No Cq	No Cq	20.58	31.38
O/NIG/KT/KT/3/2020	O/EA3	No Cq	No Cq	No Cq	13.78	32.19
O/NIG/BAU/TR/1/2020	O/EA3	No Cq	No Cq	No Cq	19.10	31.70
O/NIG/PL/RY/1/2020	O/EA3	No Cq	27.13	No Cq	24.91	31.63
SAT2/NIG/PL/RY/2/2020	SAT2/VII	No Cq	17.09	No Cq	15.79	31.49
SAT2/NIG/PL/RY/3/2020	SAT2/VII	No Cq	28.58	No Cq	21.78	31.00
O/NIG/KD/KD/1/2020	O/EA3	No Cq	No Cq	No Cq	21.86	31.22
O/NIG/KD/KD/2/2020	O/EA3	No Cq	No Cq	No Cq	21.04	30.89
O/NIG/KD/KD/3/2020	O/EA3	No Cq	No Cq	No Cq	15.74	31.35
O/NIG/KD/KD/4/2020	O/EA3	No Cq	No Cq	No Cq	16.86	31.56

(Continued)

TABLE 5 (Continued)

Isolate	Serotype/Topotype/Lineage	SAT1 Cq	SAT2 Cq	SAT3 Cq	FMDV 3D Cq	Xeno Cq
O/NIG/KD/KD/5/2020	O/EA3	No Cq	No Cq	No Cq	14.70	31.42
A/NIG/KD/KGR/1/2020	A/WAG/IV	No Cq	No Cq	No Cq	23.04	31.92
A/NIG/KD/KGR/2/2020	A/WAG/IV	No Cq	No Cq	No Cq	22.21	31.98
A/NIG/KD/KGR/3/2020	A/WAG/IV	No Cq	No Cq	No Cq	11.91	32.07
A/NIG/KD/KGR/4/2020	A/WAG/IV	No Cq	No Cq	No Cq	26.40	31.90
A/NIG/KD/KGR/7/2020	A/WAG/IV	No Cq	No Cq	No Cq	17.28	32.06
A/NIG/KD/KGR/8/2020	A/WAG/IV	No Cq	No Cq	No Cq	16.31	31.71
A/NIG/KD/KGR/9/2020	A/WAG/IV	No Cq	No Cq	No Cq	23.03	31.59
A/NIG/KD/KGR/10/2020	A/WAG/IV	No Cq	No Cq	No Cq	18.33	31.26
A/NIG/KD/KGR/11/2020	A/WAG/IV	No Cq	No Cq	No Cq	21.92	32.27
A/NIG/KD/KGR/14/2020	A/WAG/IV	No Cq	No Cq	No Cq	21.68	32.11
O/NIG/PL/KAN/1/2020	O/EA3	No Cq	No Cq	No Cq	18.67	32.88
O/NIG/PL/KAN/2/2020	O/EA3	No Cq	No Cq	No Cq	14.07	32.48
O/NIG/PL/KAN/3/2020	O/EA3	No Cq	No Cq	No Cq	15.12	32.35
O/NIG/PL/KAN/4/2020	O/EA3	No Cq	No Cq	No Cq	12.61	32.37
O/NIG/PL/KAN/5/2020	O/EA3	No Cq	No Cq	No Cq	14.17	32.28
O/NIG/PL/KAN/6/2020	O/EA3	No Cq	No Cq	No Cq	15.89	31.84
O/NIG/AD/GMB/2/2020	O/EA3	No Cq	No Cq	No Cq	19.98	32.24
O/NIG/AD/GMB/3/2020	O/EA3	No Cq	No Cq	No Cq	24.28	31.49
O/NIG/AD/GMB/4/2020	O/EA3	No Cq	No Cq	No Cq	23.79	31.99
O/NIG/AD/GMB/5/2020	O/EA3	No Cq	No Cq	No Cq	22.12	31.97
O/NIG/PL/KA/1/2020	O/EA3	No Cq	No Cq	No Cq	16.53	32.39
O/NIG/PL/KA/2/2020	O/EA3	No Cq	No Cq	No Cq	16.60	32.58
SAT2/NIG/PL/BL/1/2020	SAT2/VII	No Cq	22.44	No Cq	16.46	33.89
SAT2/NIG/PL/BL/2/2020	SAT2/VII	No Cq	13.11	No Cq	10.17	32.56
SAT2/NIG/PL/BL//3/2020	SAT2/VII	No Cq	15.71	No Cq	11.45	32.82
SAT2/NIG/PL/BL/4/2020	SAT2/VII	No Cq	19.22	No Cq	14.75	33.54
SAT2/NIG/PL/BL/6/2020	SAT2/VII	No Cq	14.02	No Cq	12.19	32.70
SAT2/NIG/PL/BL/7/2020	SAT2/VII	No Cq	16.	No Cq	12.03	32.90
SAT2/NIG/PL/BL/8/202	SAT2/VII	No Cq	19.95	No Cq	15.62	32.45
SAT2/NIG/PL/BL/9/2020	SAT2/VII	No Cq	22.35	No Cq	16.75	32.29
SAT2/NIG/PL/BL/10/2020	SAT2/VII	No Cq	15.67	No Cq	11.64	32.97
SAT2/NIG/PL/BL/11/2020	SAT2/VII	No Cq	12.81	No Cq	9.92	32.97
SAT2/NIG/PL/BL/12/2020	SAT2/VII	No Cq	24.16	No Cq	19.50	32.76
O/NIG/PL/JE/13/2020	O/EA3	No Cq	34.90	No Cq	28.71	33.25
O/NIG/PL/JE/16/2020	O/EA3	No Cq	35.56	No Cq	29.15	32.76
O/NIG/PL/JE/17/2020	O/EA3	No Cq	No Cq	No Cq	15.51	33.26
O/NIG/PL/JE/19/2020	O/EA3	No Cq	38.54	No Cq	15.42	32.29
O/NIG/PL/JN/20/2020	O/EA3	No Cq	40.46	No Cq	20.96	32.11
O/NIG/PL/JN/21/2020	O/EA3	No Cq	30.87	No Cq	18.87	32.11
O/NIG/PL/JN/22/2020	O/EA3	No Cq	No Cq	No Cq	24.12	31.80
O/NIG/PL/JN/23/2020	O/EA3	No Cq	No Cq	No Cq	22.48	32.55
O/NIG/PL/JN/24/2020	O/EA3	No Cq	30.11	No Cq	16.45	32.31
O/NIG/PL/JN/25/2020	O/EA3	No Cq	No Cq	No Cq	23.03	32.10
O/NIG/PL/JN/26/2020	O/EA3	No Cq	No Cq	No Cq	23.87	31.97
O/NIG/PL/JN/27/2020	O/EA3	No Cq	No Cq	No Cq	19.80	32.13
O/NIG/PL/JN/28/2020	O/EA3	No Cq	33.30	No Cq	18.69	32.08
O/NIG/PL/JN/29/2020	O/EA3	No Cq	No Cq	No Cq	23.24	31.19

Next-generation sequencing was utilized to identify FMDV serotype. Samples are considered positive when the quantification cycle (Cq) is ≤35.99 for SAT1, SAT2, topotype VII, SAT3, FMDV pan-serotype 3D and Xeno control assays.

The colours used to indicate each of the Foot-and-Mouth Disease virus serotypes. Serotype O is blue, serotype A is red, serotype Asia1 is grey, serotype SAT1 is yellow, serotype SAT2 is purple and serotype SAT3 is orange. When the Cq value cells are highlighted with either yellow, purple or orange, it means that a Cq value was produced for this particular viral isolate or sample. If the yellow, purple or orange is darker and the Cq value is <35.99 then the sample was positive when evaluated by the corresponding assay. If the filled cell is the lighter version of the colour, it indicates that although a Cq value was produced, it is >35.99 and the sample is considered negative.

Currently, the most sensitive validated first-line FMDV diagnostic assays are serotype-independent, able to define the presence of the virus in a sample. After FMDV is detected it is important to identify which of the seven antigenically distinct serotypes the virus belongs to. This is because FMDV serotyping is the crucial first step in establishing a VP1 Sanger-based sequencing plan and identifying an appropriate vaccine match. Currently, the most commonly used method for FMDV serotyping is the Ag-ELISA. However, with a sensitivity as low as 80%, it often requires isolation of the virus to produce enough analyte for detection and requires an overnight incubation step (3, 10, 11). Sanger sequencing of the FMDV VP1 coding region also requires prior knowledge of viral serotype to select appropriate amplification and sequencing primers (14). rRT-PCR methodologies represent an attractive method to facilitate FMDV serotyping due to its lower resource and technical requirements. This can only be accomplished if genetic signatures that are both unique to the FMDV serotype and all-encompassing to the intra-serotype strains are identified, a task that is quite difficult given the extreme genetic diversity of FMDV.

The Neptune bioinformatics tool was developed to identify genetic signature sequences that are conserved within a defined inclusion group yet absent from defined exclusion groups (37). In this study, Neptune was used for the first time to identify genetic sequences unique to a viral serotype, specifically the FMDV SAT1, SAT2 and SAT3 serotypes. The Neptune-generated SAT1, SAT2 and SAT3 genetic signatures were all predicted to have high sensitivity and specificity generating scores >87%. Each of these SAT serotype-specific genetic signatures mapped to the VP1 coding regions of the FMDV genome. This is not surprising, as the VP1 coding locus displays the greatest amount of diversity with ∼30%−50% discrepancy between serotypes (46). As such, SAT serotype-specific-TaqMan-based rRT-PCR assays were designed within the Neptune-generated signature sequences.

However, the extent of FMDV's genetic diversity extends beyond viral serotype and down into viral strains/subtypes. To facilitate the design of SAT-specific primers and probes, SAT1, SAT2 and SAT3 viral sequences published between 2011 and Fall 2021 were retrieved from NCBI and aligned to ascertain the level of genetic conservation. As foreseeable, there was significant nucleotide level diversity displayed by the intra-serotype strain/subtype sequence alignments. To reconcile these discrepancies, degenerative nucleotides were incorporated into the primers and probes to expand genetic coverage in an attempt to increase the intra-serotype sensitivity of the assay. However, no more than three degenerative nucleotides were incorporated into any of the oligonucleotides to not sacrifice assay specificity. While there is no defined limit of degenerate nucleotides in a primer or probe, expansive usage can lead to decreased assay specificity and the potential for the assay oligonucleotides to self-anneal or bind to each other. As such, successful rRT-PCR assay generation with incorporated degenerative nucleotides in the primers and/or probes must be tested empirically as was done in this study.

Repeatability analysis revealed the robustness of the SAT1, SAT2 and SAT3 rRT-PCR assays. Triplicate independent RNA extractions and rRT-PCR analysis revealed the resilience of each of the SAT-specific assays as the standard deviations produced were at most 2.15 (Figure 1).

The analytical sensitivity, LoD testing of the SAT-specific rRT-PCR assays revealed that the dynamic range was the greatest with the SAT3 rRT-PCR assay, followed by the SAT1 and then the SAT2 topotype VII (Figure 2). It is difficult to report singular assay specificity when the genetic diversity of the analyte is so great. Typically, DNA–based plasmids are utilized for analytical sensitivity analysis, however, they do not control for the variability introduced from the RNA extraction and reverse transcriptase processes that are integral upstream rRT-PCR assay procedures. As such, this study utilized FMDV isolates that were serially diluted prior to RNA extraction to account for those variables. As expected, utilizing this model for analytical sensitivity testing demonstrated the variability in the LoD and the rRT-PCR efficiency both within and between the different SAT-specific and FMDV pan-serotype 3D assays. Nonetheless, all three of the SAT-specific assays demonstrated that there was sufficient dynamic range to detect a variety of FMDV strains albeit less than the FMDV pan-serotype 3D rRT-PCR assay (Figure 2). Despite differences in assay robustness, the intention of the SAT-specific assays are to be used to evaluate samples that had been previously identified as positive by the FMDV pan-serotype 3D assay, as a way to identify serotype, not as a first-line diagnostic tool.

Genetic *in silico* predictive methodologies provide an appropriate starting point when defining genetic signatures. However, they are only as good as the data that is supplied to them and there is potential for them not to translate into viable *in vitro* reagents. Fortunately, that was not the case with the Neptune-based, SAT-specific rRT-PCR assays as the serotype specificity of each assay was 100% when tested against heterologous serotype O, A and Asia1 viruses (Table 3).

The serotype specificity of the SAT-specific rRT-PCR assays against heterologous SAT serotypes was 100% for SAT1 and SAT2 assays (Table 4). Though the SAT3 rRT-PCR demonstrated cross-reactivity with one of the nine SAT1 isolates, SAT1/BOT/12/2006, the Cq value for the SAT1/BOT/12/2006 produced by the SAT1-specific assay was 22.60 as opposed to the 35.30 produced by the SAT3 assay. Since the SAT-specific rRT-PCR assays are intended to be utilized in tandem on a single sample, the lower Cq value produced would define the viral serotype and therefore, SAT1/BOT/12/2006 would be serotyped as a SAT1 virus. Each of the SAT-specific rRT-PCR assays also demonstrated 100% analytical specificity when tested against VSNJV, VSIV, SVDV, and SVA. It should be noted that the intended use of the serotyping assays would be secondary after

the detection of FMDV genomic RNA by the pan-serotype 3D rRT-PCR and exclusion of vesicular disease differentials.

When the diagnostic sensitivity of the assays was evaluated, both the SAT1 and SAT3-specific assays were able to detect eight out of the nine viruses of a homologous serotype. The SAT1/ETH/3/2007 isolate (accession number: FJ798154.1) not detected by SAT1 rRT-PCR assay failed to bind the SAT1 primers and probe when the sequences were aligned. The SAT1/ETH/3/2007 was the only SAT1, topotype IX virus that the SAT1 rRT-PCR was evaluated against. Therefore, the assay may have limitations for that particular topotype or specifically this viral isolate given that it was submitted to NCBI prior to the 2011 cut-off used for assay oligonucleotide design. The SAT3-specific rRT-PCR assay also failed to detect one of the SAT3 isolates, SAT3/UGA/10/1997. This is likely since the sequences retrieved from NCBI to build the SAT3 alignments were limited to a 10-year window (2011-Fall 2021). This was done to design primers and probes that were likely to bind to currently circulating FMDVs.

Interestingly, when the SAT2-specific rRT-PCR assay was evaluated against the panel of 22 SAT2 isolates, a topotype VII specificity was revealed. This was not the original intention of the assay, but of the 22 SAT2 isolates examined, eight were topotype VII viruses and were all detected with Cqs ranging from 12.71 to 23.88, demonstrating a strong topotype VII specificity (Table 4). As such, the assay was redefined as the SAT2, topotype VII specific rRT-PCR assay. It has been noted previously that SAT2 viruses exhibit high genetic intra-serotype diversity within their VP1 sequences diverging by ∼20% (47). SAT2 topotype VII viruses also have the furthest geographic distribution in comparison to the other SAT2 topotypes encompassing most of the Northern part of Africa (48). It is plausible that SAT2 topotype VII viruses are retrieved more frequently resulting in sequences being reported more than the other topotypes, thus leading to an overabundance of SAT2 topotype VII sequences in the NCBI database.

Diagnostic specificity of SAT1, SAT3 and SAT2 topotype VII rRT-PCR assays was revealed to be 100% when evaluated against 18 negative clinical samples and two mock viral infections. These results support that there is no off-target amplification of host nucleic acid and that only when there is the presence of the specific FMDV SAT genome, there is template detection.

The SAT-specific rRT-PCR assays were evaluated against bovine tissue samples collected from Nigeria (Table 5). This was to determine assay utility with true clinical veterinary samples without a prior isolation step to mimic a clinical diagnostic scenario. All samples were screened utilizing the FMDV pan-serotype 3D rRT-PCR to ensure the presence of the FMDV genome, and viral serotyping was accomplished utilizing NGS. The SAT-specific rRT-PCR assays retained high sensitivity and specificity for SAT2 topotype VII, correctly identifying 100% of the SAT2 topotype VII samples and potentially cross-reacting with only five of 63 serotype O samples. The O-specific bovine tissue samples were collected from the same location as SAT2 samples. As such, these results may be true positive for SAT2 potentially due to a mixed infection or cross-contamination during sample collection. The SAT1 and SAT3-specific rRT-PCR assay also demonstrated high analytical specificity for field samples. The only sample producing a positive Cq value for SAT3 produced a much lower Cq value with the SAT2-topotype VII rRT-PCR assay, thus, correctly identifying the sample as a SAT2, topotype VII virus.

To the best of our knowledge, this study was the first to utilize the novel Neptune bioinformatics tool to generate unique FMDV serotype signatures to build rRT-PCR assays. Interestingly, Neptune produced serotype-specific signatures in the VP1 locus of the FMDV genome. Previous FMDV rRT-serotyping assays also exploited the diversity of the VP1 coding region to design primers and probes. However, due to the vast genetic diversity of FMDV, most of the current rRT-PCR serotyping assays target geographically distinct lineages. Bachanek-Bankowska et al., 2016 developed sensitive and specific TaqMan-based rRT-PCR assays for the detection of A/AFRICA/G-I, O/EA-2, O/EA-4, SAT1/I and SAT2/IV FMDVs circulating in East Africa (27). rRT-PCR capable of detecting and distinguishing serotype O, A and Asia1 serotypes circulating in West Eurasia and the Middle East have also demonstrated serotyping utility (25, 26). Most recently, Lim et al. (32) were able to serotype O, A, and Asia viruses with a VP1-directed rRT-PCR evaluated against Asian FMDV isolates (32). The SAT1 and SAT3-specific rRT-PCR assays tested in this study appear to have no preference for topotype or lineage, with the caveat that testing multiple lineages would need to be expanded but was restricted due to viral isolate availability. Unexpectedly, the SAT2-specific rRT-PCR assay designed in this study resulted in an assay with SAT2, topotype VII tropism. As such, the design of FMDV rRT-PCR that are intentionally geographically restricted continues to represent the most viable method to utilize rRT-PCR to serotype FMDV. Even so, with the dynamic evolutionary nature of circulating FMDVs, it is crucial to continue to update genomic databases and continue to evaluate these assays against contemporary strains. It is likely that over time new strains/subtypes and the introduction of mutations by an RNA-dependent-RNA polymerase will demand that additional primers and/or probes be added to the current SAT1, SAT3 and SAT2, topotype VII rRT-PCR assays presented in this work.

Data availability statement

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

Author contributions

Conceptualization: CN, OL, and TC. Methodology and writing—review and editing: TC, CN, OL, KH, HU, MN, and PS. Validation: TC, PS, MN, and KH. Formal analysis: TC, CN, PS, and MN. Investigation, data curation, and supervision: TC and CN. Writing—original draft preparation: TC. Project administration: CN. Funding acquisition: CN and OL. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.977761/full#supplementary-material

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

REVIEWED BY
Esther Blanco,
Instituto Nacional de Investigación y Tecnología Agroalimentaria (INIA), Spain
Gisselle N. Medina,
Agricultural Research Service (USDA), United States

*CORRESPONDENCE
Artur Summerfield
artur.summerfield@ivi.admin.ch

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Relationship between neutralizing and opsonizing monoclonal antibodies against foot-and-mouth disease virus

Artur Summerfield[1,2]*, Heidi Gerber[1], Rebeka Schmitt[1], Matthias Liniger[1], Santina Grazioli[3] and Emiliana Brocchi[3]

[1]Institute of Virology and Immunology, Köniz, Switzerland, [2]Department of Infectious Diseases and Pathobiology (DIP), Vetsuisse Faculty, University of Bern, Bern, Switzerland, [3]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy

Previous studies demonstrated that polyclonal antibodies against foot-and-mouth disease virus (FMDV) generated by vaccination can mediate immune functions not only through virus neutralization but also through promoting virus uptake by macrophages and dendritic cells that are otherwise resistant to FMDV infection. This causes abortive infections resulting in activation, enhanced antigen presentation but also cell death. Here we report the use of RAW264.7 cells representing a murine macrophage cells line to characterize opsonizing functions of a collection of monoclonal antibodies (mAbs) against FMDV O and A serotypes. We demonstrate that all neutralizing immunoglobulin G isotype mAbs are able to opsonize FMDV resulting in increased cell death of RAW264.7 cells. In contrast, neutralizing IgM antibodies did not possess this activity. Opsonization was observed with broader reactivity within the serotype when compared to neutralization. Importantly, the anti-O serotype D9 mAb reacting with the continuous epitope within the G-H loop of VP1 that contains the RGD binding site of FMDV, opsonized several FMDV serotypes despite its restricted neutralizing activity within the O serotype. Furthermore, by generating RAW264.7 cells expressing bovine CD32, an easy-to-use cell-based assay system to test for bovine antibody-dependent enhanced infection of FMDV was generated and tested with a collection of sera. The data indicate that opsonizing titers correlated better with vaccine dose when compared to neutralizing titers. On the other hand, neutralization and opsonization titers were similar predictive of protection. We conclude that low avidity interactions are sufficient to mediate Fcγ receptor-mediated immune functions that could contribute to protective immune responses against FMDV.

KEYWORDS

FMDV, opsonization, antibodies, monoclonal antibody, Fc gamma receptors

Introduction

Foot-and-mouth disease virus (FMDV) is a member of the *Picornaviridae* family, and causes the high impact and very contagious foot-and-mouth disease (FMD) affecting cloven-hoofed animals. Although the disease is preventable by inactivated vaccines, proper vaccine selection is of crucial importance and needs to take into consideration not only seven known serotypes of FMD virus (FMDV; O, A, C, Asia-1, South African Territories 1, 2, and 3) but also the enormous antigenic variation within one serotype. This is caused by a high mutation rate of this RNA virus that strongly affects viral proteins targeted by neutralizing antibodies. Nevertheless, despite the central importance of neutralizing antibodies in protective immunity against FMDV, animals can be protected with low levels or in absence of neutralizing antibodies (1, 2). Previous studies demonstrated that polyclonal serum antibodies generated by vaccination can mediate immune functions not only through virus neutralization but also through promoting virus uptake by Fc gamma receptors (FcγR) expressed on macrophages and dendritic cells (DC) that are otherwise resistant to FMDV infection (3–5). In fact, opsonization of virus antibody complexes has been demonstrated to be a crucial component of the immune response in a mouse model, required for the final *in vivo* destruction of the virus by phagocytes (5–7). In the case of plasmacytoid dendritic cells (pDC), such sera can greatly enhance FMDV-induced interferon-α secretion and could therefore be associated with a direct antiviral effect as well as the potent adjuvant effect of activated pDC for adaptive immune responses. Interestingly, sera with such activities were broadly cross-reactive even across different serotypes of FMDV (3).

Considering the above and the fact that field conditions usually represent heterologous challenge situations, analyzing such opsonizing antibodies (mAbs) is of relevance to understand protective immune responses. Using monoclonal antibodies, we therefore performed the present study to understand the relationship between neutralization and opsonization as well as the degree of cross-reactivity of opsonizing mAbs. To this end, we established a RAW264.7 cells-line based assay for both murine mAbs and bovine antibodies. Our data demonstrate that even at the monoclonal level opsonizing antibodies can be highly cross-reactive even across serotypes. All opsonizing mAbs identified were neutralizing against homologous viruses, indicating that low affinity is sufficient to mediate opsonization but not neutralization of FMDV. Considering these results, we also generated RAW264.7 cells expressing bovine CD32, to test the relationship of opsonizing with neutralizing activities and with the outcome of vaccination.

Materials and methods

Viruses

The following viruses were used: O_1/SWI/65 (O_1 Lausanne), O/BUL/1/91, O/GRE/22/96, O/GRE/ 21/94, O/VIE/7/97, A/MCD/6/96, A/MAY/6/96, A/TUR/99, A_{24}/Cruzeiro/55, A/SAU/17/92, and Asia-1/TUR/6/2000, C-S8cl (C_1 SPA/7/79). Viruses were kindly provided by the World Reference Laboratory for Foot-and-Mouth Disease of The Pirbright Institute, UK), with the exception of S8cl representing a plaques-purified C_1 virus kindly obtained from Francisco Sobrino (Centro de Biología Molecular Severo Ochoa, Madrid, Spain). Virus stocks were made using Baby Hamster Kidney (BHK) 21 cells that were grown in Glasgow's minimum essential medium (GMEM, Thermofisher, Gibco) supplemented with 5% v/v Fetal Bovine Serum (FBS, Biowest S05595S1810). Isolates of FMDV were propagated in BHK-21 cells and viral titres were determined by end-point titration on BHK-21 cells as previously described (8). Mock antigen was prepared from uninfected BHK-21 cells in the same manner as FMDV.

Monoclonal antibodies

The present study employed mAbs against FMDV O_1/SWI/65, O_1/Manisa/TUR/69, A_{24}/Cruzeiro/55 and A/MAY/6/96 (Table 1). All mAbs were generated at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER) using standard methods of mouse immunization, hybridoma technology and screening (14–16). The mAbs employed originated from mouse ascites fluids (generated over 20 years ago), except for mAb B2 which was from hybridoma cell culture supernatant.

Virus neutralization and opsonization assays

The virus neutralization assay was performed based on previously published protocols (17). Briefly, the different mAbs were incubated at different log2-fold dilutions with 100 $TCID_{50}$ of the different viruses in a total of 100 µl for 1 h a 37°C. Then, the mixture was added to BHK-12 cell monolayers and scored daily for cytopathogenic effects for maximum 4 days.

For the opsonization assays we employed the murine macrophages cell line RAW264.7 (ATCC) cultured in DMEM (Thermofisher Gibco 32430), 10% heat-inactivated FBS. The cells were seeded in 48 well plates at 2×10^5 cells/well in

TABLE 1 Monoclonal antibodies.

mAb	Antigen	Isotype	Neutralization	Epitope	Source/reference
1C12	O1/Manisa/TUR/69	IgM/IgG1	Yes	Site 2	IZSLER
1C6	O1/SWI/65	IgG2a	Yes	Site 2	IZSLER; (9, 10)
2A10	O1/Manisa/TUR/69	IgG1	Yes	Site 2&3	IZSLER
3C8	O1/SWI/65	IgM	Yes	Site 3	IZSLER; (9, 10)
B2	O1/SWI/65	IgG1	Yes	VP1, site 1	IZSLER; (9, 10)
D9	O1/SWI/65	IgG2a	Yes	VP1, site 1	IZSLER; (9, 10)
3B11	O1/Manisa/TUR/69	IgM/IgG1	No		IZSLER; (11)
A8	O1/SWI/65	IgG1	No	VP1	IZSLER; (10, 12)
4B10	A/MAY/6/96		Yes		IZSLER
4B12	A/MAY/6/96		Yes		IZSLER
4E9	A/MAY/6/96		Yes		IZSLER
4H2	A/MAY/6/96		Yes		IZSLER
4H8	A24/Cruzeiro/55		Yes		IZSLER
5G3	A/MAY/6/96	IgM	Yes		IZSLER
2C7	A/MAY/6/96		No		IZSLER
4E10	A24/Cruzeiro/55		No	VP1	IZSLER
5F7	A24/Cruzeiro/55		No		IZSLER; (13)

400µl medium and cultured for 4 days of culture at 37°C, 5% CO_2. To generate immune complexes, FMDV (multiplicity of infection 5 $TCID_{50}$/cell) was mixed with the mAbs at three different concentrations (10, 1 and 0.1 µg/ml; virus without antibody as negative control), or with the cattle sera dilutions (1:10, 1:100, 1:1000) in a total volume of 250 µl and incubated for 30 min at room temperature. After removal of the culture medium from the RAW264.7 cells, the immune complexes, virus and mock controls were added to the cells (250 µl/well) for 1h incubation at 37°C, 5% CO_2. Then, the cells were washed twice with 0.5 ml medium, and 400 µl of fresh medium per well was added, and the plates were incubated for 48 h at 37°C, 5% CO_2. The cells were harvested as previously described (18), centrifuged at 250 g, 4°C, 5 min and resuspended in 100–200 µl CellWash® (Becton Dickinson) at 4°C and analyzed by flow cytometry for propidium iodide (PI) incorporation to determine the percentages of dead cells. For each culture, the values obtained with mock-infected cells was subtracted from the FMDV-infected cells. The opsonization assay for the mAbs was validated for background reactions using a pool of mouse IgG1 and IgG2a of unknown specificity (MOPC-21 and MOPC-173, Sigma-Aldrich) at the same concentrations as the FMDV specific antibodies. Based on the results, a negative cut-off of 3% PI^+ cells was defined. For the cattle sera, the PI^+ values of the pre-immune sera at the corresponding dilutions were subtracted from the post-vaccination sera. Opsonizing and neutralizing titres of sera was calculated using the Reed and Muench formula (19).

Trapping ELISA

MAb reactivities against the different FMDV isolates were evaluated by a trapping ELISA (20). Briefly, mAbs were incubated with pre-titrated concentrations of viruses (supernatant of infected cells) that had been trapped using a rabbit immune serum raised against different FMDV isolates. The reactivity of field isolates with each mAb, used at the double saturating concentration, was expressed as a percentage of the corresponding reaction with the parental strain, assumed to be 100 % (15).

Cloning of bovine CD32, plasmid construction, and transfection

The nucleotide sequence of the open reading frame encoding bovine CD32 FcγR was obtained from NCBI NM_174539.2. RNA was extracted from bovine PBMCs using the Nucleo-Spin RNAII kit (Macherey-Nagel, Switzerland). Reverse transcription was done with SuperScriptIII reverse transcriptase (Life Technologies) followed by PCR amplification, gel-purification of the PCR product of expected size and ligation into the TOPO vector (Life Technology) according to manufacturer's protocol. Plasmids were amplified in XL-1 blue *E. coli*. After verification of the CD32 sequence, the gene was subcloned into the pEAK8_HIS vector (21) at the restriction sites EcoR1/Xbal using standard cloning techniques to generate the pEAK8_CD32

plasmid, which was amplified and purified using NucleoBond kits (Macherey-Nagel). For protein expression, HEK293 cells were transfected with MirusTrans IT293 transfection agent (Mirus, USA). At 48 h post transfection cells were stained with mouse-anti human CD32 (clone AT10, Biorad) followed by rabbit-anti-mouse immunoglobulin (Ig)-RPE conjugate (DAKO, Denmark) and detection was performed by flow cytometry (FACSCalibur, Becton Dickinson, Basel Switzerland).

The lentivirus expression system utilized was as previously described (22, 23) using plasmids obtained from the laboratory of Dr. Didier Trono (http://tronolab.epfl.ch/ Ecole Polytechnique Federale de Lausanne, Switzerland) through Addgene (Cambridge MA, USA). For subcloning of CD32 into the lentiviral transfer plasmid pWPT the pEAK8_CD32 plasmid was amplified by PCR using primers to insert the MIuI and SalI restriction sites present in the pWPT vector. The PCR product was digested using MIuI and SalI and the CD32 gene ligated into the pWPT vector using standard techniques. The plasmid was amplified in chemo-competent bacteria (Stbl2) and sequenced. HEK293 cells were transfected using the calcium phosphate precipitation method with the envelope plasmid (pMD2G), the packaging plasmid (pCMV-R8.74) and the transfer vector encoding bovine CD32 (pWPT _CD32). Medium was changed after overnight incubation at 37°C and the supernatant harvested after 48 h, centrifugated and filtered. The virus was purified and enriched by centrifugation on a 20% sucrose cushion at 28,000 *g* for 90 min, 4°C. For the generation of the RAW264.7 cell lines the cells were transduced twice with 1:100 dilution of the purified lentiviruses in 1 ml serum free medium of a T25 cell culture flask followed by culture overnight at 37°C and medium change between the transductions. Transduction efficiency was controlled by staining with anti-CD32 as described above and found to remain stable over at least three passages (76% positive, Supplementary Figure 1), during which the experiments were performed.

Immunization of animals and source of serum samples

The sera used in the present study originated from vaccine trials performed in 2011. The first study was designed to test the protective capacity of the quadrivalent FMD vaccine Aftovaxpur® T-1978A (O Middle East, Asia-1 Shamir, SAT2 Saudi Arabia, C Noville antigens; Merial, France; now Boehringer Ingelheim) to protect against FMDV O/BUL/2011 challenge. Following guidelines of the European Pharmacopeia, 6-month old cattle were vaccinated with four animals receiving the full dose (containing ≥3 protective doses 50 per valency), five animals ¼ dose and again five animals 1/20 dose. A second vaccination trial was performed with a vaccine (identical formulation as first trial) containing O3039, O_1 Manisa or both O_1 Manisa and O3039 antigens (full doses). For both studies, after 21 days a challenge infection with FMDV O/BUL/2011 (10,000 ID_{50}) was done intra-dermo-lingually. Animals showing FMDV lesions were immediately euthanized and recorded as non-protected. Serum was collected before vaccination and weekly up to 7 weeks post vaccination.

Ethics statement

The animal experiments were performed in compliance with the Swiss animal protection law (TSchG SR 455; TSchV SR 455.1; TVV SR 455.163). The procedures were reviewed by the committee on animal experiments of the canton of Bern, Switzerland, and approved by the cantonal veterinary authority (Amt für Landwirtschaft und Natur LANAT, Veterinärdienst VeD, Bern, Switzerland) under the license numbers BE95/10.

Results

Relationship between neutralization and opsonization by mAbs

To determine the relationship between neutralization and opsonization, we tested the opsonizing activity of six anti-O serotype mAbs generated toward O_1/SWI/65 or O_1/Manisa/TUR/69 against a collection of O serotype FMDV isolates. Figure 1A shows the opsonizing activities of mAbs with neutralizing activity (at least against homologous FMDV, see Table 1). All neutralizing anti-O serotype mAbs with neutralizing activity against a particular FMDV isolate also possessed opsonizing activity, except for the IgM mAb 3C8. MAbs 1C12, 1C6, and 2A10 were unable to neutralize certain FMDV O isolates (row labeled "VN neg"). Nevertheless, in two cases these mAbs still had opsonizing activity (e.g., 1C12 and 1C6 opsonized O/GRE/96 in absence of neutralization). In one case we also had opsonizing activity for antibodies that lacked ELISA reactivity to that FMDV isolate (1C6, blue dot).

Figure 1B shows the data for two mAbs which are highly reactive in ELISA but non-neutralizing (including homologous virus): both also lacked opsonizing activity.

Considering the above results, we also tested a collection of mAbs generated against A/MAY/6/96 or A_{24}/Cruzeiro/55 for activities against a collection of FMDV A isolates. Again, as visible in Figure 2A, the mAbs with neutralizing activity 4B10, 4E9, 4H2, and 4H8 also possessed the ability to opsonize FMDV. Nevertheless, although 5G3 was neutralizing it did not opsonize FMDV. Like 3C8 (Figure 1A), 5G3 is an IgM isotype. The upper row of Figure 2A confirms that for four of the mAbs opsonizing activity against certain FMDV isolates was found in absence of neutralization. This confirmed the opsonization was broader in its activity with antigenically divergent isolates within a serotype as previously reported using sera (3).

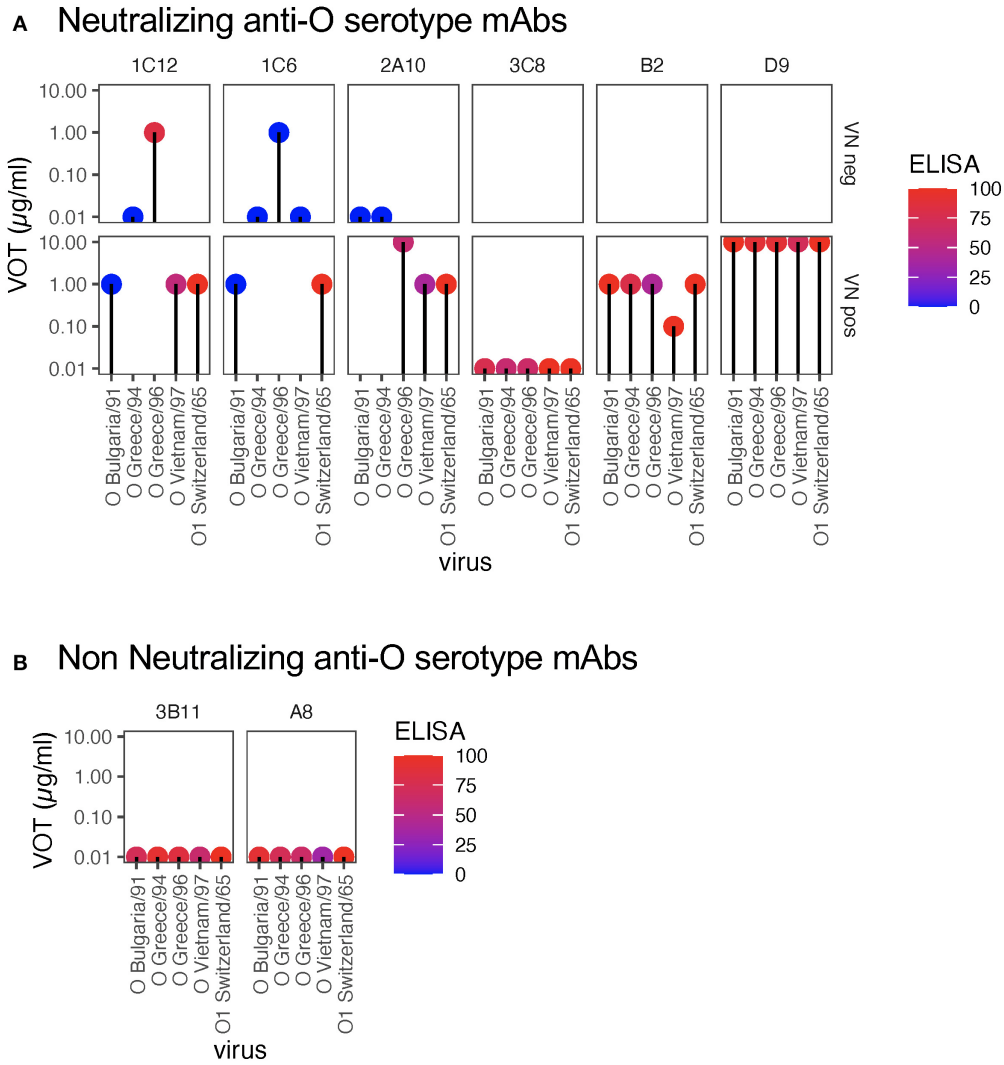


FIGURE 1
Virus opsonizing titers of mAbs against serotype O FMDV. VOTs were determined based on RAW264.7 cell death following incubation with mAbs (used at 10, 1, or 0.1 µg/ml) and virus (5 $TCID_{50}$/cell). For each virus (x-axis), the virus opsonization titres (VOT) are shown by the y-axis levels of the dots. Each plot shows the data for a different mAb (all anti-O serotype, see Table 1). The colors of the dots reflect the ELISA reactivity as indicated in the legend. The panels in **(A)** show the data for mAbs that neutralize homologous virus. To visualize the relationship of VOT to neutralization, the data was separated by the ability of a specific mAb to neutralize the different O FMDV viruses. The upper panels show the VOT data for mAb-virus pairs without neutralization ("VN neg"), while the lower panels represent mAb-virus pairs with neutralization ("VN pos"). In **(B)**, the data for mAbs with no neutralizing activity against homologous virus are shown.

Figure 2B shows that non-neutralizing anti-A serotype mAbs were not able to opsonize despite high activity in the ELISA.

Taking together, the data indicate that at the monoclonal level only or mainly neutralizing IgG antibodies can opsonize FMDV. Furthermore, opsonization reactivity for many mAbs is broader than neutralization, and occasionally reactivity is even found in absence of ELISA positivity.

Broad opsonizing activity of mAb D9 for A and O FMDV serotypes

Considering the broad reactivity of mAb D9 within the tested O serotype viruses for both neutralization and opsonization, we also tested FMDV belonging to other serotypes. Strikingly this antibody that was generated against O_1/SWI/65 was highly opsonizing for four of the five tested FMDV A viruses and even opsonized a FMDV C virus

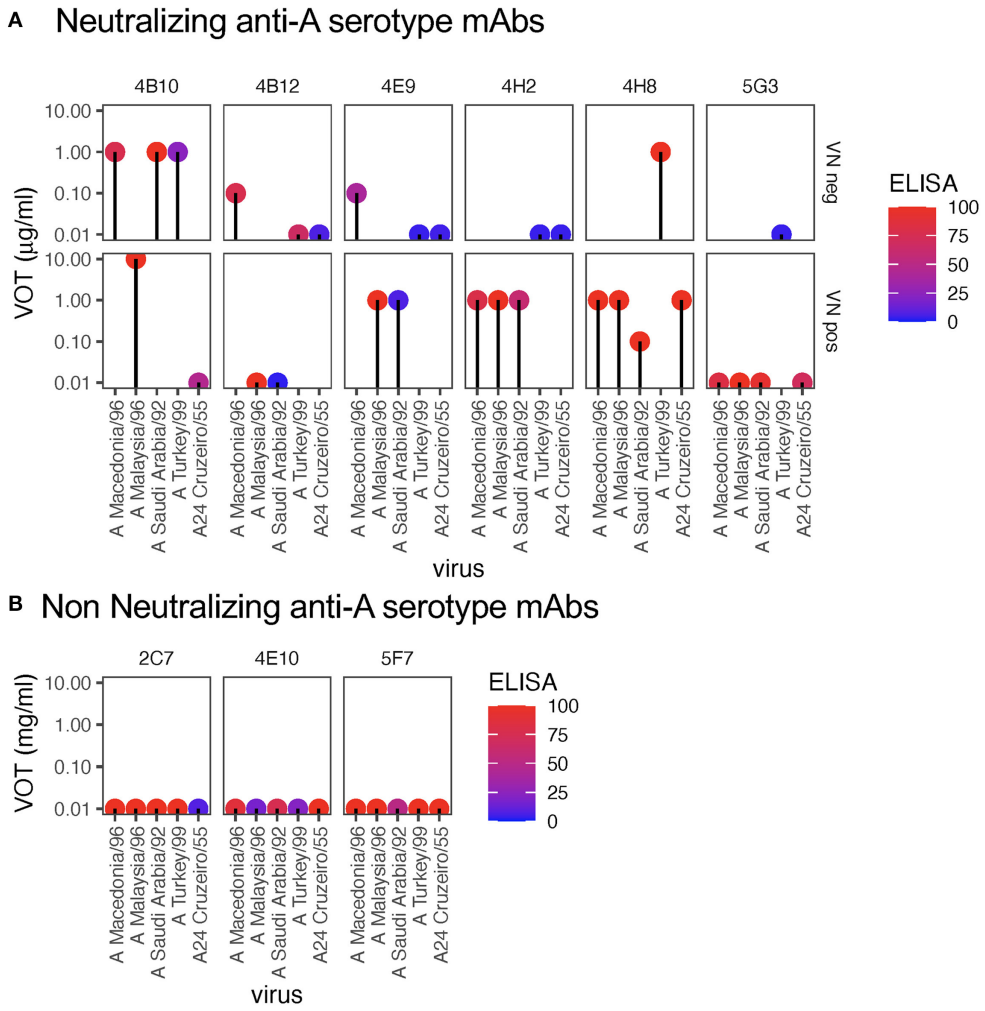


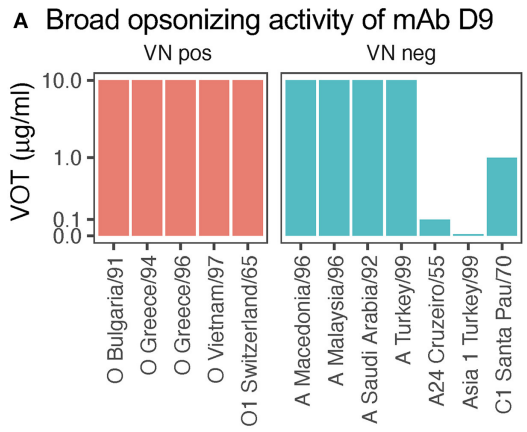
FIGURE 2
Virus opsonizing titers of mAbs against serotype A FMDV. For each virus (x-axis), the dots show the virus opsonization titers (VOT, y-axis), with the panels representing different anti-A serotype mAbs (see Table 1) as in Figure 1. The colors of the dots reflect the ELISA reactivity (legend). The panels in **(A)** show the data for mAbs that neutralize homologous virus. The upper panels show the VOT data for mAb-virus pairs without neutralization ("VN neg"), while the lower panels represent mAb-virus pairs with neutralization ("VN pos"). In **(B)**, the data for mAbs with no neutralizing activity against homologous virus are shown.

(Figure 3A). Nevertheless, as expected D9 was not able to neutralize non-O FMDV (Figure 3B).

The D9 represents one of the mAb generated in the early days of hybridoma technologies against O_1/SWI/65, and its binding site was established to be a linear epitope near the integrin binding site of FMDV (24). By sequencing of D9 escape mutants and using synthetic peptides, the minimal epitope for binding was found be amino acid position 141–154 of VP1. More precisely, D9-escape mutants retain the conserved RGD motif (position 141–143) but had mutations of the leucines on position 144/147 and of the lysin on 150 [Figure 3B; (9, 24, 25)]. Also, our data confirmed that O FMDV isolates that were neutralized by D9 were conserved for the RGDLQVL–K–R amino acid stretch. In contrast, the common motif for opsonization only was found to be RGDL–L (9, 24). These results demonstrate that opsonization of FMDV by antibodies requires less stringent binding, which explains their relatively broad reactivity even across different serotypes.

Relationship of opsonizing and neutralizing activity in cattle sera

We next employed the same methodology to sera from vaccinated cattle with the aim to understand the relationship between neutralization and opsonization with polyclonal sera in a relevant biological context. To this end we generated RAW264.7 expressing bovine CD32 to ensure efficient



B Relationship of D9 epitope to neutralization versus opsonization

FMDV isolate	VP1 site 1	Neut	Ops	Ref.
D9 escape mutants	RGDS R M	-	?	(18,19)
O1/SWI/65	VPNLRGDLQVLAQKVARTLP	+++	+++	(20)
O/BUL/1/91	VTAVRGDLQVLARKAARTLP	+++	+++	ACC63127
O/GRE23/94	VTAVRGDLQVLARKAARTLP	+++	+++	KU726614
O/GRE/22/96	ATNVRGDLQVLAQKAARTLP	+++	+++	AJ303491
O/VIE/7/97	LANVRGDLQVLAQKAARPLP	+++	+++	KJ831680
A/SAU/22/92	TGVRRGDLGPLAAHVTAQLP	-	+++	MT981284
A/MAY/13/97	PGTRRGDLGSLAARVAAQLP	-	+++	MT981286
A/MCD/6/96	SGHTRGDLGQLAARTAAQLP	-	+++	EU553869
A24/Cruzeiro/55	GSGRRGDMGSLAARVVKQLP	-	+	APHA24VP
Asia-1/TUR/6/2000	TTPRRGDMAALTQRLSGQLT	-	-	EU553916
C/SPA/7/79	TASARGDLAHLTFTTTHARH	-	++	MT219948

FIGURE 3
Virus opsonizing titers of mAb D9 against heterologous serotypes and its epitope relationship. In **(A)**, VOTs were determined as for Figure 1 but included viruses from the A, Asia-1, and C serotypes of FMDV. The data was separated by the ability of a specific mAb to neutralize the different FMDV viruses tested in neutralization negative mAb-virus pairs (right panels in blue, "VN neg") and neutralization positive mAb-virus pairs (left panels in red, "VN pos"). In **(B)**, the amino acid sequences of the D9 epitope from the viruses used in **(A)** are shown together with their neutralizing and opsonizing activities. The amino acids depicted in red and green are common to all viruses neutralized by D9. The leucine depicted in blue is found in all viruses that are well opsonized by D9.

interaction of bovine immune complexes with the murine macrophage cell line.

The first set of sera employed were from a vaccination-challenge experiment in which the multivalent vaccine Aftovaxpur® T-1978A (Merial), was tested in three different doses in cattle following European Pharmacopeia guidelines. Only three out of four animals with the full dose, two out of four with the quarter dose and none of the 1/20 dose were protected against O/BUL/2011 challenge. In general, all vaccinated animals had only low or no neutralizing activity. Nevertheless, while none of the protected animals lacked neutralizing activity, three of the seven non-protected animals had VNT's at levels comparable to protected animals (Figure 4A). This contrasted with the VOT that reached levels that were ~10x higher and correlated with the vaccine dose. However, also in this test the VOT levels did not separate protected from unprotected animals (Figure 4B). In this scenario of insufficient matching of vaccine antigen with challenge virus, a linear regression analyses indicated that vaccine dose would impact the VOT, but not the VNT (Figures 4A,B).

We also applied the same tests to sera from a second vaccination/challenge experiment performed in cattle, that was as a follow-up to the first challenge experiment with the aim to identify matching vaccine antigens against O/BUL/2011. In this experiment, all animals except one were protected, and animals receiving a vaccine containing the O Manisa FMDV antigen were all protected. These animals induced higher levels of neutralizing antibodies as compared to the O3039 antigen (Figure 4C). In contrast, the levels of VOT were similar with all three FMDV antigens. Interestingly, the only non-protected animal had lower levels of VOT as compared to the protected cattle (Figure 4D).

Considering these results, we performed further statistical evaluations of the cattle data. As expected, there was no

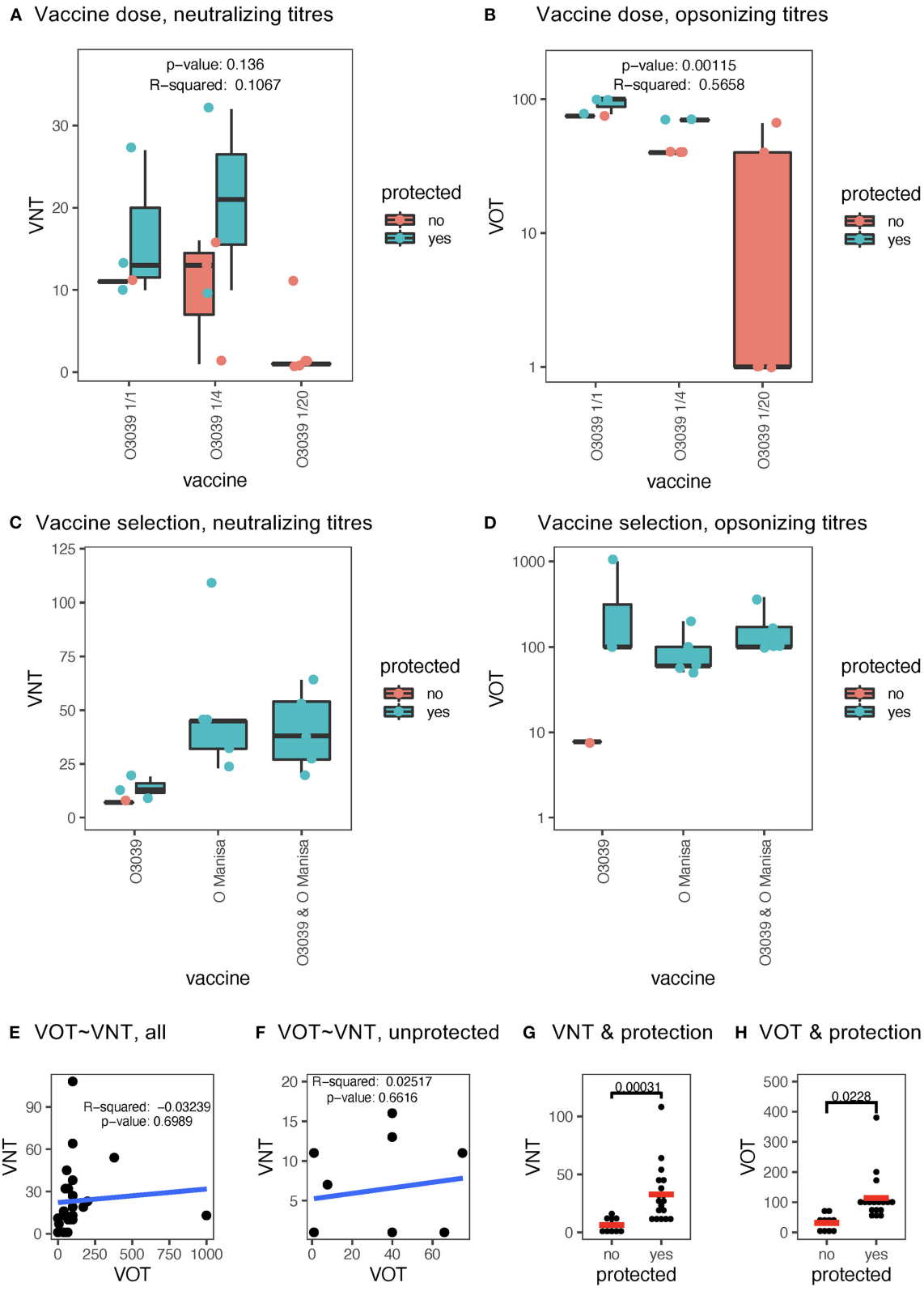


FIGURE 4
Relationship of VNT and VOT to vaccine dose, vaccine matching and protection. **(A)** Shows a boxplot of the VNT of cattle sera collected 21 days post vaccination using a multivalent FMD vaccine (T-1978) at full (:1), quarter (:4), and 1:20 dose (:20). In **(B)**, the same sera were analyzed for VOT. For the VOT's the propidium iodide positive values obtained with pre-immune sera at each serum dilution and from the same animal

(Continued)

FIGURE 4 (Continued)
was subtracted from the sera obtained after vaccination. The p and r^2 values in **(A,B)** originate from a linear regression analyses of the vaccine dose vs. the antibody tests. In **(C,D)**, boxplots of the VNT and VOT, respectively, induced by three different vaccine antigens (x-axis) are shown. Statistical significance was calculated with the Kruskal-Wallis test with corrections for multiple comparisons using the Benjamini and Hochberg test. For the boxplots in **(A–D)** the black horizontal bar shows the median, with the lower and upper hinges of the box plot indicating the 25 and 75th percentiles. The ends of the whiskers show the maximum and minimum values being at most 1.5 times the inter-quartile range the of the hinge. Dots represent values of individual animal (turquoise = protected; red = non-protected). **(E,F)** represent dot plots for the VOT vs. VNT tests for all data points and only for non-protected cattle, respectively (r^2 and p-values from Spearman correlation analyses). In **(G,H)**, shows dot plots comparing protected and non-protected animals for their VNT and VOT, respectively. The p-values were determined by a Mann-Whitney t-test.

correlation between VNT and VOT, when all data were used or only the data from the non-protected cattle (Figures 4E,F, respectively). Nevertheless, when all data available were pooled, significantly higher levels of both VNT and VOT were found in protected compared to non-protected animals (Figures 4G,H).

Taken together, the cattle data indicate that VOT represent a sensitive measurement for vaccine-induced antibodies that correlate with vaccine antigen dosing but does not appear to be superior compared to VNT measurements in prediction protection.

Discussion

Virus neutralization mediated by antibodies represents an *in vitro* measured functional phenotype mediated by high affinity interactions between the antigen binding (Fab) region of the antibody and a viral structure that typically prevents infection of cells and viral spreading within a cell culture. While neutralization assays are most useful and important functional assays often related to the protective value of antibodies, they are unable to measure antibody functions mediated by the Fc region of the antibody molecules through binding to FcR and complement. By binding to FcγR, IgG-antigen complexes can mediate endocytosis or phagocytosis followed by destruction of immune complexes, as well as activation of innate immune functions in Fc receptor bearing cells. These are typically myeloid cells, such as neutrophils, monocytes and macrophages, DC and natural killer cells (26). For FMDV, FcγR-mediated antibody functions related to macrophage or DC activation were previously demonstrated (3–7). In mouse models, these functions have been shown to be important in protection against FMDV (5, 6) and other virus infections (27).

For one of our aims, which was understanding the relationship of neutralization and opsonization of mAbs, we selected the murine RAW264.7 based on their resistance of FMDV infection and the fact that they had been employed for FcγR based functions (28). Of note, in these cells FMDV antibody complexes promoted cell death, as previously observed with bovine monocyte-derived DC (4). With the bovine DC model, antibodies mediated FMDV infection of these otherwise virus-resistant cells, resulting in cell death. In the present study we did not further investigate the mode of RAW264.7 cell death, as we used the cell line solely as a tool to measure opsonizing antibody.

The data with the mAbs permitted two main observations. First, only neutralizing IgG antibodies were identified as able to opsonize FMDV. Second, opsonization reactivity with FMDV strains of varying antigenicity was often broader than neutralization. The anti-O serotype mAb D9 was even found to cross-react with serotype A FMDV isolates.

A possible explanation for these observations is based on the very small size of FMDV of 20 nm. Taking the distance between the two F(ab) fragments of an antibody molecule of 10 nm into consideration, half of the diameter of the virion is covered by only one antibody molecule. Consequently, antibodies binding any structure to the outside surface of the virion with sufficient affinity (or concentration) will sterically inhibit the interaction of FMDV with its receptor on the target cell. It can therefore be argued that antibody affinity, and not *per se* epitope targeting, should represent the main determinant of neutralization strength. Now, while neutralization will require a binding competition between the virus-receptor and the antibody-virus interactions, this competition is absent during opsonization, which is mediated by Fc-FcγR interactions. In addition, myeloid cells and DC expressing FcγR lack FMDV receptors. Therefore, opsonization of FMDV occurs both with high and low affinity antibodies, providing they can bind the surface of the capsid. In contrast, antibodies against the internal side of the capsid or against non-structural proteins of FMDV obviously cannot opsonize intact virions for structural reasons.

Based on these simple and fundamental structural facts, it is understandable that several reports are available that have employed affinity/avidity measurements of sera to predict the protective values following vaccination (29–33). Nevertheless, as explained above while such tests are suitable for high-throughputs testing, they do not quantify other important functions of antibodies, in particular opsonization. For these reasons, we also tested a collection of bovine sera from vaccination-challenge trials in the RAW264.7 cell-based assay. The results obtained confirmed the work with monoclonal antibodies in the sense that opsonization was still observed with higher serum dilution and even with a vaccine antigen badly matched with the challenge

virus. This was in accordance with previous observations demonstrating a high level of cross-reactivity with porcine sera in a DC-based opsonization test (3). In the present study, we found that the correlation of opsonizing antibody titer to vaccine dose was superior than that of neutralizing antibodies. For predicting protection both tests appeared to provide useful but not absolute information. Nevertheless, the number of animals analyzed was too low to permit a final conclusion or even define cutoffs. Future studies are required to address the question is a combination of the two tests could improve vaccine matching and vaccine quality assessment.

In conclusion, the present study demonstrates that low affinity interactions are sufficient to mediate Fcγ receptor-mediated immune functions that could play a role in protective immune responses against FMDV. This novel test developed provides the bases to collect more data to determine the value of such antibodies as correlate of protection following vaccine-induced immune responses against FMDV.

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 experiments were performed in compliance with the Swiss animal protection law (TSchG SR 455; TSchV SR 455.1; TVV SR 455.163). The procedures were reviewed by the committee on animal experiments of the canton of Bern, Switzerland and approved by the cantonal veterinary authority (Amt für Landwirtschaft und Natur LANAT, Veterinärdienst VeD, Bern, Switzerland) under the license numbers BE95/10.

Author contributions

AS designed the study and wrote the manuscript with corrections from EB. HG, RS, ML, SG, and EB performed laboratory work or contributed essential reagents and results. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1033276/full#supplementary-material

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

REVIEWED BY
Manuel Jose Sanchez Vazquez,
Pan American Health Organization, United States
Rebecca Lee Smith,
University of Illinois at Urbana-Champaign, United States

*CORRESPONDENCE
Amy H. Delgado
amy.h.delgado@usda.gov

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Epidemiologic and economic considerations regarding persistently infected cattle during vaccinate-to-live strategies for control of foot-and-mouth disease in FMD-free regions

Shankar Yadav[1,2,3], Amy H. Delgado[2]*, Amy D. Hagerman[4], Miranda R. Bertram[1,3], Karla I. Moreno-Torres[1,2,3], Carolina Stenfeldt[1,5], Lindsey Holmstrom[2] 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]Center for Epidemiology and Animal Health, Animal and Plant Health Inspection Service, United States Department of Agriculture, Fort Collins, CO, United States, [3]Plum Island Animal Disease Center Research Participation Program, Oak Ridge Institute for Science and Education, Oak Ridge, TN, United States, [4]Department of Agricultural Economics, Oklahoma State University, Stillwater, OK, United States, [5]Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, United States

Development of a foot-and-mouth disease (FMD) carrier state following FMD virus (FMDV) infection is a well-established phenomenon in cattle. However, the proportion of cattle likely to become carriers and the duration of the carrier state at a herd or population-level are incompletely understood. The objective of this study was to examine the epidemiologic and economic impacts of vaccination-to-live strategy in a disease-free region or country. We developed and simulated scenarios of FMD spread and control in the US livestock population, which included depopulation for a limited period, followed by a vaccinate-to-live strategy with strong biosecurity and movement restrictions. Six scenarios of FMD spread and control were simulated in the InterSpread Plus (ISP) modeling tool. Data on the number of infected and depopulated cattle (by operation types) from ISP model runs were used to estimate the monthly number of infected but not depopulated (potential carrier) cattle after the infection. Using available literature data on the FMD carrier state, we estimated the monthly proportion of carrier cattle (from infected but not depopulated cattle) over time following infection. Among the simulated scenarios, the median (25th, 75th percentile) number of infected cattle ranged from 43,217 (42,819, 55,274) head to 148,907 (75,819, 205,350) head, and the epidemic duration ranged from 20 (11, 30) to 76 (38, 136) days. In general, larger outbreaks occurred when depopulation was carried out through longer periods, and the onset of the vaccination was late ($p > 0.05$). The estimated proportion of surviving cattle, which were infected and not depopulated and

had the potential to become persistently infected ranged from 14 to 35% of total infected cattle. Production losses in beef and dairy sectors were higher when outbreaks started in multiple states simultaneously, but production losses were small compared to trade losses and consumer avoidance losses. These results can be used to inform the consideration of a vaccinate-to-live strategy for FMD outbreaks and the development of appropriate post-outbreak management strategies. Furthermore, this output will enable a more detailed examination of the epidemiologic and economic implications of allowing convalescent cattle to survive and remain in production chains after FMD outbreaks in FMD-free regions.

KEYWORDS

foot-and-mouth disease, FMD, vaccination, carrier, virus, economic model, simulation model, persistent

Introduction

Challenges in foot-and-mouth disease (FMD) control are numerous and complex. While some of these are social and/or political, many are directly linked to the inherent characteristics of the virus—extreme contagiousness, a wide range of affected hosts, and multiple viral serotypes that do not confer cross-protection (1–4). As a result, the introduction of FMD into a previously free region requires a rapid response, which synergistically addresses all of these factors while considering the practical and logistical aspects of emergency response and the concurrent economic impacts. Given the intricacies of FMD outbreak responses in the United States, both consumers and livestock producers are increasingly expecting that every tool, including vaccination, will be considered in the event of an outbreak.

Vaccination has been an important tool in controlling and eradicating FMD, particularly in endemic settings, but also during outbreaks in previously free countries (1, 5, 6). Vaccination offers several benefits to an emergency response, including reducing the need for large-scale depopulation of animals and the environmental impact associated with the disposal of depopulated animals. However, vaccination as a response tool in a previously FMD-free country also brings challenges, particularly related to decisions on how to manage vaccinated animals. Under the US Department of Agriculture's (USDA) FMD response plan, the management of vaccinated animals varies across four different response strategies — emergency vaccination to kill, emergency vaccination to slaughter, emergency vaccination to live, and emergency vaccination to live without stamping out of infected animals or vaccinates (7). In geographically constrained outbreaks where the disease can be controlled and eradicated in a short amount of time, there are few economic incentives for a vaccinate-to-live strategy. Guidelines suggest trade embargos for six months when the vaccinated animals are allowed to live versus three months when the vaccinated animals are culled. In addition, there are additional resources required to manage vaccinates in a vaccinate-to-live strategy including costs and effort required for testing and tracking vaccinated animals through their productive life spans (8–10).

However, there are benefits to vaccinate-to-live approaches. These alternative approaches to vaccinate-to-kill and stamping-out can reduce the costs of on-farm responses, such as depopulation, carcass disposal, and indemnity paid to producers for animals taken during the response. In addition, genetically valuable animals may be preserved, and disruptions in production chains could be reduced. Lastly, there are scenarios in which vaccinate-to-live is unavoidable, most notably when the number of vaccinated animals exceeds the practical capacity for depopulation. In addition, for countries that are not highly dependent on livestock and meat export markets, the economic advantage associated with reduced trade embargoes of vaccinate-to-kill, may be overcome by the value of protein saved due to reduced depopulation. However, the potential use of vaccinate-to-live is confounded by some of the biological properties of the virus, particularly the carrier state (11).

Foot-and-mouth disease virus (FMDV) is highly transmissible, and while vaccination can substantially reduce transmission and the development of clinical signs, vaccinated animals exposed to the virus may still become infected and develop antibodies (12). In addition, regardless of vaccination status or virus strain, a substantial proportion ($\geq$50% of infected cattle) of infected ruminants will develop a subclinical persistent phase of infection (1, 11). This carrier state is identified by detection of infectious virus (FMDV) in oropharyngeal fluid (OPF) more than 28 days post-infection (dpi) (12, 13). Most genetic diversity of FMDV (14) develops during the carrier state (15); however, the epidemiologic significance of carriers in relation to disease transmission remains unclear (11).

Concerns about the risks posed by FMDV carriers remain due to historical accounts of FMD outbreaks believed to have resulted from transmission between persistently infected carriers and susceptible animals. Although published experimental studies have almost uniformly reported a lack of transmission from carriers to contact exposed animals (16, 17), some studies have demonstrated that OPF from carriers is capable of infecting naïve cattle (18). The duration of the carrier state is likely influenced by numerous factors, as evidenced by the diversity of reported lengths in the literature (19–22). For example, the carrier state has been shown to last for up to 4–5 years in African buffalo (*Syncerus caffer*), 4 months to 3.5 years in cattle (19, 20), and up to 9 months in sheep (21–24). The discrepancies in the duration of the carrier state may be due to differences in virus (serotype, strain or virulence), host (genetic differences, nutritional or immunological status), environment (climate, mineral intake), and methods of virus detection across studies (14).

Despite the limited epidemiologic evidence, carriers have been recognized as a barrier to re-establishing disease freedom following an outbreak (8, 25). Proving disease freedom is a critical step to the gradual or immediate resumption of international trade. As a result, when vaccination is used as part of the outbreak response strategy, vaccinated herds must be monitored and managed to reduce the risk associated with the presence of infectious virus. With increasing expectations from livestock producers and the general public regarding the use of vaccination, additional information on the duration and relevance of the carrier state is needed in order to design monitoring plans for vaccinated herds, particularly for large or complex livestock operations. While some work has been done to model the prevalence of carriers after reactive vaccination, recent studies offer new insights into the prevalence and duration of the carrier state (19, 26).

The objective of this study was to examine the epidemiologic and economic impacts of alternative approaches for FMD control that limit the use of depopulation, while taking into account the presence of carrier cattle over time in the affected population. To achieve this, we developed and simulated scenarios of FMD spread and control in the US livestock population, which included depopulation for a limited period, followed by a vaccinate-to-live strategy with strong biosecurity and movement restrictions. Based on currently available data from the literature, we estimated the prevalence and duration of the carrier state over time for varied cattle production systems. The resulting scenarios were also used to estimate the economic consequences of deviating from a stamping-out strategy, including production losses in infected herds, market impacts, and response costs. Many questions remain about the social and trade ramifications of managing carrier and vaccinated animals.

Materials and methods

Study population

The FMD model scenarios developed in this study were based on the national livestock population of the United States. The farm data was obtained using a micro-simulation model called the Farm Location and Animal Population Simulator (FLAPS), which generates synthetic farm populations with production types, herd sizes, and geographical coordinates, while taking into account livestock census data (27). To account for the geographical diversity in demographics of livestock operations, animals, and their movement, the mainland of the United States was divided into five discrete regions: Pacific (PC), Midwest (MW), Great lakes (GL), North East (NE) and South East (SE). Altogether 1.82 million livestock operations, which included bison (0.14%), cattle (84%), goats (7%), sheep (3%), and pigs (0.2%) were included in the model. Operation characteristics, including movement destinations and frequencies, varied based on the herd size (Table 1 and Supplementary Table S1). Cattle farms were categorized into four different production types based on the age and management practices of animals—cow calf, stocker, dairy, and feedlot (Supplementary Table S1). Among cattle farms in the population dataset, 2% were classified as large farms ($\geq$200 head for cow calf, $\geq$500 head for dairy, and $\geq$1,000 head for feedlot). The stocker farms were identified as small operations. The cow calf (small: 96%, large 4%), dairy (small: 95%, large: 5%), feedlot (small: 93%, large: 7%), and stockers (all small) constituted 47, 4, 2, and 47% of the total cattle farms in the population file.

Among the pig farms, 83% were small and 17% were large farms. There were seven different operation types included in the population file: small swine enterprises (75%), farrow to feeder (1%), farrow to finish (6%), farrow to wean (2%), grow to finisher (13%), nursery (2%), and others (2%) (Table 1). Large operations were considered to be those with $\geq$1,000 head for all production types (Supplementary Table S1). The small swine enterprises operations were backyard and hobby swine farms, with $<$100 heads. Bison, goat, and sheep operations were recognized as small farms (Table 1 and Supplementary Table S1). Dealers were also included in the model. Dealers were considered to be small operations which could have different species present that represented frequent aggregation and dispersion points outside of normal livestock markets.

Epidemic model description

Disease spread

InterSpread Plus (ISP) *version 6.0* model software was used to simulate the between-herd spread of FMD in the US livestock population (28). ISP is a state-transition, stochastic and spatial modeling tool for the simulation of FMD and other similar

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TABLE 1 Descriptive statistics of foot-and-mouth disease susceptible livestock population incorporated in the InterSpread Plus (ISP) model for the simulation of model scenarios.

Farm type	Operation type	Number of farms		Median (5th, 95th) herd size	
		Small	Large	Small	Large
Cattle	Cow calf	700,655	25,980	14 (2, 103)	293 (210, 1,038)
	Dairy	60,715	3,342	40 (1, 231)	1,257 (575, 4,071)
	Feedlot	24,813	1,772	59 (12, 430)	2511 (1,072, 25,031)
	Stockers	717,330		9 (2, 161)	
Bison	Bison	2,547		10 (1, 223)	
Goat	Goat	127,954		11 (6,58)	
Sheep	Sheep	87,935		13 (3, 148)	
Swine	Small swine enterprises	47,062		6 (1, 45)	
	Farrow to feeder	353	119	193 (105, 765)	3,921 (1,387, 28,739)
	Farrow to finish	2,234	1,480	289 (110, 802)	4,291 (1,331, 15,152)
	Farrow to wean	322	762	309 (106, 848)	4,501 (1,283, 17,126)
	Grower to finisher	2,134	6,063	466 (134, 850)	4,373 (1,394, 13,760)
	Nursery	203	1,030	595 (160, 880)	4,638 (1424, 14,219)
	Others	271	945	320 (107, 815)	4,362 (1,411, 13,472)
Dealer	Dealer	3,427		56 (5, 114)	

diseases (29). The unit of interest in the model was individual livestock operations. FMD epidemics were either simulated from a single farm or two farms depending on the model scenarios. The simulation proceeded by the time-step of 1 day. The herd-level disease parameters (such as incubation phase duration and maximum infectiousness) were assigned stochastically in the models specific to animal species. The herd-level incubation phase durations (in days) assigned were Poisson (3.8) for bison (30), Poisson (5.9) for cattle (31), Poisson (6.59) for goat and sheep (30), and Poisson (5.58) for pigs (32). The infectious phase duration was assigned to be [Triangular (30, 34, 42)] days. Model parameters were based on transmission characteristics of FMDV serotype O, and all outbreaks were assumed to start in January, for the purposes of livestock placement. Depending on the model scenarios, disease spread initiated within a single state (California or Texas) from a single farm, or within two states simultaneously (California and Texas) from two farms (Table 2).

Transmission of FMDV between farms could occur through multiple routes, including direct contacts such as animal movements, indirect contacts, such as shared vehicles or personnel, airborne transmission, and local area spread at short distances. Movement frequencies, distances, and destination types were unique to each production type, and these varied for movements to markets (Supplementary Table S2), and by the region of farm location and size (small vs. large) (Table 3, Supplementary Tables S3–S6). The daily probability of transmission of FMDV after contact between an infected and susceptible farm was estimated based on the hypergeometric probability of shipping at least one infected animal off of an infected farm given the average herd size, shipment size, and the number of infected animals in a herd on a given day. This parameter was estimated and assigned for each of the animal species (bison, cattle, goat, sheep, pig), dealers, and markets (Supplementary Figure S1).

Indirect contacts between infected and susceptible farms included connections such as shared farm workers, veterinarians, vehicles, equipment. Indirect contacts were modeled as high or low risk based on the potential for viral contamination and contact with animals. High-risk indirect contacts included veterinarians, customers, dealers, employees with livestock at residence, extension agents, livestock haulers including those used for dead box pick-ups, and manure haulers. Low risk indirect contacts included commodity/feed trucks, shared equipment, drivers of livestock haulers, nutritionist, feed company consultants, other vehicles such as postal deliveries, and visitors. The indirect contact rates assigned in the ISP model scenarios are summarized in Table 4. The probability that an indirect movement occurs within a certain distance varied by production type, with most movements occurring within 20 km of the original farm (Supplementary Table S7). The probability that infection occurs was estimated for high risk and low risk indirect contact movements separately, and we assumed that low risk indirect contacts followed basic biosecurity protocols, leading to a reduced risk of disease transmission (Supplementary Figure S2).

Local area spread was assumed to occur at short distances (within 4 km of an infected farm) through insects, rodents, or other unknown factors. The probability of disease transmission

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TABLE 2 Descriptions of the foot-and-mouth disease model scenarios.

Characteristics	Scenarios					
	1	2	3	4	5	6
Index herd location	CA	TX	CA & TX	CA	TX	CA & TX
Index herd type	Large dairy	Large feedlot	Large dairy & large feedlot	Large dairy	Large feedlot	Large dairy & large feed lot
Index herd size	3,596	42,806	3,596 & 42,806	3,596	42,806	3,596 & 42,806
Onset of depopulation	Day 14					
Depopulation duration	28 days				14 days	
End of depopulation	Day 42				Day 28	
Onset of vaccination	Day 43				Day 29	

CA: State of California, TX: State of Texas. In scenarios 3 and 6, the epidemic was initiated on day 0 from a farm in CA and 7 days later, another farm in TX was modeled to infect.

TABLE 3 The distributions of the average frequency of direct contact movements originated from respective farm types and operations in the various regions of the United States.

Farm/operation	Frequency per day
Bison	Poisson (0.0021)
Cow calf (large) in MW & PC region	Poisson (0.009)
Cow calf (large) in another region	Poisson (0.005)
Cow calf (small) in MW& PC region	Poisson (0.004)
Cow calf (small) in another region	Poisson (0.002)
Dairy (large)	Poisson (0.0986)
Dairy (small)	Poisson (0.0356)
Dealer	Poisson (0.1471)
Feedlot (large)	Poisson (0.03)
Feedlot (small)	Poisson (0.03)
Goat	Poisson (0.0022)
Sheep	Poisson (0.0026)
Stockers	Poisson (0.007)
Small swine enterprises	Poisson (0.0023)
Swine farrow to feeder	Poisson (0.1049)
Swine farrow to finish	Poisson (0.0209)
Swine nursery	Poisson (0.0868)
Swine farrow to wean	Poisson (0.4068)
Swine grow to finish	Poisson (0.0015)
Swine other	Poisson (0.0413)

MW, Mid-West; PC, Pacific region of the United States.

due to local spread was modeled separately based on the status of the infected farms. Undetected, infected farms were given highest risk for disease transmission in compared to detected but not depopulated farms, or depopulated farms which still needed to complete carcass disposal (Supplementary Figures S3A–C). The airborne spread of FMDV was assumed to occur within 10 km of infected swine farms after the onset of clinical signs, with the probability of transmission declining over distance (Supplementary Figure S3D).

Control measures

Initial detection was fixed on day 11 for all scenarios and after that it was based on background passive surveillance. The probability of detection during passive surveillance varied by days post-onset of clinical signs and species affected, with swine having the highest probability of detection and dealers having the lowest (Supplementary Figure S4). Following detection of an infected farm, control measures were initiated on day 1 after the detection, including the establishment of control zones. Two types of radial control zones (inner and outer) were included in the model. The inner control zone was from 0 to 10 km of the infected farms, and the outer control zone was from 10 to 20 km away from the infected farms. After the first detection, direct contact tracing, indirect contact tracing, and surveillance of all farms within the 10 km zone of the detected farms were initiated. Movement restrictions were imposed on all farms within inner control zones immediately after detection. The percentage of animal movements restricted ranged from 60% for swine up to 85% for cattle, while only 25% of indirect contact movements were restricted.

In all scenarios, depopulation of infected animals was initiated following detection on day 14; however, the duration of depopulation efforts varied between 14 and 28 days (Table 2). While initial depopulation capacity was assumed to be small (4 farms/day for the first 2 days of control activities), depopulation capacity ramped up quickly and varied by operation type. From day 3 to the end of the depopulation effort, the assigned depopulation capacity distributions were Betapert I4, 6, 10) for large cattle farms, Betapert (2, 4, 6) for small cattle, goat and sheep farms, Betapert (4, 8, 28) for large feedlots, Betapert (1, 2, 4) for large swine farms, and Betapert (2, 2, 6) for small swine farms. After 14 or 28 days of depopulation efforts, all depopulation was ceased and vaccination was initiated at day 29 or 43 post-introduction, respectively (Table 2). All cattle, bison, and swine in the 10 km zone of the detected farms were vaccinated at the rate of

TABLE 4 Distributions of indirect contact rates among the livestock farms in the United States used to incorporate in the InterSpread Plus model.

Operations	Pacific region		Other regions	
	High risk	Low risk	High risk	Low risk
Bison	Poisson (1.9167)	Poisson (0.5747)	Poisson (1.9617)	Poisson (0.5747)
Cow calf (large)	Poisson (0.11)	Poisson (0.2244)	Poisson (0.11)	Poisson (0.2244)
Cow calf (small)	Poisson (0.053)	Poisson (0.098)	Poisson (0.053)	Poisson (0.098)
Dairy (large)	Poisson (1.5873)	Poisson (0.3891)	Poisson (1.5873)	Poisson (0.3891)
Dairy (small)	Poisson (0.4596)	Poisson (0.1252)	Poisson (0.4596)	Poisson (0.1252)
Dealer	Poisson (0.147)	Poisson (0.164)	Poisson (0.147)	Poisson (0.164)
Feedlot (large)	Poisson (1.48)	Poisson (6.46)	Poisson (1.48)	Poisson (6.46)
Feedlot (small)	Poisson (0.15)	Poisson (0.28)	Poisson (0.15)	Poisson (0.28)
Stockers	Poisson (0.006)	Poisson (0.017)	Poisson (0.006)	Poisson (0.017)
Goats	Poisson (0.335)	Poisson (0.0452)	Poisson (0.335)	Poisson (0.0452)
Sheep	Poisson (01961)	Poisson (0.0428)	Poisson (0.1961)	Poisson (0.0428)
Small swine enterprises	Poisson (0.002)	Poisson (0.0940)	Poisson (0.002)	Poisson (0.0940)
Commercial swine (large)	Poisson (2.214)	Poisson (1.3053)	Poisson (2.214)	Poisson (1.239)
Commercial swine (small)	Poisson (0.3486)	Poisson (0.3402)	Poisson (0.3387)	Poisson (0.2119)

85,000 cattle, 1,000 bison, and 14,000 pigs per day. Sheep and goats were not vaccinated in these scenarios. All vaccinated cattle, bison, and pigs were assumed to live out their normal production periods.

FMD model scenarios

Based on the previously described model structure, six different model scenarios were simulated (Table 2). Briefly, the model scenarios were run from either one-farm (scenarios 1, 2, 4, and 5) or two-farms (scenarios 3 and 6). The characteristics of index farms differed in herd size, location, and operation types. The detected farms in a scenario were depopulated for either for 28 days (scenario 1, 2, 3) or 14 days (scenario 4, 5, 6). Vaccination was initiated after the cessation of depopulation activities, i.e., at 43rd day in scenario 1, 2, and 3 and 29th day in scenario 4, 5, and 6. A shorter duration of depopulation in the model scenarios was designed intentionally to allow vaccination-to-live strategy and thereby to estimate the risks and challenges due to emergence of persistence infection in cattle of the US livestock population. The models were simulated for 200 iterations; each iteration was simulated for 730 days (maximum). The major outcomes of the ISP model were to estimate the epidemic duration, epidemic size, number of infected and depopulated farms and animals, and the number of infected cattle potentials for the emergence of the persistent infection. To test for significance among model scenarios we used the Kruskal-Wallis test with Bonferroni correction for multiple comparison.

Estimation of persistently infected cattle

In this study, we quantified the number of persistently infected cattle and extinction of persistent infection over time after infection. First, the numbers of infected but not depopulated cattle (all cattle, cow calf, dairy, and feedlot and stockers combined) were estimated for each month after onset of infection using the infection data and depopulation data from ISP model outcomes for each of the scenarios. These were the monthly numbers of cattle with potential for persistent infection. Second, using literature data (Table 5), an equation ($y = 0.59–0.021x$) was derived to estimate prevalence of persistently infected cattle over the succeeding month after the infection. In the equation, y is the prevalence of the persistent infection and x is the month after infection. The equation demonstrated that the prevalence of persistently infected cattle was 57% after the first month of infection (28 days post infection), 55% in the second month (56 days post infection), and consequently the persistent infections were cleared by 29 months post-infection. This equation was used to estimate the numbers of persistently infected cattle at a month after FMD infection. For example, in scenario 1, altogether 28,505 cattle remained that had been infected and not depopulated; these are the cattle with potential of establishing persistent infection after 28 days of infection. Using the equation, it was estimated that after the first month of infection, 57% of these cattle (16,248) were persistently infected. When these cattle reached the third month post infection, 55% (15,678) remained persistently infected indicating that 570 cattle (2%) had cleared the persistent infection within this period. Consequently, all of the persistently infected cattle in

TABLE 5 Published studies from which data on the prevalence of persistently infected cattle were extracted to derive an equation.

References	Prevalence	Months after outbreak	Species
de Carvalho Ferreira et al. (33)	10.8%	12 months	Cattle and buffalo
Hayer et al. (34)	38%	7.5 months	Dairy cattle
	14%	10.5 months	Dairy cattle
Hayer et al. (20)	67%	6 months	Dairy catt
	55%	14 months	Dairy cattle
	51%	11 months	Dairy cattle
Tenzin et al. (17)	62%	28 days	Cattle
	52%	7 months	Cattle
Hedger (35)	20%	7 months	Cattle

this scenario had cleared infection by 29 months post onset of infection.

ISP model outcomes inputs for economic model

In order to facilitate comparison of economic impact results to epidemiologic outcomes, an index was created based on ranking iterations by key outcomes from the ISP model within each scenario. The index was comprised of an ordinal ranking herds infected, head infected, duration, and states affected, and creating an index for each iteration based on equal weighting of each epidemiological outcome. The median, 25th percentile, and 75th percentile based on this index was analyzed in the United States Partial Equilibrium Model (36). Although the economic model has many outcomes, the ones reported for this study are the change in returns to capital and management from livestock and agricultural product sales, the change in returns to dairy cattle and milk, and the change in returns to beef cattle and beef. In each case, the scenario specific values for each quarter are subtracted from the quarterly no-disease base from 2019 to 2021.

Cost of response and economic model

Economic impacts for animal health outbreaks were categorized as production losses, costs of disease response on farms, and market impacts. Production losses included the loss of animals available to the market due to mortality and depopulation, as well as reduced weight gain, milk production and fecundity that resulted from clinical infection. Observations of production losses in the published literature were used in the absence of observations from FMD outbreaks in the US (Table 6). Details on the production loss parameters can be found in Supplementary material.

The ISP disease spread results in infection by herd type, depopulation by herd type, duration of outbreak, and states with infected livestock, which were used as inputs in the economic modeling. Losses were tracked across time based on the quarter in which infection occurred for each herd in ISP results. The reduced beef supply available from fed cattle, the increased beef supply from culled dairy cows that aborted, and the reduced fluid milk supply for processing were incorporated as production shocks, along with the meat and milk removed from supply due to depopulation and calf deaths, in the US Partial Equilibrium Model by quarter (36).

Total on-farm costs of disease response included surveillance, depopulation and indemnification of depopulated animals, disposal of carcasses and potentially contaminated materials, cleaning and disinfection of facilities, and vaccination. Response costs were estimated in US dollars per head by production type. The response cost burden to producers for farm labor and equipment used to manage disease was not included, recognizing that some costs are part of normal herd management and that not all of the costs to producers can be foreseen. In addition, we recognize the existence of additional costs in an outbreak, but this study will focus solely on those costs associated with disease response activities on farms that are designated as infected, vaccinated and/or under surveillance at some point in the outbreak and recovery period. Total on-farm costs of disease response was carefully differentiated from the "total cost of the outbreak" which would include a variety of other costs to producers, agribusinesses, and the government. For example, the cost to a feed company of cleaning and disinfecting trucks making feed deliveries in surveillance zones, or the cost of state and federal animal health laboratory personnel. Thus, this estimate is limited to a taxpayer cost for on-farm response activities. Additional details on costs of on-farm response can be found in Supplementary materials document.

In addition to production losses and costs of response on farms that were directly impacted, losses may also accrue due to market responses. It is unknown how US trade partners or domestic consumers would respond to a vaccinate to live strategy, but literature and historical experiences for other diseases offer a place to begin developing market shocks for

TABLE 6 Published studies from which data on production and demand impacts in cattle were extrapolated to estimate economic impacts of an FMD vaccinate-to-live without stamping out response.

References	Production loss	Description	Type of loss
Ferreira et al. (37)	−1.4%	Reduced rate of gain during clinical infection. Normal weight gain post- clinical infection	Average daily weight gain in beef cattle
Lyons et al. (38)	−35% 5% increase in lbs milk per month	During clinical infection 7 month recovery period in which milk production increases steadily from the 35% loss until it is back to normal. Uniform recovery gains were assumed.	Pounds of milk production in dairy cattle Pounds of milk production in dairy cattle
Doel (39)	−10%	Rate of abortion in pregnant cows during clinical infection	Dairy and beef cows and heifers pregnancy losses
Rufael et al. (40)	−2.8%	Death rate in calves under 2 years of age	Unweaned beef and dairy cattle
Mu et al. (41)	−0.5%	Consumer avoidance of beef, pork and lamb due to risk perception.	Beef, pork and lamb domestic consumption

both trade embargoes and domestic consumer avoidance of animal products from susceptible species. Trade embargoes for beef, pork and limited dairy products were derived from the literature and World Animal Health Organization (OIE) trade guidelines (8). This is consistent with Schroeder et al. (42), the only other published study to compare vaccinate to live and vaccinate to kill. Domestic demand can also be affected by consumer avoidance, although these effects have been found to be relatively small in percentage terms and of short duration as in Mu et al. (41) examination of highly pathogenic avian influenza and bovine spongiform encephalopathy Based Mu et al.'s findings, a shallow, negative shock (−0.5%) was imposed to US beef and pork demand that was sustained through the outbreak. Recovery was allowed to occur quickly afterward. Although not modeled directly, there may actually be a positive perception by consumers of a vaccinate to live strategy since images of mass depopulation was associated with a negative public response in the UK in 2001 as found in Thompson 2002 (43). The impact of production losses, depopulation and death losses, trade embargoes and domestic consumption losses on markets were estimated using the United States Partial Equilibrium Model (USPEM) (36). This model is a national price-endogenous economic model that endogenously estimates changes in market prices and economic welfare in calendar quarter time steps. Production losses and demand shocks, as described above, were imposed on the model as exogenous shocks. Output includes market prices and producer welfare, which is defined as the difference between the schedule of prices at which producers are willing and able to supply a good in varying quantities supplied, and the price they actually realize in the market for those quantities supplied. It is different from profit in that producer welfare accounts for fixed, or sunk, costs of production.

Results

Livestock demographics

The simulation model consisted of 1.82 million livestock farms distributed across animal production types as follows: 0.14% (bison), 84% (cattle), 7% (goat), 5% (pig), and 3% (sheep). Of the 84% designated as cattle farms, cow-calf and stockers made up 47% each, while dairy and feedlot farms made up 4 and 2%, respectively. Of the 5% designated as pig farms, 75% were small swine enterprises and 13% were grower to finisher farms. The majority of cattle and pig farms (98% each) were small holdings (Table 1), and the herd size across farm types ranged from: 1 to 50,528 head (bison), 1 to 100,734 head (cattle), 1 to 4,837 head (goat), 1 to 422,475 head (pig), and 1 to 48,160 head (sheep).

Number of infected and depopulated farms and animals

Among the simulated scenarios (Table 2), the median number of infected farms ranged from 5 to 38 farms, whereas the median number of infected animals ranged from 43,256 to 150,572 animals (Figure 1). Across the six scenarios, we found that the number of infected farms estimated from scenarios 2 and 5 were significantly smaller than the other scenarios ($p < 0.0001$), while the comparison among the remaining scenarios showed no significant difference in outbreak size ($p > 0.05$). Additionally, the number of infected farms was not significantly different between the 28- and 14-day depopulation strategies ($p = 0.705$). However, a

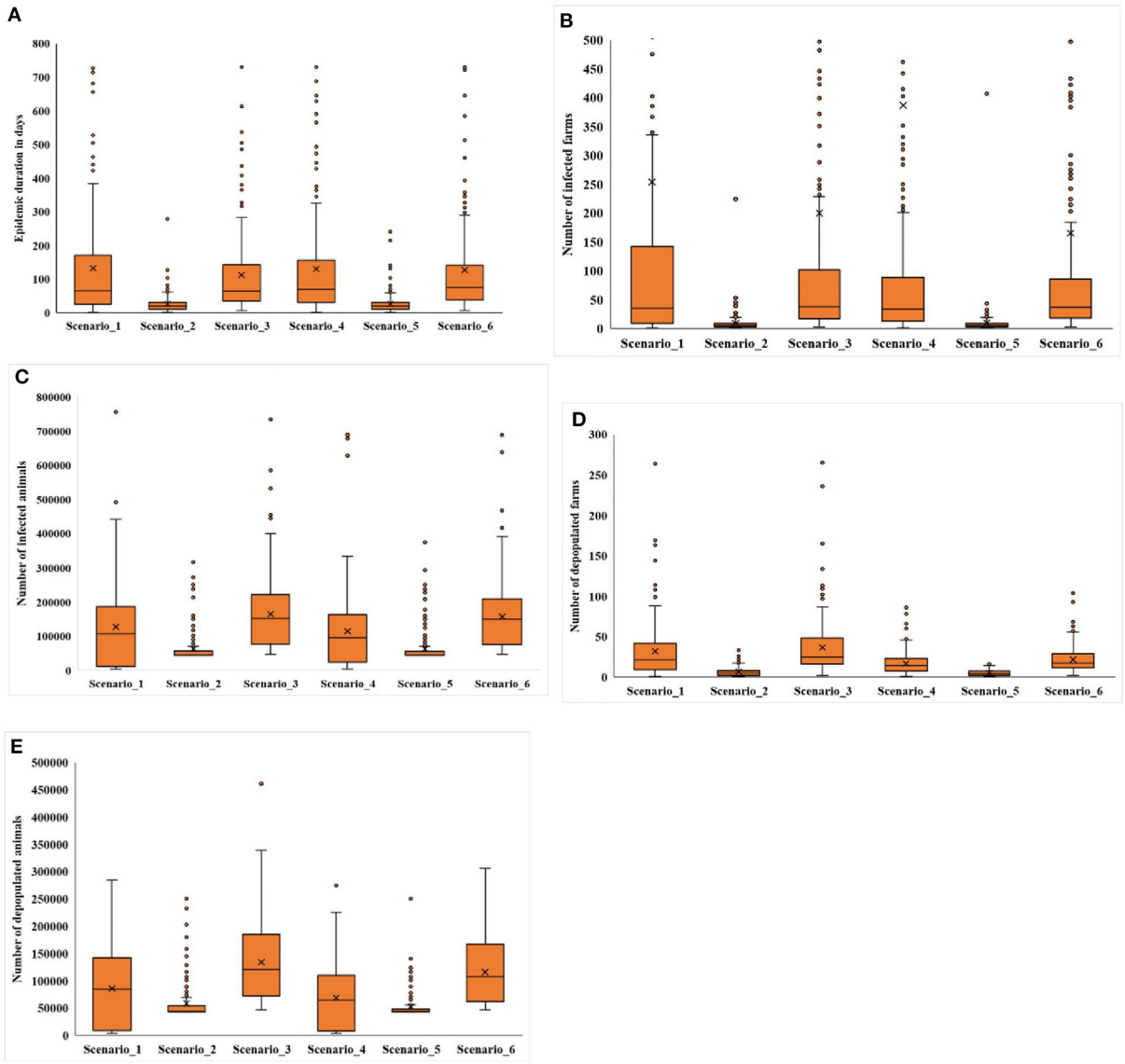

FIGURE 1
Box plots for **(A)** epidemic duration, **(B)** number of infected farms, **(C)** number of infected animals, **(D)** number of depopulated farms, and **(E)** number of depopulated animals obtained from InterSpread Plus model scenarios. The middle, lower, and the upper line of the box represents the median, 25th, and 75th percentile. The whiskers represent 1.5 times of the interquartile range. The sign × represents the mean, and the dots are the outliers detected by the analytic tool.

slightly higher number of animals were infected in the 28-day depopulation scenarios when compared to the 14-day depopulation scenarios (p =0 .54).

The median number of depopulated farms among the simulated scenarios ranged from 4 to 25 farms (Figure 1), and the number of depopulated farms was significantly higher in the scenarios using the 28-day depopulation strategy ($p < 0.05$) except in scenarios 2 and 5 ($p = 0.4538$). The median number of depopulated animals among the simulated scenarios ranged from 43,162 to 120,282 animals, while the number of depopulated cattle ranged from 43,134 to 106,625 head. Like depopulated farms, a significantly higher number of animals ($p = 0.0058$) and cattle ($p = 0.0073$) were depopulated under the 28-day depopulation strategy compared to the 14-day strategy.

Epidemic duration

The median epidemic duration ranged from 20 to 76 days among the simulated scenarios (Figure 1). The shortest

epidemic duration was observed when the outbreak was initiated in a feedlot herd (scenario 2 or 5), while the longest epidemic duration occurred when infection was initiated in two herds simultaneously (scenario 6) ($p < 0.0001$). The epidemic duration was not significantly affected by the duration of depopulation ($p = 0.4966$).

Persistently infected cattle

In this study, we simulated a shorter duration depopulation strategy to examine scenarios in which exposed cattle could remain in the population for the full duration of their production life. It was found that 20–38% of infected cattle were not depopulated and had the potential to progress to the FMD carrier state, which is defined as maintaining detectable virus after 28 days of infection. Amongst non-depopulated and infected cattle, 57% transitioned into the carrier state after the first month of infection with the potential to remain in the population from 30 to 52 months post infection. The monthly cumulative number of persistently infected cattle, across farm types, is a function of the total number of infected animals, and correspondingly, these values were highest in scenarios where the outbreak size was large (Scenarios 1, 3, and 6) (Figure 2). Over the epidemic period, the cumulative number of infected cow-calf, dairy, and feedlot/stockers cattle ranged from 98 to 2,266 head for cow calf; 332–5,333 head for dairies; and 8,637–32,953 head for feedlot/stocker operations. The estimated number of potentially persistently infected cattle varied across scenarios, consistent with differences in outbreak size and depopulation capacity within a farm type. For example, in scenarios 2 and 5, around 5,004 cattle were estimated to be persistently infected, compared to an estimate of 18,655 in scenarios 3 and 6, after the first month of the outbreak [Figure 3 (all cattle)]. Among the different types of cattle farms, we found that feedlots and stocker farms accounted for the highest proportion of cattle with the potential to become persistently infected, followed by dairies and cow calf operations (Figure 3). These findings are consistent with the breakdown of the overall simulated cattle population by production setting, while also reflecting the difficulties of depopulating large herd sizes seen in US feedlots.

Economic model outcomes

Lost beef production due to clinical disease was small for any given quarter (<1% per quarter), but losses aggregated over time as outbreak duration increased. The aggregate milk losses were larger than beef losses due to the assumed time to milk production recovery, however, the milk production losses represented a small proportion of the total milk produced in the United States (<1%) (Table 7). Scenarios that incorporated the Texas panhandle resulted in minimal milk loss, with ∼22% of iterations, originating in the Texas panhandle, resulting in disease spread to dairy production sites. The median (25th, 75th percentile) pounds of reduced milk production, due to disease, ranged from 3.33 million lbs. (41,206 lbs., 6.74 million lbs.) to 8.15 million lbs. (3.10 million lbs., 16.63 million lbs.) among scenarios. For context, annual US production of fluid milk was 217.6 billion lbs. in 2018 (44). Scenarios originating in California (scenarios, 1 vs. 4 and 3 vs. 6) resulted in statistically significant milk losses ($p = 0.044$) at the 5% level between scenarios 1 and 4 (depopulation at 28 vs. 14 days).

Once culling was accounted for, longer-term impacts on beef production are more ambiguous with no clear-cut outcome across all scenarios. When evaluating the near term, cattle being fed for slaughter that were assumed to have experienced reduced ADG resulted in aggregate beef reductions ranging from 179,413 lbs. (177,841 lbs., 225,340 lbs.) to 426,915 lbs. (237,195 lbs., 589,874 lbs.) of beef never realized. Further complicating beef production impacts is the fact that many dairy bred steer calves and cull dairy cows are fed out for the beef market, meaning that both CA and TX have robust beef production. The estimated beef losses from scenarios involving both the Panhandle of Texas and California dairy production (3 and 6) were significantly larger when compared to the losses in the Panhandle scenarios alone (2 and 5) ($p < 0.0001$). However, when comparing the two depopulation strategies of 28- or 14- days, we did not find a significant difference in beef losses ($p = 0.381$).

The median cost of disease response in US dollars ranged from $76 million ($70 million, $93 million) to $230 million ($139 million, $339 million) (Table 7). For all scenarios except scenario 4 (CA, 14-day depopulation), indemnities paid on depopulated livestock represented the largest portion of outbreak response cost. When outbreaks originated in the Texas Panhandle indemnities accounted for up to 64% of the response cost. The median indemnity per outbreak ranged from $47 million ($12 million, $82 million) to $119 million ($71 million, $163 million). This was not an unexpected outcome considering that the region of interest was in a cattle dense area and fed beef cattle and lactating dairy cows are highly valued on a per head basis compared to other livestock types.

During the outbreak the second highest total on-farm response cost category was surveillance for all scenarios except scenario 4 (CA, 14-day depopulation), where it was the highest response cost category. Surveillance cost ranged from $8 million ($4 million, $13 million) to $62 million ($29 million, $106 million). With the reduced duration of depopulation to either 14 or 28 days, the cost of depopulation, disposal, and cleaning and disinfection did not represent a large portion of the total on-farm response cost. To illustrate this point, depopulation, disposal and cleaning and disinfection represented 16% of the estimated total on-farm response cost per head for each animal infected in this study; however, in these scenarios they represent only 12% and 10% of overall total on-farm response

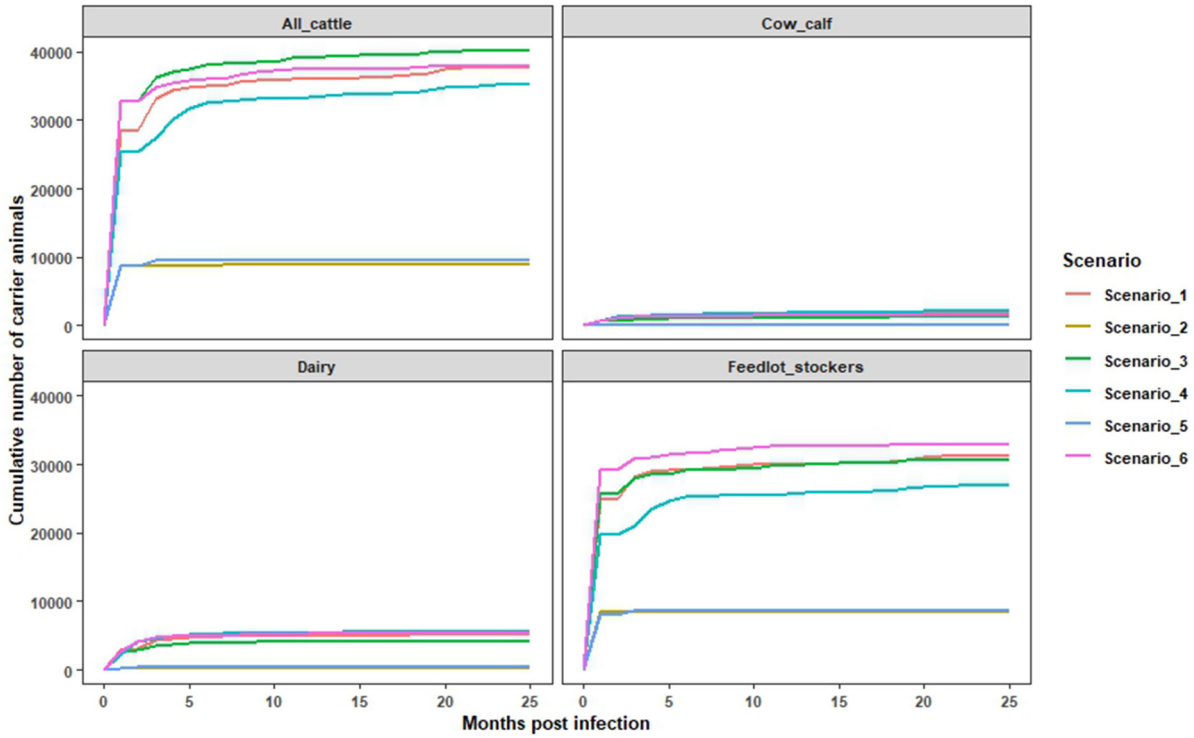

FIGURE 2
The estimated monthly cumulative numbers of infected but not depopulated cattle, corresponding to potential FMDV carriers. The estimates were obtained using the infection file and depopulation file data from InterSpread Model specific to all cattle, cow calf, dairy, and feedlot and stocker combined.

cost on average for 14 day scenarios and 28 day scenarios respectively. Spending shifts toward surveillance instead. As would be expected, the outbreaks with the lowest on-farm response cost resulted from the shortest and smallest outbreaks, which occurred in the Texas panhandle scenarios. Whereas, the highest on-farm response cost resulted from the longest and largest simulated outbreaks, which occurred in scenarios started in both CA and TX. Comparisons of total on-farm response cost between scenarios produced significantly different results ($p < 0.05$) for all scenarios based on start location and depopulation strategy except those originating in the Texas Panhandle.

Quarterly economic impacts were estimated over a 4-year period (2018 to 2021) and aggregated (Table 7). When comparing the median (25th, 75th) lost returns to capital and management (producer welfare), every scenario resulted in a statistically significant change from the pre-disease economic baseline ($p < 0.05$). Over the course of the 4-year period the markets did not recover to the pre-disease forecast of production returns for any of the 6 simulated scenarios. It has not been uncommon to see multi-year recovery periods in other countries after FMD outbreaks (45).

The pre-disease baseline quarterly returns to capital and management from sales of agricultural products averaged $23 billion. The median (25th, 75th percentile) outbreaks' average quarterly economic impacts for the lost returns to capital and management from sales ranged from $1,611 million ($1,595 million, $1,610 million) to $2,097 million ($1,618 million, $2,508 million), representing an average quarterly reduction of 7% to 11% in returns to producers and agribusinesses, across the agricultural sector. However, there was not a significant difference in the economic impacts, when evaluated at the median, 25th, or 75th percentile outbreaks. This could result from the influence of the export and consumer demand shocks on the economic impacts, which were similar across scenarios because of insignificant differences in epidemic duration.

In comparison, when the individual livestock industries were examined, the beef cattle sector's quarterly returns to beef cattle production were reduced in all 6 scenarios. Those reductions in returns ranged from $403 million ($397 million, $510 million) to $649 million ($402 million, $522 million). The swine and pork sector had the greatest loss to capital and management on sales of agricultural products at 61–64% while, the beef sector return reductions ranged from a 15–23%, when compared to the pre-disease baseline. The beef sector losses were the second highest industry specific component to the total (25–26%) followed by the red meat processing sector (14–17%). In contrast, the median

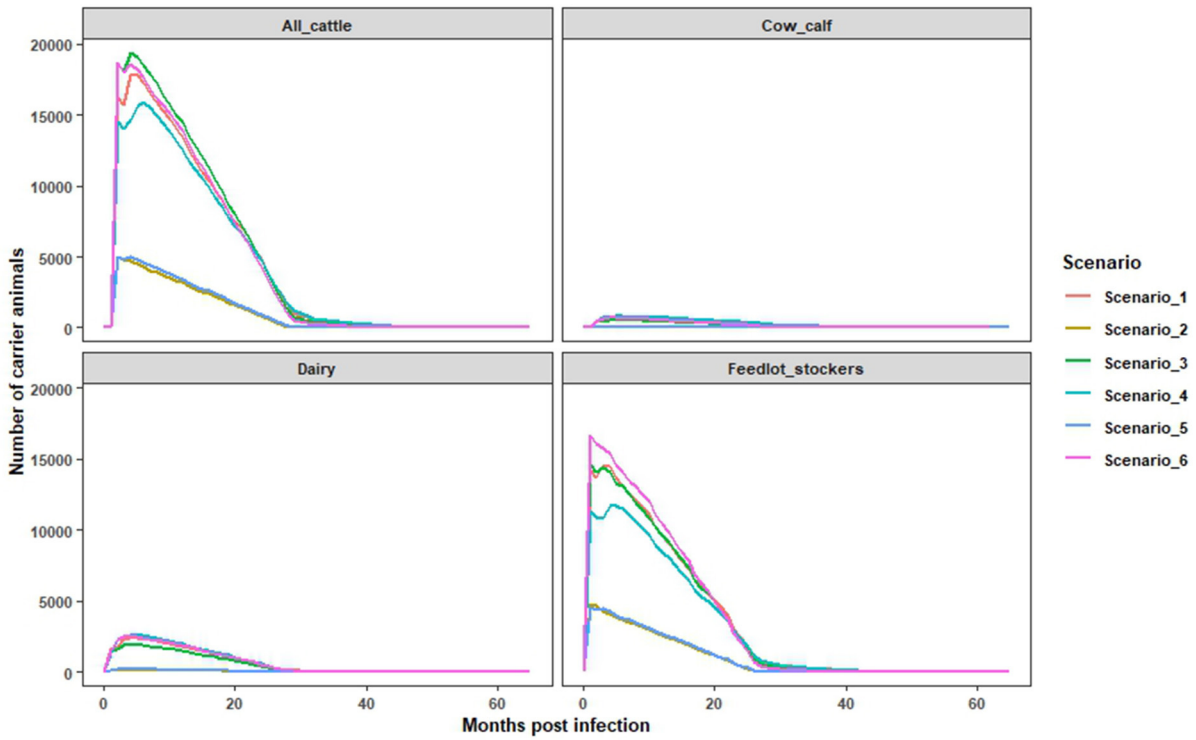

FIGURE 3
The estimated numbers of monthly persistently infected cattle and their extinction over the time. The estimates were obtained using the Microsoft Excel based model for all cattle, cow calf, dairy, feedlots and stockers (combined).

TABLE 7 Total economic outcomes from 2018 to 2022 associated with median (25th percentile, 75th percentile) disease outbreaks for: production losses of beef (thousands of pounds) and milk (millions of pounds) due to disease; on-farm government response cost (millions of dollars); and economic impact as measured by producer welfare (millions of dollars).

Outcomes	Scenarios					
	1	2	3	4	5	6
Milk losses (million pounds)	6.73 (3.10, 12.70)	7.64 (3.17, 14.21)	3.33 (0.04, 6.74)	7.63 (3.22, 16.99)	8.15 (3.20, 16.63)	3.18 (0.04, 5.54)
Beef Losses (thousands pounds)	196 (106,384)	402 (229, 579)	179 (178, 225)	246 (41, 450)	427 (237, 590)	179 (178, 228)
Cost of response (millions $)	$120 ($50, $189)	$76 ($70, $90)	$197 ($127, $276)	$136 ($44, $259)	$77 ($70, $100)	$228 ($138, $334)
Change in quarterly returns to beef cattle (millions $)	–$520 (–$405, –$640)	–$403 (–$397, –$510)	–$649 (–$402, –$522)	–$533 (–$405, –$634)	–$403 (–$397, –$402)	–$517 (–$405, –$638)
Chang in quarterly returns to dairy cattle and milk (millions $)	$7.48 ($6.82, $7.40)	$9.62 ($9.56, $12.07)	$11.58 ($7.05, $1.81)	$10.24 ($4.84, $4.22)	$9.73 ($9.58, $9.71)	$9.39 ($6.99, –$2.08)
Change in quarterly returns to capital and management on sales (millions $)	–$2,062 (–$1,614, –$2,508)	–$1,611 (–$1,595, –$2,034)	–$2,532 (–$1,611, –$2,070)	–$2,097 (–$1,616, –$2,501)	–$1,611 (–$1,595, –$1,610)	–$2,056 (–$1,618, –$2,508)

The numbers in the table are the economic impacts for a variety of economic measures across a 4-year period (2018 to 2022) based on the median, 25th percentile and 75th percentile iterations of the disease spread outcomes as measured by head infected. For more details on economic outcome calculations, see the online Supplementary material.

(25th, 75th percentile) outbreaks for the dairy cattle and milk sector resulted in insignificant differences in producer returns compared to the pre-disease baseline; dairy sector economic impacts were moderated by the ability to export pasteurized and processed dairy products.

Discussion

The potential use of vaccinate-to-live approaches for FMDV eradication is closely related to understanding the epidemiology and economic impacts of the carrier state; these considerations are highly specific to variations of specific outbreak contexts. In order to explore this question in a US production setting, we developed scenarios utilizing a vaccination-to-live strategy subsequent to depopulation of infected animals for a limited period (14 or 28 days). Overall, we found that economic production impacts varied across sectors, but were overshadowed by trade impacts associated with the estimated duration that carriers would be present in the population. Vaccinate-to-live may be attractive in terms of animal welfare, conservation of limited resources during response or for preserving valuable animal genetics. The long-term consequences on industry viability and farm and ranch longevity should be the subject of further research.

The model outcomes demonstrated that the epidemic size and durations estimated from a single index (dairy cattle) herd located in California (Figure 1) resulted in similar findings to that of a previous FMD modeling study conducted in the same state, as well as simulated outbreaks in European countries (46–49). Specifically, a review of FMD outbreaks conducted using real outbreak data in non-endemic countries reported that ∼46% of epidemics had <5 infected farms, 16% of epidemics had more than 150 infected farms, and another 16% of epidemics were extensive (>2,000 infected farms) (48). In the current study, outbreaks initiating from a feedlot in the Panhandle Region of Texas resulted in a smaller number of infected farms (median = 5), which is similar to the findings of the previously review (48). Another modeling study predicted smaller outbreaks when the index herd was beef cattle as was found in scenarios 2 and 5 of this study (46). Differences in movements associated with both direct and indirect contacts on dairy vs. feedlot operations likely drove this difference in outbreak size.

We found that, in most scenarios (iterations), early onset of vaccination reduced the epidemic size and depopulation burdens. For example, outbreaks with >100 infected farms were found in 31% of iterations in scenario 1 (vaccination onset on day 43), whereas only 23% of iterations reached this level when the vaccination was initiated on day 29 of simulation (Scenario 4). This is consistent with previous studies looking at FMD control in California and in Denmark (6, 49), which also found a similar epidemic duration and outbreak size. Though the epidemic size and length was reduced by early vaccination, the overall impact of vaccination on controlling an outbreak is influenced by several factors, such as available resources for vaccination and other control programs, compliance with movement restrictions and on farm biosecurity standards, and efficacy of the vaccines. As such, the use of vaccination must be considered in the context of the specific outbreak. In some settings, a particular vaccination strategy could result in overwhelming resource demands (humans, financial, and logistics) or result in extensive economic impacts (46). For example, the culling of vaccinated animals could increase the number of animals depopulated and be counterproductive considering the environmental impacts and resource allocations for carcass disposal and post-disposal activities. Additionally, there could be a shift in resource allocation and on-farm response costs as suggested in the economic model output, with indemnities paid out absorbing the largest portion of cost for all scenarios except scenario 4 where the outbreak was initiated on a dairy site in CA and depopulation was started at 14 days. In this scenario, surveillance absorbed the largest portion of cost. A vaccinate-to-live strategy could extend the trade ban period and result in the establishment of FMDV carrier animals (cattle, sheep and goats) in the population, which necessitates additional consideration for resource allocations for their management. As a result, the efficacy of vaccination in reducing outbreak size and duration should be balanced with an understanding of the additional resources and long-term implications of managing or disposing of vaccinated animals.

In this study, implementation of vaccinate-to-live strategy allowed up to 35% of infected cattle to remain in the population, and these cattle had the potential to become asymptomatic carriers of FMDV. Based on our modeling approach, the majority (57%) of non-depopulated, infected cattle transitioned into the carrier state after the first month of infection with the potential to remain in the population from 30 to 52 months post infection. Under such circumstances, the management of carrier animals would surely place an additional resource demand on response personnel. However, it is possible that this additional demand could be offset by reducing the resources required for depopulation and carcass disposal under a stamping-out or vaccinate-to-die strategy.

The results from the economic analysis suggested a reduction in overall on-farm response cost of 12% and 10% with the implementation of 14- or 28-day depopulation, respectively, from an estimated 16% under stamping-out. During a shorter and smaller epidemic (scenario 2 and 4), vaccination may not be beneficial as compared to stamping-out. Market impact analysis including the international and domestic trade consequences further affects the decisions regarding vaccination, depopulation and management of potential carrier animals (47). FMD is a disease that has the potential to cause considerable and lasting damage in export markets. In the case of outbreaks that are shorter and smaller, the

economic damages from consumer avoidance and trade more than offset savings from reduced response costs. However, with the development of vaccines that can differentiate infected from vaccinated animals (DIVA), improved testing for carrier animals, and an improved understanding of the risks associated with carrier animals, an opportunity exists to refine trade embargo guidelines and regionalization agreements to account for alternative response strategies that may be needed in the event of resource constraints.

There was no conclusive impact on economic losses from the early onset of vaccination for on-farm response costs or economic returns as compared to later onset of vaccination. Government response costs were primarily associated with the indemnification of high value beef and dairy cattle and surveillance costs, including the costs of testing in vaccinated herds. Surveillance would be critically important to establish regionalization with key trade partners, and consequently limit trade impacts where possible. In this study, only Canada and Mexico were assumed to regionalize trade bans. Trade ban duration was linked to epidemic duration, based on the OIE standards, and epidemic duration was also used as the period of consumer avoidance. ISP results indicated that the only scenarios with significantly different simulated durations were scenarios 2 and 5, which had shorter durations than other outbreaks. Consequently, the trade embargo and consumer demand results were not greatly different except for scenarios 2 and 5. The economic losses in the Panhandle outbreaks were only 1% lower than other start locations in the 25th percentile but could range much higher (22%-36%) in larger simulated outbreaks. The greatest contributor to national economic loss was not the cost of managing carrier animals, but rather trade losses and consumer reaction; this coincides with studies of FMD vaccination in the US (50) and also with evidence from FMD outbreaks in other countries where vaccinate-to-live was practiced (51). As more scientific gaps are filled regarding FMDV persistence and transmission, there will be revision in FMD economic impact based upon how managers and consumers will respond to alternatives to stamping-out approaches. Further research is needed to address these gaps and refine analyses of vaccinate-to-live strategies given the potential of improved tracking and management carrier animals.

In executing this study, limited information was available on which to base assumptions of production losses in FMD recovered cattle, and these estimates could be improved by additional research on production losses in FMD-recovered herds. Although not explicitly examined in this study, it is possible that carrier animals would be removed from the herd more rapidly due to emergence of hoof deformity issues or other sequelae (14). A producer weighing the cost of monitoring and managing herd health in herds with carriers may not reap enough profit from recovered cows to keep those animals in production. Instead, those cows might be culled and replaced with new stock. Further, breeding stock producers with the highest potential gains associated with protecting genetic advances may also have the highest value associated with their brand and reputation. It may be more difficult to sell replacement animals out of vaccinated herds; however, there is no information on which to develop additional analyses regarding early culling due to reputation concerns at this time. Thus, the potential for livestock operations to accelerate removal of recovered livestock, or even go out of business, should be investigated more explicitly to fully understand the potential economic consequences of maintenance of carrier animals.

It is unknown at this time how US consumers would react to a vaccinate-to-live strategy without stamping out. Communication of scientific information on the safety of FMD-recovered animals living out their productive life and entering the US food chain would be crucial. It is also unknown how trade partners would react to FMD-recovered animals being allowed to continue production, given surveillance and tracing of recovered and vaccinated herds. However, even with relatively conservative trade embargo and consumer avoidance assumptions as compared to other studies (42, 50), beef markets did not recover to the pre-disease returns in the 4-year period examined. The uncertainty surrounding market recovery in the United States livestock industry, from a vaccinate-to-live without stamping-out strategy, could mean that there would be additional losses beyond the time period analyzed in this study. Improved understanding of the risks of carrier animals, along with higher potency vaccines and companion diagnostic tools, may contribute to shorter durations of risk-based trade embargoes in future outbreaks (8, 11). Further research that contributes to the understanding of FMD carrier risk may help align trade recovery guidelines, and perhaps reduce the economic burden associated with allowing recovered and vaccinated animals to live out a productive life.

The major caveat of this study is that the estimated outcomes are largely dependent on the input parameters and livestock demographics of the United States; therefore, extrapolation of these findings should be conservative. Further, it was necessary to assume that once animals entered the carrier phase, there was no transmission of FMDV; although this reflects the consensus of the published literature, it is also possible that low-level transmission does occur (11), which could have various downstream impacts on the findings herein. Similarly, simulations were conducted using serotype O-based transmission parameters, which may not reflect the full diversity of FMD viruses and transmission dynamics. Additionally, economic impacts were largely dependent on the parameters and baseline economic returns of the economic model, and the assumptions on trade and consumer avoidance. Both of these reactions may be influenced by risk perceptions associated with an individual outbreak (41, 52), and are very difficult to predict. Thus, these economic results should also be extrapolated cautiously.

Conclusion

These results can be used to inform the consideration of vaccinate-to-live and controlled slaughter strategies for FMD outbreaks and the development of appropriate post-outbreak surveillance. Furthermore, this output will enable more detailed examination of the epidemiologic and economic implications of allowing convalescent cattle to survive and remain in production chains after FMD outbreaks in FMD-free regions. With the development of next generation DIVA vaccines, improved diagnostic tests to identify carriers, and an improved understanding of the risks associated with carrier animals, an opportunity exists to refine trade embargo guidelines and regionalization agreements to account for alternative response strategies to FMD outbreaks. It is envisioned that further improvement of vaccine and diagnostic technologies will contribute toward greater confidence in vaccinate-to-live strategies for FMD control.

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

SY and AD: conceptualization of research and study design. SY: model scenarios development, data analysis and visualization, and drafting of the manuscript. AH: economic assessment design and analysis. CS, AD, MB, KM-T, LH, and JA: contributions to writing, reviewing, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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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.2022.1026592/full#supplementary-material

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Teresa de los Santos,
Agricultural Research Service (USDA), United States

REVIEWED BY
Michael Eschbaumer,
Friedrich-Loeffler-Institute, Germany
Richard Bradhurst,
The University of Melbourne, Australia
David Dazhia Lazarus,
Agricultural Research Council of South Africa, South Africa

*CORRESPONDENCE
Aurelio H. Cabezas
disease.status@woah.org
Min-Kyung Park
disease.status@woah.org

†PRESENT ADDRESSES
Neo J. Mapitse,
Regional Activities Department, World Organization for Animal Health, Paris, France

Matthew Stone,
SPADE Solutions, Wellington, New Zealand

Paolo Tizzani,
Data Integration Department, World Organization for Animal Health, Paris, France

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Analysis of suspensions and recoveries of official foot and mouth disease free status of WOAH Members between 1996 and 2020

Aurelio H. Cabezas[1]*, Neo J. Mapitse[1†], Paolo Tizzani[2†], Manuel J. Sanchez-Vazquez[3], Matthew Stone[4†] and Min-Kyung Park[1]*

[1]Status Department, World Organization for Animal Health, Paris, France, [2]World Animal Health Information and Analysis Department, World Organization for Animal Health, Paris, France, [3]Pan American Center for Foot-and-Mouth Disease and Veterinary Public Health, Communicable Diseases and Environmental Determinants of Health, Pan American Health Organization/World Health Organization, Duque de Caxias, Rio de Janeiro, Brazil, [4]International Standards and Science, World Organization for Animal Health, Paris, France

Foot and mouth disease was the first disease for which, in 1996, the World Organisation for Animal Health (WOAH; founded as OIE) established an official list of disease-free territories, which has helped to facilitate the trade of animals and animal products from those territories. Since that year, there have been a number of suspensions of FMD-free status which have impacted the livestock industry of the territories affected. The objective of this study is to identify factors associated with the time taken to recover FMD-free status after suspension. Historical applications submitted (between 1996 and the first semester of 2020) by WOAH Members for recognition and recovery of FMD-free status were used as the main source of data. Only FMD-free status suspensions caused by outbreaks were considered. Data on the Member's socio-economic characteristics, livestock production systems, FMD outbreak characteristics, and control strategies were targeted for the analysis. The period of time taken to recover FMD-free status was estimated using Kaplan–Meier survival curves. A Cox proportional hazard model was used to identify factors associated with the time taken to recover FMD-free status after suspension. A total of 163 territories were granted official FMD-free status during the study period. The study sample consisted of 45 FMD-free status suspensions. Africa and the Americas accounted for over 50% of FMD-free status suspensions, while over 70% of these occurred in formerly FMD-free territories where vaccination was not practiced. The study noted that implementing a stamping-out or vaccination and remove policy shortened the time to recover FMD-free status, compared with a vaccination and retain policy. Other variables associated with the outcome were the income level of the Member, Veterinary Service capacity, time taken to implement control measures, time taken until the disposal of the last FMD case, whether the territory bordered FMD-infected territories, and time elapsed since FMD freedom. This analysis will contribute toward the understanding of the main determinants affecting the time to

recover the FMD free status of WOAH Members and policy processes for FMD control and elimination.

KEYWORDS

foot and mouth disease, official FMD-free status, suspension of FMD-free status, recovery of FMD-free status, WOAH Members, survival analysis

Introduction

The World Organisation for Animal Health (WOAH) is the intergovernmental organization responsible for improving animal health, veterinary public health and animal welfare throughout the world (1, 2). WOAH is recognized by the World Trade Organization (WTO) as the global authority for defining sanitary rules in relation to animal health and zoonoses to facilitate the safe international trade of animals and animal products while avoiding unnecessary impediments to trade (3, 4). Among its other mandates, and since 1994, WOAH officially recognizes countries and zones[1] as being free from disease for the purposes of international trade.

Foot and mouth disease is a highly infectious disease that affects cloven-hoofed animals, and it is considered one of the most devastating diseases for livestock as the virus spreads easily among susceptible populations. Beyond its implications for animal health, FMD threatens national economies and the economic livelihoods of millions of people who depend on livestock for their income (5). In 1996, FMD became the first disease in WOAH's official list of disease-free countries and zones, based on a transparent, science-based and impartial procedure for the recognition and maintenance of FMD-free status. The voluntary procedure for official recognition of FMD-free status allows WOAH Members (Members) to apply for two categories of FMD-free status for their country or a zone within their country: FMD-free status where vaccination is not practiced and FMD-free status where vaccination is practiced. Members requesting official recognition of their FMD-free country or zone status must submit an application that follows the Standard Operating Procedures established by WOAH and provide documented evidence demonstrating compliance with the Terrestrial Animal Health Code (Terrestrial Code). The FMD-free status granted by WOAH represents a milestone in the economy of Members as it facilitates the trade of animals and animal products from those territories to attractive markets that require FMD-free status (1).

Foot and mouth disease outbreaks in FMD-free recognized countries or zones would result in suspension of that territory's FMD-free status. This loss of the status results in an immediate loss of export markets that require FMD-free status, which can only be recovered once the status is restored. Moreover, the process for regaining FMD-free status could involve significant investment and activity by the Member. A number of studies have estimated the costs associated with FMD outbreaks in non-endemic countries (5–14). These costs are incurred at the production level as stamping-out polices are often implemented to combat the disease, and through disease eradication efforts and losses in revenue because of trade restrictions (8). The FMD-free status of the country or zone can be recovered by submission of an application by the Chief Veterinary officer to WOAH providing sufficient evidence that the country or zone complies with the provisions in the Terrestrial Code. In short, it is necessary to present sufficient evidence to demonstrate the absence of FMD in that country or zone and to show that there are appropriate measures in place to avoid introduction.

Foot and mouth disease has been widely distributed around the world, as discussed by Grubman and Baxt (15), Paton et al. (16), and Brito et al. (17). While FMD mostly affects countries to which the disease is endemic, countries with an FMD-free status have also been impacted by the incursion of the virus. The FMD outbreaks in Chinese Taipei (6, 18); South Korea (19–22); Japan (23); the United Kingdom (UK) (24–26); France (27); Ireland (28); the Netherlands (29); South Africa (30); Uruguay (31); and Argentina (32) represent a few examples in which an FMD outbreak has led to the suspension of officially recognized FMD-free status. However, despite the research conducted to describe and understand the epidemiology of these outbreaks, the circumstances that led to the suspension and the strategies used for the subsequent reinstatement of FMD-free status have not yet been comprehensively described.

Several studies can be found in the literature that attempt to evaluate strategic approaches that could affect FMD-free status recovery periods. One study assessed the quality of higher potency vaccines and the performance of DIVA (differentiating infected from vaccinated individuals) assays on post-outbreak serosurveillance (33). Other authors explored the impact of using emergency vaccination during an epidemic in endemic and non-endemic countries (34), and the impact of emergency vaccination on the waiting period to recover FMD-free status (35). The effects of post-outbreak management strategies for vaccinated animals on market trade have also been explored (36). Studies using mathematical modeling have

1 A part of the country defined by the Veterinary Authority, containing an animal population or subpopulation with respect to its FMD status.

been conducted to simulate outbreaks in the Netherlands, with the application of a vaccination and retain policy, to evaluate the dynamics of the simulated outbreaks and to assess that policy's effect on regaining FMD-free status (37, 38). Finally, there have been studies evaluating surveillance methods to substantiate the absence of disease and viral circulation after FMD outbreaks in FMD-free territories (39–41). However, it is difficult to extrapolate conclusions from the above studies that would apply to a range of different scenarios, as they evaluated specific cases. It is also important to consider the intrinsic characteristics of a country or zone and the capability of a country to manage these emergency events when making informed recommendations on control strategy policies.

The objectives of this study are to identify factors associated with the time taken to recover a country or zone's FMD-free status after its suspension as the result of an outbreak, and to use that information to make informed recommendations on areas that should be strengthened for better preparedness and contingency planning against a potential incursion of FMDv. This is the first study that utilizes all the historical records available on the Member submissions to WOAH for FMD-free status recovery.

Materials and methods

Case selection

A country or zone was considered as the study unit. The source population consisted of all study units officially recognized as FMD-free (with or without vaccination) between 1996 and the first semester of 2020 (inclusive). Study units that had been granted an official FMD-free status, had their FMD-free status suspended as the result of an FMD outbreak and had applied for recovery of FMD-free status were included in the study. Study units that applied a zoning strategy—after the suspension—which resulted in the recovery of FMD-free status in only a part of the initially recognized country or zone and study units with no records available were excluded from the study.

Data collection

The main source of data for this study were the dossiers submitted to WOAH by Members for recognition and recovery of FMD-free status during the study period. Other important sources of data were the immediate notifications and follow-up reports of exceptional epidemiological events submitted to WOAH during the study period, retrieved from two digital interfaces: Handistatus II[2] which records data between 1996 and 2004 and WAHIS[3] which records data between 2005 and 2020. Other sources of data were FAOSTAT,[4] DataBank,[5] and other relevant WOAH reports. The analysis targeted variables in three main groups: agricultural characteristics of the study units, characteristics of the FMD outbreak, and emergency response and preparedness of the study unit (see Table 1 for more detail about targeted variables). All data collected were contemporaneous with the period of suspension/recovery of FMD-free status in the study unit. Data were compiled in Microsoft Excel® 365 (Microsoft, Redmond, WA, USA).

Statistical analysis

Descriptive statistics were computed to explore variables gathered from the data sources. Means, medians, and percentiles were computed for continuous variables while frequency tables were computed for categorical variables.

The association of potential risk factors affecting the time taken to recover FMD-free status (the outcome) was determined by conducting a survival analysis. The outcome was modeled in months and calculated from the date of the suspension of FMD-free status until the date of submission of the application for recovery of FMD-free status (see Figure 1). The date of submission of the application for the recovery of FMD-free status was used instead of the date of the official recovery to avoid administrative procedures by WOAH affecting the analysis. Thus, the date of application was taken as the moment when the country/zone were ready to fulfill the requirements for status recovery.

The 2020 edition of the FMD Chapter in the Terrestrial Code stipulates that Members can apply for the recovery of FMD-free status within 24 months after the date of suspension. If FMD-free status cannot be recovered within this period, Members would need to follow the general provisions for recognition of FMD-free status. However, this deadline of 24 months was only described in the Terrestrial Code editions of 2002 and from 2015 onwards. It was noted that the time-to-application for recovery after suspension was within 24 months in 75% of study units; within 36 months in 90% of the study units and up to 5 years for the remaining 10% of study units. Considering that this deadline was not described in the FMD Chapter of all the

2 Handistatus II can be accessed at: https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/data-before-2005/.

3 WAHIS Interface can be accessed at: https://wahis.woah.org/#/home.

4 FAOSTAT can be accessed at: http://www.fao.org/faostat/en/#data.

5 DataBank can be accessed at: https://databank.worldbank.org/reports.aspx?source=world-development-indicators.

TABLE 1 Target variables for the analysis.

Group	Variable	Scale of measurement
Agricultural characteristics of the study unit	Epidemiological unit	Farm, village, other
	Livestock density	Number of livestock per km^2 of agricultural land
	Shared borders with neighboring FMD-infected countries or zones	Binary
Characteristics of the FMD outbreak	FMDv serotype	A, O, C, SAT 1, SAT 2, SAT 3, or ASIA 1
	Species in which FMD was first detected	Bovines, swine, or small ruminants
	Species affected during the FMD outbreak	Bovines, swine, small ruminants, multiple
	Percentage of at-risk livestock during the outbreak	Percentage of confirmed FMD cases, and percentage of animals culled (if only stamping-out was applied) or proportion of vaccinated animals (if only emergency vaccination was applied) or percentage of animals culled and vaccinated (if stamping-out and emergency vaccination were applied) in relation to the total livestock population
Emergency response and preparedness of the study unit	Income level	Higher, upper-middle, lower-middle, low
	Time since FMD freedom[a]	Number of years
	Capacity of official Veterinary Services	Number of official veterinarians per number of livestock
	Time taken to implement control measures after FMD detection	Number of days
	Time between first detection of FMD and culling or vaccination of the last case	Number of weeks
	Time since adoption of FMD legislation or latest revision prior to suspension of FMD-free status	Number of years
	Control strategy used during the outbreak	Stamping-out, emergency vaccination and retain[b], emergency vaccination and remove[c]
	Conduction of simulation exercises prior suspension	Binary
	Conduction of simulation modeling studies prior suspension	Binary
	Existence of a public private partnership[d]	Binary

[a]Refers to the time elapsed since the date of initial recognition for countries or zones that had only one suspension, or since the date of last suspension for countries or zones with more than one suspension.
[b]Refers to letting vaccinated animals complete their production cycle after the application of emergency vaccination to control FMD outbreaks (protective vaccination).
[c]Refers to the slaughter of vaccinated animals after the application of emergency vaccination to control FMD outbreaks (suppressive vaccination).
[d]A joint approach in which the public and private sectors agree responsibilities and share resources and risks to achieve common objectives that deliver benefits in a sustainable manner.

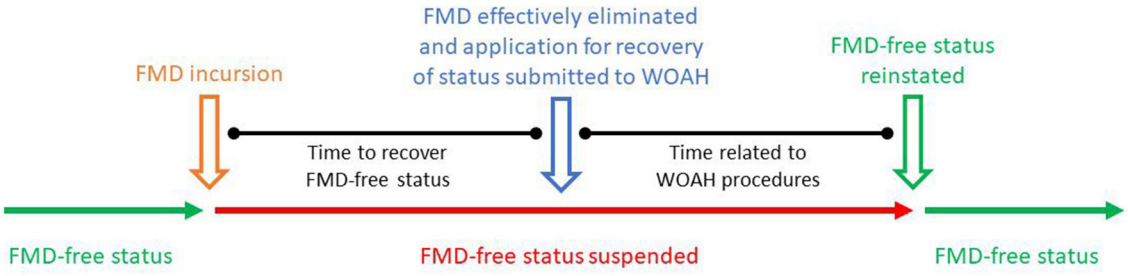

FIGURE 1
Schematic figure showing the relevant timelines in the study.

TABLE 2 Number of official FMD-free status recognitions and suspensions during the study period.

Official category of FMD-free status recognized	Number of official FMD-free status recognitions (%)	Number of suspensions and recoveries of FMD-free status (%)
Free country without vaccination	73 (44%)	21 (47%)
Free country with vaccination	6 (4%)	1 (2%)
Free zone without vaccination	42 (26%)	12 (27%)
Free zone with vaccination	42 (26%)	11 (24%)

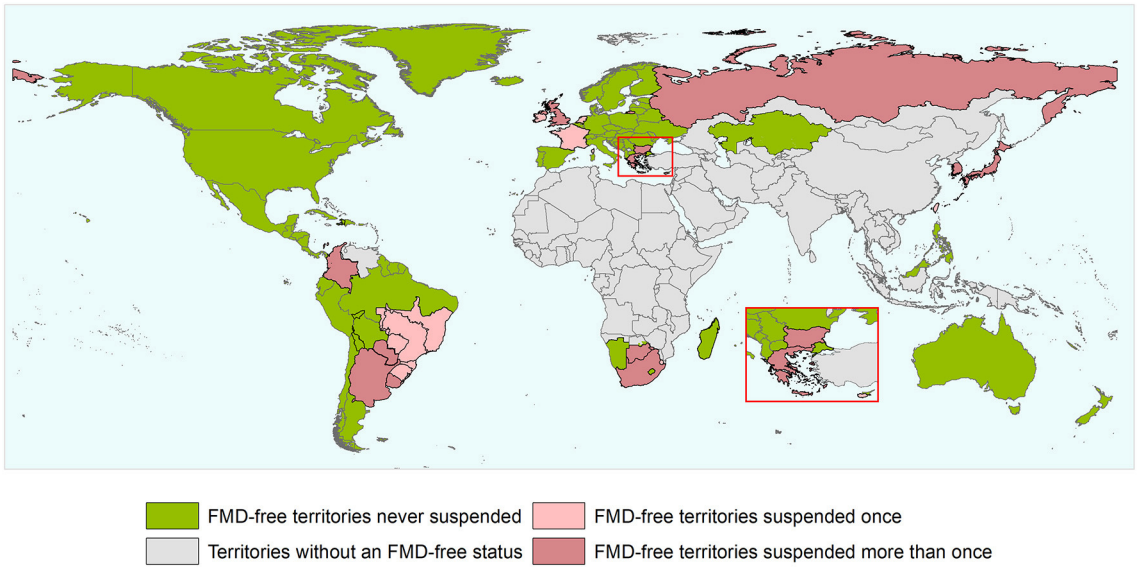

FIGURE 2
World map depicting the FMD-free status of countries and zones during the study period (1996 and the first semester of 2020). Territories suspended for reasons others than an outbreak and/or that did not meet the inclusion criterium are not depicted in the map as suspended territories. The authors highlight that the countries and zones depicted in the map does not necessarily represent the list of countries and zones having an official recognized FMD-free status by WOAH after the study period.

editions of the Terrestrial Code of the study period, a threshold of 36 months was used for the purposes of the study. Study units for which an application for recovery was not submitted within 36 months after suspension or by the end of the study period were progressively right censored.

The time taken to recover FMD-free status upon suspension according to the different factors was explored using Kaplan–Meier survival curves. In a further analysis, a Cox proportional hazard model was constructed. Variables with a large proportion (over 60%) of missing values were excluded from the analysis, and pair-wise correlations were also explored to assess for collinearity. Univariable models with each predictor and the outcome were determined to be fit to assess for unconditional associations, and associations with a liberal p-value ≤ 0.2 were selected for the multivariable model. Selection for retention in the model was carried out by the manual forward selection process, using a level of 0.05 as a criterion for statistical significance. Two-way interaction terms were evaluated. Evaluation of the proportional hazard assumption was conducted by estimating the Schoenfeld residuals and a test for significance for non-zero slope (log hazard–ratio function is constant over time) (42). The overall fit of the model was evaluated by computing the Groennesby and Borgan goodness-of-fit test, while the predictive ability of the model was assessed by computing the Harrell's C concordance statistic (42). Outliers and influential observations were evaluated by computing deviance and score residuals. Shared frailty models were also fitted for the assumption of non-independence between study units. "Member" was included as a frailty term to deal with the lack of independence for multiple failures within a Member—a Member having more than one suspension of FMD-free status, or more than one zone having a suspension of FMD-free status. The "edition of the Terrestrial Code" was also included as a frailty term with the assumption that study units for which applications for recovery were assessed under the requirement in a specific edition of the Terrestrial Code were

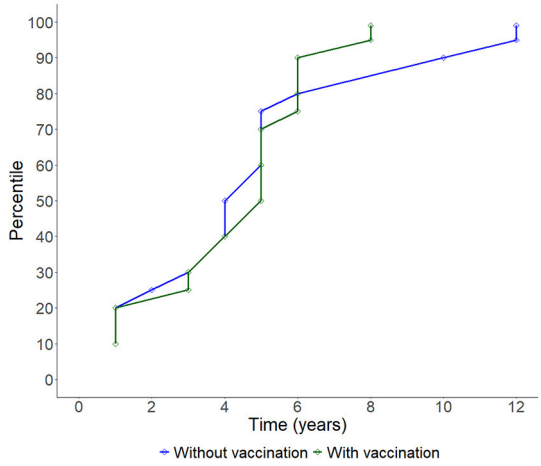

FIGURE 3
Line plot showing the cumulative progression of study units having their FMD-free status suspended over time.

likely correlated (see Supplementary Table A1 for a summary of the variation of waiting periods in the Terrestrial Code since 1996). The contribution of the frailty component to the model was evaluated by the log-likelihood test of $\theta = 0$ (equal variances) for evidence of within-cluster correlation. If there was no statistical significance (p-value > 0.05), then the simpler model was preferred. Results for significant variables are presented with hazard ratios, 95% confidence intervals, p-values, and medians for the time between FMD-free status suspension and application for recovery of FMD-free status. Data cleaning and statistical analysis were conducted in STATA 13 (StataCorp LP, College Station, TX, USA), the output figures were done in R (43) using the ggplot package, and the map was drawn in ArcGIS 10.3.1 (Esri, Redlands, CA, USA).

Results

Descriptive analysis

During the study period, there have been 163 official FMD-free statuses granted to countries or zones (see Table 2). FMD-free countries and zones without vaccination represent 44 and 26%, respectively, while FMD-free countries and zones with vaccination represent 4 and 26%, respectively. A total of 52 suspensions of FMD-free status have taken place, of which 45 suspensions met the inclusion criteria and were therefore part of the analysis ($n = 45$ study units). Zones accounted for 51% (23) of suspensions while countries for 49% (22). Seventy-three percent (33) of suspensions took place in study units with FMD-free status without vaccination; thus, 27% (12) of suspensions were in study units that were FMD-free with vaccination (see Table 2). The number of suspensions in each study unit ranged from 1 to 4, with a median of 1 (see Figure 2). It was found that in 80% of the study units (free with and without vaccination), the status was suspended within 6 years after recognition (see Figure 3). The time from suspension of FMD-free status to application for recovery ranged from 3 to 106 months, although in 90% of study units, this time was <36 months (see Figure 4). The cumulative time to recover the status in 90% of study units was <72 months (see Figure 5).

Most of the outbreaks that led to suspensions were caused by FMD serotype O (71%). The population reported to be at-risk during the FMD outbreak(s) was <8% of the total population in the study units. Over 40% (18) of study units that had their FMD-free status suspended applied stamping-out alone as a strategy to control the FMD outbreak(s), while 4% (2) applied emergency vaccination only, and 56% (25) applied a combination of stamping-out and emergency vaccination. In 49% (22) of the outbreaks that led to suspensions, bovines were the only species affected while in 40% (18) FMD infected multiple species. Simulation exercises were conducted in 8% (4) of study units prior to the suspension while simulation modeling studies to explore control strategies against potential FMD outbreaks were conducted prior to suspension in 18% (8) of study units. In study units that conducted simulation exercises, these occurred 4–8 years prior suspension. A public private partnership (PPP) relevant to FMD was in place in 24% (11) of the study units. In 9% (4) of the study units, a PPP was in place but the year of start of the PPP could not be determined. A concise summary of the data collected can be found in Supplementary Tables A2, A3.

Survival analysis

A total of 45 suspensions of FMD-free status were included in the analysis, from which 88% (40) recovered FMD-free status and 12% (5) of those were right censored. The total time at risk (that study units had their FMD-free status suspended until the status was recovered or until the study units were right censored) was 723 months. Study units of Members with a high-income level had a median survival time of 6 months, compared to 14 (upper-middle income) and 26 (low-middle income). The use of stamping-out (only), or stamping-out combined with emergency vaccination and remove policy, had a median survival time of 6 months, compared to 21 months in which stamping-out was implemented in combination with emergency vaccination and retain policy. Study units in which suspension of FMD-free status occurred after a year or longer than when the FMD-free status was recognized, had median survival time of 8 months compared to 14 months for those units in which suspension occurred within 1 year after FMD-free status recognition. More detail on the median and interquartile range (IQR) of survival times are displayed in Table 3. The Kaplan–Meier survival functions are presented in Figure 6.

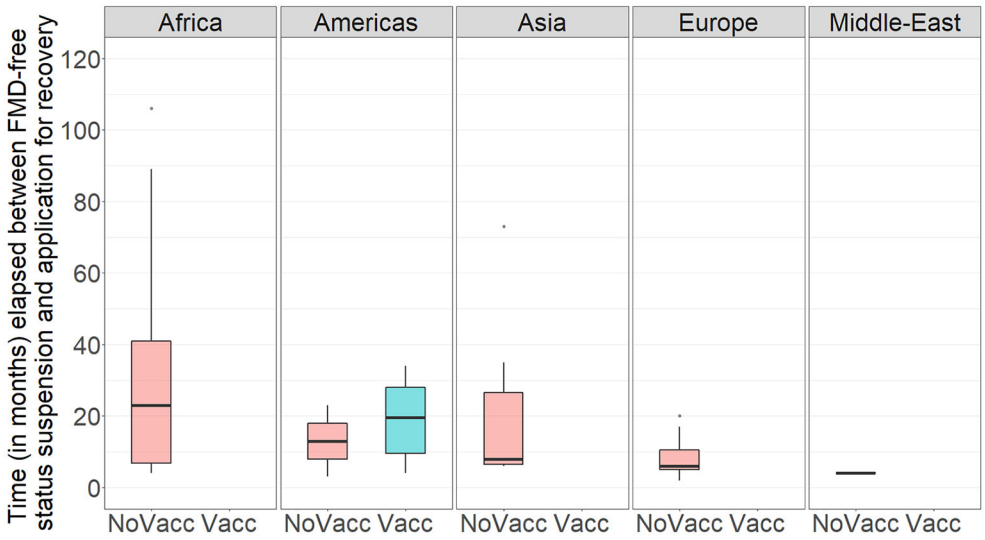

FIGURE 4
Boxplot showing the time (in months) between suspension of FMD-free status and application for recovery of FMD-free status in the study population per WOAH Regional Representation. NoVacc means FMD-free status without vaccination, Vacc means FMD-free status with vaccination.

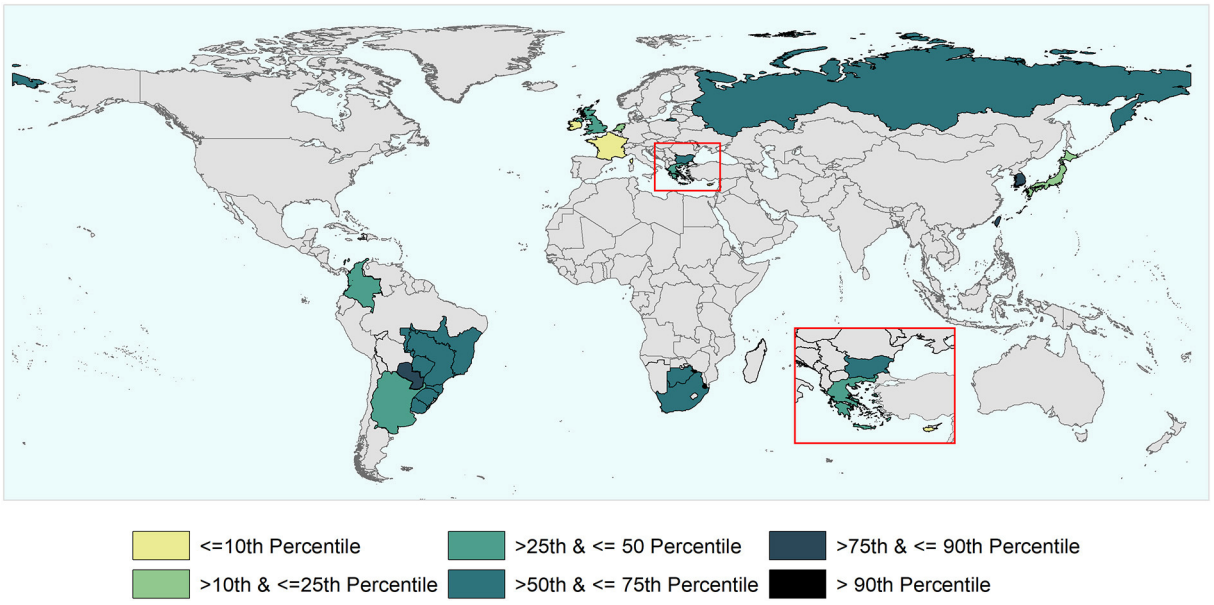

FIGURE 5
World map depicting the cumulative time taken to recover (i.e., to submit to the WOAH the official request) the FMD-free status suspended in the study units that were part of the analysis. The time to recover ranged between 3 and 106 months and percentiles were used for the categorization of study units.

The results of the Cox proportional hazards model univariable analysis are presented in Table 4. A total of 8 variables were selected for the multivariable Cox proportional hazard model (see Table 5). The inclusion of "Member" and "Edition of the Terrestrial Code" as a frailty term in the multivariable model were not statistically significant, so a simpler model was chosen, and those results are described.

In reporting hazards of recovery resulting from this survival analysis, note that shorter survival represents faster recovery of freedom, and therefore higher hazards of recovery represent the

TABLE 3 Summary statistics for significant categorical variables in the univariable analysis, showing the median and interquartile range for the survival time (months) taken to recover FMD-free status.

Variable	Category	Number of study units	Survival time[a] (months)		
			25th percentile	Median	75th Percentile
Income level	Lower middle	8	18	26	32
	Upper middle	23	8	14	25
	High	14	5	6	10
Time between first detection of FMD and culling or vaccination of the last case	Within 1 week	6	3	4	14
	Within 1 month	5	4	4	5
	Within 3 months	14	6	7	10
	More than 3 months	20	17	26	34
Control strategy used during the outbreak	Stamping-out	18	4	6	11
	Stamping-out + vaccination and remove	8	6	6	18
	Stamping-out + vaccination and retain	19	10	21	35
Shared borders with neighboring FMD-infected countries or zones	None	10	5	6	23
	At least 1	35	6	14	27
Time since FMD-freedom	1 year	11	6	14	35
	More than 1 year	34	6	8	26

[a]The survival time is the time in months between suspension of FMD-free status and application for recovery of FMD-free status.

more favorable outcome. In other words, higher hazard ratios are indicative that study units in a given category were more likely to have a faster recovery when compared to study units in the baseline category. For study units of a Member with an upper middle- or high-income level, the hazards of recovery of FMD-free status were 5.5 (95% CI, 1.5–8.08) and 6 (95% CI, 1.59–9.04) times greater than for study units of Members with a lower middle-income level, that is, study units with an upper-middle or high-income level had faster recoveries. The hazard for recovery of FMD-free status in study units that managed to slaughter or vaccinate the last FMD case within 3 months was 0.08 (95% CI, 0.04–0.12) times the hazard (a 90% decrease) when compared to the baseline (slaughter or vaccination of the last FMD case within a week). The implementation of stamping-out combined with emergency vaccination and remove policy was not significantly different from the implementation of stamping-out alone. However, the hazard for recovery for stamping-out in combination with emergency vaccination and retain policy was 0.11 (95% CI, 0.08–0.41) times the hazard (an 89% decrease) when compared to implementing stamping-out only. Study units that shared no borders with FMD-infected countries or zones had 2.2 (95% CI, 0.83–6.94) times the hazard to recover their FMD-free status, when compared to study units that shared a border with an FMD-infected country or zone. Study units in which FMD-freedom (either initial recognition or recovery) was achieved longer than a year prior to the suspension of status had 6 times the hazard (95% CI, 1.51–10.63) to recover their FMD-free status when compared to study units in which FMD-freedom was achieved within a year of the suspension. An increase of one official veterinarian in charge of the animal health situation in the country or zone per 100,000 livestock increased the hazard to recover FMD-free status by 5% (95% CI, 2–8%). Moreover, an increase of 1 day in implementing measures after FMD detection decreased the hazard to recover FMD-free status by 11% (95% CI, 2–17%). An increase in 1% of the livestock population at risk in the study unit decreased the hazard to recover FMD-free status by 4% (95% CI, 2–6%).

Interaction terms included in the multivariable model were not found to be statistically significant and were therefore removed from the final model. The statistical test to evaluate the assumption of proportional hazards suggested that there was no evidence that the assumption was violated (p-value 0.92). The Groennesby and Borgan goodness-of-fit test produced a p-value of 0.7, which suggested that there was no evidence of

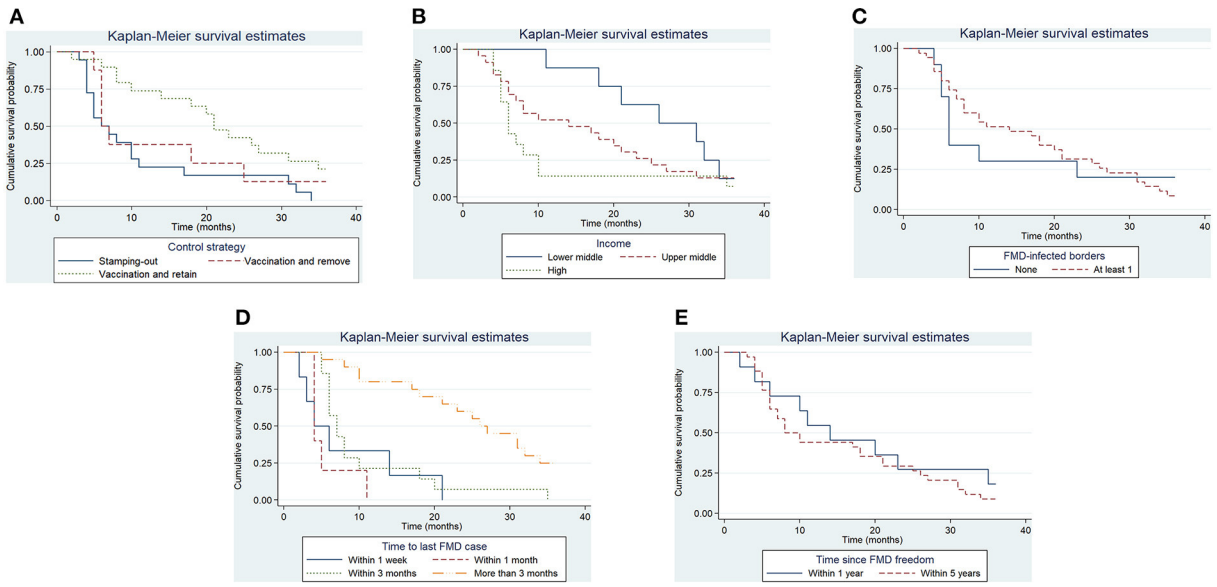

FIGURE 6
Kaplan–Meier survival function for significant categorical variables. Control strategy implemented to control the outbreak **(A)**, Income level of Member **(B)**, whether the study unit shared at least one border with an FMD-infected country or zone **(C)**, the time elapsed in the study unit between the detection of FMD and the slaughter or vaccination of the last FMD case **(D)**, the time since the study unit was last recognized as having FMD-free status **(E)**.

lack of fit. The Harrell's C concordance static was 0.89, which suggested that the model correctly predicted the findings 89% of times. There were no outliers and/or substantial influential observations identified.

Discussion

The current study documents the suspensions, and recoveries of FMD-free status from 1996, when WOAH first started granting official FMD-free status to its Members, until the first semester of 2020. Information has been synthesized from official documentation submitted by Members to WOAH and some external sources. This unique study has allowed to explore and understand the risk factors that could affect the time taken to recover FMD-free status after it had been suspended, and to use that understanding to help national veterinary services to make informed decisions to manage FMD at the country level. This discussion starts addressing general findings at the descriptive analysis, then continues to discuss the main findings at the survival analysis, and concludes with limitations of the study.

General findings

Our results show that 89% of the FMD-free status suspensions occurred between 1996 and 2011. After 2011, there have been only sporadic suspensions, which reflects the effort and progress made by Members in the control and prevention of FMD, potentially including better implementation of the expanded range of risk management options provided in the Terrestrial Code. For instance, in South America, significant progress has been made over recent years and continues to be made, thanks to an eradication program led by the Pan American Foot and Mouth Disease Center (PANAFTOSA), which targets improvements in veterinary infrastructure, mass vaccination campaigns, and PPPs to eliminate FMD (31, 44–46). In other regions, on the contrary, the management and control of FMD has been more challenging. For instance, in Asia the disparities across the continent in the financial resources allocated to Veterinary Services have had a direct impact on efforts to control and eliminate FMD (47). In parts of Africa and Eastern Europe, the role of seasonal transhumance (48) and wildlife species such as African buffalo (*Syncerus caffer*) and wild boar (*Sus scrofa*) in the epidemiology of FMD have also affected FMD control (49–52). Serotype O has been found to be responsible for most of the suspensions of FMD-free status (66%), which is not surprising as it is the most widely spread serotype around the world (15–17, 53).

In regard to the percentage of livestock population at risk during FMD outbreaks, it was found that fewer than 8% of the total livestock population in each study unit were considered at risk, and 75% of outbreaks were localized events, which means that they were restricted to a limited area of the study unit.

TABLE 4 Results of the univariable analysis to estimate the association between targeted variables and the time taken (in months) to recover FMD-free status.

Variable	Category	Hazard ratio	*p*-value	95% CI
Income level	Lower middle			
	Upper middle	1.67	0.244	0.70–3.98
	High	2.58	0.049	1.01–6.58
Species in which FMD was first detected	Bovines			
	Small ruminants	3.76	0.037	1.08–13.09
	Swine	0.90	0.814	0.37–2.18
	Wild	1.41	0.741	0.19–10.56
	Multiple	2.45	0.149	0.73–8.28
Time taken to cull or vaccinate the last FMD case after FMD detection	Within 1 week			
	Within 1 month	1.78	0.356	0.52–6.06
	Within 3 months	0.63	0.357	0.24–1.68
	More than 3 months	0.17	0.001	0.06–0.46
Control strategy used during the outbreak	Stamping-out			
	Stamping-out + vaccination and remove	0.68	0.394	0.28–1.64
	Stamping-out + vaccination and retain	0.38	0.006	0.18–0.76
Shared borders with neighboring FMD-infected countries or zones	At least 1			
	None	1.03	0.19	0.44–2.09
Time since FMD freedom	1 year			
	More than 1 year	1.32	0.16	0.63–2.79
Time since adoption of FMD legislation or latest revision prior to suspension of FMD-free status (years)		0.97	0.042	0.96–0.99
Capacity of official veterinary services[a]		1.01	0.021	1.01–1.03
Time taken to implement control measures after FMD detection (days)		0.95	0.122	0.88–0.01
Percentage of at-risk livestock during the outbreak		0.98	0.215	0.95–1.01

[a]The capacity of official Veterinary Services for the purpose of this study was estimated as the number of official veterinarians per 100,000 livestock in the study unit.

Considering the relatively low percentage of livestock affected and at risk, and the localized nature of these outbreaks, it is pertinent to ask why Members did not opt to apply for the establishment of a containment zone (CZ) as a strategy to hasten the recovery of at least part of their territories. Since the inclusion of provisions for the establishment of a CZ in the 2008 edition of the Terrestrial Code, this approach has been implemented in only three cases (17%).

One notable approach that has been used in the past few years, in a range of different territories and livestock production systems, is the application of network analysis using routinely or specifically collected traceability data to understand patterns of livestock movements (54–65). These methods can be helpful to identify areas within a country that are at a higher risk of the spread of FMDv or of being infected during an FMDv incursion. This information could be useful in determining the boundaries of a CZ that could be established as a strategy to quickly recover FMD-free status in part of a Member's territory.

Main findings

Based on the data and methods used, there was evidence of an inverse association between the income level of Members and the time taken to recover FMD-free status. This could be due to

TABLE 5 Results of the multivariable analysis to estimate the association between selected variables from the univariable analysis and the time taken (in months) to recover FMD-free status.

Variable	Category	Hazard ratio	*p*-value	95% CI
Income level	Lower middle			
	Upper middle	5.51	0.001	1.35–8.08
	High	6.08	0.022	1.59–9.04
Time taken to cull or vaccinate the last FMD case after FMD detection	Within 1 week			
	Within 1 month	0.92	0.928	0.16–5.34
	Within 3 months	0.33	0.164	0.07–1.57
	More than 3 months	0.08	0.001	0.04–0.12
Control strategy used during the outbreak	Stamping-out			
	Stamping-out + vaccination and remove	0.41	0.218	0.01–1.69
	Stamping-out + vaccination and retain	0.11	0.001	0.08–0.41
Shared borders with neighboring FMD-infected countries or zones	At least one			
	None	2.22	0.068	0.83–6.94
Time since FMD freedom	1 year			
	More than 1 year	5.8	0.011	1.51–10.63
Capacity of official veterinary services		1.05	0.03	1.02–1.08
Time taken to implement control measures after FMD detection (days)		0.89	0.015	0.83–0.98
Percentage of at-risk livestock during the outbreak		0.96	0.003	0.94–0.98

Members with a higher income level having more resources to devote to surveillance and early detection systems that lead to rapid FMD detection and the swift implementation of control measures, in addition to more resources being available for an emergency response in the event of disease outbreaks. Although the authors consider this finding plausible, this should not be over-emphasized because the study made use of an overall classification published by the World Bank, which may not represent the actual resources devoted to Veterinary Services or to emergency preparedness and response. Shorter time periods from the detection of FMD to the elimination/vaccination of the last case (depending on the control strategy) in the study unit were also found to increase the likelihood of rapid recovery times after the suspension of FMD-free status. In other words, the shorter the time from detection to elimination/vaccination of the last FMD case, the shorter the time to recover FMD-free status. This demonstrates the critical importance of the capacity of Veterinary Services during the onset of the emergency, to detect and diagnose FMD, including their ability to track and trace cases both backwards and forwards, and operational efficiency and effectiveness in implementing controls on infected places. Such operations will likely reduce the scale and duration of outbreaks. Nevertheless, after elimination/vaccination of the last FMD case, the country or zone must still provide evidence of the absence of FMD, in accordance with the relevant provisions of the Terrestrial Code.

An increase in the percentage of the livestock population at risk in the study unit during the outbreak contributed to a delay in recovery time, again re-affirming the importance of controlling the size of outbreaks. This variable may have been affected by the time taken to implement control measures in the study unit (no evidence of statistical significance). Experiences of previous outbreaks in Chinese Taipei and the UK (66) provide evidence that a delay in the implementation of movement bans and shutting down of markets contributes to an increase in the size of the epidemic, which suggests that FMD spread occurred through the movement of animals in the subclinical stage of infection.

There was also evidence that the study units which shared borders with FMD-infected countries or zones were less likely to recover their FMD-free status rapidly. This is an important finding for Members to consider. They may consider national strategies that implement targeted or heightened surveillance in these border areas aimed at the early detection of FMDv introduction, as well as stricter prevention strategies and controls in the movement of animals and animal products to

and from these areas, potentially (but not necessarily) within a zoning approach. The finding emphasizes the importance of regional collaboration in transboundary FMD risk management, both in preventing outbreaks and also during control operations during outbreaks.

Interestingly, it was observed that the study units that had their FMD-free status suspended within 12 months after recognition or recovery, were more likely to take a longer time to recover from a subsequent outbreak. This finding illustrates the vulnerability of countries and zones in the period after FMD recognition/recovery, and the need for follow-up work to be done to maintain the FMD-free status. This may suggest a need to prioritize resources and activities and maintain vigilance against FMD, particularly during the first year of FMD-free status, for successful or continuous maintenance of that status. This is also a relevant consideration for WOAH to put a particular emphasis in following up countries or zones during the first year after attaining FMD-free status.

The availability of public veterinarians, measured as the number of official veterinarians per 100,000 livestock, was also linked to the time taken to recover FMD-free status in the study population. If increasing the number of official veterinarians is not possible, an effective strategy might be to train and allocate more veterinarians or veterinary para-professionals in areas with a higher density of livestock or higher risk of outbreaks (e.g., border areas). In fact, through modeling exercise, it has been shown that the success of the outbreak control was impacted by the number of staff available for surveillance activities in the early phase of the emergency (67).

In terms of the impact of control strategies implemented during the outbreak(s), the application of stamping-out (only) led to shorter recovery times when compared to stamping-out with emergency vaccination to live. There was no statistically significant difference in the time taken to recover between the application of stamping-out (only) and stamping-out with emergency vaccination and remove policy. Many studies have investigated the potential impact of emergency vaccination and retain policy in FMD-free areas without vaccination. Based on the experience of FMD outbreaks in the Netherlands in 2001, Backer et al. (38) suggest that vaccination and retain policy can be a viable alternative to stamping-out, even in situations where resources are scarce. The authors suggest targeting densely populated areas for vaccination. While mentioning the economic and ethical implications of stamping-out and emergency vaccination and remove policies, Parida (68) points out that the success of emergency vaccination and retain policy is highly dependent on good traceability systems and record-keeping. Other authors argue that implementation of a vaccination and retain policy should be avoided, based on the assumption that cattle persistently infected animals could act as a disease reservoir (35). However, evidence indicates that transmission from persistently infected animals in the field is rare (69–74). Other studies have also investigated vaccination strategies and their impact on trade, and suggest that the costs of implementing emergency vaccination and retain policy lowered the overall costs of controlling the outbreak (in comparison to using stamping-out and emergency vaccination and remove policy), but that these costs were nowhere near close to the losses in trade (7, 36). For this reason, Members with significant export markets may decide that emergency vaccination and retain policy is not the most economic strategy. In addition, Paton et al. (39) reviewed the use of non-structural protein tests in substantiating freedom from disease and suggested that, while a vaccination and retain policy is feasible, it may involve greater financial costs due to the components of the surveillance system needed to demonstrate freedom. Another important factor to consider when planning control strategies is the psychological impact that these policies can have on producers and the major opposition by the citizens to these kind of interventions, as suggested by Davies (25) in his description of the 2001 UK epidemic.

The analysis could not find any association between the existence of a PPP related to FMD activities and the time to recover the FMD-free status. Nevertheless, the authors noted the importance of PPPs in the maintenance of animal health status and disease control in Members through increasing awareness and incentivising risk management, and Members should therefore be encouraged to provide such data when applying for official recognition or recovery of FMD-free status. In addition, the authors recommend WOAH to develop a harmonized methodology to record this type of data or develop indicators to evaluate the impact of this kind of collaboration in applicant Members in the future.

Similarly, the analysis could not find any associations between having conducted simulation exercises or simulation modeling studies prior to the suspension and the time to recover the FMD-free status. Regardless, the authors considered important that Members should be encouraged to conduct simulation exercises on a regular basis to test and increase awareness and capacity in their emergency response to control FMD outbreaks more effectively and report updates on this topic. A study by Westergaard (75) summarizes all the components needed to manage and conduct simulation exercises for highly infectious diseases and more recently WOAH developed guidelines for simulation exercises that could be used by its Members (76). With regard simulation modeling studies, their use and application has significantly increased in the past decades in developed countries that have lost their FMD-free status, such as the UK, Japan, the Netherlands and South Korea (14, 77–84). It is important to note that the studies mentioned above were conducted during or after the outbreaks. Nevertheless, simulation modeling is a useful tool to explore management strategies to control outbreaks and facilitate policy making.

In assessing the different variables involved in the time taken to recover FMD-free status, the duration of the period

from suspension of FMD-free status to submission of the application for the recovery of the free status was measured as the outcome. The reason for selecting this period as the outcome was to avoid incorporating the time taken by WOAH procedures in the evaluation of the recovery application. The current outputs might have suffered from misclassification bias due to uncertainty in the categorization of some variables. This misclassification may occur as a result of differences in the production system and FMD epidemiological situation between study units during the study period. Misclassification bias has also been discussed by McLaws and Ribble (66) in their review of outbreaks in non-endemic countries. There was no evidence of non-independence (clustering) in the study subjects; however, caution should be applied because there were many study units that suffered more than one FMD-free status suspension. The assumption is that study units of a Member, shared some similarities because some variables are measured at the country level and not at the zone level, and that these should be taken into account in the analysis. Perhaps other statistical methods could be explored in the future. One possible justification for the lack of evidence of clustering could be that FMD-free status suspensions in the majority of the study units were temporally far apart during the study period, and thus their epidemiological situations could have changed. In a similar way, differences in the nature of outbreaks, such as those species affected and the magnitude of FMD spread, could also have influenced the choice of control strategies adopted in the study units and their effectiveness.

Limitations

The following limitations of the analysis should also be noted. The FMD Chapter in the Terrestrial Code (2002 edition, and editions since 2015) indicates a 24-month deadline to apply for recovery after the suspension of FMD-free status; this was not included in other editions of the Terrestrial Code. Therefore, some study units had a longer period from suspension to recovery (up to 5 years). To avoid those study subjects with prolonged periods between the suspension of their FMD-free status and their application for recovery affecting the analysis, a period of 36 months was chosen as a threshold for the inclusion of the study units. This threshold was also chosen to include a larger proportion of study units, since 90% fall within this range. The current study does not have a large sample size ($n = 45$), and this could affect the power to detect associations between variables and the time taken to recover FMD-free status.

A zonal approach to recover the FMD-free status in only a part of the initially recognized country or zone had been shown to be a reasonable strategic approach to consider for many Members—especially in South America. Whilst the zonal approach was not included as part of this study, depending on the prevailing epidemiological situation, it could be considered to gradually recover the FMD-free status of a country or zone. One important variable that was not considered in this analysis was the effect of the season on the time taken to recover FMD-free status. In a study to evaluate factors affecting the time taken to eliminate porcine epidemic diarrhea virus (PEDv) in Canada, the authors found that PEDv was eliminated faster in the spring, summer and fall than in winter (85). The reason why season was not included in this analysis was due to the variability of climates in the Members that formed part of the study sample, which did not allow the authors to make a sound comparison. Other variables were removed from the study because of the large number of missing values. Dohoo (86) and Pedersen et al. (87) have described methods to deal with missing values during the analysis. The same authors have explored the use of multiple imputation to account for missing values in the data (87, 88), although this method has been questioned by other authors because biased estimates have been noted in the association between predictors of interest and outcome (89, 90). Owing to the significance of the outputs of this report, methods to deal with missing values were not implemented. Nonetheless, it would be interesting to use the multiple imputation approach to conduct future analyses and assess the behavior of the models. Finally, the outputs generated by this analysis should be interpreted cautiously because of continuous improvements in the performance of surveillance and early warning systems, and increased capacity building in Members' emergency management capacity since the last suspension of their FMD-free status.

Conclusions

This is the first project that attempts to describe the suspensions and recoveries FMD-free status in WOAH Members and to evaluate the effects of different risk factors on the time taken to recover official FMD-free status. The analysis identified important areas to be strengthened for better preparedness and contingency planning against a potential incursion of FMDv into Members' territories. Nevertheless, the authors emphasize that the findings should be considered carefully as the study made a retrospective analysis and many of the areas discussed in the sections above are likely to have improved in the years after the suspension and recovery of FMD-free status. The study also emphasizes the challenges encountered by the authors when collecting, cleaning, and analyzing the data. For this reason, the authors recommend WOAH to develop better data management strategies so that similar studies can be more readily repeated in the future and make

more efficient use of the data available and produce more robust findings.

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 authors.

Author contributions

This study was conceived by WOAH. The data collection, entry, and cleaning were done by AC. AC, PT, and MS-V contributed to the statistical analyses. All authors contributed to the study design, wrote the report, read, and approved the final version for publication.

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Conflict of interest

The 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.

The reviewer ME declared a past co-authorship with one of the author MS-V to the handling editor.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1013768/full#supplementary-material

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EDITED BY
Artur Summerfield,
Institute of Virology and Immunology (IVI), Switzerland

REVIEWED BY
Shankar Yadav,
European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy
Florencia Celeste Mansilla,
IVIT, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina

*CORRESPONDENCE
Wilna Vosloo
wilna.vosloo@csiro.au

†These authors have contributed equally to this work and share first authorship

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Investigation into the protective ability of monovalent and bivalent A Malaysia 97 and A_{22} Iraq 64 vaccine strains against infection with an A/Asia/SEA-97 variant in pigs

Jacquelyn Horsington[1†], Nagendrakumar Singanallur Balasubramanian[1†], Charles K. Nfon[2], Hilary Bittner[2] and Wilna Vosloo[1*]

[1]Australian Centre for Disease Preparedness, Transboundary Disease Mitigation, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Health and Biosecurity, Geelong, VIC, Australia, [2]Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, MB, Canada

Over the last 15 years, FMDV serotype A viruses in South-East Asia (A/ASIA/SEA-97 lineage) have diverged into several clusters. Variants from Thailand in 2011-2013 have caused vaccine failures and returned poor r_1-values (<0.30) to A_{22} Iraq 64 (A22) and A Malaysia 97 (A May) vaccine strains. We investigated the protective ability of monovalent and bivalent A Malaysia 97 and A22 Iraq 64 vaccine strains against infection with an A/Asia/SEA-97 variant in pigs. Pigs were challenged with a variant of A/Asia/SEA-97 lineage either 21- or 7- days post-vaccination (V21 or V7) using the heal-bulb challenge. Only one in five pigs were protected in the V21 monovalent vaccine groups. Less severe clinical signs were observed in the A22 IRQ group compared to the A MAY 97 group. In the V21 combination group, 4 out of 5 pigs were protected and viraemia was significantly reduced compared to the monovalent V21 groups. V7 vaccine groups were not protected. The neutralising antibody response was below the detection limit in all groups on the challenge day, showing a poor correlation with protection. There was no evidence that the pigs protected from systemic disease had protective antibody responses sooner than other pigs in the study, implying other immune mechanisms might play a role in protecting these animals. FMDV was detected in the nasal and oral swab samples between 1 and 6 dpc. Viral loads were lower in the nasal swab samples from the V21 combination group than the other groups, but there was no difference in the oral swab samples. Since all unvaccinated controls were euthanised by 6-day post-challenge for ethical reasons, the 'area under the curve (AUC)' method was used to compare the viraemia and virus excretion in different groups. We recommend that for the A/Asia/SEA97 variants, a combination vaccine with A Malaysia 97 and A22 Iraq 64 vaccine strains would be ideal compared to monovalent vaccines.

KEYWORDS

foot-and-mouth disease, vaccines, A/Asia/SEA-97, protection, area under a curve, pigs

Introduction

Foot-and-mouth Disease (FMD) is an important transboundary animal disease of cloven-hoofed livestock and wild ungulates (1). It is highly infectious, has a high morbidity rate and causes significant loss in livestock production. Mortality due to the disease is seen predominantly in young animals. Most of the economic impact of FMD is because of restriction to trade with FMD-free countries, particularly where a trade barrier is imposed on livestock and their products (2). The disease is caused by the FMD virus (FMDV), a single-stranded, positive-sense RNA virus, which belongs to the genus *Aphthovirus* within the family *Picornaviridae,* and comprises the serotypes O, A, C, Asia1, and Southern African Territories (SAT) 1, 2 and 3 (3, 4).

FMDV is widely distributed and is currently maintained in three continental reservoirs: in Asia, Africa, and some parts of South America. The viruses in these continents are subdivided into seven major virus pools of infection that contain different serotypes and lineages (5). South-East Asia (SEA) is endemic to viruses belonging to Pool 1, comprising serotypes O and A, with a few historical occurrences of serotype Asia1. The viruses of SEA have evolved distinctly from the other regions in Asia with the emergence of O/SEA/CAM-94 lineage in 1994 and 1998 (6); A/ASIA/SEA-97 in 1997 (7) and O/SEA/Mya-98 in 1998 (8, 9) respectively. However, recently we have seen incursions of virus strains belonging to Pool 2 into the region, such as O/ME-SA/PanAsia and O/ME-SA/Ind-2001 (7).

Although there is heavy disease burden in SEA, the control of FMD has been hampered by many factors such as the geo-political situations, unrestricted animal movements, poorly resourced veterinary services, lack of funding for FMD control programs and producers' apathy (10). With increase in population and demand for animal protein, there is an increase in the number of small and marginal farmers who access transboundary markets through traders. This has resulted in unrestricted movements of animals within the region (11). Added to these problems is the non-availability of quality vaccines and poor vaccination rates. Different commercial vaccines incorporating different virus strains are sold in the region and there is insufficient data on vaccine matching in the region.

Thailand uses a locally manufactured vaccine with strains for serotype O and A that were previously collected in the country and that differs from other countries in the region. While the choice of serotype O vaccine strains depend on the different producers, there is some synergy in which serotype A vaccine strains are used in SEA. Both the vaccine strains, A/Sakolnakorn/97 and A/Malaysia/97, belong to the same genetic lineage and year of isolation, with the former isolated from the outbreaks in Thailand and the latter from Malaysia (25). These two strains continued to be a part of the FMD vaccines in the region for almost two decades. However, since 2012, serotype A viruses in the region, especially in Thailand, had diverged resulting in a distinct SEA-97 variant. These variants demonstrated a poor antigenic match with the serotype A vaccine strains (12). Some of these variants were antigenically so diverged that the routine antigen ELISA used in the region failed to detect them. Soon after the OIE-Regional Reference Laboratory for FMD (OIE-RRL), Pakchong in Thailand reported the emergence of this new SEA-97 variant, the National Laboratory for FMD, Thailand developed a new vaccine strain, A Lopburi 2012, from one of the isolates (13). This strain is now incorporated into the FMD vaccines manufactured in Thailand (13). Studies carried out at the OIE/FAO World Reference Laboratory (WRL) for FMD, the Pirbright Institute, United Kingdom, and at OIE-RRL, Pakchong indicated that these A/ASIA/SEA-97 variants from SEA had poor relative homology (r_1) values in vaccine matching studies with the serotype A vaccines including A/Malaysia/97, but good matching with the new vaccine strain, A Lopburi 2012 (12–14).

Since viruses of this sub-lineage have spread to other countries such as Lao PDR, Vietnam, and Cambodia (12, 14), it was important to reassure stakeholders, including endemic countries in the region and free countries/regions holding vaccine banks, on the efficacy of the vaccine strains against these variant viruses. We report the results of the vaccine efficacy studies in pigs vaccinated with different serotype A vaccine strains and challenged by a field virus belonging to A/Asia/SEA-97 variant. Our aim was to establish if a monovalent vaccine or a combination vaccine would be effective in preventing clinical disease in pigs and if the vaccines will impart early protection soon after administration.

Materials and methods

Animals

Cross-bred landrace pigs 7–8 weeks of age and of mixed sex were obtained from a registered supplier in Canada. They were kept at the National Centre for Foreign Animal Diseases (NCFAD), Winnipeg, Canada, facility under quarantine for 2 weeks before the commencement of the experiment. This study was approved by the Australian Centre for Disease Preparedness (ACDP) Animal Ethics Committee (AEC 1774 and AEC 1801) and the Canadian Centre for Human and Animal Health Animal Care Committee (AUD# C-15-007) and performed in strict accordance with the recommendations of the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes and the Canadian Council for Animal Care Guidelines.

Vaccines and challenge virus

Monovalent A Malaysia 97 (A May) and A22 Iraq 64 (A22) double oil adjuvant vaccines with antigen payloads of at least 6

PD_{50}/ml, and combination (Combo: A May and A22) double oil adjuvant vaccine with an antigen payload of at least 6 PD_{50} of each strain were prepared from the Australian FMD vaccine reserve by Merial Company Limited, United Kingdom (now Boehringer Ingelheim). The vaccines were imported to Canada and stored at NCFAD, Winnipeg, under controlled conditions.

The challenge virus, FMDV isolate A/TAI/15/2013, which belongs to the new A/Asia/SEA-97 variant, was obtained from the FAO/OIE World Reference Laboratory for FMD (WRL), Pirbright, United Kingdom. The virus was originally isolated from cattle on 21/10/2013 in the Lampang province of Thailand. Vaccine matching studies showed that the r_1 values were 0.05 and 0.10 for A22 Iraq 64 and A Malaysia 97, respectively (14). It was passaged twice using BHK-21 cells at the WRL and imported into NCFAD. The isolate had a poor relative homology (r_1) value of 0.05 and 0.01 against A22 Iraq 64 and A Malaysia 97 vaccine strains, respectively (14). To prepare for the pig challenge virus, the cell culture supernatant containing the isolate was passed once in two pigs by inoculation into the bulb of the heel (15, 16) of the left forelimb at two sites (0.1 ml/site), intravenously (1 ml) into the ear vein and intramuscularly (1 ml) on the mid-neck region as described (17, 18). Vesicular material was collected at 2 days post infection and a 10% w/v suspension prepared in phosphate buffered saline (PBS; pH 7.4 ± 2). The aliquots were stored at−80°C until use. One of the aliquots was titrated using LFBK-αVβ6 (αVβ6-expressing foetal porcine kidney) cells (19, 20).

Study design

The vaccine efficacy experiment consisted of eight vaccine groups and unvaccinated controls (Supplementary Table 1) and was carried out in two phases. In Phase 1, the efficacy of the monovalent vaccines (A22 and A May) was studied in groups of five pigs and five control pigs and in Phase 2 the combination vaccine (A22 + A May) was tested in two groups of five pigs with five additional control pigs, resulting in ten unvaccinated control pigs (UVC). With each vaccine formulation, one group was vaccinated 21 days prior to challenge (V21) and one group was vaccinated 7 days prior to challenge (V7). Finally, a group of pigs was vaccinated either with A22 and A May vaccine ($n = 2$ each) or the Combo vaccine ($n = 5$), along with the V21 groups but were not challenged with virulent virus (VO). The vaccines were administered intramuscularly in the left side of the neck (2 ml/dose). Vaccination was staggered so that all pigs (except A May VO, A22 VO and Combo VO groups) were challenged on the same day.

All vaccinated pigs and the UVC group (except VO groups), were challenged by the heel bulb route using 0.2 ml of virus inoculum (equivalent to 10,000 $TCID_{50}$) divided equally between two sites on one foot as previously described (17, 18). The animals were monitored for development of clinical signs consistent with infection by FMDV such as pyrexia (rectal temperature >40°C), lameness and development of vesicles on the surface of the tongue and snout, up to 6 days post-challenge (dpc). Lesion scores were calculated by scoring one for each site where lesions formed, except the inoculation site (1 per foot and 1 for any oral/snout lesions) resulting in a maximum score of 4. Whole blood in K2-EDTA vials was collected from all pigs at the time of vaccination,−4 dpc and daily between 0 and 14 dpc, at which point the experiment was terminated for RT-qPCR. Clotted blood for serology was collected from pigs in VO groups on all days synchronous with the challenge groups i.e.,−21 dpc,−7 dpc, 0 dpc, 5 dpc, 7 dpc, 10 dpc and 14 dpc, corresponding to 0-, 14-, 21-, 26-, 28-, 31- and 35-day post-vaccination (dpv). The serum was inactivated at 56°C for 30 min and stored in aliquots under −70°C until use. Small, sterilised cotton buds were used to collect nasal and saliva secretions daily between 0 and 14 dpc for virus isolation and RT-qPCR. Swabs were placed in tubes containing 500 µl of PBS for RT-qPCR or 500 µl Dulbecco's modified Eagle's media (DMEM) containing 5% foetal bovine serum and antibiotics (Gibco, Cat. No. 15240062) for virus isolation. All samples were stored at −70°C until processing.

Pigs were sedated using isoflurane gas anaesthesia, during heel bulb inoculation and collection of samples. If deemed necessary, Flunixin Meglumine (1.1–2.2 mg/kg) and Buprenorphine (0.005–0.01 mg/kg) was administered every 12–24 h to manage pain. Pigs were humanely euthanised when they reached the ethical end points or end of the experimental period; pigs were sedated first (0.8 mL xylazine at 20 mg/ml and 4.5 mL ketamine at 100 mg/ml) followed by intravenous barbiturate injection (sodium pentobarbital at 100 mg/kg).

Virus isolation

Serum, nasal swab, and saliva (oral swab) samples were tested for the presence of live virus using LFBK-αVβ6 cells [LFBK cells; (19, 20)]. Monolayers of LFBK cells grown in 96-well cell culture trays were inoculated with 100 µl sample and incubated for 30 mins at 37°C. The cells were washed with PBS and overlayed with DMEM containing 5% foetal bovine serum and antibiotics (Gibco, Cat. No. 15240062) and examined for cytopathic effect (CPE) after 24, 48 and 72 h incubation at 37°C with 5% CO_2. If no CPE was observed, cells and supernatant were collected, freeze-thawed and inoculated onto fresh LFBK monolayers. The presence or absence of FMDV was confirmed using an FMDV antigen enzyme-linked immunosorbent assay (ELISA) as described by Hamblin et al. (21).

Detection of FMDV RNA by RT-qPCR

The FMDV RNA levels in serum, nasal and oral swabs were quantified by a TaqMan RT-qPCR assay as described previously (22). Viral RNA was extracted from 50 µl of sample with the

MagMAX™-96 Viral RNA Isolation Kit (Life Technologies) using the MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies). One-step RT-qPCR was performed using the AgPath ID One-Step RT-PCR reagents (Life Technologies) on the Applied Biosystems 7500 Real-Time PCR Instrument. All samples were tested in duplicate and samples with poor Ct value correlation in the duplicate reactions were repeated. Samples with a Ct <40 (equivalent to $1 \times 10^{3.5}$ copies RNA/ml blood or $1 \times 10^{3.2}$ copies RNA/swab) were considered positive (23).

Determination of neutralising antibody titre

Virus neutralisation test (VNT) in LFBK cells was performed on heat inactivated (56°C, 30 min) serum samples using either A/MAY/97 or A22/IRQ/64 virus (provided by NCFAD) and the LFBK-αVβ6 cell adapted A/TAI/15/2013 virus (24). Titres >1.2 log10 (1:16) were considered positive (25).

Detection of antibodies to structural proteins by ELISA

The presence of antibodies against structural proteins (SP) of serotype A was assayed using a serotype A-specific solid-phase competition ELISA (SPCE) using reagents homologous to A22/IRQ/64 following a protocol described by Mackay et al. (26) with some modifications with respect to the antigens and control sera. We did not have a system with homologous reagents for A Malaysia 97 and so not performed.

Statistical analysis

Data on clinical scores, virus RNA levels and VNT antibody titres were used for statistical analysis using R version 4.0.2 (27). Clinical protection based on count data was compared using the two-sided Fischer exact test. Group means and standard deviations were calculated and expressed as Mean ± SD. Mean survival time and probability of protection were estimated using Kaplan-Meier survival analysis [(28), "*survival*" and "*survminer*" libraries in R]. Longitudinal data for continuous outcomes in multiple vaccine groups were compared using a linear mixed effects model ("*lme*" library in R). All plots were drawn using the library "ggplot2" in R. ANOVA was used to test the statistical differences between groups with Holm's *post-test* if a statistical difference was found. Longitudinal data (virus isolation, RT-PCR results and NSP response) were analysed using animal number as random variable and dpc, group and vaccination (yes or no) as possible explanatory variables. Using forward selection, the best model with the lowest AIC (Akaike's Information Criterion) was chosen. Pig number was added as a random variable while dpc (as a factor) and vaccine group and the interactions were considered as explanatory variables. In all models, explanatory variables were selected based on the lowest AIC using forward selection. Area under the curve (AUC) was used to compare estimated virus loads in serum, nasal and oral swabs, and the duration of viraemia in a single parameter (29, 30). A new variable, AUC units, was constructed to measure the FMDV load in pigs from day of challenge to end of experiment or removal of pig in terms of duration and quantity of excretion (log10 copy numbers/ml). The median and mean AUC units for each animal were calculated following the trapezoidal rule using "*rgeos*" and library in R. Group-wise comparison of median and mean AUC units were performed using one way ANOVA with *post hoc* Bonferroni's test (31) using "*car*" library in R.

Results

Clinical signs

All vaccinated and unvaccinated pigs, except those in the VO group, were challenged with A/TAI/15/2013 and monitored for up to 14 dpc. All unvaccinated control pigs developed clinical signs and since they reached the ethical endpoint, they were euthanized between 3 and 5 dpc. Systemic disease (vesicular lesions on the non-inoculated feet, snout and/or tongue) was observed in all V7 pigs, regardless of vaccine used. Several animals in these groups were euthanized between 4 and 6 dpc after reaching humane endpoint. The mean maximum lesion score was lower in all vaccinated groups compared to the control group (Figure 1A), however, when the different vaccine groups were compared, significant protection was only observed in the Combo V21 group (4/5 pigs protected: Fisher's Exact $p = 0.01794$). In the A May V21 and A22 V21 groups, 1/5 pigs were protected from systemic disease (Figure 1B). The individual daily lesion scores and time of euthanasia are presented in Table 1). Elevated rectal temperatures lasting 2–4 days were observed in the pigs in all groups between 1 and 6 dpc (Figure 1B).

Kaplan-Meier survival analysis was performed on the day pigs showed lesions post-challenge in each group; the mean probability of protection (0-1) and median time to appearance of lesions was estimated (as dpc). In the UVC group the median time until lesions appeared was 5 dpc (4–5 dpc) and the mean probability of protection was 0.06 (0.01-0.42; mean and 95% CI) by 5 dpc. The pigs in the Combo V21 group performed the best with the median protection time until lesions develop indeterminable (∞ dpc) and the mean probability of protection from clinical disease 0.80 (0.52–1.00; mean and 95% CI) by 14 dpc. For the other vaccine groups, the median time until lesions appeared was between 9 (A May V7) and 14 days (A22

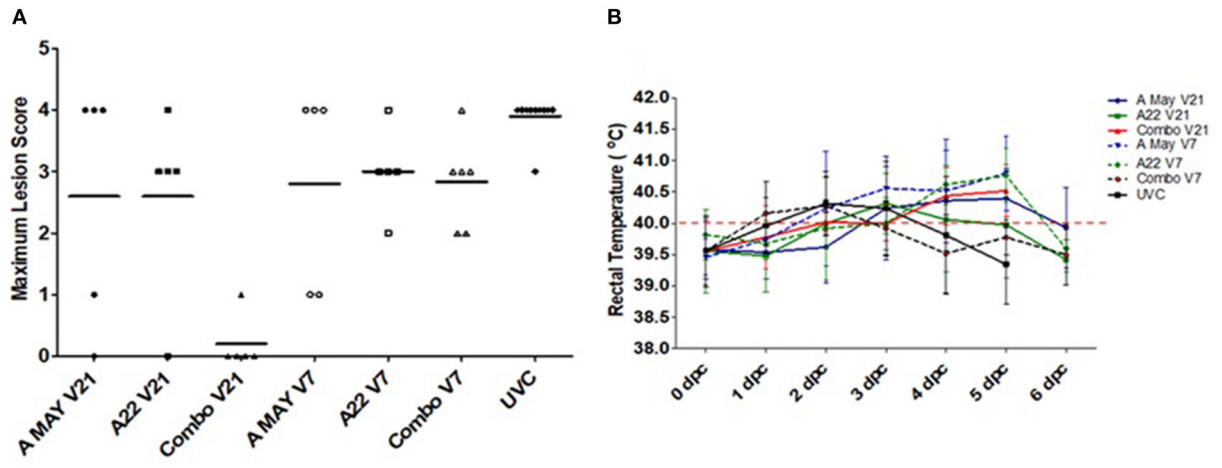

FIGURE 1
Lesion scores and rectal temperatures. **(A)** Maximum lesion scores in pigs challenged with A/Asia/SEA-97 variant virus post-vaccination. Horizontal lines indicate the group mean scores. **(B)** Average rectal temperatures in pigs vaccinated and challenged during the experiment. Temperatures >40°C were considered Horpyrexia (red dotted line).

V21). For the A May V21 group the median time was 10 days compared to 11 days for the A22 V7 group. The probability of protection progressively decreased from 5 dpc to 14 dpc (Figure 2, Supplementary Tables 2A,B).

Detection of neutralising antibodies using VNT in the challenged groups

Complete results of the homologous and heterologous (challenge virus) neutralising antibody titres in different vaccine groups are in Supplementary Tables 3A–C. At the time of challenge (21 or 7 dpc), none of the vaccinated pigs had measurable homologous or heterologous neutralising antibodies to the vaccine or challenge strain. As a result of the pigs that reached endpoint and had to be euthanised, only limited data were available to investigate the anamnestic response up to 14 dpc.

Detection of antibodies to structural proteins using an FMD A22 CELISA in the challenged groups by SPCE

Antibodies to FMDV A structural proteins were detected by SPCE using A22 as antigen (Supplementary Table 4, Figure 3). A stronger antibody response was observed in the Combo V21 pigs compared to the A May V21 or A22 V21 pigs post-vaccination. All Combo V21 pigs and two of the A May V21 pigs were seropositive at the time of challenge. None of the V7 pigs had seroconverted by the day of challenge. An anamnestic response was observed in the vaccinated pigs from 3 dpc, when compared to the UV controls.

Antibody responses in unchallenged vaccinated pigs (VO groups) using VNT and SPCE

At the same time as the groups that were vaccinated 21 days prior to challenge, two pigs each were vaccinated with A22 and A May 97 vaccines (A22 VO and A May VO respectively). An additional 5 pigs were vaccinated with the Combo vaccine (Combo VO). There animals were not challenged but their antibody titres were measured at the same days as the V21 challenge groups.

The neutralising antibody titres to A May 97, A22 and A TAI/15/2013 are presented in Supplementary Tables 3A–C. The A May VO group did not have homologous or heterologous antibody titres throughout the course of the experiment. One of the A22 VO group animals had low detectable homologous antibody titres by 7 dpc (28 dpv) and both had variable levels of heterologous antibody titres to A/TAI/15/2013 by 31 dpv and 35 dpv.

In the Combo VO group, the homologous and heterologous responses were generally low with a small number of pigs having measurable antibodies by 7 dpc (28 dpv). One pig (#046) did not sero-convert up to 35dpv.

With the SPCE assay that used A22 specific reagents, the animals in the VO groups had detectable antibodies as early

TABLE 1 The individual lesion scores for the first 7 dpc and the time of euthanasia as well as the protection outcome.

Vaccine	Day of vaccination	Group	Pig ID	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	Status
A Malaysia 97	−21 dpc†	A May V21	001	0	0	3	3	4	E	E	E	Not protected
			002	0	0	2	3	4	2	E	E	Not protected
			003	0	0	0	0	0	0	0	0	Protected
			004	0	0	4	4	4	4	4	E	Not protected
			005	0	0	0	0	0	1	1	E	Not protected
A22 Iraq 64	−21 dpc	A22 V21	006	0	0	0	2	3	2	3	E	Not protected
			007	0	0	0	0	0	0	0	0	Protected
			008	0	0	0	1	3	2	2	2	Not protected
			009	0	0	1	2	4	4	E	E	Not protected
			010	0	0	1	1	2	3	2	E	Not protected
A Malaysia 97 + A22 Iraq 64	−21 dpc	Combo V21	030	0	0	0	0	1	1	1	1	Not protected
			031	0	0	0	0	0	0	0	0	Protected
			032	0	0	0	0	0	0	0	0	Protected
			033	0	0	0	0	0	0	0	0	Protected
			034	0	0	0	0	0	0	0	0	Protected
A Malaysia 97	−7 dpc	A May V7	011	0	0	3	4	4	4	E	E	Not protected
			012	0	0	0	1	1	1	E	E	Not protected
			013	0	0	3	4	4	4	E	E	Not protected
			014	0	0	0	0	1	1	E	E	Not protected
			015	0	0	3	4	4	E	E	E	Not protected
A22 Iraq 64	−7 dpc	A22 V7	016	0	0	0	3	3	1	E	E	Not protected
			017	0	0	0	1	2	2	E	E	Not protected
			018	0	0	0	0	1	2	2	2	Not protected
			019	0	0	0	1	1	1	1	E	Not protected
			020	0	0	3	3	3	3	E	E	Not protected
A Malaysia 97 + A22 Iraq 64	−7 dpc	Combo V7	035	0	0	0	0	1	2	E	E	Not protected
			036	0	0	0	0	2	3	E	E	Not protected
			037	0	0	0	0	2	3	E	E	Not protected
			038	0	0	0	3	4	4	E	E	Not protected
			039	0	0	0	1	2	3	E	E	Not protected
Unvaccinated controls		UV	021	0	0	3	4	4	E	E	E	Not protected
			022	0	0	4	4	4	E	E	E	Not protected
			023	0	0	3	4	4	E	E	E	Not protected
			024	0	0	3	3	4	E	E	E	Not protected
			025	0	0	3	4	4	E	E	E	Not protected
			040	0	0	4	4	E	E	E	E	Not protected
			041	0	0	2	3	3	3	E	E	Not protected
			042	0	0	3	4	4	4	E	E	Not protected
			043	0	0	4	4	4	E	E	E	Not protected
			044	0	0	3	4	3	E	E	E	Not protected

† dpc, days post-challenge; Score of 1 for each site where lesions form besides inoculation site (maximum of 4). E, euthanized (animals where E is not indicated were euthanized at the end of the study, 14 dpc).

as 25 dpv (−4 dpc) in most of the animals (6/9 pigs) and by 35 dpv (14 dpc) eight out of nine pigs had seroconverted (Supplementary Table 4). Using this assay, antibodies were detected in pig #046.

Viraemia

Viraemia was detected by both real-time RT-qPCR and virus isolation (Table 2). Infectious virus was detected in only four of

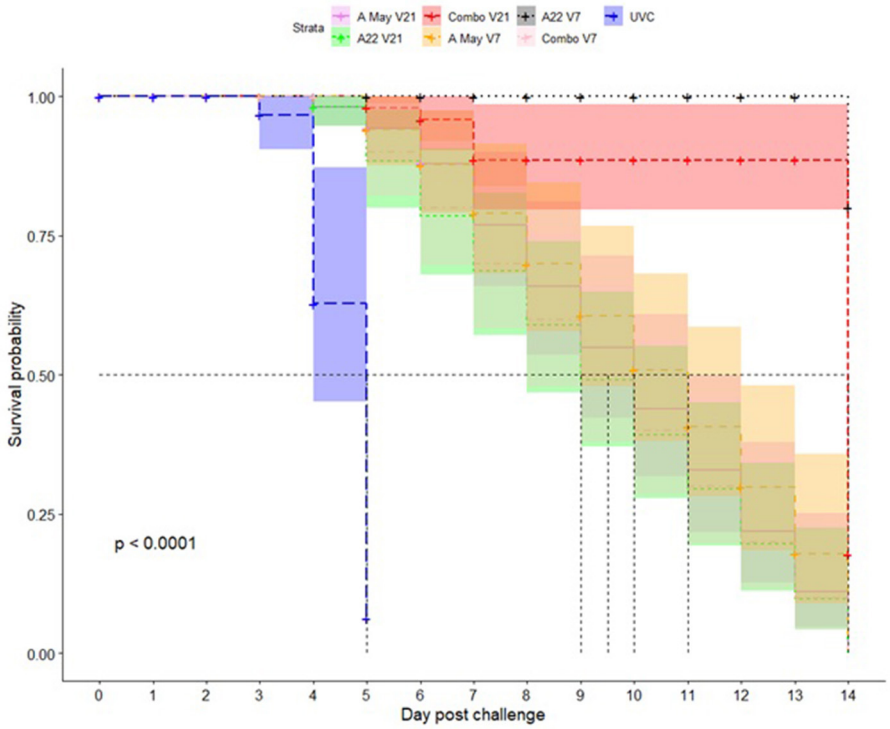

FIGURE 2
Kaplan-Meier survival plots for protection against A/TAI/15/2013 virus in pigs vaccinated with A May 97 and A22 Iraq 64 vaccines in monovalent or combination formulations. The x-axis represents time in days, and the y-axis shows the probability of surviving or the proportion of pigs surviving the virus challenge post-vaccination in different vaccine groups. The lines represent survival curves of the seven vaccine groups (Vaccine = 1: A May V21; Vaccine = 2: A22 V21; Vaccine = 3: Combo V21; Vaccine = 4: A May V7; Vaccine = 5: A22 V7; Vaccine = 6: Combo V7; Vaccine = 7: UVC). A vertical drop in the curves indicates an event (pigs showing clinical signs of FMD). The vertical tick mark on the curves means that a pig was censored at this time.

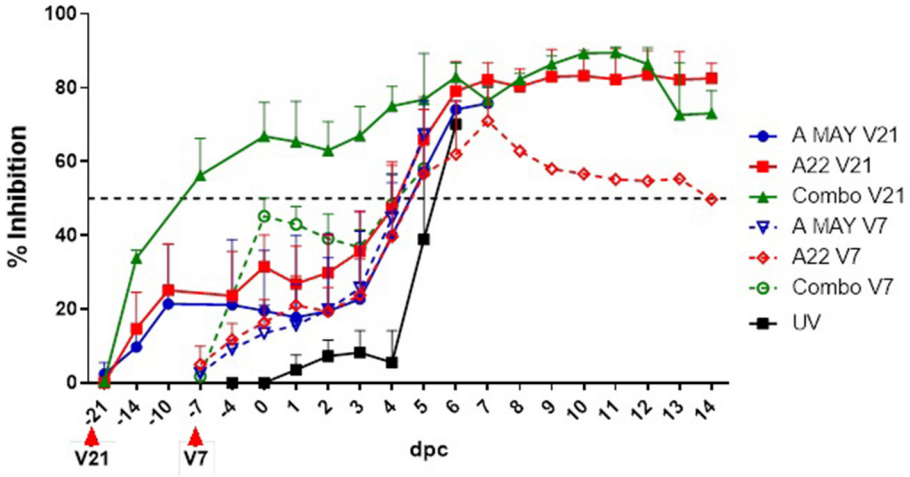

FIGURE 3
Antibody responses to FMDV structural protein based on a solid-phase competition ELISA. Per cent Inhibition values are shown in the y-axis and day post-challenge (dpc) in the x-axis. The error bars represent the standard deviation of mean PI values. The horizontal dashed line indicates the cut-off level for a sample to have a positive response to vaccination. V21 and V7 indicate the day the vaccines were administered prior to the challenge.

TABLE 2 Viral RNA concentration measured as log_{10} copy numbers/ml by a reverse transcription and quantitative PCR (RT-qPCR) in sera from animals challenged with A/TAI/15/2013 virus.

Group	Pig ID	0 dpc†	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc
A May V21	001	-	4.91*	7.02	6.59	3.92	E
	002	-	4.33	3.55	5.43	4.05	-
	003	-	-	-	3.76	-	-
	004	-	5.65	7.21	4.68	-	-
	005	-	-	-	-	-	-
A22 V21	006	-	-	3.76	-	-	-
	007	-	3.78	-	-	-	-
	008	-	-	-	-	-	-
	009	-	-	-	5.69	4.93	-
	010	-	-	-	3.76	-	-
Combo V21	030	-	-	-	-	4.51	-
	031	-	-	-	-	-	-
	032	-	-	-	-	-	-
	033	-	-	-	-	-	-
	034	-	-	-	-	-	-
A May V7	011	-	4.25	6.04	5.55	-	-
	012	-	-	3.82	3.76	-	-
	013	-	-	5.52	4.49	-	-
	014	-	-			-	-
	015	-	-	6.17	5.64	-	E
A22 V7	016	-	3.76	-	-	-	-
	017	-	-	-	-	-	-
	018	-	-	-	4.09	-	-
	019	-	-	-	-	3.81	-
	020	-	4.66	5.50	-	-	-
Combo V7	035	-	-	-	-	-	-
	036	-	-	-	4.86	4.60	-
	037	-	-	4.91	4.65	-	-
	038	-	-	-	4.39	-	-
	039	-	5.09	-	5.41	4.83	-
UV	021	-	4.03	7.74	6.17	3.97	E
	022	-	6.28	8.22	6.76	5.41	E
	023	-	5.76	8.71	6.53	4.06	E
	024	-	5.07	8.26	6.68	4.22	E
	025	-	5.11	8.93	6.13	-	E
	040	-	6.75	8.16	7.18	E	E
	041	-	7.04	9.22	5.87	5.51	E
	042	-	7.63	9.93	6.99	6.16	-
	043	-	8.04	9.53	4.48	4.89	-
	044	-	6.43	8.44	4.96	4.55	E

† dpc, day post-challenge; *Log_{10} FMDV RNA copies/ml serum; Red square = VI positive; E = animal had been euthanized.
Samples positive by virus isolation on $LFBK_{\alpha5\beta6}$ cells are highlighted with grey colour. - indicates samples where the viral RNA was below the detection limit of the RT-qPCR.

the vaccinated pigs up to 3 dpc, except in groups Combo V21, A22 V7 and Combo V7.

FMDV RNA was detected in the serum of most animals on at least 1 day between 1 and 4 dpc, except for the Combo V21 group, where only one pig was positive on 1 day, 4 dpc (Table 2; Figure 4A). The highest mean viraemia was in the UV pigs with FMDV RNA detected in all animals during 1–4 dpc with most pigs in this group removed from the study

on 5 dpc. No viral RNA was detected in the blood of any vaccinated pigs after 4 dpc (not shown in Figure 4A). With 21 days between vaccination and challenge, the combination vaccine was most effective at reducing viraemia and the A May vaccine appeared less effective at reducing viraemia compared to the A22 vaccine. There was statistically insignificant difference in viral RNA between the groups challenged−7 dpv ($P < 0.05$). Peak viraemia was observed as early as 2 dpc ($p = 0.005$) and 3 dpc ($p = 0.045$) in A May V7 vaccine group when compared to the other vaccinated groups. All vaccinated animals showed a decrease in detectable viraemia compared to the unvaccinated animals ($P < 0.001$).

Excretion of virus in nasal and oral swabs

FMDV RNA was detected in nasal and oral swabs from all pigs between 1 and 7 dpc. Viral RNA levels in oral swabs were similar in all groups (UVC and vaccinated and challenged; Figure 4B). Peak excretion in nasal secretion was observed at 3 and 4 dpc ($p = 0.004$) in all groups (Figure 4C). Compared to the other vaccine groups reduced virus excretion in nasal swabs was observed in the Combo 21 group ($p < 0.001$; Figure 4C). As many of the pigs from the vaccinated and UVC groups animals were removed at 4 or 5 dpc, comparison of duration of excretion between groups was not possible.

Statistical comparison of median and mean AUC values for viral RNA in serum, oral and nasal secretions

Due to ethical reasons, several pigs from each group were euthanised before the end of the trial at 14 dpc and the Area Under Curve was used to compare the viral RNA loads in serum, nasal, and oral swabs post-challenge. The median and mean AUC values for levels of viral RNA in blood, nasal and oral secretions were estimated for individual pigs in each group (Supplementary Tables 5A–C) and between group comparisons were performed using one-way ANOVA (including all groups) or two-way ANOVA (excluding the UVC group). The results of ANOVA on the median and mean AUC for viraemia, virus excretion in nasal and oral secretions are presented in Figures 5A–F and Supplementary Tables 6A,B.

A significant difference in the median and mean AUC value for viral RNA in sera was observed in the different groups (one-way ANOVA $p = 1.2e\text{-}07$ and $p = 7.8e\text{-}09$ respectively, Figures 5A,B) and the difference was attributable to the differences between the vaccine and the control groups ($p < 0.001$). A significant difference in mean viral RNA in the blood was also present between the Combo V21 and A May V7 groups (Figure 5B, $p < 0.05$).

The overall differences in median and mean values for viral RNA in the oral swabs were significant between the different groups (ANOVA $p = 0.052$ and $p = 0.051$ respectively; Figures 5C,D). *Post hoc* tests with Bonferroni correction (Supplementary Table 5A) showed that the median AUC values differed significantly between the A May V7 and UVC group only ($p < 0.05$) and the mean AUC values were also significantly different ($p < 0.1$).

The median and mean AUC values for the nasal swabs differed between various experimental groups ($p = 0.00083$ and $p = 0.00027$ respectively; Figures 5E,F). The values for the Combo V21 group differed significantly from the Combo V7 ($p < 0.05$ and $p < 0.01$ respectively; Supplementary Table 5A), A May V21 ($p < 0.01$ and $p < 0.01$ respectively) and the UVC group ($p < 0.05$ and $p < 0.05$ respectively; Supplementary Table 5A).

Since we lost all the unvaccinated control pigs by 6 dpc, we compared the median and mean AUC values in vaccinated groups excluding the unvaccinated groups to identify differences in viraemia and viral excretion using a two-way ANOVA (vaccine vs challenge day i.e., V21 or V7). The results are presented in Supplementary Table 5B. The AUC values for viraemia in vaccine groups differed significantly ($p = 0.0728$ and $p = 0.090$ resp.) and the difference was prominent between A May 97 vs. Combo vaccine groups (V21; adjusted $p = 0.098$ and 0.062 resp.). There was no significant difference in the AUC values for virus excretion in oral swabs between the vaccine groups ($p < 0.100$). However, significant differences were noticed in the median AUC values for virus excreted in the nasal secretions ($p < 0.0001$) and the difference was prominent between the Combo groups (V21 vs V7; adjusted $p = 0.006$) and between A May 91 and Combo groups (V21; adjusted $p = 0.0006$). The mean AUC values were also significantly different ($p < 0.0001$) and could be attributed to Combo V21 vs A22 V21 (adjusted $p = 0.0006$), Combo V7 vs A22 V7 (adjusted $p = 0.00708$), Combo V21 vs V7 (adjusted $p = 0.001$), A May 97 and Combo (V21; adjusted $p < 0.0001$) and A May 97 V7 vs. Combo V21 (adjusted $p = 0.0045$).

Discussion

The emergence of novel variants of FMDV in South and South-East Asia continues to pose a threat to Australia's biosecurity and livestock industries. The continued evolution of O/ME-SA/Ind2001 lineage and spread to FMD-free countries like Indonesia and the appearance of new sublineages of A/Asia/SEA-97 in the region are of grave concern. Unlike serotype O there are limited vaccine strain options for serotype A, due to significant antigenic diversity and lack of cross protection. Therefore, it is important to continuously evaluate

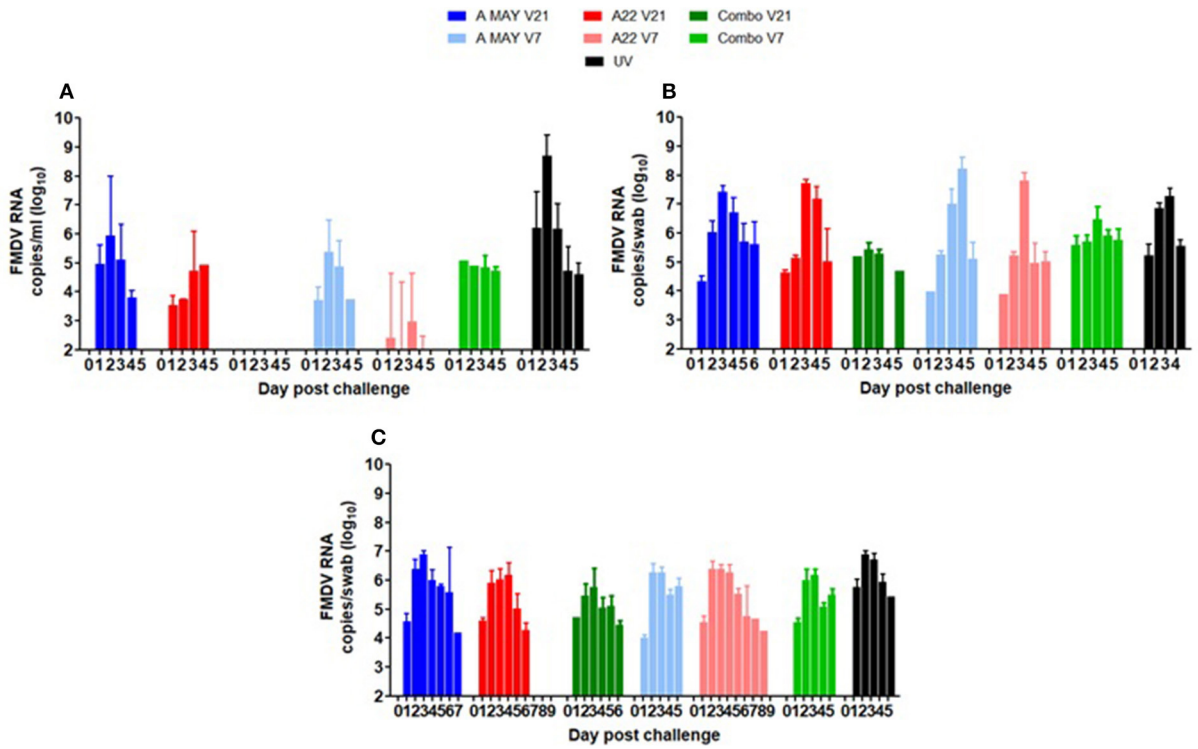

FIGURE 4
Mean viral loads as **(A)** Log_{10} FMDV RNA copies/ml in serum indicative of viraemia and Log_{10} FMDV RNA copies/swab indicative of virus shedding in **(B)** oral swabs and **(C)** nasal swabs. The error bars show the standard deviation of the mean. Since many pigs were euthanised due to the appearance of lesions and attaining humane endpoints, the numbers in the day post-challenge differ with different groups.

the existing vaccine strains against variants emerging in the region. The emergence of a new Thai variant of A/Asia/SEA-97 lineage is a serious threat and there is a paucity of information if the internationally recognised vaccine strains, A Malaysia 97 and A22 Iraq 64, would offer protection in animals infected with this sub-lineage of viruses. In this study, we compared the outcomes of virus challenge at 7 and 21 dpv in pigs vaccinated with the above-mentioned vaccine strains as monovalent vaccines or as a combined vaccine.

The challenge virus, A/TAI/15/2013, was highly virulent in pigs and for ethical reasons many pigs had to be euthanised before the end of the study. This resulted in fewer animals for all observable time points. It was challenging to obtain a meaningful outcome when comparing the viraemia and virus shedding in nasal and oral secretions between the different groups using traditional statistical methods (ANOVA). A method of using AUC of the viral load integrated over time was used in this study to compare the response in pigs vaccinated and challenged with FMDV with unvaccinated and challenged pigs. The AUC approach is a universal means of assessing the interrelationship among initial viral load, rate of increase of viral load and peak viral load (32). By combining the absolute viral load and the duration of viraemia into a single parameter, the AUC concept provides a means of combining the determinants of viraemia (29). This approach was shown to be a valuable method to access PCV2 infections in pigs and their effect on the average daily weight gain and viral load. Such a method was used to compare virus loads in patients infected with Respiratory Syncytial Virus in the past (33). Comparisons were also made between the AUC during viraemia and the absolute virus load for cytomegalovirus infections in human patients undergoing kidney transplants (29). Since this method considers the duration of viraemia/shedding and the load of the virus we can map the success of the vaccine-induced protection in pigs when pigs are removed from experiments due to ethical reasons or on reaching the humane endpoint. The assumption is that not only the virus load but also the duration of shedding is important when the vaccine quality is assessed.

Vaccination with high potency A22 Iraq or A May 97 protected 20% of pigs from clinical FMD following challenge with FMDV/A/TAI/15/2013 when administered 21 days prior to challenge. Overall, clinical signs were less severe in animals that received the A22 Iraq vaccine compared to those that received the A May 97 vaccine in both V7 and V21 groups. The A22 vaccinated pigs also had a reduced viraemia when compared to the A May 97 vaccinated pigs. In contrast, using the combination

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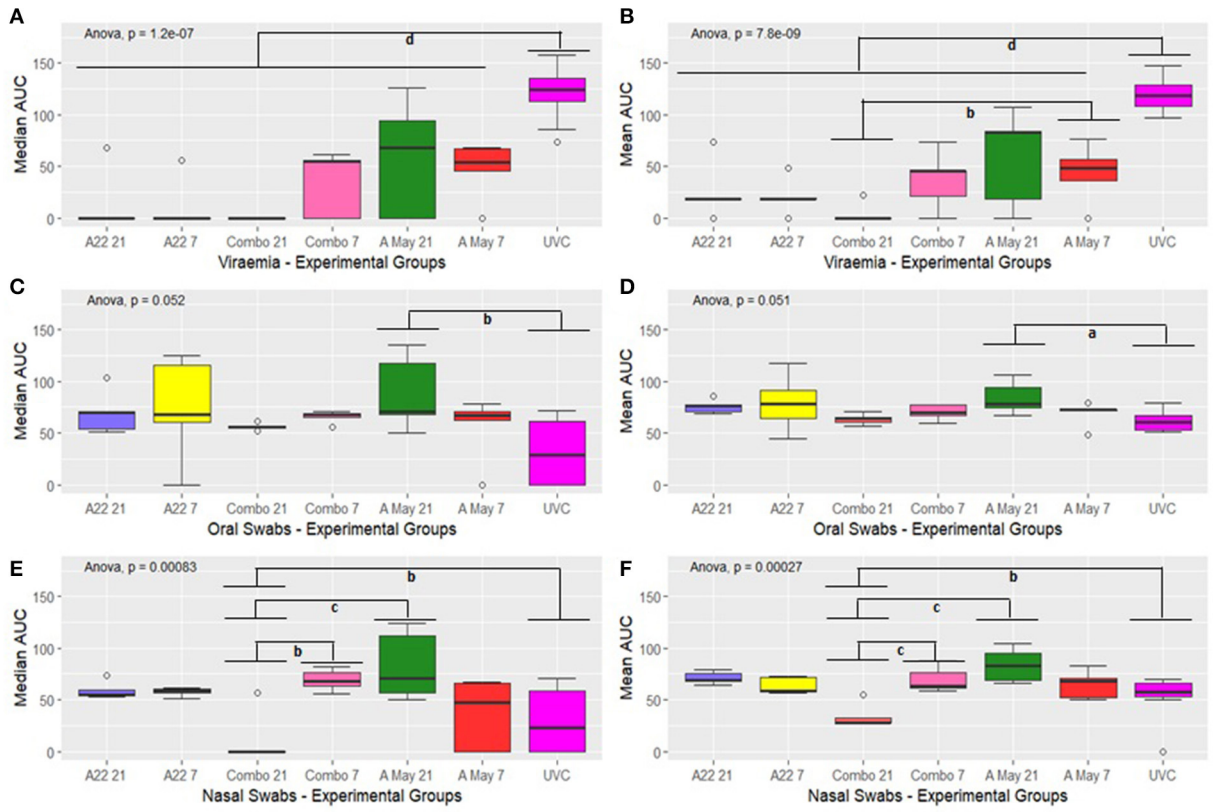

FIGURE 5
Box plot showing median and mean AUC of virus load in the blood **(A,B)**, oral fluids **(C,D)** and nasal secretions **(E,F)**. The boxplots show the interquartile range (median represented as the thick horizontal line within the box, and the first and third quartile of the data) and the minimum and maximum values for each group connected to the boxes with the vertical line. Groups with subscripts differ significantly. a = $p < 0.1$; b = $p < 0.05$; c = $p < 0.01$ and d = $p < 0.001$.

A22/A May vaccine, 80% (4 of 5) of the V21 pigs were protected from disease. Neither vaccine protected pigs when administered just 7 days prior to the challenge. All V7 pigs developed a systemic disease and were euthanized between 3 and 5 dpc. Viraemia was notably reduced in the Combo V21 pigs (only one animal positive), however, in the Combo V7 pigs, results were comparable to those seen in the A May V7 and A22 V7 pigs.

None of the vaccines induced a protective neutralising antibody response by the time of challenge, 7 or 21 dpv, showing poor correlation between neutralising antibody levels and protection. There was also poor correlation between neutralising titres and SP ELISA results. In ELISA, all Combo V21 pigs and two A May V21 pigs had seroconverted by the day of challenge; however, these results were also not entirely concordant with clinical protection. As most vaccinated pigs had seroconverted by 4 dpc, and only 1/10 UV pigs had seroconverted at this time, there is some indication of an anamnestic response, however with many pigs culled at 4 or 5 dpc, this result is not conclusive.

Virus was detected in the nasal and oral swab samples from all pigs between 1 and 6 dpc. Viral loads were lower in the nasal swab samples from the Combo V21 pigs compared to the other groups, but there was no difference in the oral swab samples.

These results suggest the combination A22 Iraq/A May 97 vaccine is more effective at providing protection from the A/TAI/19/2013 strain than the individual strain vaccines, with 21 days between vaccination and challenge. Kaplan-Meier survival analysis showed that a combination vaccine with A22 and A May 97 vaccine strains will be suitable for use against A/Asia/SEA-97 variants with a high probability of protection followed by A22 monovalent vaccine.

There was no evidence that the pigs protected from the systemic disease had protective antibody responses sooner than other pigs in the study, implying other immune mechanisms might play a role in the protection of pigs. All Combo V21 pigs and two of the A May V21 pigs were seropositive at the time of challenge. We did not have a homologous ELISA system with A May 97 reagents to compare the results with one performed using A22 Iraq homologous reagents. Therefore, we could not address the issue with one pig #046 that had no detectable antibodies in VNT but was positive throughout the experimental period in the ELISA using A22 Iraq reagents.

Conclusion

FMD viruses continue to evolve and pose a significant challenge to both endemic and FMD-free countries. Development of new vaccine strains is time consuming and expensive while control of the disease by vaccination using the existing vaccine strains can be a challenge. This study shows that by combining vaccine strains we can increase the efficacy of vaccines against variant FMD viruses. Though these results are based on a small number of pigs and with a virulent virus challenge, we still can get valuable information by employing novel methods of analysis like the AUC method and the Kaplan-Meier probability of survival statistics. Given the epidemiological situation in South-East Asia and the co-circulation of different variants of the A/Asia/SEA-97 lineage, we recommend that both A Malaysia 97 and A22 Iraq 64 are included in the vaccines.

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.

Ethics statement

This study was approved by the Australian Centre for Disease Preparedness (ACDP) Animal Ethics Committee (AEC 1774 and AEC 1801), the Canadian Centre for Human and Animal Health Animal Care Committee (AUD# C-15-007), and performed in strict accordance with the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the Canadian Council for Animal Care Guidelines.

Author contributions

WV, NSB, and JH: conceptualization, formal analysis, and writing–original draft preparation. JH and NSB: methodology and data curation. JH and HB: animal studies. NSB, JH, CKN, and WV: writing–review and editing. WV: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1027556/full#supplementary-material

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EDITED BY
Shawn Babiuk,
National Centre for Foreign Animal Disease (NCFAD), Canada

REVIEWED BY
Charles K. Nfon,
National Centre for Foreign Animal Disease (NCFAD), Canada
Nagendrakumar Singanallur Balasubramanian,
Australian Animal Health Laboratory (CSIRO), Australia

*CORRESPONDENCE
Teresa de los Santos
teresa.delossantos@usda.gov
Fayna Díaz-San Segundo
fayna.diaz-sansegundo@usda.gov

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Mutation of FMDV L^{pro} H138 residue drives viral attenuation in cell culture and *in vivo* in swine

Paul A. Azzinaro[1], Gisselle N. Medina[1,2], Devendra Rai[1,3], Elizabeth Ramirez-Medina[1], Edward Spinard[1], Monica Rodriguez-Calzada[1,4], James Zhu[1], Elizabeth Rieder[1], Teresa de los Santos[1]* and Fayna Díaz-San Segundo[1]*

[1]Plum Island Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Greenport, NY, United States, [2]National Bio and Agro-Defense Facility (NBAF), Agricultural Research Service (ARS), U.S. Department of Agriculture (USDA), Manhattan, KS, United States, [3]Pfizer Worldwide Research, Development and Medical, Pearl River, NY, United States, [4]Oak Ridge Institute for Science and Education, PIADC Research Participation Program, Oak Ridge, TN, United States

The foot-and-mouth disease virus (FMDV) leader proteinase (L^{pro}) is a papain like protease that cleaves the viral polyprotein and several host factors affecting host cell translation and induction of innate immunity. Introduction of L^{pro} mutations ablating catalytic activity is not tolerated by the virus, however, complete coding sequence deletion or introduction of targeted amino acid substitutions can render viable progeny. In proof-of-concept studies, we have previously identified and characterized FMDV L^{pro} mutants that are attenuated in cell culture and in animals, while retaining their capacity for inducing a strong adaptive immunity. By using molecular modeling, we have now identified a His residue (H138), that resides outside the substrate binding and catalytic domain, and is highly conserved across serotypes. Mutation of H138 renders possible FMDV variants of reduced virulence *in vitro* and *in vivo*. Kinetics studies showed that FMDV A12-L_{H138L} mutant replicates similarly to FMDV A12-wild type (WT) virus in cells that do not offer immune selective pressure, but attenuation is observed upon infection of primary or low passage porcine epithelial cells. Western blot analysis on protein extracts from these cells, revealed that while processing of translation initiation factor eIF-4G was slightly delayed, no degradation of innate sensors or effector molecules such as NF-κB or G3BP2 was observed, and higher levels of interferon (IFN) and IFN-stimulated genes (ISGs) were induced after infection with A12-L_{H138L} as compared to WT FMDV. Consistent with the results in porcine cells, inoculation of swine with this mutant resulted in a mild, or in some cases, no clinical disease but induction of a strong serological adaptive immune response. These results further support previous evidence that L^{pro} is a reliable target to derive numerous viable FMDV strains that alone or in combination could be exploited for the development of novel FMD vaccine platforms.

KEYWORDS

FMDV, foot-and-mouth disease virus, leader proteinase, L^{pro}, attenuated virus

Introduction

Foot-and-mouth disease virus (FMDV) is the prototype member of the *Aphthovirus* genus of the *Picornaviridae* family. The virus contains a positive single-stranded RNA genome of approximately 8,500 nucleotides surrounded by a non-enveloped icosahedral protein capsid. Upon infection, the viral RNA is released in the cytoplasm of the cell followed by rapid translation into a single polyprotein that is co-temporarily processed into intermediate and mature proteins, by three viral-encoded products, leader (L^{pro}), 2A and $3C^{pro}$. Final viral cleavage products include four structural, VP1, VP2, VP3, and VP4, and 10 non-structural (NS) proteins (L^{pro}, 2A, 2B, 2C, 3A, $3B_{1,2,3}$, $3C^{pro}$, and $3D^{pol}$) although, as mentioned above, several intermediate protein products are also detected during the viral infectious cycle (1). In particular, L^{pro} cleaves itself off from the rest of the viral polyprotein at its carboxyl terminus (2, 3). FMDV displays high genetic variation and exists as a quasipecies in seven distinct serotypes including A, O, C, Asia and South African Territories (SAT)−1, 2, and 3, and multiple strains. Such a genetic variability is probably the result of its error-prone RNA polymerase ($3D^{pol}$), the multiple receptor usage, and the virus adaptation evolved to successfully infect over 70 cloven-hoof animal species (4, 5).

Outbreaks of FMD can cause devastating economic loses in FMD-free countries and, at the same time, they can jeopardize development of nations that rely on agriculture for subsistence (6). Control of FMD outbreaks is mainly achieved through a strong veterinary surveillance, physical isolation of endemic areas, and use of an inactivated whole virus vaccine in endemic/high risk areas as well as in some FMD-free areas to maintain the "free" status (7). Although this vaccine has proven successful in reducing the number of outbreaks worldwide, it has fallen short in eradicating FMD probably due to intrinsic limitations of the vaccine *per se*, resource crunch and social and political instability in endemic areas (8). There is interest in developing alternative vaccines that could be tailored for using in different environments (9). One of the main limitations of the current vaccine is the need of 7 days to induce protection. Historically, it has been shown that live attenuated vaccines (LAVs) can act rapidly post inoculation inducing strong immunity as early as 3 days post- immunization (10). In fact, viral disease eradication has only been achieved for smallpox and rinderpest by using LAV (11, 12). Thus far, no attenuated vaccine has been successfully developed for FMDV (9). However, previous studies showed that viable attenuated FMDV could be derived using reverse genetics by deleting the entire L^{pro} coding region (13). Unfortunately, cattle and swine studies showed limited replication of this virus, poor immune response and lack of protection in efficacy trials (14, 15). Nevertheless, leaderless strains (LLV) have been further developed by inclusion of markers that allow differentiation of infected from vaccinated animals (DIVA) and convenient restriction sites for easy genetic manipulation, leading to the development of an effective platform for production of inactivated FMDV vaccine (16). Moreover, the LLV strain has been of extreme utility in studies aimed to deciphering the mechanisms by which FMDV counteracts the immune response in the host. It is currently known that L^{pro} blocks the induction of interferon (IFN) mRNA and the expression of IFN protein (17–21). In addition, the molecular mechanisms implicated in this inhibition have been further elucidated, and to this end many signaling molecules have been identified as L^{pro} specific targets in a direct or indirect manner [reviewed in (22–25)].

We and others have previously shown that viable attenuated FMDV could also be derived after introduction of specific mutations in the conserved L^{pro} SAP domain (26–28) or in other residues that abolish specific L^{pro} enzymatic activities such as deUbiquitinase and deISGylase activity (22, 24, 29). Interestingly, and differently than for leaderless virus, inoculation with SAP mutant FMDV induces a strong adaptive immune response and animals are completely protected against challenge with wild type virus as early as 2 days post-vaccination (dpv) and for at least 21 dpv. Similarly, a W105A mutation that obliterates deUb- and deISG- activity, resulted in a virus attenuated in cell culture and in a mouse model of FMD (22).

In this work, we have identified H138 as another highly conserved amino acid within L^{pro} that lies outside of the substrate-binding pocket, but plays a critical role in FMDV virulence. While a conserved change such as H138N did not dramatically affect virus growth properties and characteristics, disruptive H138L did result in a virus with slower kinetics of growth in cell culture allowing for the induction of higher levels of IFN and IFN stimulated genes (ISGs). Interestingly, inoculation of swine with this FMDV A12-L_{H138L} mutant induced mild or no disease, albeit induction of significant and protective levels of neutralizing antibodies. These results highlight the plasticity of L^{pro} as a candidate target within the viral genome, to derive multiple virus strains with potential for development as novel live attenuated, or relatively safer seeds of inactivated vaccines, for the control of FMD.

Materials and methods

Cells and viruses

BHK-21 cells (baby hamster kidney cells strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC, Rockville, MD) were used to propagate virus stocks and to measure virus titers. Porcine kidney (LF-BK, SK6, and IBRS-2) cells were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL), Animal, Plant,

and Health Inspection Service (APHIS) at the PIADC and/or ATCC (Manassas, VA). Primary/secondary porcine kidney (PK) cells were provided by the APHIS National Veterinary Service Laboratory, Ames, Iowa. LF-BK, IBRS-2, SK6 and PK cells were maintained in minimal essential medium (MEM, Thermo Fisher, Waltham, MA) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and non-essential amino acids. BHK-21 were maintained in similar media but bovine calf instead of fetal bovine serum was used, and the media was also supplemented with 10% Tryptose phosphate broth (Thermo Fisher). All cell cultures were incubated at 37°C in 5% CO_2.

FMDV A12-WT was generated from the full-length serotype A12 infectious clone, pRMC35 (30). FMDV A12-LLV (leaderless virus) was derived from the infectious clone by deleting the Lb coding region, pRM-LLV2 (13). A12-L_{H138} mutant viruses were constructed by site directed mutagenesis using the QuickChange® kit (Agilent, La Jolla, CA). All viruses were derived by electroporation of RNA in BHK-21 cells, passed 4 times in the same cells, followed by propagation, concentration by polyethylene glycol precipitation, and titration by plaque assay (pfu/ml) or end point dilution ($TCID_{50}$/ml) on BHK-21 cells (30). Viral full-length sequences were confirmed by DNA sequencing of derived viral cDNA using an ABI prism 7,000 apparatus (Applied Biosystems, Thermo Fisher).

Molecular modeling

Initial structural analysis was performed using the crystal structure of the Protein Data Bank (PDB): 4QBB (31). The model was manipulated using Discovery Studio visualizer (v21.1.0.20298). Structural analysis identified internal interacting residues which we predicted to effect protein stability. The identified critical residue was mutated *in silico* and the effects of substitutional mutations were simulated.

Western blotting

Total cell lysates were prepared as described previously (20, 23). Proteins were resolved by SDS-PAGE and analyzed by western blotting. eIF4G was detected with a rabbit polyclonal antibody (Ab) #A300-502A (Bethyl Laboratories, Montgomery, TX), p65 with a rabbit polyclonal Ab #RB-1638 (NeoMarkers, Lab Vision, Freemont, CA), G3BP2 with a rabbit polyclonal Ab #OALA09398 (Aviva Systems Biology, San Diego, CA), VP1 with a rabbit polyclonal Ab made at PIADC and control tubulin-α, with mouse monoclonal Ab Ab-2 MS-581 (NeoMarkers, Lab Vision, Freemont, CA). Secondary Abs anti-mouse or anti-rabbit, conjugated to horseradish peroxidase (HRP) were obtained from Pierce (Rockford, IL). Protein bands were detected with ECL chemiluminescence Kit (Biorad, Hercules, CA) and images acquired with Gel Doc c300 digital imager (Azzure Biosystems, Dublin, CA).

Detection of interferon (IFN) stimulated genes (ISGs) by real time PCR

Expression of several ISGs was analyzed by qRT-PCR as previously described (27). RNA was extracted from PK cells infected at a multiplicity of infection (MOI) of 10, with wild type (WT), leaderless (LLV) or mutant H138L FMDV. Porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the values for each sample. Reactions were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by comparative cycle threshold analysis (user bulletin 2; Applied Biosystems) utilizing as a reference the samples at 0 dpi.

Analysis of IFN-α protein

A porcine IFN-α (pIFN-α) double capture ELISA previously developed in our laboratory was used to quantitate pIFN-α protein in the supernatants of infected cells (32). Anti pIFN-α mAb K9 and F17 were purchased from R&D Systems (Minneapolis, MN). MAb K9 (1 μg/ml) was used for antigen capture and biotinylated mAb F17 (0.35 μg/ml) in conjunction with horse-radish-peroxidase-conjugated streptavidin (streptavidin-HRP) (KPL, Gaithersburg, MD) were used for detection. pIFN-α concentrations were determined by extrapolation on a standard curve prepared with recombinant pIFN-α (PBL Biomedical Laboratories, Piscataway, NJ).

Indirect immunofluorescence analyses (IFA)

Sub-confluent cell monolayers prepared in 12 mm glass coverslips were infected with the different FMDV strains at a MOI = 10 for the indicated time. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100® (Sigma) in PBS, blocked with blocking buffer (PBS, 2% bovine serum albumin [BSA], 5% normal goat serum, 10 mM glycine) and then incubated overnight at 4°C with the respective primary antibodies. FMDV VP1 was detected with mouse mAb 6HC4 (33), L^{pro} with a rabbit polyclonal Ab elicited against bacterially expressed recombinant protein (de 20), and NF-κB -p65/RelA- with rabbit polyclonal Ab-1 RB-1638 (NeoMarkers, Lab Vision). Alexa Fluor 488 and

Alexa Fluor 594 (Molecular Probes, Invitrogen) conjugated secondary Abs were used for detection. Nuclei were visualized by DAPI staining included in ProLong Gold Antifade mounting media (Invitrogen). Cells were examined in an Olympus BX40 fluorescence microscope and the images were taken with a DP-70 digital camera and DP-BSW v2.2 software (Olympus America, Central Valley, PA).

Animal experiment

Animal experiments were performed in the high-containment facility of the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee (Protocol No: 151-13R). Nine Yorkshire barrows (5 weeks old, castrated males and weighing approximately 40 lbs each) were divided in three groups of three animals each. Animals were inoculated intradermally (ID) in the heel bulb of the right rear foot with FMDV A12-WT ($5x10^5$ pfu/animal) [group 3, control group] or two different doses of A12-L_{H138L} ($1x10^6$ or $1x10^7$ pfu/animal) [group 1 and 2, respectively] (Table 1). Rectal temperatures and clinical signs, including lameness and vesicular lesions, were monitored daily during the 1st week post-inoculation and samples of whole blood, serum and nasal swabs (BD universal viral transport [UVT] system, BD, Franklin Lakes, NJ) were collected on a daily basis to monitor complete blood counts (CBC), *in vivo* viral replication and spreading. Also, serum samples were collected at days 4, 7, 14, and 21 post-inoculation (dpi) to evaluate the development of neutralizing antibodies. Clinical scores were determined by counting the number of FMD-vesicles in the toes (max 4 vesicles per foot; 16 per animal), plus one, for one or more lesions detected in the snout and/or mouth. The maximum possible score was 17, and lesions restricted to the site of challenge were not counted (34). CBC data was done using a Hemavet® analyzer (Herba Diagnostics Miami Lakes, FL), to evaluate lymphopenia.

Virus titration in sera and nasal swabs

Sera and nasal swabs were assayed for the presence of virus by plaque titration on BHK-21 cells. Briefly, serial 10-fold dilutions of the samples were allowed to adsorb on monolayers of BHK-21 cells grown in 6-well plates. After 1 h adsorption, overlay was added, and the plates were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2 and then stained with a crystal violet-formalin solution to visualize the plaques. Virus titers were expressed as $\log_{10}$ plaque forming units (pfu) per ml of serum or nasal swab media. Detection level $\geq$5 pfu/ml of serum or nasal swab media.

Determination of neutralizing antibody titer

Neutralizing antibody titers were determined in mice or swine sera samples by end-point titration according to the Spearman-Kärber method (35). Antibody titers were expressed as the log10 value of the reciprocal of the dilution that neutralized 100 $TCID_{50}$ in 50% of the wells.

Analysis of cytokines in serum

IFN-α, IL-1β, IL-10 and TNF-α protein concentration was determined in sera from infected animals using an ELISA. IFN-α was detected as described above (Section 2.5). IL-10 Cytoset ELISA (Biosource-Invitrogen, Carlsbad, CA) and IL-1β, IL-6 and TNF-α Duo Set ELISAs (R&D Systems, Minneapolis, MN) were performed following the manufacturer's directions. All ELISAs were developed with 3, 3', 5, 5', tetramethylbenzidine (TMB) from KPL (Gaithersburg, MD). The absorbance at 450 nm was measured in an ELISA reader (Varioskan Lux, Thermo Fisher Scientific). Cytokine concentrations were calculated based on the optical densities obtained with the standards.

Statistical analyses

Data handling, analysis and graphic representation were performed using Prism 5.0 (GraphPad Software, San Diego, CA) or Microsoft Excel. Statistical differences were determined using a Student's *t*, comparing the same parameter in the different groups or change of the parameter over different timepoints as compared to a baseline; Gehan-Breslow-Wilcoxon test was used to analyze clinical disease onset. Statistically significant differences were expressed with asterisks ($P < 0.05$[*]).

Results

Mutation of A12 L^{pro} H138 affects normal FMDV growth in cell culture

A detailed structural analysis of L^{pro} revealed the presence of an aromatic pocket outside of the substrate binding domain which consists of three Tyr (Y) residues coordinated by an internal His (H138), a residue that is absolutely conserved across all FMDV serotypes (36) (Figure 1). Simulations were performed using Discovery Studio visualizer to predict the effect of different point mutations replacing residue H138 and the effect on perturbation of the aromatic pocket (leucine and asparagine shown as an example), showing that incorporation of the different mutations introduced selectively, preserved or

TABLE 1 Clinical performance of swine inoculated with varying doses of FMDV A12-L_{H138L} or A12-WT.

Group# and Dose[a]	Clinical results after mutant or WT virus inoculation in swine								
	Pig #	Clinical Score[b]	Fever[c]	Lymphopenia[d]	Viremia[e]	Viremia-PCR[f]	Shedding Virus[g]	Shedding RT-PCR[h]	SN[i]
1: A12-L_{H138L} 1×10^6	29,044	3/9	Y	Y	0 / 0 / 0	Y	2 / 5.3×10^2 / 3	Y	0/2.7
	29,045	0/0	N	N	0 / 0 / 0	N	3 / 1.5×10^1 / 2	Y	0/3.3
	29,046	0/0	N	Y	0 / 0 / 0	N	3 / 1.5×10^1 / 2	Y	0/3.0
2: A12-L_{H138L} 1×10^7	29,047	3/5	N	Y	3 / 5.0×10^1 / 1	Y	1 / 5.5×10^1 / 1	Y	0/2.7
	29,048	3/3	Y	Y	0 / 0 / 0	Y	2 / 9.3×10^1 / 2	Y	0/3.3
	29,049	2/12	Y	Y	2 / 5.0×10^3 / 1	Y	2 / 4.2×10^2 / 2	Y	0/3.3
3: A12-WT 5×10^5	29,050	2/14	Y	Y	2 / 7.8×10^4 / 2	Y	1 / 7.8×10^1 / 1	Y	0/3.3
	29,051	2/7	Y	Y	2 / 7.5×10^1 / 1	Y	3 / 1.5×10^1 / 2	Y	0/3.3
	29,052	2/15	Y	Y	2 / 7.5×10^2 / 2	Y	3 / 1.5×10^1 / 1	Y	0/3.3

[a]Dose of inoculum per animal expressed as number of pfu in a total volume of 0.4 ml.
[b]Days post inoculation (dpi) first signs of lesions are detected /highest lesion score achieved throughout the entire experiment.
[c]Rectal temperature of $\geq$40°C at any time after inoculation.
[d]Reduced percentage of lymphocyte to $\leq$30 % of lymphocytes/mL of blood.
[e]First dpi that viremia was detected using virus isolation techniques; maximum amount of viremia in pfu/ml detected in sera samples; and the duration (days) of viremia.
[f]Any analyzed serum sample during 1 to 7 dpi was positive using Real Time-PCR (Y, yes or N, no).
[g]First dpi that shedding virus was detected using virus isolation techniques; maximum amount of shedding virus in pfu/ml detected in nasal swab samples; and the duration (days) of shedding.
[h]Any analyzed nasal swab sample during 1 to 7 dpi was positive using Real Time-PCR (Y, yes or N, no).
[i]SN=serum neutralizing antibody response reported as Log10 TCID50 at 0 and 21 dpi, respectively.
[#]Number.

disrupted different components of the interaction. A collection of mutants changing this specific residue was selected including H138W, H138N, and H138L, being H138W the least disruptive. By using reverse genetics, these mutations were introduced in the infectious clone but only H138N and H138L rendered viable viruses after transfection of RNA in BHK-21 cells. Viable FMDV A12-L_{H138N} and A12-L_{H128L} were used for further characterization and compared to FMDV A12-wild type (WT) and A12-Leaderless (LLV). Kinetics of growth in multiple cells lines, including BHK-21, LF-BK, IBRS-2 and SK6 cells, demonstrated that mutants of H138 have an altered growth phenotype when compared to WT virus (Figure 2). Overall, the rate of growth for H138 mutants fell between WT virus and LLV mutant virus. Also, as observed in Figure 2, A12-L_{H138L} mutant showed a lower endpoint titer specifically in LF-BK cells when compared to A12-L_{H138N}. Based on these results, this mutant was further selected for additional characterization.

Cleavage of translation initiation factor eIF-4G is only slightly affected by mutation of A12 L^{pro} H138 residue

One of the hallmarks of picornavirus infection is the cleavage of the host translation initiation factor eIF-4G, an event that results in the shut-off of host cap-dependent mRNA translation (37–39). In order to determine if mutations in H138 residue affected eIF-4G cleavage, we performed western blot analysis on protein extracts obtained from LF-BK cells infected with FMDV A12-L_{H138L}, in comparison to FMDV A12-WT. We also run a parallel infection with FMDV A12-LLV which is known as unable to process eIF-4G (13). As shown in Figure 3, by 4 h post infection, cellular factor eIF-4G (p220) was almost completely processed by A12-WT virus. Interestingly only a slight delay, ~2 h, was observed in cells infected with A12-L_{H138L} although cleavage was complete thereafter. As expected, little or no processing was observed in the time lapsed when A12-LLV was used. This result suggested that alterations of viral growth of A12-L^{pro}H138L were probably not due to the overall shut-off of cellular translation imposed by L^{pro}.

NF-κB (p65/RelA) and G3BP2 are not cleaved upon infection with FMDV A12-L_{H138L}

We have previously demonstrated that during FMDV infection there is L^{pro} dependent degradation of NF-κB that correlated with nuclear accumulation of L^{pro} (20). Further, mutations in L^{pro} SAP (**S**AF-A/B, **A**cinus and **P**IAS) domain had an effect in L^{pro} nuclear retention and degradation of NF-κB. Analysis of protein profiles in lysates of infected cells indicated that mutation H138L in L^{pro} directly affects degradation of NF-κB. As seen in Figure 3, by 4 hpi, p65/RelA

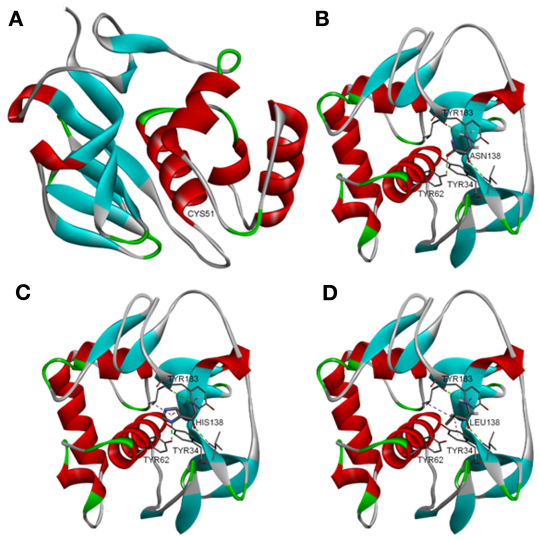

FIGURE 1
Molecular modeling of L^{pro}. Structural analysis of L^{pro} reveals an aromatic pocket located opposite from the substrate binding domain. **(A)** This pocket consists of three tyrosines coordinated by an internal histidine residue (H138), which is absolutely conserved across serotypes. Simulations were performed to assess the effects of different point mutations replacing the histidine at position 138. **(B–D)** Incorporation of different mutations selectively preserved or disrupted different components of the interaction. Simulations were used to generate a collection of mutants with varying degrees of functional conservation.

signal was significantly reduced in the cytoplasm of LF-BK cells infected with A12-WT. In contrast, the p65/RelA signal did not decrease in the extracts of A12-L_{H138L} infected cells, and signal resembled that of mock infected cells (Figure 3). Parallel analysis of NF-κB by indirect immunofluorescence analysis to evaluate the localization of p65/RelA in infected cells demonstrated that by 4 hpi, the p65/RelA signal was almost absent from the nucleus of A12-WT infected cells while it accumulated in the nucleus of A12-LLV infected cells (Supplementary Figure 1), as previously reported (20). Interestingly, the pattern for A12-L_{H138L} infected cells resembled the pattern for A12-LLV, with a bright p65/RelA staining concentrated in the nuclei (Supplementary Figure 1). The same image was observed even after 8 hpi (data not shown), time point considered a relatively late stage of infection in LF-BK cells which are known to be very susceptible to FMDV infection (40, 41).

It has also been reported that FMDV antagonizes the innate immune response, by modulating the stress response (24). In particular, it has been known that L^{pro} causes degradation of the scaffold proteins Ras GTPase-activating protein-binding proteins 1 and 2 (G3BP1 and G3BP2), preventing stress granules formation and accumulation (24). Interestingly, no cleavage of G3BP2 was detected in FMDV A12-L_{H138L} infected cells (Figure 3).

Mutation in A12 L^{pro} H138 residue does not affect L^{pro} nuclear retention

Previous results reported by our lab suggested that nuclear accumulation of L^{pro} is required for p65/RelA degradation, since during infection with L^{pro} SAP mutant, no L^{pro} could be detected in cell nuclei at relatively late times post infection while little or no NF-κB degradation could be detected in the same compartment (26). We analyzed the sub-cellular localization of L^{pro} during infection with A12-WT, A12-LLV2, and A12-L_{H138L}. As observed in Figure 4, cells infected with A12-WT showed an early signal (2 hpi) of L^{pro}, that rapidly appeared and accumulated in cell nuclei. A similar pattern was observed after infection with A12-L_{H138L}, although detection of mutant L^{pro} was slightly delayed in comparison to WT L^{pro}. As expected, no L^{pro} was detected in cells infected with leaderless A12-LLV virus.

In sum, the results of this study indicated that disruption of the L^{pro} H138 residue did not affect the ability of L^{pro} to translocate to the nucleus of infected cells or to target the general translation factor eIF-4G, but selectively prevented, at least, p65/RelA and G3BP2 proteolytic processing.

Mutation of A12-L^{pro} H138 residue prevents L^{pro} inhibition of IFN expression

It is well-stablished that L^{pro} antagonizes the innate immune response by blocking the expression of IFN and NF-κB signaling (23, 25, 42). To test whether mutation of L^{pro} H138 residue affects the FMDV capability of blocking IFN expression, we analyzed the levels mRNA for IFN-β, the pro-inflammatory cytokine TNF-α, the chemokine RANTES and the IFN stimulated genes (ISGs) Mx1 and IRF7. For this analysis we used cells known to have intact IFN responses such as primary/secondary porcine kidney (PK). For comparison we also infected cells with A12-WT and A12-LLV2 (Figure 5). Although by 4 hpi the differences among expression varied among the analyzed genes a clear pattern of upregulation was seen in all analyzed transcripts at 8 hpi, with significantly higher expression in cells infected with A12-L_{H138L} and A12-LLV than in cells infected with A12-WT (Figure 5A). Interestingly, most of the analyzed genes, except for IRF-7 and Mx1, showed higher expression upon infection with A12-L_{H138L} as compared to A12-LLV. ELISA quantitation of secreted IFNα protein in the supernatants of infected PK cells, followed similar kinetics (Figure 5B). By 24 hpi, 5 to 8-fold higher amounts of IFN-α protein were detected in A12-LLV and A12-L_{H138L}, respectively, as compared to A12-WT. These results indicated that mutation on A12-L^{pro} H138 residue prevented the inhibitory effect of L^{pro} on NF-κB dependent transcriptional activity and IFN protein expression.

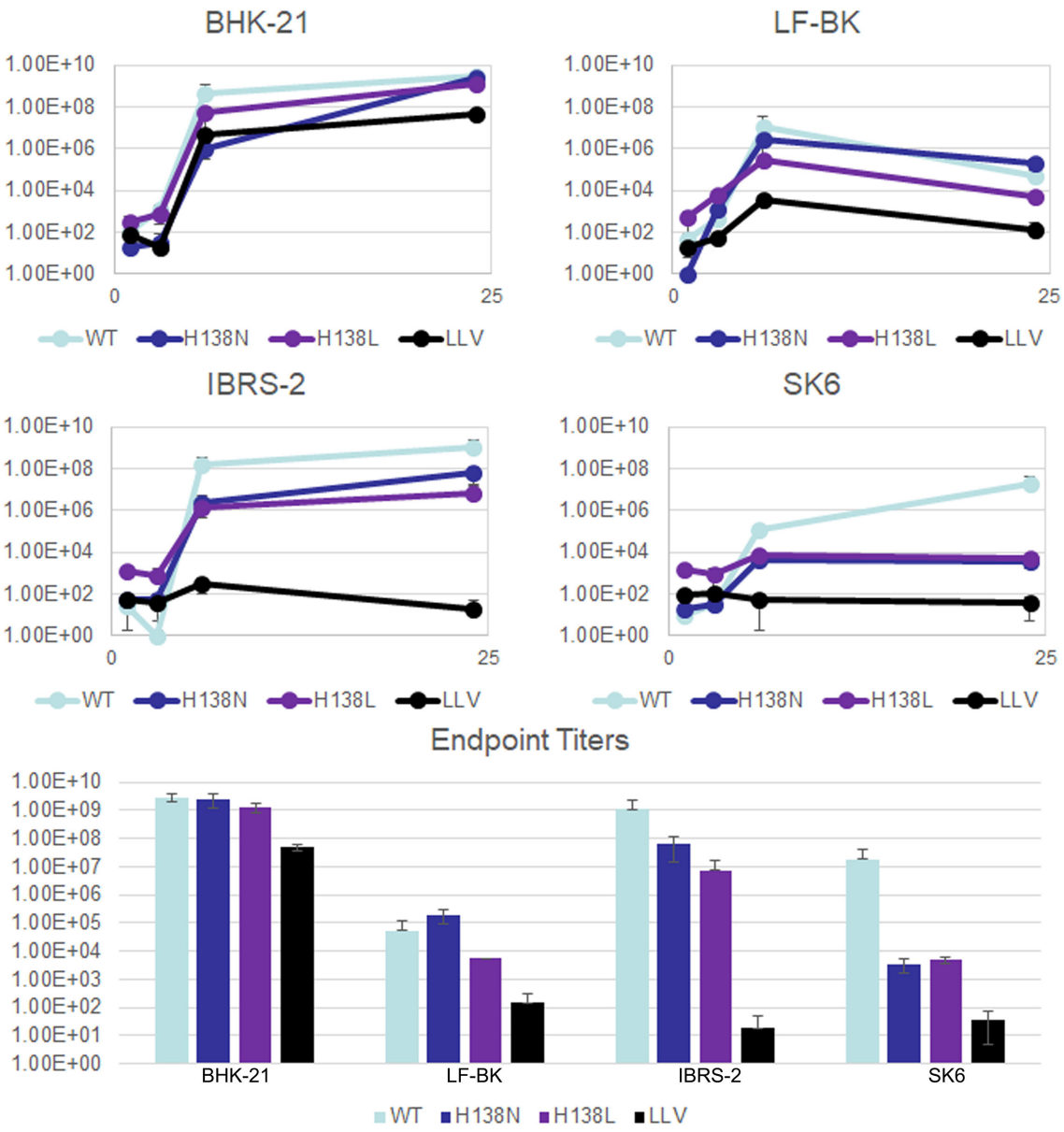

FIGURE 2
Growth kinetics for A12-L_{H138} mutants. Growth curves on BHK-21, LF-BK, IBRS2, or SK6 cells. Cells were infected with FDMV A12 wild type (WT), FMDV A12 leaderless (LLV), and FMDV A12-L_{H138} mutants (H138 N and H138 L) at MOI =10. After 1h, unabsorbed virus was removed by washing with 150 mM NaCl, 20 mM MES pH = 5.5 followed by addition of complete media. Samples were taken at 1, 3, 6 and 24 hpi and virus titers were determined by log10 TCID50 on BHK-21 cells.

FMDV A12-L_{H138L} mutant is attenuated in swine

To compare virulence of A12-L_{H138L} vs. A12-WT *in vivo*, we inoculated animals intradermally (ID) in the rear heel bulb (n = 3) with either 10^5 pfu/animal of A24-WT (group 3/control group) or two different doses of A12-L_{H138L} (group 1: 10^6 pfu/animal and group 2: 10^7 pfu/animal) (Table 1). A12-WT inoculated animals showed vesicles as early as 2 days post-inoculation (dpi) (Table 1) and transient fever and lymphopenia were also detected on the day of disease onset or 1 day later, as previously described (43). However, when animals were inoculated with a 10-fold higher concentration of the A12-L_{H138L} virus, only one out of three animals showed vesicles, lymphopenia and fever with 1 day delayed disease onset. Furthermore, even when the animals were inoculated with a 100-fold higher dose of A12-L_{H138L} than WT virus, disease onset was statistically significantly delayed on those animals as

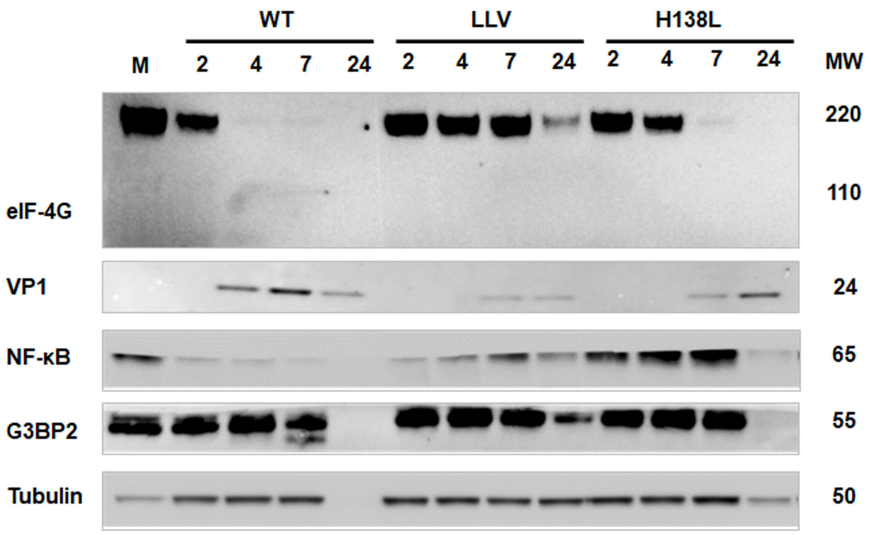

FIGURE 3
Processing of cellular proteins. LF-BK cells were infected with FMDV A12-WT, A12-LLV (leaderless) or FMDV A12-L_{H138L} at MOI = 10. Protein cytoplasmic extracts were collected at 2, 4, 7, and 24 h post infection, followed by SDS PAGE and Western Blot analyses using anti-eIF4G (p220), anti-FMDV VP1, anti-NF-κB (p65/RelA), anti G3BP2, and anti-tubulin-α antibodies. M stands for mock infected cells.

compared to animals inoculated with A24-WT virus ($P < 0.05$) (Table 1).

Animals inoculated with A12-WT developed viremia concomitantly with the appearance of clinical signs. Interestingly, none of the animals inoculated with A12-L_{H138L} at $1x10^6$ pfu had detectable viremia measured either by virus isolation or by rRT-PCR, and one out of three animals inoculated with 1×10^7 pfu of A12-L_{H138L} did not show any viremia (Table 1). On the other hand, virus shedding was detected in nasal swabs in all inoculated animals regardless the virus or dose used (Table 1).

All together, these data indicate that A12-L_{H138L} FMDV displays significantly reduced virulence in swine as compared to A12-WT.

FMDV A12-L_{H138L} mutant and A12-WT elicit equivalent adaptive immune responses

It has previously been demonstrated that animals inoculated with an attenuated strain of FMDV with mutations or complete deletion of L^{pro}, developed significant increase of antibody titers against viral proteins in swine serum (15, 27). In the current experiment, we observed that regardless the presence or absence of viremia, all the animals inoculated with A12-L_{H138L} developed significant levels of FMDV-specific neutralizing antibodies starting at 7 dpi with a peak at 14 dpi (Table 1), and there was not statistically significant difference in the virus titers between A12-WT or any of the A12-L_{H138L} inoculated groups ($P > 0.05$).

Mutations in A12-L^{pro} H138 residue has an effect on cytokine profile in swine

We have previously demonstrated that L^{pro}, is an immune response antagonistic factor, limiting the expression of IFN and ISGs (17–21), and reducing the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in swine (27). Furthermore, FMDV WT infection induces production of the anti-inflammatory cytokine IL-10, thus impairing T-cell proliferation (44) with a consequent induction of an anti-inflammatory state. To understand if H138 residue is involved in this effect, we analyzed the expression of pro- and anti-inflammatory cytokine protein levels in the sera of animals inoculated with A12-L_{H138L} mutant and A12-WT for 5 days after infection. In the case of A12-WT inoculated animals, despite a high variability among individuals, a statistically significant decrease was observed of all analyzed pro-inflammatory cytokines (IFN-α, TNF-α, and IL1-β) between days 1 and 2 post-inoculation (Figure 6). Furthermore, there was an increase in the levels of IL-10 with a peak at 3 dpi (Figure 6). Similarly, animals inoculated with A12-L_{H138L} showed a peak of IL-10 at 3 dpi, regardless of the dose of virus inoculated. However, when analyzing the expression of pro-inflammatory cytokines, a different profile was detected. None of the animals inoculated with A12-L_{H138L} showed a

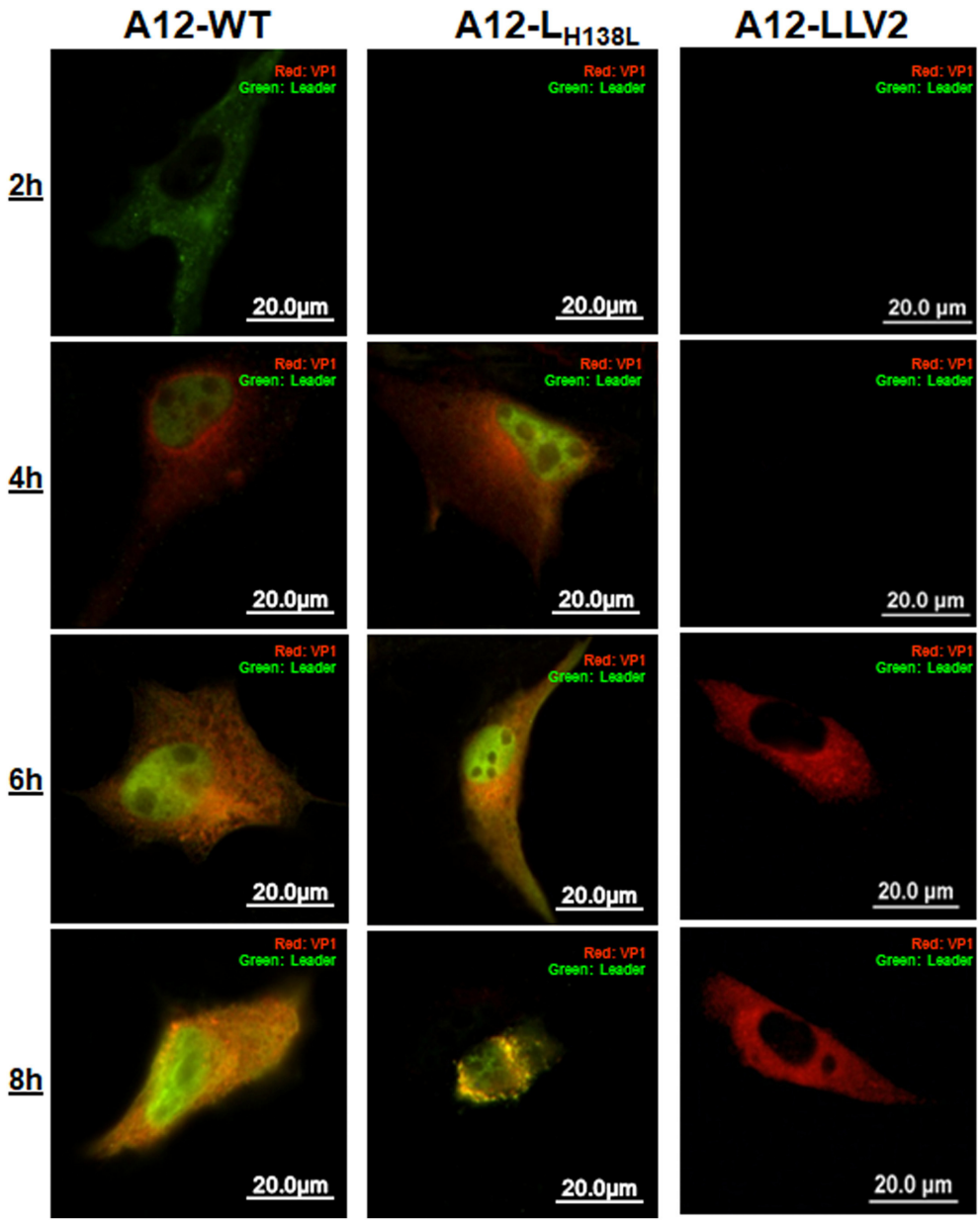

FIGURE 4
Translocation of L^{pro} is not affected in A12-L$_{H138L}$. LF-BK cells were infected with A12-WT, A12-L$_{H138L}$ or A12-LLV FMDV at MOI = 10. At the indicated times cells were fixed and viral proteins L^{pro} (green) and VP1 (red) were detected by IFA.

decrease in any of the pro-inflammatory cytokines analyzed. On the contrary, in the case of IFN-α, animals inoculated with 1×10^6 pfu of A12-L$_{H138L}$ showed a consistent increase starting at day 3 post-inoculation (Figure 6). These results indicate that mutations in L^{pro} residue H138 alter the capacity of FMDV to counteract the *in vivo* pro-inflammatory immune response.

Discussion

As all viruses, FMDV has co-evolved with its host to successfully antagonize the immune response. In the particular case of innate responses, despite one protein, L^{pro} stands out as the most effective IFN suppressor (23, 42, 45, 46). As Vadim Agol described several years ago, FMDV L^{pro}, and most picornaviral

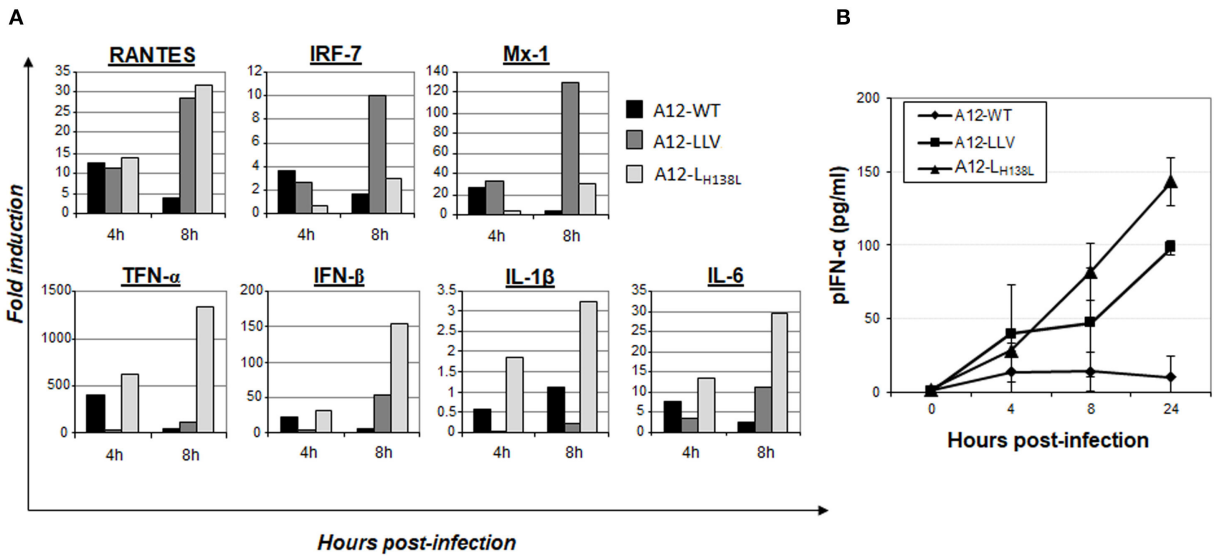

FIGURE 5
Induction of IFN and ISGs during infection. **(A)** Expression of IFN-β, TNF-α, RANTES, Mx1, IL-1β and IL-6 and IRF7 mRNAs was measured by real-time RT-PCR in primary porcine kidney cells infected with A12-WT, A12-LLV and A12-L_{H138L} at MOI = 10. Graphs represent results of one out three independent studies with parallel results. **(B)** Porcine IFNα ELISA in supernatants of PK cells infected with A12-WT, A12-LLV and A12-L_{H138L}, plotted as AVER±. STDEV of three independent studies.

2A proteins, could be grouped as "security proteins, dedicated specifically to counteract host defenses," bearing non-essential activities on the viral polyproteins (47). Consistently, deletion of FMDV L^{pro} is tolerated and results in a viable virus that can grow well in immortalized cells with no selective immune pressure (13); however, infectiousness in the animal host is severely affected (14, 15). In contrast, mutations that target the catalytic pocket of the enzyme result inactive protein and non viable viruses (13, 38). Interestingly, mutations that lie outside the L^{pro} catalytic pocket and at the 3' end of the protein are usually tolerated (3, 22, 26, 27, 48).

Here we demonstrate as a proof-of-concept that another L^{pro} mutation that resides outside the catalytic site, H138L, is tolerated by FMDV, resulting in a mutant virus with an attenuated phenotype *in vitro* and *in vivo,* in swine. This mutation lies in a His residue that seems to stabilize an aromatic pocket of the protein containing three Tyr residues and that is highly conserved across all FMDV serotypes (36). Indeed, earlier work by Piccone et al. had reported that viruses containing this mutation were not attenuated in cell culture displaying a WT virus phenotype (13). However, the authors had only used immortalized cells (BHK-21) that lack features of a natural host (49, 50).

We also observed that in SK6 cells, an immortalized cell line with an apparently normal IFN pathway, A12-L_{H138L} mutant is significantly more attenuated than in cell lines that display an altered IFN induction or transduction pathways (18). Consistent with this observation, L^{pro} dependent degradation of innate immunity molecules such as p65 (NF-κB), or stress granules marker G3BP2, was less evident in cells infected with A12-L_{H138L} mutant, while only a mild delay was observed in cleavage and degradation of the translation initiation factor eIF-4G. As a result, higher levels of IFN and ISGs were detected in cells infected with A12-L_{H138L} as compared to WT virus (Figure 3). These results resembled previous observations made with the FMDV mutant L^{pro}W105A that mostly affected the deISGylation capability of L^{pro} without affecting the specific cleavage of translation factors (22, 51). Analysis of deUbiquitinase activity indicated that similarly to LLV, A12-L_{H138L} had a reduced ability to decrease the ubiquitination profile of host proteins (data not shown). Perhaps reduced stability or misfolding of A12-L_{H138L} protein partially affects ubiquitin dependent signaling in response to viral infection. Further research including detailed biochemical studies is warranted to confirm this hypothesis. Similar results have been observed for other RNA viruses such as Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV-1 and SARS-CoV-2), mouse hepatitis virus (MHV), and porcine reproductive and respiratory syndrome virus (PRRSV) (52–54), emphasizing the role of posttranslational modification in intrinsic protein function or interaction with other factors.

Overall, our results confirmed that intact/unmutated L^{pro} is required for an effective suppression of the cellular innate response against FMDV infection. While only a slight delay was detected in the processing of eIF-4G, a significant increase in

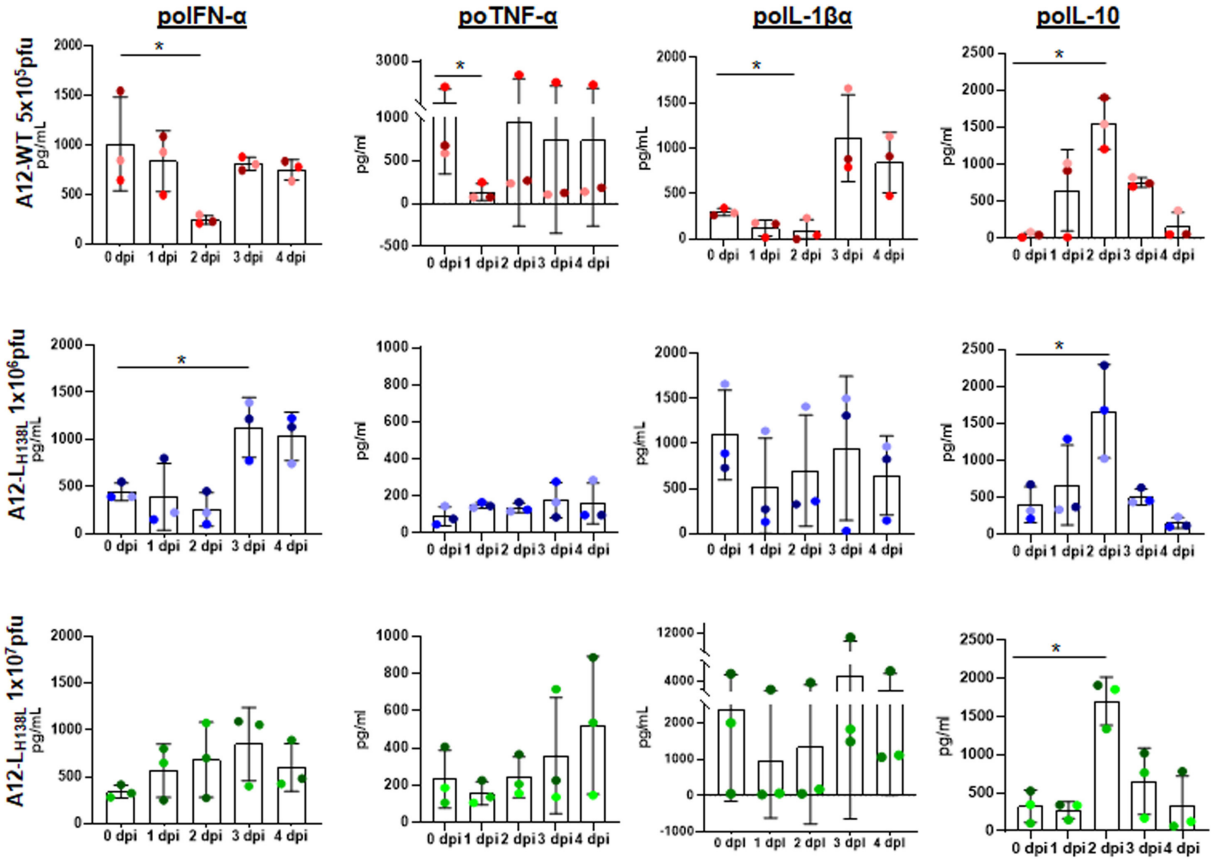

FIGURE 6
Cytokine profile in animals inoculated with FMDV A12-WT or A12-L_{H138L}. Serum levels of porcine IFN-α, IL-10, IL-1β, and TNF-α were detected by ELISA at different times post virus inoculation. Results are expressed in pg/ml. Error bars represent the variation within the three animals from each group. Each color grade dot represents the same animal throughout the graphs ($P < 0.05$[*]).

the expression of IFN and ISG was detected in cells infected with A12-L_{H138L} as compared to WT. However, differently than for other L^{pro} mutations, the mutation H138L did not affect the ability of the protein to accumulate in the nucleus of infected cells and interestingly, no p65 degradation could be detected, despite the normal processing of eIF-4G. Similar results had previously been shown for L^{pro} SAP mutant, but it is worthy to mention that this mutant did not accumulate in the nucleus of infected cells, suggesting that this behavior might have affected p65 degradation (26). Interestingly, another L^{pro} mutant (L^{pro} L143A) known to affect substrate specificity at least *in vitro*, (39) did not induce degradation of p65 or cleavage of RLR signaling proteins (i.e., MAVS, TBK), but preserved the induction of high levels of IFN-β mRNA transcripts (51). Hence, mutation in H138 may interfere or prevent the interaction with p65 or other protein of a multiprotein complex thus far unknown. In parallel, A12-L_{H138L} mutant failed to cause cleavage/degradation of the stress granule component G3BP2. Analysis of ISG mRNA and IFN protein expression revealed that this mutant behaved similarly to FMDV LLV. Both, LLV and A12-L_{H138} mutants induced higher levels of ISGs and IFN than the WT virus. These results are consistent with our previous experiments using LLV and the SAP L^{pro} mutants (20, 26) but different than those obtained for A12-L^{pro}W105A or other nearby mutants in which the deUb/deISG activity was significantly impaired while ISG expression was not blocked (22, 51). Although induction of innate immune responses based on the examination of some mRNA transcripts (Mx1 and IRF7) was better induced by A12-LLV, overall A12-L_{H138L} produced a stronger response (higher upregulation in RANTES, TNF-α, IFN-β, IL-1β, and IL-6 transcripts). It is possible that not all IFN dependent promoters do get activated at the same time, or a higher replication rate for A12-L_{H138L} as compared to LLV must have induced the pathway more efficiently. In fact, levels of replication of A12-L_{H138L} were always higher than LLV in all cell lines used. On the other hand, the picture was different when A12-L_{H138L} and WT titers were compared. While no differences in viral titers were detected in cell lines with impaired IFN

signaling systems, the titers of A12-L_{H138L} were significantly lower than those for WT in cells which display a competent IFN system (49, 50) such as SK6 cells.

These results may also explain the phenotype observed in the animal experiments in which two out of three pigs inoculated with twice the dose of A12-L_{H138L} virus as compared to WT virus, did not develop clinical signs of FMD, neither they had viremia. Further, two pigs inoculated with 20 times higher dose also showed less severe disease and in one of them no virus could be isolated from serum. Consistent with these results, animals inoculated with the mutant virus that did not get sick or had lower scores in the evaluation of clinical signs and developed higher levels of systemic IFN protein. These results were also similar to those previously obtained with the SAP FMDV mutant (27). The levels of systemic IFN significantly increased early after inoculation of swine with FMDV A12-L_{H138L}, while they decreased or remained unchanged in animals inoculated with WT virus. In contrast, the levels of proinflammatory cytokines (TNF-α and IL-1β) were not affected in animals infected with the mutant virus while they significantly decreased in animals inoculated with WT virus prior to the appearance of clinical signs. It has recently been proposed that the IL-1 family of cytokines may act as an important backup antiviral system in the host with a critical role in skin defense (55). Production of IL-1 by keratinocytes induces an antiviral state in neighboring stromal cells including among others, fibroblasts and endothelial cells. Of interest, this effect was seen in tissues infected with fully virulent strains of VSV and Zika virus. It is possible then that FMDV has also evolved to block this response in the host, causing significant damage in skin tissues, and L^{pro} may contribute a pivotal role for the pathogenesis of the virus.

Similarly, it has been shown that TNF promotes a dual function: it provides protective antiviral immunity; and, at the same time, it enhances inflammation (56). For example, poxviruses and herpesviruses A, produce soluble or secreted versions of TNF like receptors that could neutralize this host cytokine. Also, regulation of IFN-β and TNF-α have previously been associated with acute and persistent phases of FMDV infection (57, 58). As seen in our experiments *in vitro*, it is plausible to think that higher expression of IL-1 and TNF-α are the result of the reduced degradation of NF-κB for the FMDV A12-L_{H138L} mutant as compared to the WT virus, however further pathogenesis studies are required to confirm this hypothesis. Though, similar increases in the levels of the cytokine IL-10 were detected for both, FMDV A12-L_{H138L} and WT virus. The role of IL-10 in modulation of dendritic cell (DC) function early post infection, has been studied (59). Presumably this cytokine induces a Th2 /cytokine-like environment, and as a consequence high levels of FMDV-specific neutralizing antibodies are induced (44). On this regard, similar levels of IL-10 were detected in the animals infected with WT and A12-L_{H138L} viruses consistent with equivalent levels of detectable neutralizing antibodies by 21 days post infection. Our results are consistent with previous studies with other FMDV mutants (27) and further support the concept of IL-10 as key regulatory cytokine during FMD (60).

In summary, our results further support the notion that manipulation of the L^{pro} coding region is perhaps the best tool to derive live attenuated strains of FMDV. Further studies with a larger number of testing individuals should be performed to demonstrate statistical power. A fine tune of attenuation is imperative to partially block the host innate responses while allowing for sufficient viral replication and induction of strong adaptive immune responses. A delicate manipulation of L^{pro} in the context of other genetic changes including the incorporation of DIVA markers, may help develop improved live attenuated vaccine candidates to be evaluated in different endemic settings.

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.

Ethics statement

The animal study was reviewed and approved by Plum Island Animal Disease Center Animal Care and Use Committee (protocol #151-13R).

Author contributions

FD-SS and TS conceived and supervised the study. PA and DR performed structural analysis, designed and/or constructed mutant viruses. PA, GM, ER-M, MR-C, ES, and FD-SS performed laboratory and/or animal experiments. JZ and ER-M provided support and critical thinking on the research design. PA, GM, FD-SS, and TS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author DR is currently employed by the company Pfizer Worldwide Research.

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.

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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.2022.1028077/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Nuclear p65/RelA (NF-κB) is not degraded during A12-LH138L early infection. LF-BK cells were infected with A12-WT, A12-LH138L or A12-LLV FMDV at MOI=10. At 4h post infection cells were fixed and p65/RelA (NF-κB) and viral protein VP1 were stained and detected by IFA. Mock and poly r[IC] treated were used as negative and positive controls to verify proper p65 response/cellular localization.

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EDITED BY
Wilna Vosloo,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

REVIEWED BY
Javier Martinez Lobo,
Universitat de Lleida, Spain
John M. Ngunjiri,
The Ohio State University, United States

*CORRESPONDENCE
David J. Paton
david.paton@pirbright.ac.uk

†These authors share first authorship
‡Retired

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Predicting cross-protection against foot-and-mouth disease virus strains by serology after vaccination

Simon Gubbins[1†], David J. Paton[1*†], Aldo Dekker[2], Anna B. Ludi[1], Ginette Wilsden[1], Clare F. J. Browning[1], Michael Eschbaumer[3], Jamie Barnabei[4], Hernando Duque[4‡], Lisa L. Pauszek[4] and Donald P. King[1]

[1]The Pirbright Institute, Pirbright, United Kingdom, [2]Laboratory Vesicular Diseases, Department of Virology and Molecular Biology, Wageningen Bioveterinary Research, Lelystad, Netherlands, [3]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald, Germany, [4]Plum Island Animal Disease Center, Greenport, NY, United States

Serology is widely used to predict whether vaccinated individuals and populations will be protected against infectious diseases, including foot-and-mouth disease (FMD), which affects cloven-hoofed animals. Neutralising antibody titres to FMD challenge viruses correlate to protection against FMD, for vaccinated cattle that are infected with the same strain as in the vaccine (homologous protection). Similar relationships exist for cross-strain protection between different vaccine and challenge viruses, although much less data are available for these heterologous studies. Poor inter-laboratory reproducibility of the virus neutralisation test (VNT) also hampers comparisons between studies. Therefore, day-of-challenge sera (n = 180) were assembled from 13 previous FMD cross-protection experiments for serotypes O (n = 2), A (n = 10), and SAT 2 (n = 1). These were tested by VNT against the challenge viruses at the FMD FAO World Reference Laboratory (WRLFMD) and the titres were compared to challenge outcomes (protected or not). This dataset was combined with equivalent serology and protection data for 61 sera from four cross-protection experiments carried out at WRLFMD for serotypes O (n = 2), A (n = 1), and Asia 1 (n = 1). VNT results and protection outcomes were also analysed for a serotype O cross-protection experiment involving 39 cattle, where the sera were not available for retesting at WRLFMD. Three categories of association between heterologous neutralising antibody titre and heterologous protection were found (Group 1–3). The $\log_{10}$ reciprocal titres associated on average with 75% protection (with 95% credible limits) were: Group 1: 2.46 (2.11–2.97); Group 2: 1.67 (1.49–1.92); Group 3: 1.17 (1.06–1.30). Further cross-protection data are needed to understand the factors that underpin this variability and to develop more robust antibody thresholds. Establishing cut-off serological titres that can be used to score the adequacy of vaccine-induced immunity will facilitate the monitoring and thereby the performance of FMD vaccination in the field.

KEYWORDS

foot-and-mouth disease, vaccination, predicting cross-protection, immune correlate, serology, virus neutralising antibody, post-vaccination monitoring

Introduction

Foot-and-mouth disease (FMD), which affects domestic and free-living ungulates, is a vesicular disease caused by an RNA virus (FMDV) in the family *Picornaviridae*, genus *Aphthovirus*. The virus is contagious and antigenically diverse, with six currently circulating serotypes (1) that do not cross-protect and multiple strains within serotypes that cross-protect to variable degrees. Consequently, infection and reinfection can be common in endemic settings and the virus may be reintroduced into countries where it has been eliminated. Vaccines are an important control option for both prophylactic and reactive responses to FMD (2).

Current vaccines are produced from inactivated cell culture grown virus capsids formulated with an oil or aqueous adjuvant. The protection afforded by FMD vaccines is relatively short-lived and may be strain dependent but can be strengthened and prolonged by increasing the vaccine's potency and by giving boosters, which will also improve the antigenic coverage of field strains but is more expensive. Before a new vaccine strain can be registered, a potency test is normally conducted by vaccinating target hosts (usually cattle, sometimes pigs) and challenging them 21–28 days later with FMDV that is the same as (homologous) to the vaccine strain. In cattle, the test involves inoculation of FMDV into the tongue and if the challenge virus is blocked from generalisation to cause vesicles on the feet, then the animal is considered protected (3). Antibodies are a major component of acquired immunity and once a correlation can be shown between protection and day-of-challenge antibody titre, then serology can be used as an indirect potency test for acceptance of subsequent vaccine batches without challenge [batch release testing (3)]. The antigenic suitability of a vaccine strain can be assessed serologically by comparing the antibody titres of sera from vaccinated animals against the vaccine strain and one or more relevant field strains. Vaccine selection is informed by this combination of verification of homologous potency and antigenic match, but there is uncertainty in how these two factors interact. A heterologous potency test that takes account of both potency and match is likely to be a better predictor of vaccine performance in the field but is laborious, expensive, and unethical for routine use. An indirect heterologous potency test could be based on heterologous serology, without a prior challenge test, if it could be shown that the titres associated with protection do not differ between strains. A study of heterologous protection with challenge for several antigenically distinct serotype A strains showed a better correlation between protection and day-of-challenge neutralising antibody titre to the challenge strain than between protection and titre to the vaccine strain (4). High-potency vaccines that elicit strong antibody responses were found to protect even against strains for which there was a poor antigenic match. Since there is poor reproducibility of virus neutralisation tests (VNT) between laboratories (5), this study attempted to quantify the variation in the titres associated with cross-protection when all of the serology was performed in a single laboratory (the World Reference Laboratory for FMD, WRLFMD, at the Pirbright Institute). Other aims were to consider if (1) the titres associated with protection after homologous challenge would be equivalent to those after heterologous challenge, provided that the heterologous virus was used in the VNT; and if (2) the titres associated with protection are not affected by boosting.

Materials and methods

Protection studies

Day-of-challenge sera ($n = 241$) were obtained or had already been tested from 17 cross-protection experiments with 245 cattle (four sera unavailable) and four serotypes [O, A, Asia 1, and Southern African Territories 2 (SAT 2)] carried out under high containment between 2007 and 2020 in Germany, the USA, the Netherlands, and the UK (Table 1). The vaccine strains and challenge viruses had been isolated between 1964 and 2015 originating from the Middle East, North Africa and South America. The cattle used were conventionally reared, of various breeds and mostly between 6 and 12 months of age. Most of the vaccines had been supplied by Merial/Boehringer Ingelheim, formulated at a potency of at least 6 PD50/dose, from antigen banks maintained by FMD-free countries and given as monovalent full or reduced-volume doses. Only in the eight experiments of Brehm et al. (4) had potency tests been performed to establish the homologous potency of the same vaccines also used to study cross-protection. Most of the vaccines were double oil emulsion (DOE) formulations that were administered intramuscularly. In the SAT 2 study, vaccination was by the subcutaneous route. In one study (9), an aqueous multivalent vaccine with a saponin adjuvant that had not been formulated from bank antigen was given subcutaneously. Another study (8) employed a multivalent vaccine from Vecol in South America, with a serotype O and a serotype A component. The studies were carried out to test the ability of vaccines to protect against challenge viruses that had an incomplete antigenic match [one-way relationship r_1 values of 0–0.64; Rweyemamu (11)] to the vaccine strain in question.

All of the challenges were by tongue inoculation of 10^4 bovine 50% infectious doses of virus, or an equivalent based on titration in cell culture (12). Of the cattle, 159 (65%) were protected by vaccination from virus generalisation to the feet, whereas 86 (35%) cattle were unprotected. The sera had been collected at 21 days post vaccination (dpv), but in one study, 10 cattle were boosted at 14 dpv and then challenged 21 days after this second vaccination (8).

TABLE 1 Summary of heterologous vaccination-challenge studies for foot-and-mouth disease included in the analysis.

Expt	Serotype	Vaccine strain	Challenge virus (strain)	r_1-value	Number challenged*	Number protected	Serology test at WRLFMD	Mean $\log_{10}$ VNT at challenge†	References
1	O	O Manisa	O/ALG/3/2014 (O/ME-SA/Ind-2001)	0.13	15	7	2015	2.31	(6)
2	O	O Manisa	O Campos	0.6	39	20	–	– (1.82)	(7)
3	O	O Manisa	O/IRN/34/2006 (O/ME-SA/PanAsia2)	0.64	15	7	2007	1.41	Pirbright Institute unpublished
4	O	O Campos‡	O/Orellana-036/ Ecuador 2010** (O/EURO-SA/unnamed)	0.24	10	9	2021	2.06	(8)
5	O	O Campos‡	O/Orellana-036/ Ecuador 2010 (O/EURO-SA/unnamed)	0.16	10	5	2021	1.40	(8)
6	A	A Iran 05/A Sau 95‡	A/IRN/22/2015 (A/Asia/G/VII)	0/0.25	16	9	2016	1.25	(9)
7	A	A22 Iraq	A/IRN/22/2015 (A/ASIA/GVII)	0.2	7	2	2017	1.26 (1.16)	(10)
8	A	A May 97	A/IRN/22/2015 (A/ASIA/GVII)	ND	22	18	2017/18	1.45 (1.33)	(10)
9	A	A22 Iraq	A Iran 96	0.09	15	9	2020	0.98	(4)
10	A	A22 Iraq	A Egypt 06	0.12	15††	11	2020	1.43	(4)
11	A	A22 Iraq	A Iran 99	0.04	15‡‡	7	2020	1.21	(4)
12	A	A Iran 99	A22 Iraq	0.10	15††	12	2020	1.23	(4)
13	A	A Iran 99	A Iran 96	0.23	15	13	2020	1.43	(4)
14	A	A Iran 96	A Iran 99	0.12	15	11	2020	1.04	(4)
15	A	A Iran 96	A22 Iraq	0.12	15‡‡	5	2020	0.86	(4)
16	A	A Iran 96	A22 Iraq	0.10	15	10	2020	1.21	(4)
17	Asia 1	Asia 1 Shamir	Asia 1/TUR/49/11 (Asia 1/ASIA/Sindh-08)	0.20	15	13	2012	1.40	Pirbright Institute unpublished
18	SAT 2	SAT 2 Sau 2000	SAT 2/LIB/40/2012 (SAT 2/VII/unnamed)	ND	15	11	2020	1.60 (1.11)	Dekker et al. unpublished

*Cattle challenged at 21 days post vaccination.
†VNT, virus neutralisation titre; titres shown out with brackets are those obtained at WRLFMD, while those within brackets are those obtained at the original laboratory; means were calculated for all cattle in the study regardless of whether or not they were protected.
‡Multivalent vaccine containing other serotypes.
**Cattle boosted at 14 days post first vaccination and challenged at 21 days post booster vaccination.
††Serum from one protected animal in the original study no longer available.
‡‡Serum from one unprotected animal in the original study no longer available.

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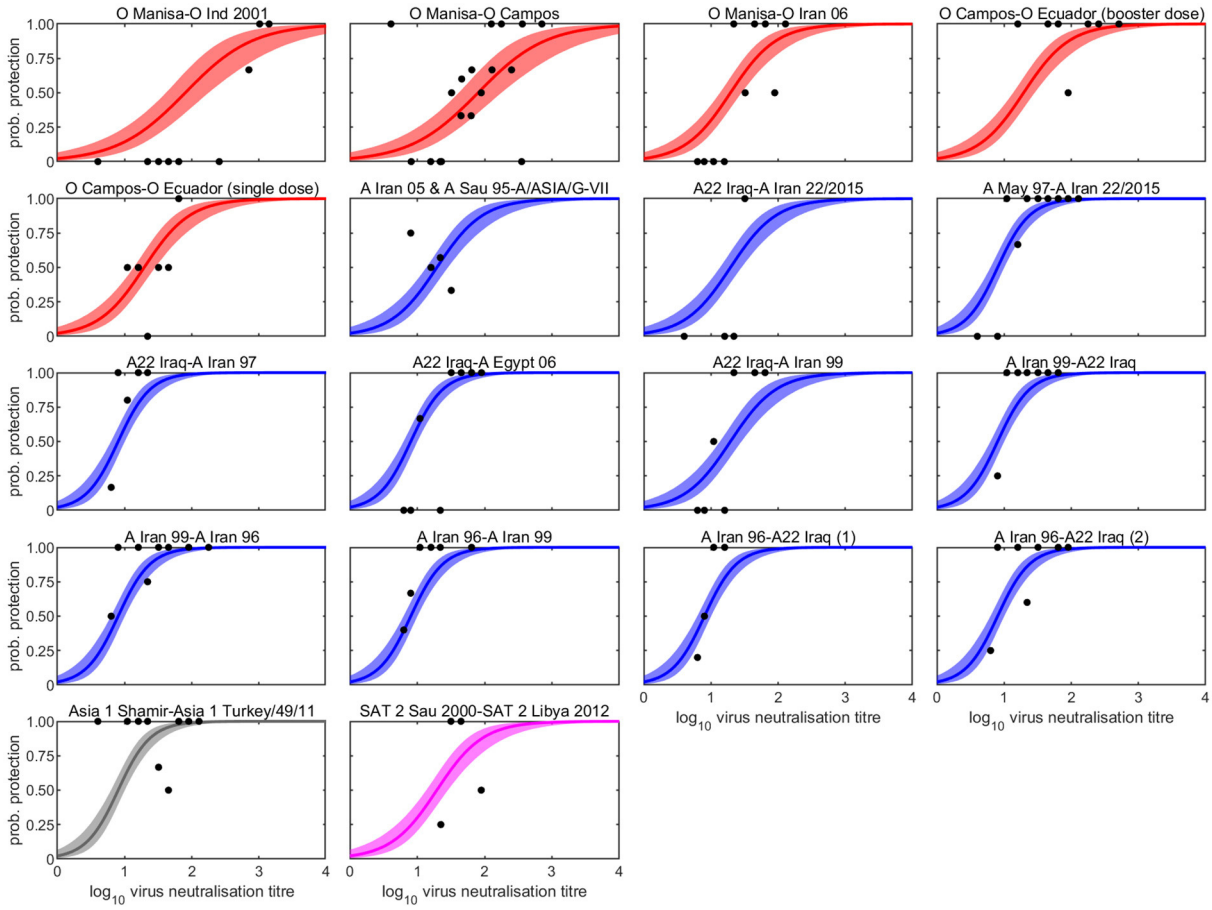

FIGURE 1
Observed and estimated probability that a vaccinated bovine animal is protected following heterologous challenge and its dependence on $\log_{10}$ virus neutralisation titre for eighteen vaccine-challenge studies. The vaccine and challenge strains are identified before and after the hyphen, respectively. Each plot shows the observed proportion of cattle protected at each titre in the study (circles) and the posterior median (line) and 95% credible interval (shading) for the probability of protection. Colour indicates serotype: O (red), A (blue), Asia 1 (grey), and SAT 2 (magenta).

VNT results from an 18th study of cross-protection were also included in the analysis (Table 1, experiment 2). In this study, 39 cattle had been immunised with a DOE formulation of O Manisa vaccine produced by Indian Immunologicals Ltd. Only twenty of the cattle (51%) had been protected from virus generalisation after challenge with O Campos despite earlier serology showing a relatively good antigenic match to O Manisa [$r_1 = 0.6$; (7)]. These 39 sera were not available for retesting by VNT at WRLFMD.

Virus neutralisation test

Archived sera ($n = 180$) were shipped to WRLFMD and tested with their in-house method, which follows the description in the WOAH Manual (3), using doubling final dilutions from 0.9 $\log_{10}$ to 3.0 $\log_{10}$, against the strains used for challenge in the respective cross-protection studies. In the case of O Ecuador 2010, the viruses used in the challenge and serology had been isolated from different but contemporaneous and epidemiologically linked outbreaks. The titre of the virus and of the positive control serum were controlled by reference to their running mean values and the Kärber method was used for titre calculation (13). For analysis, titres of <0.9 $\log_{10}$ were scored as 0.8. Sera collected from experiments at WRLFMD ($n = 61$, Table 1: 1, 3, 6, 17) had been tested according to this method between 2007 and 2021.

Statistical methods

A logistic regression model was used to relate the probability of protection to VNT. Specifically, the probability (p) that an animal with a titre $\log_{10}$ T was protected after challenge was given by $\log[p/(1-p)] = a + b\log_{10} T$, where a is the intercept and b is the slope. To explore how the level of protection for a given

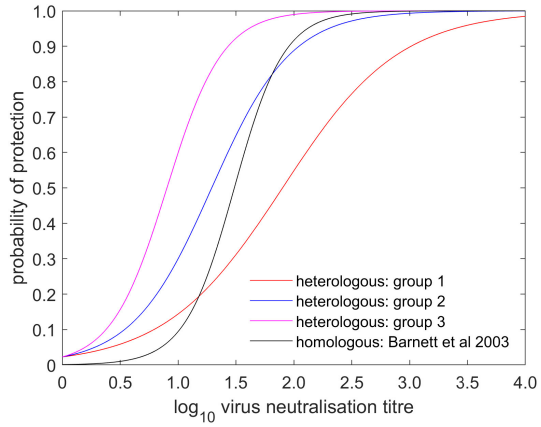

FIGURE 2
Estimated probability that a vaccinated bovine animal is protected following challenge and its dependence on $\log_{10}$ virus neutralisation titre. The plot shows the posterior median for the probability of heterologous protection for group 1 (comprising experiments 1 & 2; red), group 2 (comprising experiments 3, 4, 5, 6, 7, 11, & 18; blue) and group 3 (comprising experiments 8, 9, 10, 12, 13, 14, 15, 16, & 17; magenta) and the probability of homologous protection as estimated by Barnett et al. (5) (black).

titre varies amongst serotypes and strains, three possibilities were considered for slope and intercept: (i) they are independent of strain/serotype; (ii) they differ amongst serotypes but are common within a serotype; and (iii) they differ amongst strains, including within a serotype. Variation amongst serotypes or strains was incorporated by including hierarchical structure in the parameters so that the parameters for serotype/strain j are drawn from higher-order normal distributions, so that $a_j \sim N(\mu_a,\sigma_a)$ and $b_j \sim N(\mu_b,\sigma_b)$, where the μs and σs are the means and standard deviations. A total of nine models was considered (Supplementary Table 1).

Parameters were estimated in a Bayesian framework. A Bernoulli likelihood was assumed for the data (i.e., whether an animal was protected or not). Here protection was defined based on the development of lesions on the feet: if no lesions developed the animal was considered protected; if lesions developed at least one foot, it was considered to not be protected. Diffuse normal priors (with mean 0 and standard deviation 10) were assumed for a and b (in a non-hierarchical model) or μ_a or μ_b (in the hierarchical model). Diffuse exponential priors (with mean 100) were assumed for σ_a or σ_b in the hierarchical models. The methods were implemented using OpenBUGS (version 3.2.3; https://www.mrc-bsu.cam.ac.uk/software/bugs/openbugs/). Two chains each of 120,000 samples were run, with the first 20,000 iterations discarded to allow for burn-in of the chain. Chains were subsequently thinned by selecting every tenth iteration to reduce autocorrelation amongst the samples. Convergence of the chains was monitored visually and using the Gelman-Rubin statistic in OpenBUGS. Different models

TABLE 2 Estimated $\log_{10}$ virus neutralisation titres at which vaccinated cattle are protected from challenge with foot-and-mouth disease virus.

	$\log_{10}$ 50% protective titre			$\log_{10}$ 75% protective titre			$\log_{10}$ 95% protective titre		
	Estimate*	95% credible limits		Estimate	95% credible limits		Estimate	95% credible limits	
		Lower	Upper		Lower	Upper		Lower	Upper
Group 1†	1.90	1.61	2.25	2.46	2.11	2.97	3.39	2.86	4.29
Group 2†	1.29	1.13	1.46	1.67	1.49	1.92	2.30	2.01	2.77
Group 3†	0.90	0.80	1.00	1.17	1.06	1.30	1.61	1.43	1.87
Homologous protection‡	1.49	1.38	1.58	–	–	–	2.12	–	–

*Posterior median.
†Group 1 comprises experiments 1 & 2; group 2 comprises experiments 3, 4, 5, 6, 7, 11, & 18; and group 3 comprises experiments 8, 9, 10, 12, 13, 14, 15, 16, & 17.
‡From Barnett et al. (5), their Table 2.

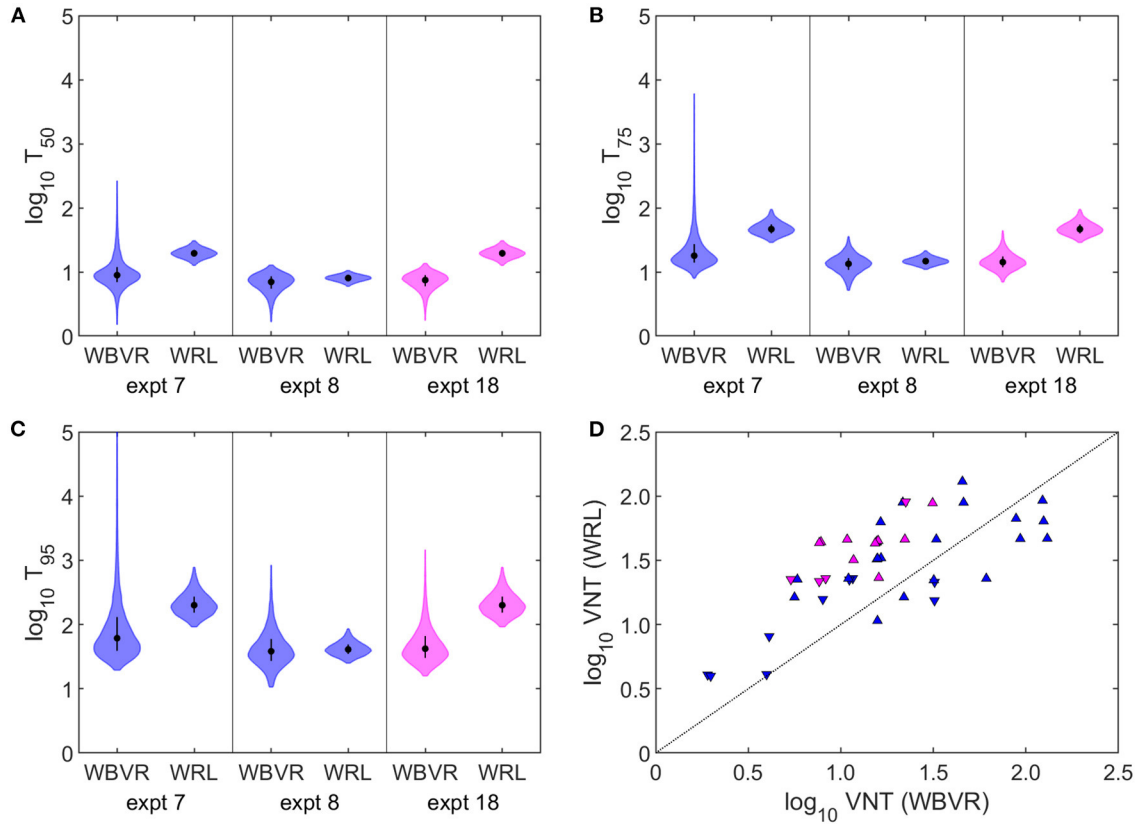

FIGURE 3
Impact of inter-laboratory variation in virus neutralisation titres (VNT) on estimates of titres required for protection. **(A–C)** VNT required for **(A)** 50%, **(B)** 75%, or **(C)** 95% of cattle to be protected from challenge with foot-and-mouth disease virus estimated using VNT obtained at WBVR or WRLFMD. Violin plots show the median (circle), interquartile range (error bar), and density (shape) for the posterior distribution. **(D)** VNT results obtained at WBVR and those obtained at WRLFMD in this study. Symbols indicate protection status of the animal: up-triangle (protected), down-triangle (not protected). The dotted line indicates equality. In all panels colour indicates serotype: A (blue) and SAT 2 (magenta). Details of the three experiments (7, 8, and 18) are shown in Table 1.

for the variation amongst serotypes/strains in parameters were compared using the deviance information criterion (DIC) (14).

Three analyses were conducted using the approach outlined above. First, the results from all studies in Table 1 were included in the analysis. Second, the results from all studies in Table 1 except those of Nagendrakumar et al. (7) were included in the analysis, to test the sensitivity of the results to the one study for which sera were not retested at WRLFMD. Finally, for the three studies where the titre results from the original laboratory were available for each animal [studies 7, 8, and 18; Wageningen BioVeterinary Research (WBVR)] the effect of using these titres was also analysed.

Results

The serology results and protection outcomes for each animal are available as Supplementary material 1. The probability of protection was best captured by a model in which the intercept was common to all studies and the slope varied amongst studies (Supplementary Table 1). However, *post-hoc* comparison of the slopes suggested the experiments could be divided into three groups: group 1 comprising experiments 1 and 2 (two O Manisa vaccine studies); group 2 comprising experiments 3, 4, 5, 6, 7, 11, 18 (serotypes O, A, and SAT 2 and also including one O Manisa study), and group 3 comprising experiments 8, 9, 10, 12, 13, 14, 15, 16, and 17 (serotypes A and Asia 1), which did indeed yield a much better fit to the data (Supplementary Table 1). In experiment 4, in which O Campos vaccinated cattle were boosted prior to challenge, the boost did not change the relationship between VN titre and cross-protection compared to single vaccination (Experiment 5), both being categorised as Group 2. The observed proportions of protected cattle and the fitted curves for probability of protection are shown for each experiment in Figure 1. In addition, the expected probabilities of protection for the three groups are shown in Figure 2 and Table 2 and the

corresponding estimates for the intercept (*a*) and slopes (*b*) are provided in Supplementary Table 2.

The best-fitting model was not influenced by the inclusion of the experiment for which the sera could not be retested at WRLFMD (7) (Supplementary Table 1). Furthermore, the same best-fitting model was selected when the results of experiments 7, 8, and 18 were analysed using the titres obtained at the original laboratory (Supplementary Table 1). However, the estimated titres required for protection were lower using the original WBVR titres compared with those obtained using the WRLFMD titres (Figures 3A–C). This reflects the fact that the titres obtained by WRLFMD were typically higher than those obtained by WBVR (Figure 3D).

Discussion

Antibody levels, often measured by VNT, are widely used predictors of protection against FMD in vaccinated animals (3, 15). Our understanding about the levels of antibody, or other immune responses (16) that are associated with protection is mainly derived from homologous potency tests in which the same virus strain is used in both the vaccine and the post-vaccination challenge. In reality, FMD vaccination must protect against field viruses that belong to the same serotype but may be antigenically different (i.e., are heterologous) from the vaccine strains to variable extents. A simple approach to assess cross-protection is to measure the amount of antibody that vaccinated animals have against the field virus of concern. This takes account of both vaccine potency and regime and antigenic suitability as well as avoiding the need to obtain proprietary vaccine strains from vaccine producers. It also has the advantage of not requiring antiserum to a monovalent vaccine, making it applicable to animals vaccinated with multiple strains of a given serotype. However, the adoption of a common heterologous serological threshold of protection will be difficult if results for different vaccine/challenge combinations are highly variable unless such variability can be controlled for. To explore this, the present study examined the correlation between day-of-challenge antibody titres to heterologous challenge viruses and the challenge outcomes. The VNT was used for serology because of the recognised correlation between neutralising antibodies and protection, and because, unlike ELISA systems, it is relatively easy to change the virus used in the test to match the threat in the field. To minimise reproducibility problems when VNT is performed in different laboratories, the sera were all tested in one place. Curves relating neutralising antibody levels to the probability of protection were established and analysed from 18 previously performed cross-protection studies with four FMDV serotypes.

The WOAH minimum standard for FMD vaccines is three 50% protective doses (PD_{50}) per full dose. For oil emulsion vaccines, this equates to an ∼71% probability of protection (17). In the present study, the average heterologous neutralising antibody titre associated with 75% protection ranged from 1.17 to 2.46 $\log_{10}$, so it was not possible to define a common threshold for all the vaccine/challenge virus combinations. Three groups were defined, but with a larger dataset either additional groups or even a continuum of results might be anticipated. In Group 1 (experiments 1 and 2), involving different challenges of O Manisa vaccinated cattle, the highest antibody levels were required for protection. The results of the other experiments fell into two groups with 75% protection thresholds of 1.17 and 1.67. It is not obvious what determines the variable antibody thresholds for protection for different vaccine/challenge combinations and this requires further study. Possible explanatory variables include virus, vaccine, host, sample and test related factors. Serotype O studies were categorised in the groups with higher thresholds (Groups 1 & 2) and were the only serotype represented in the highest threshold group (Group 1). The serotype A studies, which were the most numerous, were evenly split between Groups 2 and 3. The single SAT 2 and Asia 1 studies were assigned to Groups 2 and 3, respectively. Strain-specific effects are not obvious, as experiments with O Manisa vaccine fall into Groups 1 and 2, and others with A22 vaccine fall into Groups 2 and 3. Similarly, use of the same challenge strain (A Iran 99) was associated with different thresholds (Groups 2 and 3). As most of the vaccines were produced as double oil emulsions by the same company, differences in formulation do not explain the variations in antibody thresholds for protection, although batch-specific differences might have had some impact. The three correlation groups also did not appear to be explained by the extent of antigenic difference between the vaccine and challenge strains (Table 1). Genetic differences between the cattle used might explain differences in their immune responses and the nature of their immune protection. It cannot be excluded that different passage histories of the FMD viruses used for cattle challenge and in the VNT might have resulted in antigenic changes that affected the relationship between *in vivo* and *in vitro* cross-protection. Furthermore, differences in virus strain growth characteristics in cell culture could affect the VNT and alter the relationship between *in vitro* and *in vivo* protection. Given the extended time over which the VNTs were performed, and the range of virus stocks used, a completely standardised test is unlikely to be achieved (due to variations in virus integrity, cell susceptibility, etc), even with testing at one location. Inter-laboratory variability of VNT results was not systematically analysed, but differences between WRLFMD and WBVR results were noted.

FMD cross-protection studies in livestock are infrequent. Brehm et al. (4) studied cross-protection for 8 different vaccine-challenge combinations, but most reports of such studies in cattle have been of small numbers or singleton experiments (6–8, 10, 18, 19). In contrast, homologous protection studies are performed as part of vaccine licencing and, over the last 40 years,

many day-of-challenge sera from these have been analysed by VNT or ELISA and the results compared to protection outcomes (5, 17, 20–24). Barnett et al. (5) included an analysis of 246 sera collected 21 days post-vaccination from cattle vaccinated with serotypes O, A and Asia 1 using the same VNT method at the same laboratory (WRLFMD) as the current study. These authors considered that the relationship between antibody levels and homologous protection was similar for the three serotypes and different strains analysed at WRLFMD, with a titre of 1.49 being associated with 50% protection. This is approximately mid-way between the titre ranges associated with cross-protection in the present study (Figure 2, Table 2). However, taken as a whole, these homologous potency studies show considerable variation in the VN titres associated with protection including significant differences between some serotypes and strains. The requirement for higher antibody titres for protection against serotype O compared to the levels required for equivalent protection against serotypes A and C has been noted (21, 22), but has not been a universal finding. VN titre differences were also noted when the same sera were analysed against the same virus strains in different laboratories (5, 24).

For registration and batch release of FMD vaccines, potency and immunogenicity trials are usually performed on sera collected from animals that have been vaccinated once, usually 21–28 days previously. However, most FMD vaccine manufacturers recommend that animals being vaccinated for the first time should receive two doses of vaccine, often at an interval of 1 month. Post-vaccination monitoring studies done at population level can be performed in 6–12 month-old animals that have had only the first vaccination. However, when immunity needs to be measured in other age groups this will involve analysis of sera collected from animals at different times after different numbers of vaccinations. It is therefore of interest to know if the same antibody thresholds that predict a certain level of cross-protection after one vaccination would be appropriate after a second vaccination. One of the analysed studies (8) involved challenge of cattle after both a single and double vaccination and this did not appear to influence the correlation between *in vitro* neutralising antibody and *in vivo* protection. However, in the current study we only looked at the titre at the day of challenge in relation to protection in cattle that had been vaccinated 21–28 days earlier. A previous study showed that the relation between antibody titre and protection 9–49 months after vaccination is different (25). This shows that antibodies alone are not responsible for protection but are a correlate of the immune response in the animal.

The experiments analysed in this study used high-potency vaccines, and where tested, some of the vaccines had homologous potency results of >32 PD50/dose (4). This may account for the relatively good protection (65%) seen against challenge strains with a mostly poor antigenic match. These vaccines may be typical of those produced from banks held by FMD-free countries, but lower potency vaccines are often used to control FMD in endemic settings, where cost is a greater constraint.

Since VNT results are poorly reproducible between laboratories, most of the sera were assembled and tested in one place, where the method has been used and standardised over many years under ISO 17025 accreditation, incorporating reference sera, and charting of result trends. Comparing the titres and correlations obtained using results from two different laboratories (Figure 3) confirmed a consistent pattern of differences, that might be eliminated by reference to the results obtained with shared standard sera (26). FMD serology by VNT is mainly carried out by the quality control departments of vaccine producers and by FMD reference laboratories with appropriate biocontainment facilities and procedures. For routine post-vaccination monitoring at population level, commercial ELISAs are recommended for their ease and simplicity but a subset of the tested sera can be sent to a reference laboratory for VNT against specific field viruses of concern (27). Some regions, such as parts of Africa, with a great diversity of strains of FMDV and inconsistent vaccine quality control would benefit considerably from a system of vaccine selection and monitoring that can account for variations in vaccine potency and antigenic match. In response to this challenge, a recent initiative has been the launch of a global prize for vaccine producers who can provide vaccines for East Africa that elicit specific antibody responses measured in terms of VN titres against a panel of representative field viruses[1]. The requirement is for three out of five vaccinated cattle to develop $\log_{10}$ 1.5 antibody titres to at least three of the four strains tested per serotype when tested at WRLFMD. This is a pragmatic approach to drive up vaccine standards but carries some risk of excluding adequate vaccines and promoting ones with a low level of protective ability.

In conclusion, testing and analysing day-of-challenge sera from vaccination-and-challenge cross-protection studies, confirms the association between *in vitro* neutralising antibody titre to the challenge viruses and *in vivo* clinical cross-protection. However, different threshold levels of heterologous neutralising antibody were associated with specific levels of protection. This makes it difficult to define serological cut-offs that can predict protection against specific threats with precision. There is a suggestion of higher antibody titres being required for serotype O but other factors influence the thresholds required and remain to be identified. Further vaccination-and-challenge studies are needed to define the thresholds with greater certainty and to better understand what causes them to differ between some studies. Given the difficulty in conducting challenge studies, efforts to collect real-world field data on cross-protection should be encouraged.

1 https://www.wrlfmd.org/sites/world/files/quick_media/Cross-neutralisation%20measure%20AgResults%20Final%20v2.1.pdf

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.

Ethics statement

The findings in this report are derived from analysis of samples or results from already published animal studies, except for three experiments. The two unpublished experiments carried out at Pirbright in 2007 (Table 1, #3) and 2012 (Table 1, #17) were compliant with the Animals (Scientific Procedures) Act 1986, EU Directive 2010/63/EU, and licenced by the Home Office after local ethical review. The unpublished experiment carried out in the animal facility of Wageningen BioVeterinary Research in Lelystad in 2020 (Table 1, #18) was performed according to the Dutch Animal Ethics Law, approved by the Ethical Committee of WBVR-Lelystad.

Author contributions

DP, DK, AL, and SG: conception. DK and DP: funding. AD, GW, CB, ME, JB, HD, and LP: data provision. SG and DP: analysis and primary authors. All authors: manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1027006/full#supplementary-material

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EDITED BY
Mariano Pérez-Filgueira,
Instituto Nacional de Tecnología Agropecuaria, Argentina

REVIEWED BY
Joshua Macdonald,
Tel Aviv University, Israel
Yiltawe Wungak,
National Veterinary Research Institute (NVRI), Nigeria

*CORRESPONDENCE
Junyuan Yang
yjyang66@sxu.edu.cn

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Temporal-spatial analysis of a foot-and-mouth disease model with spatial diffusion and vaccination

Junyuan Yang[1,2]*, Xiaoyan Wang[3] and Kelu Li[4]

[1]Complex Systems Research Center, Shanxi University, Taiyuan, China, [2]Shanxi Key Laboratory of Mathematical Techniques and Big Data Analysis on Disease Control and Prevention, Shanxi University, Taiyuan, China, [3]School of Information, Shanxi University of Finance and Economics, Taiyuan, China, [4]School of Mathematics and Information Science, Henan Normal University, Xinxiang, China

Foot-and-mouth disease is an acute, highly infectious, and economically significant transboundary animal disease. Vaccination is an efficient and cost-effective measure to prevent the transmission of this disease. The primary way that foot-and-mouth disease spreads is through direct contact with infected animals, although it can also spread through contact with contaminated environments. This paper uses a diffuse foot-and-mouth disease model to account for the efficacy of vaccination in managing the disease. First, we transform an age-space structured foot-and-mouth disease into a diffusive epidemic model with nonlocal infection coupling the latent period and the latent diffusive rate. The basic reproduction number, which determines the outbreak of the disease, is then explicitly formulated. Finally, numerical simulations demonstrate that increasing vaccine efficacy has a remarkable effect than increasing vaccine coverage.

KEYWORDS

foot-and-mouth disease, the basic reproduction number, vaccination coverage, diffusion, latent period

1. Introduction

The foot-and-mouth disease virus (FMDV), a spherical, capsule-free, single-stranded RNA virus, is an infectious disease that causes foot-and-mouth disease (FMD). Both domestic and wild animals with cloven hooves are susceptible to FMDV infection (1). FMD frequently causes dairy cattle to produce less milk and beef cattle and pigs to lose weight. The efficacy of vaccination against infection is frequently weakened by temporal and spatial variations in FMDV antigenicity (2). Consequently, the presence of FMD poses a significant barrier to international trade, has a negative impact on the livestock industry, and results in significant economic losses for animal products (3). Therefore, the World Organization for Animal Health (WOAH) has ranked FMD as the top animal disease.

Animals with a clinical infection always have FMDV in their excretions and secretions, contaminating the environment (4). There are three different types of FMD transmission routes: (1) Direct transmission: the infection spreads through direct contact between infected animals and naive animals (5); (2) Indirect transmission: the infection spreads through indirect contact *via* fomites (6); (3) Airborne transmission: the transmission of virus-carrying particles through aerosols (7). It has been shown that FMDV can survive various conditions and maintain a longer survival cycle. Therefore, it is important to acknowledge that contaminated environments can transmit FMD infection to animals as a risk factor. Colenutt et al. found that the $\mathcal{R}_0$ estimated reproduction number is 1.65, which is significantly lower than the amount for direct animal to animal transmission. However, it would be sufficient to sustain an outbreak even if control measures to prevent direct transmissions, including animal movement and culling restrictions, are implemented (6).

Vaccination is a very effective measure of preventing FMD outbreaks in field conditions and lab settings (8). Evidence has shown that FMDV was radically eliminated in cattle after vaccination (4, 9). It can effectively lower the cost of agricultural production and the cost of health from an economic standpoint. According to reports, FMD has seven serotypes: A, O, C, Asial, SAT1, SAT2, and SAT3, all of which are highly mutagenic (2). Generally, vaccination with one serotype of these seven strains does not protect against other serotypes and does not provide complete protection from a single shot. Hence, to limit FMD infection, emergency ring vaccination and culling of infected animals have been executed. Vaccinating and restricting the movement of infected animals and their products is crucial when dealing with an outbreak of FMD transmission (10, 11). Several studies have quantified the efficacy of FMD vaccinations and evaluated the comprehensive economic consequences from a statistical point of view. The major concern is whether vaccinating all susceptible animals is required to limit the spread of FMD or if vaccinating only against certain agents could be adequate. It is essential to employ mathematical models to qualitatively assess the comprehensive efficacy of FMD vaccination and provide guidance for policymakers. For example, Mushayabasa et al. proposed a basic compartment model to investigate the effects of vaccination and the impact of seasonal conditions on the spread of foot-and-mouth disease (12). De Rueda et al. estimated that in mixed cattle-sheep populations with at least 14% of cattle, vaccination of cattle is sufficient to lower $\mathcal{R}_0$ to be less than 1 (8). The causes of FMD outbreaks have been explained in detail by Lyons et al. to demonstrate the effectiveness of vaccines for FMD control (13).

Many dynamic models have been explored for examining long-term FMD behaviors according to their transmission mechanisms. Mathematical models can be used to build preparedness plans in advance of an outbreak epidemic, anticipate outbreaks, and evaluate the efficacy of control measures. Researchers proposed several models to forecast FMD development trends in response to the UK's 2001 FMD epidemics (14–17). For instance, Ferguson et al. built an empirical model to forecast changes in the foot-and-mouth disease outbreak (18). Keeling et al. used the Cambridge–Edinburgh model to address the long tail property of foot-and-mouth disease cases in the UK in 2001 (19, 20). Morris et al. developed the inter-spread model to evaluate the transmission of temporal-spatial foot-and-mouth disease (21). Lewis and Ward adopted a logistic regression model to ascertain whether a collection of explanatory factors was associated with an outbreak of foot-and-mouth disease (22). Ringa and Bau created a pair approximation model to examine the role of vaccination in the optimal long-term prevention of the spread of foot-and-mouth disease (23). Most of these models ignore animal heterogeneities and assume all animals are mixed homogeneously. Jolles et al. found that FMD viruses cannot persist among infected hosts without environmental transmission through experimental and theoretical methods (24). Colenutt et al. found that environmental transmission has been linked to long-lasting FMD outbreaks (6). Bravo de Rueda et al. quantified the FMDV transmission process and showed that the environment is responsible for approximately half of FMDV transmission (25).

Animal movements significantly impact the FMD transmission pattern since it was revealed that FMD had displayed geographical diversity. Mathematical models must be used to reveal the mechanisms of spatial transmission for FMD infection. Three basic models are being used to analyze such temporal-spatial features. Spatial diffusive models investigate the temporal-spatial dynamics described by partial differential equations (26). The main focus of percolation theory is the impact on the farming landscape. Network models examine short- or long-distance transmissions starting from stochastic events (27). The information for the last two models was frequently obtained from a statistical physics point of view (28).

In this paper, we build a linked model of FMD transmission from animal to animal and from FMD virus to animal with an age-space structure. We offer a diffusive mathematical model with partial immunity from vaccination, which implies that the vaccinated animals may catch infection again once they come in contact with the infected ones. The FMD vaccine cannot provide total immunity against FMD transmission. According to numerical analysis, increasing vaccine efficacy has a greater impact than increasing vaccination coverage.

2. Method

The qualitative analysis of the evolution of FMDV transmission relies heavily on mathematical models since they offer a conceptual framework for understanding a particular system's language and making a large-scale prediction. FMDV prevalence is significantly influenced

by spatial effects, animal movements, and vaccine efficacy. Identifying the FMDV transmission mechanisms in the UK can be done with the help of a spatial diffusion model (19, 20). In this paper, we used a spatial diffusion model to investigate the efficacy of the vaccination against FMDV infection. The model complies with the "compartmental concepts" proposed by Kermack and McKendrick (29), which couples with the Laplace operator Δ to describe an animal's random movements. This model provides the most accurate representation of spatial FMDV propagation due to diffusion.

2.1. Model formulation

The main concern of this paper is to reveal the temporal and spatial patterns of FMD transmission. According to the compartmental modeling rules, we categorize the total cattle population $N(t,x)$ into three subgroups: susceptible animals, vaccinated animals, and infected animals. $S(t,x)(V(t,x))$ denotes the space density of susceptible (vaccinated) animals at time t in position $x \in \bar{\Omega}$, where $\Omega \subset \mathbb{R}^n$ is a bounded subset of $\mathbb{R}^n$.

The early detection of the incursion as well as the ability to efficiently trace and identify animals that have been exposed to the source of infection is crucial for curbing FMD transmission. There exists a high-risk period from the first infection to the detected first case, which lasts about 0.5 days after susceptible animals contact infected animals. During such period, there are potentially subtle or unapparent clinical signs of infection and it causes an underestimation of the infection. For the description of such period, we employ an age of infection to investigate the preclinical transmission process. $i(t,a,x)$ represents the age-space density of infected animals with since infection age a, at time t in position x. $B(t,x)$ denotes the space density of foot-and-mouth virus (FMDV) in a contaminant environment at time t in position $x \in \Omega$.

We hypothesize that susceptible cattle directly contact infected cattle and get an infection at rate $\beta(a)$, where a is the age since infection, and moreover, susceptible cattle can get infection indirectly contacting by fomites in the contaminated environment at rate $\frac{\beta_B}{\kappa+B(t,x)}$. Conversely, we assume that vaccinated cattle can be infected both by infected cattle and FMDV at a discount rate σ compared with the original infection. The infection force is defined by

$$\lambda(t,x) = \left(\int_0^\infty \beta(a) i(t,a,x) da + \frac{\beta_B B(t,x)}{\kappa + B(t,x)} \right).$$

Motivated by the above, the mechanisms of a foot-and-mouth disease model are characterized in the following equations (see Figure 1):

$$\begin{cases} \dfrac{\partial S(t,x)}{\partial t} = d_S \Delta S(t,x) + \Lambda - (\mu + \psi) S(t,x) \\ - S(t,x)\lambda(t,x), x \in \Omega, \\ \dfrac{\partial V(t,x)}{\partial t} = d_V \Delta V(t,x) + \psi S(t,x) - \mu V(t,x) \\ - \sigma V(t,x)\lambda(t,x), x \in \Omega, \\ \dfrac{\partial i(t,a,x)}{\partial t} + \dfrac{\partial i(t,a,x)}{\partial a} = d_i(a) \Delta i(t,a,x) \\ - (\mu + \alpha(a)) i(t,a,x), x \in \Omega, \\ i(t,0,x) = (S(t,x) + \sigma V(t,x)\lambda(t,x), x \in \Omega, \\ \dfrac{\partial B(t,x)}{\partial t} = d_B \Delta B(t,x) + \displaystyle\int_0^\infty p(a) i(t,a,x) da \\ - cB(t,x), x \in \Omega, \\ \dfrac{\partial S(t,x)}{\partial \mathbf{n}} = \dfrac{\partial V(t,x)}{\partial \mathbf{n}} = \dfrac{\partial i(t,a,x)}{\partial \mathbf{n}} = \dfrac{\partial B(t,x)}{\partial \mathbf{n}} = 0, x \in \partial\Omega, \end{cases} \tag{1}$$

where $d_j (J = S, V, i, B)$ denotes the diffusion coefficients of susceptible, vaccinated, infected animals, and foot-mouth viruses, Λ denotes the produce rate, μ and c denote slaughter rate of cattle and the degradation rate of FMDV, respectively. $\alpha(\cdot)$ stands for the death rate caused by FMD. The infected animals release the FMDV into the environment at rate $p(\cdot)$. $\partial/\partial \mathbf{n}$ denotes the derivative along the outward unite normal vector **n.**

By Theorem 1.5 in Pazy (33), the operator $d_j \Delta (j = S, V, i, B)$ with the zero flux boundary condition generate the following compact and strongly positive semigroups

$$(T_j(t)[\phi])(x) = \int_\Omega \Gamma_j(t,x,y)\phi(y)dy, j = S, V, i, B.$$

where $\Gamma_j (j = S, V, i, B)$ are Green functions. Assume that the latent period is τ, then we can separate the infected cattle into two subgroups:

$$E(t,x) = \int_0^\tau i(t,a,x)da, \quad I(t,x) = \int_\tau^\infty i(t,a,x)da,$$

where $E(t,x)$ represents the space density of latent cattle at time t in position x, $I(t,x)$ denotes the space density of infected cattle at time t and position x. Integrating the third equation of model (1) with its initial and boundary conditions, we have that

$$i(t,a,x) = \begin{cases} \displaystyle\int_\Omega \Gamma_i(a,x,y) i(t-a,0,y) dy \pi(a), & t \geq a, \\ \displaystyle\int_\Omega \Gamma_i(a,x,y) i_0(a-t,y) dy \frac{\pi(a)}{\pi(a-t)}, & t < a, \end{cases} \tag{2}$$

where

$$\pi(a) = e^{-\int_0^a (\mu + \alpha(s)) ds}$$

TABLE 1 List of parameter values.

Parameters	Biological meanings	Values	Unit	References
Λ	Produce rate	38,340	day^{-1}	(30)
β_B	The transmission rate from FMDV to cattle	1.3348×10^{-6}	day^{-1}	(30)
κ	The half-saturation concentration of the FMDV	10^8	copies/cattle	(30)
μ	The slaughter rate	0.0018	day^{-1}	(5)
α	The death rate due to FMD	1/3.5	day^{-1}	(30)
c	The natural decay rate of FMDV	1/30	day^{-1}	(31)
p	The pathogen production rate of an infected cattle	$10^{4.3}$	day^{-1}	(30)
ψ	the vaccinated rate	0.8	day^{-1}	(32)
$1-\sigma$	the efficacy of vaccination	0.5	day^{-1}	(11)
d_S	The diffusion coefficient of susceptible cattle	0.0005	-	Assumed
d_V	The diffusion coefficient of vaccinated cattle	0.0005	-	Assumed
d_I	The diffusion coefficient of infected cattle	0.0003	-	Assumed
d_B	The diffusion coefficient of FMDV	0.001	-	Assumed

represents the probability of an infected animal survives until infect age a. If we set

$$d_i(a) = \begin{cases} d_E, & 0 \le a \le \tau, \\ d_I, & \tau < a < \infty, \end{cases} \quad \beta(a) = \begin{cases} \beta_E, & 0 \le a \le \tau, \\ \beta, & \tau < a < \infty, \end{cases}$$

$$\alpha(a) = \begin{cases} \alpha_E, & 0 \le a \le \tau, \\ \alpha, & \tau < a < \infty, \end{cases} \quad p(a) = \begin{cases} p_E, & 0 \le a \le \tau, \\ p, & \tau < a < \infty. \end{cases}$$

Based on the above assumptions on $\beta(\cdot), d_i(\cdot)$, and $\alpha(\cdot)$, then the evolution of the latent and infected cattle satisfies

$$\begin{aligned} \frac{\partial E(t,x)}{\partial t} &= d_E \Delta E(t,x) - (\mu + \alpha_E) E(t,x) \\ &\quad - \int_\Omega \Gamma_i(\tau, x, y)(S(t-\tau, x) + \sigma V(t-\tau, x)) \\ &\quad \times \left(\beta_E E(t-\tau, y) + \beta_I I(t-\tau, y) \right. \\ &\quad \left. + \frac{\beta_B B(t-\tau, x)}{1 + \alpha B(t-\tau, y)} \right) dy e^{-(\mu+\alpha_E)\tau} \\ &\quad + (S(t,x) + \sigma V(t,x)) \\ &\quad \left(\beta_E E(t,x) + \beta I(t,x) + \frac{\beta_B B(t,x)}{1 + \alpha B(t,x)} \right), \qquad (3) \\ \frac{\partial I(t,x)}{\partial t} &= d_I \Delta I(t,x) da - (\mu + \alpha) I(t,x) \\ &\quad + \int_\Omega \Gamma_i(\tau, x, y)(S(t-\tau, x) + \sigma V(t-\tau, x)) \\ &\quad \times \left(\beta_E E(t-\tau, y) + \beta_I I(t-\tau, y) \right. \\ &\quad \left. + \frac{\beta_B B(t-\tau, x)}{1 + \alpha B(t-\tau, y)} \right) dy e^{-(\mu+\alpha)\tau}. \end{aligned}$$

The detailed derivations of E and I are enclosed in Appendix A. From Equation (3), it is easy to see that the compartment E is decoupled, but the latent information is inclosed in the term

$$\int_\Omega \Gamma_i(\tau, x, y)(S(t-\tau, x) + \sigma V(t-\tau, x))$$

$$\left(\beta_I I(t-\tau, y) + \frac{\beta_B B(t-\tau, x)}{1 + \alpha B(t-\tau, y)} \right) dy e^{-(\mu+\alpha_E)\tau},$$

where we have assumed that the latent cattle has no infected ability. Replacing i in Equation (1) and ignoring equation E, one arrives at

$$\begin{cases} \frac{\partial S(t,x)}{\partial t} = d_S \Delta S(t,x) + \Lambda - (\mu + \psi) S(t,x) \\ - S(t,x) \left(\beta I(t,x) + \frac{\beta_B B(t,x)}{\kappa + \nu(t,x)} \right), x \in \Omega, \\ \frac{\partial V(t,x)}{\partial t} = d_V \Delta V(t,x) + \psi S(t,x) - \mu V(t,x) \\ - \sigma V(t,x) \left(\beta I(t,x) + \frac{\beta_B V(t,x)}{\kappa + B(t,x)} \right), x \in \Omega, \\ \frac{\partial I(t,x)}{\partial t} = d_I \Delta I(t,x) da - (\mu + \alpha) I(t,x) \\ + \int_\Omega \Gamma_i(\tau, x, y)(S(t-\tau, x) + \sigma V(t-\tau, x)) \\ \quad \times \left(\beta_I I(t-\tau, y) + \frac{\beta_B B(t-\tau, x)}{1 + \alpha B(t-\tau, y)} \right) dy e^{-(\mu+\alpha)\tau}, \\ x \in \Omega, \\ \frac{\partial B(t,x)}{\partial t} = d_B \Delta B(t,x) + p I(t,x) - c B(t,x), x \in \Omega, \\ \frac{\partial S(t,x)}{\partial \mathbf{n}} = \frac{\partial V(t,x)}{\partial \mathbf{n}} = \frac{\partial i(t,a,x)}{\partial \mathbf{n}} = \frac{\partial B(t,x)}{\partial \mathbf{n}} = 0, x \in \partial\Omega, \end{cases} \qquad (4)$$

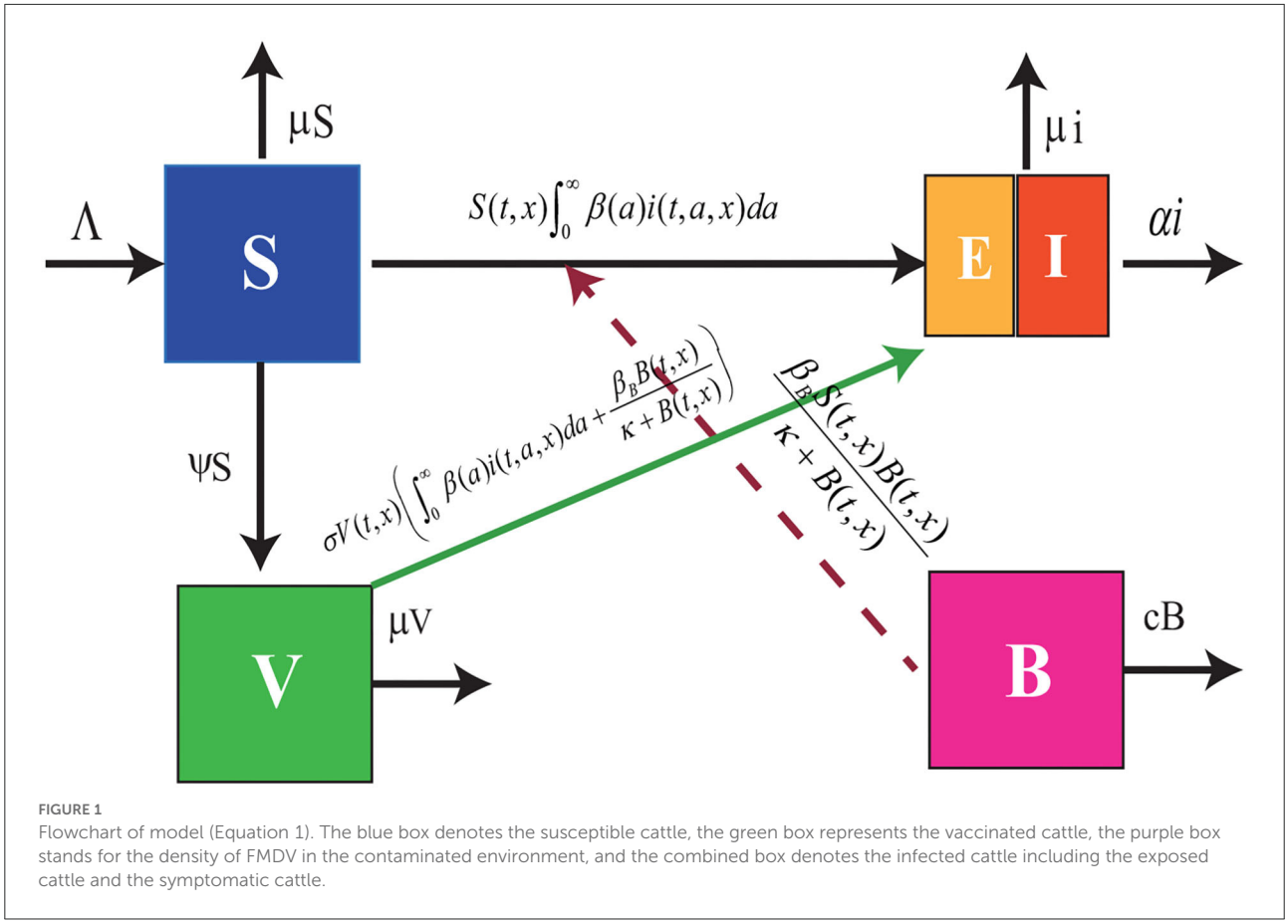

FIGURE 1
Flowchart of model (Equation 1). The blue box denotes the susceptible cattle, the green box represents the vaccinated cattle, the purple box stands for the density of FMDV in the contaminated environment, and the combined box denotes the infected cattle including the exposed cattle and the symptomatic cattle.

where we have assumed that $p_E = 0$ suggesting that latent cattle do not release the FMDV into the environment. In what follows, we will focus on the efficacy of vaccination and the diffusion of the latent cattle on the temporal-spatial patterns of FMD transmission.

2.2. Basic reproduction number

The basic reproduction is the average number of secondary cases produced by an infected individual at a completely susceptible environment during his infectious period, which provides an overall measure of the potential for transmission of an infection in a population. Generally, if it is less than one, the disease dies out; otherwise, it invades the host population.

Lemma 0.3 in Appendix B implies that system (Equation (4)) has a disease-free steady state $E_0 = (S^0, V^0, 0, 0) = (\frac{\Lambda}{\mu+\psi}, \frac{\psi\Lambda}{\mu(\mu+\psi)}, 0, 0)$. Linearizing system (Equation (4)) around the disease-free equilibrium E_0, we obtain

$$\begin{cases} \frac{\partial I(t,x)}{\partial t} = d_i\Delta I(t,x) - (\mu+\alpha)I(t,x) \\ + (S^0 + \sigma V^0)\int_\Omega \Gamma_i(\tau,x,y) \\ \quad \times \left(\beta_I I(t-\tau,y) + \frac{\beta_B B(t-\tau,y)}{\kappa}\right) dy e^{-(\mu+\alpha)\tau}, x \in \Omega, \\ \frac{\partial B(t,x)}{\partial t} = d_B\Delta B(t,x) + pI(t,x) - cB(t,x), x \in \Omega, \\ \frac{\partial I(t,x)}{\partial \mathbf{n}} = \frac{\partial B(t,x)}{\partial \mathbf{n}} = 0, x \in \partial\Omega. \end{cases} \tag{5}$$

Let us introduce a newly infection operator

$$F[\phi](x) = (F_1[\phi], F_2[\phi])(x),\ \forall \phi = (\phi_3, \phi_4) \in Y^2, x \in \bar{\Omega},$$

where

$$F_1[\phi](x) = (S^0 + \sigma V^0)\int_\Omega \Gamma_i(\tau,x,y)\left(\beta_I\phi_3(y) + \frac{\beta_B}{\kappa}\phi_4(y)\right)$$

$$dy e^{-(\mu+\alpha)\tau}, F_2 = p\phi_3(x).$$

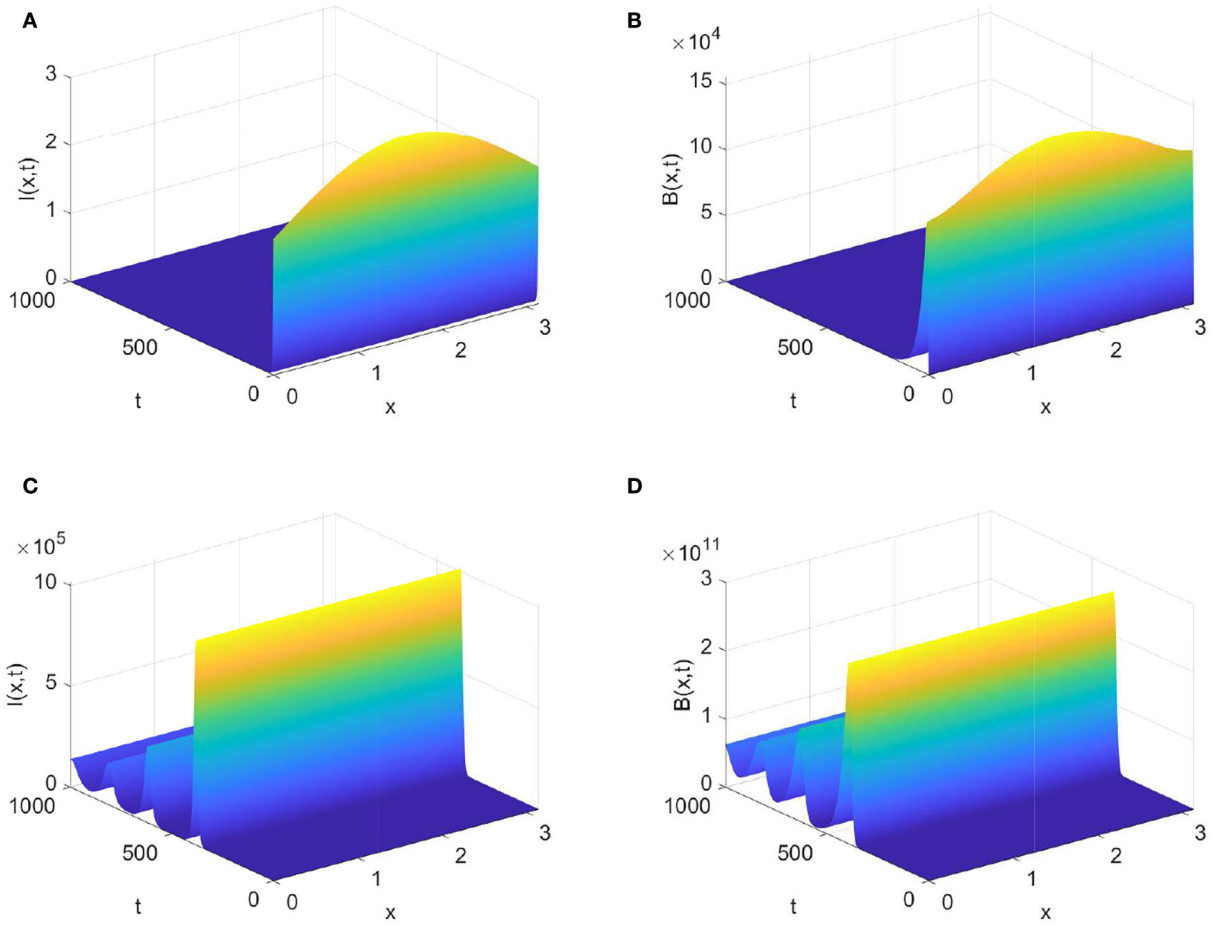

FIGURE 2
Evolution of infected animals and FMD virus with parameters in Table 1. **(A,B)** with $\mathcal{R}_0 \approx 0.8381 < 1$. **(C,D)** with $\mathcal{R}_0 \approx 4.1778 > 1$.

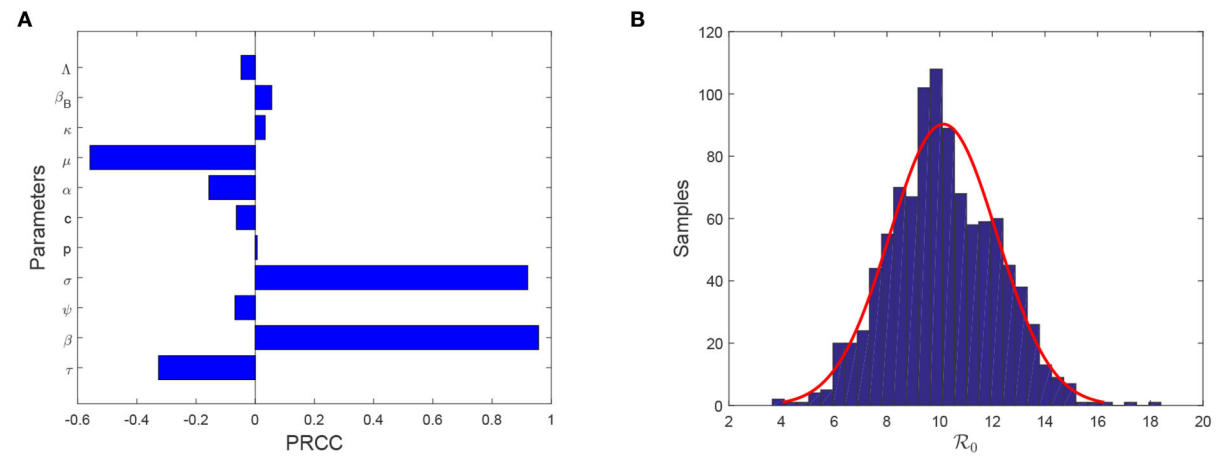

FIGURE 3
(A) Tornado plot of PRCC for model parameters associated with $\mathcal{R}_0$. **(B)** Samples of $\mathcal{R}_0$.

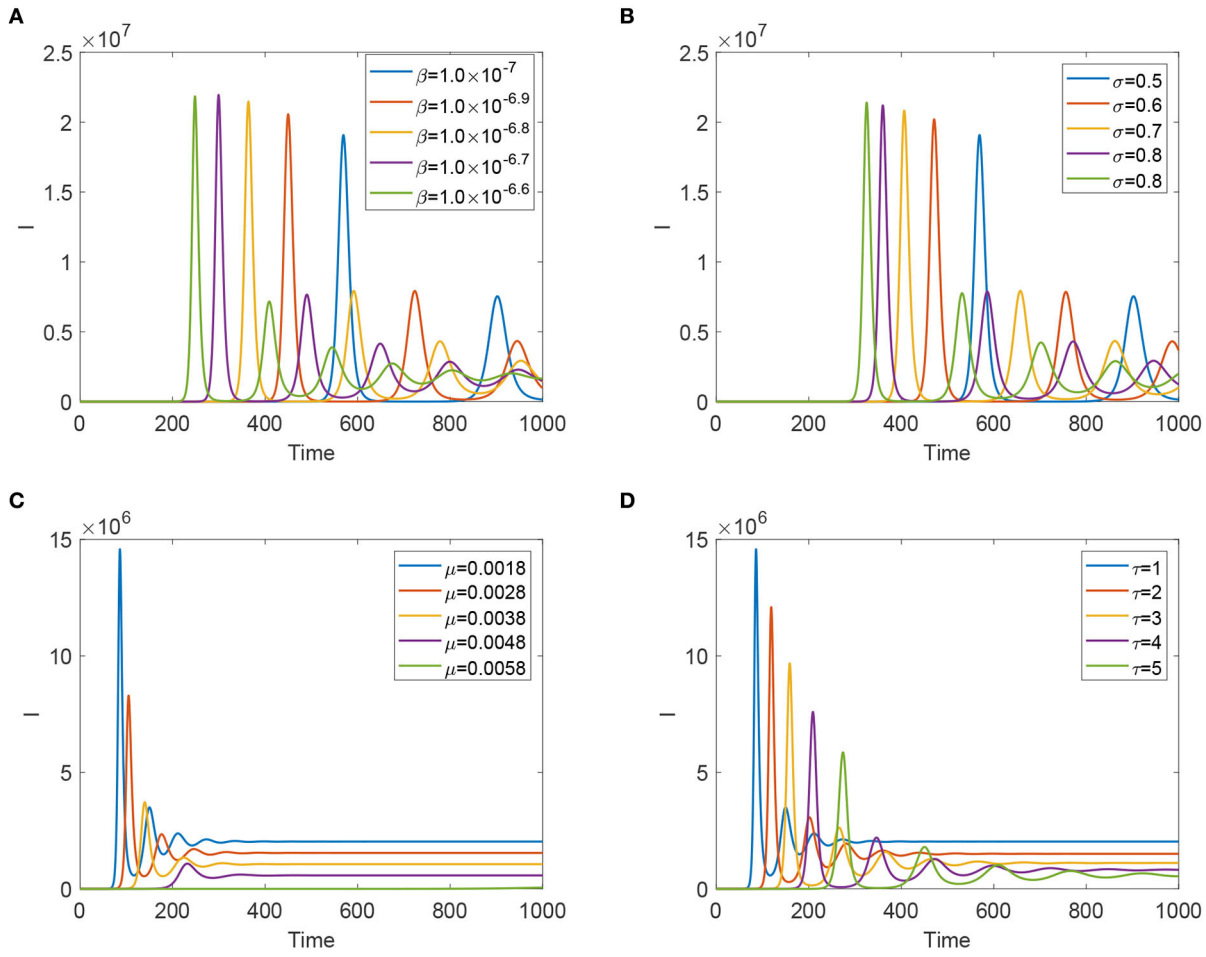

FIGURE 4
Evolution of infected animals and FMD virus with some significant parameters. **(A)** With different β. **(B)** With different σ. **(C)** With different μ. **(D)** With different τ.

Moreover, let us introduce a transition operator

$$B[\phi](x) = (B_1[\phi], B_2[\phi])(x),\ \forall \phi \in Y^2,$$

where

$$B_1[\phi] = d_i\Delta\phi_3(x) - (\mu+\alpha)\phi_3(x), B_2[\phi] = d_B\Delta\phi_4(x) - c\phi_4(x).$$

Besides, the transit operator B generates the following positive and compact semigroup

$$T([\phi](x))(t) = (e^{-(\mu+\alpha)t}\int_\Omega \Gamma_i(t,x,y)\phi_3(y)dy, e^{-ct}$$

$$\int_\Omega \Gamma_B(t,x,y)\phi_4(y)dy)^T, \forall t \in \mathbb{R}_+.$$

Then

$$(-B)^{-1}[\phi](x) = \int_0^\infty T[\phi](t)dt.$$

Hence, the next-generation operator G can be defined by

$$G[\phi](x) = F(-B)^{-1}[\phi](x) = (F_1(-B_1)^{-1}[\phi](x),$$

$$F_2(-B_2)^{-1}[\phi](x))^T,$$

where

$$F_1(-B_1)^{-1}[\phi](x) = (S^0 + \sigma V^0)\int_0^\infty \int_\Omega \Gamma_i(\tau,x,y)\left(\beta_I e^{-(\mu+\alpha)t} \int_\Omega \Gamma_i(t,x,y)\phi_3(y)dy + \frac{\beta_B}{\kappa}e^{-ct}\int_\Omega \Gamma_B(t,x,y)\phi_4(y)dy\right) dydte^{-(\mu+\alpha)\tau},$$

$$F_2(-B_2)^{-1}[\phi](x) = p\int_0^\infty e^{-(\mu+\alpha)t}\int_\Omega \Gamma_i(t,x,y)\phi_4(y)dydt.$$

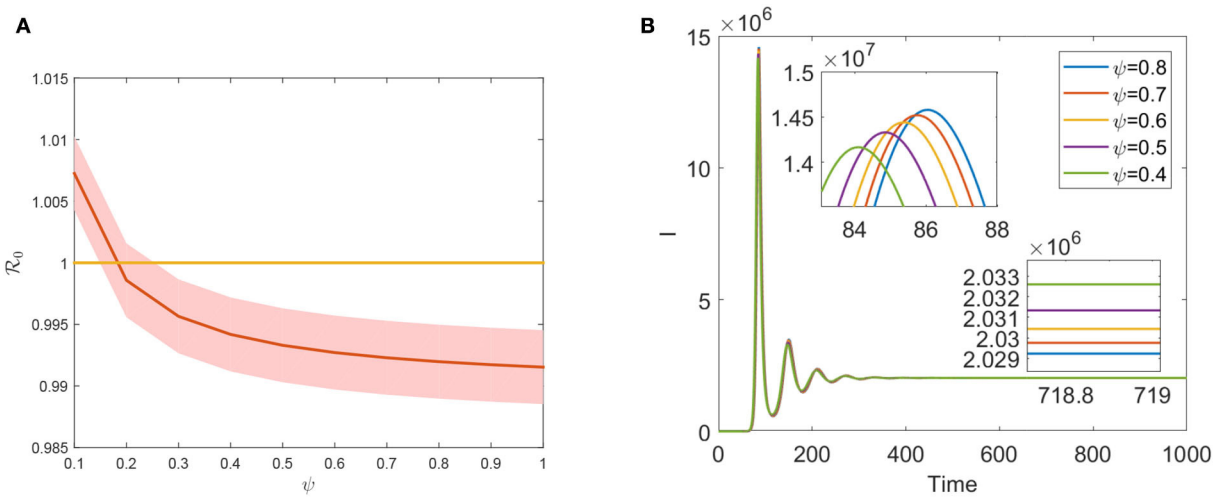

FIGURE 5
(A) The plot of $\mathcal{R}_0$ *via* ψ. **(B)** Evolution of total infected cattle varies with different ψ.

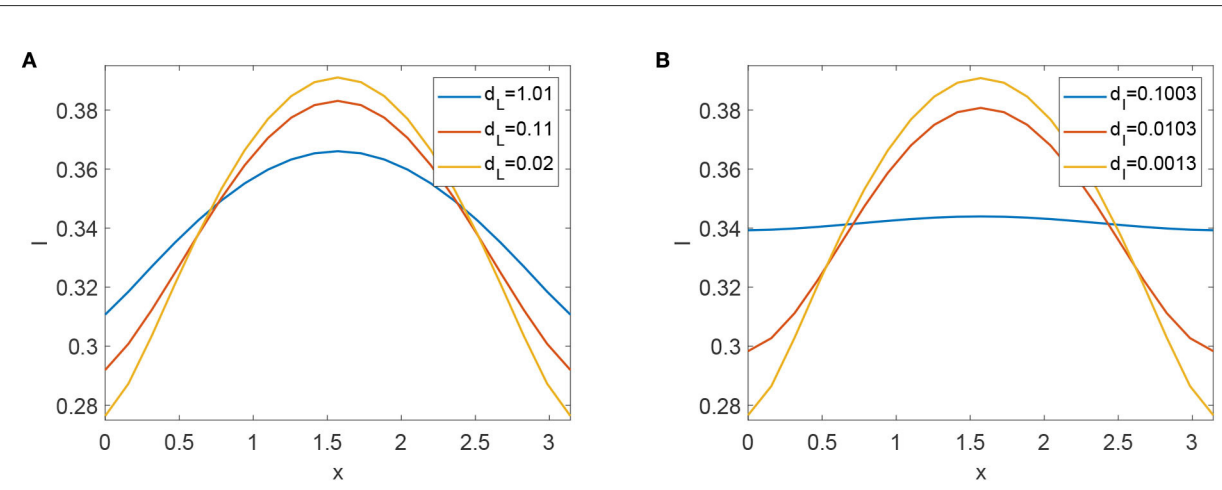

FIGURE 6
(A) Profiles of infected animals with different diffusive coefficients d_L. **(B)** Profiles of infected animals with different diffusive coefficients d_I.

Therefore, the basic reproduction number is defined by

$$\mathcal{R}_0 = \rho(G)$$

From the property of Γ_j, we have concluded that the next operator G is positive and compact. Employing Krein–Rutman Theorem, $\mathcal{R}_0$ is a positive eigenvalue with respect to a positive eigenvector ϕ, which suggests that

$$G[\phi](x) = \mathcal{R}_0[\phi](x).$$

Letting $\phi = \mathbf{1}$, then

$$\mathcal{R}_0 = \frac{(S^0 + \sigma V^0)\frac{\beta}{\mu+\alpha}e^{-(\mu+\alpha)\tau} + \sqrt{(S^0 + \sigma V^0)^2\frac{\beta^2}{(\mu+\alpha)^2}e^{-2(\mu+\alpha)\tau} + 4(S^0 + \sigma V^0)\frac{\beta_B}{c\kappa}\frac{p}{\mu+\alpha}e^{-(\mu+\alpha)\tau}}}{2}. \quad (6)$$

From the epidemiological view of points, we introduce the other reproduction number by

$$\hat{\mathcal{R}}_0 = (S^0 + \sigma V^0)\left(\frac{\beta}{\mu+\alpha} + \frac{\beta_B p}{\kappa(\mu+\alpha)c}\right)e^{-(\mu+\alpha)\tau} = \hat{\mathcal{R}}_{0a} + \hat{\mathcal{R}}_{0b}, \quad (7)$$

where

$$\hat{\mathcal{R}}_{0a} = (S^0 + \sigma V^0)\frac{\beta}{\mu+\alpha}e^{-(\mu+\alpha)\tau},$$

$$\hat{\mathcal{R}}_{0b} = (S^0 + \sigma V^0)\frac{\beta_B p}{\kappa(\mu+\alpha)c}e^{-(\mu+\alpha)\tau}$$

Theorem 2.1. *Let $\mathcal{R}_0$ and $\hat{\mathcal{R}}_0$ be defined by Equations (6) and (7). The following statements are true:*

(1) $\mathcal{R}_0 > 1 \Leftrightarrow \hat{\mathcal{R}}_0 > 1$;
(2) $\mathcal{R}_0 < 1 \Leftrightarrow \hat{\mathcal{R}}_0 < 1$;
(3) $\mathcal{R}_0 = 1 \Leftrightarrow \hat{\mathcal{R}}_0 = 1$.

From what has been discussed, we return to give a detailed explanation for $\hat{\mathcal{R}}_0$. In fact, $\beta e^{-(\mu+\alpha)\tau}$ gives the average number of the secondary cases produced by one infected animal and it is still alive after the latent period τ. Hence, $\hat{\mathcal{R}}_{0a}$ gives the average number of the secondary infected animals produced by an infected animal during its infectious period. Similarly, $\hat{\mathcal{R}}_{0v}$ means that the average number of the secondary cases produced by a typical FMDV during its period.

3. Results

3.1. Theoretical results

In this section, we will show the basic reproduction number is a threshold index for disease extinction or persistence.

Lemma 3.1. *For any $\phi \in C_\tau^+$, the following items hold.*

(1) For any $t \in \mathbb{R}_+$, $S(t,\cdot) > 0$ and $V(t,\cdot) > 0$. Moreover, there exists a positive value $\bar{\epsilon}$ such that

$$\liminf_{t\to\infty} S(t,\cdot) \geq \bar{\epsilon}, \quad \liminf_{t\to\infty} V(t,\cdot) \geq \bar{\epsilon},$$

(2) If there exists some $t_0 \geq 0$ such that $I(t_0,\cdot) \not\equiv 0$ or $B(t_0,\cdot) \not\equiv 0$, then

$$I(t,\cdot) > 0, \quad B(t,\cdot) > 0,$$

Proof. In the proof of Lemma 0.1 in Appendix B, there exist two positive constants T and M such that for any $(t,x) \in (T,\infty)\times\bar{\Omega}$

$$I(t,\cdot) \leq M, B(t,\cdot) \leq M, \forall t > T.$$

In view of the first equation of (4), we note that

$$\begin{aligned}
&\frac{\partial S(t,x)}{\partial t} \geq d_S \Delta S(t,x) + \Lambda - (\mu+\psi \\
&+ (\beta+\beta_B)M)S(t,x), (t,x) \in (T,\infty)\times\Omega, \\
&\frac{\partial V(t,x)}{\partial t} \geq d_V \Delta V(t,x) + \psi S(t,x) \\
&- (\mu + \sigma(\beta+\beta_B)M)V(t,x), (t,x) \in (T,\infty)\times\Omega, \\
&\frac{\partial S(t,x)}{\partial \mathbf{n}} = \frac{\partial V(t,x)}{\partial \mathbf{n}} = 0, x \in \partial\Omega.
\end{aligned}$$

By Lemma 4.1, the following system

$$\begin{aligned}
&\frac{\partial \bar{S}(t,x)}{\partial t} = d_S \Delta \bar{S}(t,x) + \Lambda - (\mu+\psi \\
&+ (\beta+\beta_B)M)\bar{S}(t,x), x \in \Omega, t \geq T_B, \\
&\frac{\partial \bar{V}(t,x)}{\partial t} = d_V \Delta \bar{V}(t,x) + \psi \bar{S}(t,x) \\
&- (\mu + \sigma(\beta+\beta_B)M)\bar{V}(t,x), \\
&\frac{\partial \bar{S}(t,x)}{\partial \mathbf{n}} = \frac{\partial \bar{V}(t,x)}{\partial \mathbf{n}} = 0, x \in \partial\Omega.
\end{aligned}$$

has a unique equilibrium $\bar{E}^* = (\bar{S}^0, \bar{V}^0) = \left(\frac{\Lambda}{\mu+\psi+(\beta+\beta_B)M}, \frac{p\Lambda}{(\mu+\psi+(\beta+\beta_B)M)(\mu+\sigma(\beta+\beta_B)M)}\right)$ which is globally asymptotically stable in $C(\bar{\Omega},\mathbb{R})\times C(\bar{\Omega},\mathbb{R})$. By the standard parabolic comparison theorem, we conclude that

$$\liminf_{t\to\infty} S(t,\cdot) \geq \bar{S}^0, \quad \liminf_{t\to\infty} V(t,\cdot) \geq \bar{V}^0.$$

From Lemma 0.3 in the Appendix B, it follows that

$$\begin{cases}
\frac{\partial I(t,x)}{\partial t} \leq d_I \Delta I(t,x) - (\mu+\alpha)I(t,x), \\
\frac{\partial B(t,x)}{\partial t} \leq d_B \Delta B(t,x) + pI(t,x) - cB(t,x).
\end{cases} \tag{8}$$

The part (2) is a direct result of Theorem 3 and Theorem 4 in Protter and Weinberger (34) replacing $t = 0$ by $t = t_0$.

Theorem 3.2. *Suppose $\mathcal{R}_0$ is defined in Equation (6). Then the following results hold.*

(1) If $\mathcal{R}_0 < 1$, then the virus-free equilibrium E_0 is globally asymptotically stable;
(2) If $\mathcal{R}_0 > 1$, then there exists a positive value $\epsilon > 0$ such that for all $\phi_3(x) \not\equiv 0$ and $\phi_4(x) \not\equiv 0$

$$\liminf_{t\to+\infty} I(t,x) \geq \epsilon, \quad \liminf V(t,x) \geq \epsilon$$

uniformly for all $x \in \bar{\Omega}$. Moreover, system (Equation 4) has at least one endemic equilibrium E^*.

The detailed proof of Theorem 3.2 is enclosed in Appendix C.

3.2. Numerical results

In this section, we have conducted numerical examples to show some significant results. First, we fix some parameters in Table 1. Hence, we pick up

$$\Gamma_i(\tau,x,y) = \frac{2}{\pi}\sum_{n=1}^{\infty}\exp(-(n^2 D_L + d + \alpha)\tau)\cos(nx)\cos(ny).$$

The initial values are chosen as follows:

$$\phi_S(\tau,x) = 4 + \sin(x)\cos(\tau), \quad \phi_V(\tau,x) = 4 + \sin(x)\cos(\tau),$$
$$\phi_I(\tau,x) = 2 + \sin(x)\cos(\tau), \quad \phi_B(\tau,x) = 2 + \sin(x)\cos(\tau).$$

3.3. The dynamics of the system

Next, if we choose $\beta = 3.0 \times 10^{-8}$, then $\mathcal{R}_0 = 0.8381 < 1$. From Theorem 3.2 (1), it follows that the virus-free steady state E_0 is globally attractive. Figures 2A,B show that the densities of infected animals and the FMD virus decay to zero as time goes to infinity. Enlarging $\beta = 1.0\times10^{7}$, we calculate $\mathcal{R}_0 = 4.1778 > 1$. Theorem 3.2 (2) ensures that the disease persists when $\mathcal{R}_0 > 1$ and $\phi \in W_0$. Figures 2C,D display that the densities of $I(t,x)$ and $B(t,x)$ gradually decay to a positive distribution when time evolves.

3.4. Sensitivity analysis

Note that model (Equation 4) contains fifteen parameters. It is necessary to find which parameters are more sensitive than other parameters in affecting evolution of FMD infection. Theorem 3.2 shows that $\mathcal{R}_0$ plays a significant role in determining the outbreak of FMD. Hence, we need to seek the sensitivity analysis of $\mathcal{R}_0$ on each parameter. To achieve this aim, we select Latin hypercube sampling (LHS) to identify the rank of key factors that affect the basic reproduction number. In this process, we use partial rank correlation coefficient (PRCC) with 1,000 samples to give a tornado plot, which provides a visible figure to show the importance of every parameter's uncertainty. Figure 3A shows that reducing the transmission from animal to animal, improving the efficacy of vaccination, enlarging the curing rate, and lengthening the latent period are helpful for reducing the size of $\mathcal{R}_0$. Moreover, reducing transmission rate from animal to animal has the most importance than other control measures. The samples of $\mathcal{R}_0$ converge a normal distribution with an average value 10.1404 [95% CI (10.014–1.02668)] and a variance 2.0369 [95% CI(1.9514–2.1304)] (see Figure 3B).

To evaluate each effective control measure, we verify parameters β, μ, σ, and τ to detect the sensitivity analysis of the dynamics of system (Equation 4). From Figures 4A,B, we find that reducing the transmission risk from animal to animal and improving the efficacy of the vaccination can delay the fist peak arrival time and reduce the sizes of peaks, but such two prevention measures enhance the frequencies of temporal oscillations. Figures 4C,D expound that improving the slaughter rate and lengthening the latent period can reduce the size of the final prevalence, delay fist peak arrival time, and decrease the size of each peak. However, lengthening the latent period enhances the frequency of the temporal oscillations; increasing the slaughter rate has a side effect on the frequency of oscillation patterns.

4. Conclusion and discussion

This paper proposes a nonlocal, diffusive foot-and-mouth disease model that couples the animal to animal and FMDV-to-animal transmission modes. We derived the basic reproduction number using the next generation operator theory, whose characteristic is equivalent to a principal eigenvalue problem. The basic reproduction number $\mathcal{R}_0$ is a threshold value determining the outbreak of FMD infection. If $\mathcal{R}_0 < 1$, the disease ends; otherwise, it persists.

Vaccination is one of the most important preventive measures for curbing FMD prevalence. However, the evaluation of the FMD vaccination's effectiveness plays a significant role in preventing disease transmission since the vaccine does not provide full immunity against FMD. Theorem 3.2 states that $\mathcal{R}_0 < 1$ is a necessary condition for eradicating FMD in a region. $\mathcal{R}_0$ is a declining function concerning ψ, as seen in Figure 5. Hence, ψ effectively decreases the size of $\mathcal{R}_0$ by increasing vaccination coverage. Moreover, increasing vaccination coverage ψ can delay the first peak arrival period and lower the final prevalence (see Figure 5B). Compared to Figures 4B, 5B, we found that increasing the efficacy of the FMD vaccine has a greater impact on preventing infection than increasing vaccine coverage. The development of more potent vaccines will offer the best defense against FMDV invasion. The first one has a notable accomplishment for reducing the value of $\mathcal{R}_0$, which suggests that slaughtering the animals and purifying the environment play an effect in the face of an outbreak of an emerging FMD. This contrasts the effects of improving the slaughter rate μ and the vaccination rate ψ. However, such measures will inevitably result in significant economic losses. Long-term, increasing vaccination coverage rates may have a greater economic impact on preventing FMD infection.

The reviews of the expression of $\mathcal{R}_0$ have no relation with any diffusive coefficient. As we know, stochastic movement's speed does have an impact on how FMD transmission scenarios develop. We conducted computational experiments to alter the values of d_L and d_I to understand how diffusive coefficients affect the dynamics of FMD. The scenarios of infected animals eventually flatten (see Figures 6A,B). Increasing the diffusive rate of infected animals is advantageous for reducing the prevalence of FMD.

The carriers of FMDV is defined by confirmed ones if the virus or viral genomes are isolated from the esophageal-pharyngeal fluid more than 28 days after infection. Several experimental evidence shows that carriers may be the main reason of the occasional cause of outbreaks (35, 36). Although the role of carriers in the occurrence of new outbreaks is still

a matter of debate (37), it is useful to study the risk of carriers on the persistence of FMDV from a cost-benefit perspective (24) and quantify the risk of infection from carriers to susceptible cattle. We will leave these work in future.

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.

Author contributions

JY wrote the manuscript. XW designed the numerical algorithms. KL reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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Supplementary material

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

REVIEWED BY
Artur Summerfield,
Institute of Virology and Immunology (IVI), Switzerland
Sabrina Galdo Novo,
University of Buenos Aires, Argentina
Fayna Diaz San Segundo,
Agricultural Research Service (USDA), United States

*CORRESPONDENCE
Melissa McLaws
melissa.mclaws@fao.org

†These authors share first authorship

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PRAGMATIST: A tool to prioritize foot-and-mouth disease virus antigens held in vaccine banks

Anna B. Ludi[1†], Melissa McLaws[2*†], Bryony Armson[2], Jessica Clark[3,4], Antonello Di Nardo[1], Krupali Parekh[1], Mark Henstock[1], Petra Muellner[5,6], Ulrich J. Muellner[5], Fabrizio Rosso[2], Joaquin M. Prada[4], Daniel L. Horton[4], David J. Paton[1,2], Keith Sumption[2] and Donald P. King[1]

[1]Vesicular Disease Reference Laboratory, The Pirbright Institute, Woking, United Kingdom, [2]The European Commission for the Control of Foot and Mouth Disease (EuFMD), Food and Agriculture Organization of the United Nations, Rome, Italy, [3]Wellcome Centre for Integrative Parasitology, Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, United Kingdom, [4]Faculty of Health and Medical Sciences, School of Veterinary Medicine, University of Surrey, Guildford, United Kingdom, [5]Epi-Interactive, Miramar, Wellington, New Zealand, [6]School of Veterinary Science, Massey University, Palmerston North, New Zealand

Antigen banks have been established to supply foot-and-mouth disease virus (FMDV) vaccines at short notice to respond to incursions or upsurges in cases of FMDV infection. Multiple vaccine strains are needed to protect against specific FMDV lineages that circulate within six viral serotypes that are unevenly distributed across the world. The optimal selection of distinct antigens held in a bank must carefully balance the desire to cover these risks with the costs of purchasing and maintaining vaccine antigens. PRAGMATIST is a semi-quantitative FMD vaccine strain selection tool combining three strands of evidence: (1) estimates of the risk of incursion from specific areas (source area score); (2) estimates of the relative prevalence of FMD viral lineages in each specific area (lineage distribution score); and (3) effectiveness of each vaccine against specific FMDV lineages based on laboratory vaccine matching tests (vaccine coverage score). The output is a vaccine score, which identifies vaccine strains that best address the threats, and consequently which are the highest priority for inclusion in vaccine antigen banks. In this paper, data used to populate PRAGMATIST are described, including the results from expert elicitations regarding FMD risk and viral lineage circulation, while vaccine coverage data is provided from vaccine matching tests performed at the WRLFMD between 2011 and 2021 (n = 2,150). These data were tailored to working examples for three hypothetical vaccine antigen bank perspectives (Europe, North America, and Australia). The results highlight the variation in the vaccine antigens required for storage in these different regions, dependent on risk. While the tool outputs are largely robust to uncertainty in the input parameters, variation in vaccine coverage score had the most noticeable

impact on the estimated risk covered by each vaccine, particularly for vaccines that provide substantial risk coverage across several lineages.

KEYWORDS

vaccination, vaccine matching, vaccine bank, foot and mouth disease (FMD), decision support tool, vaccine selection

Introduction

Foot-and-mouth disease virus (FMDV) exists as seven serotypes: O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3, although serotype C has not been reported since 2004 (1, 2). The world is divided into FMD-free and endemic countries and regions (3), with virus widespread in Africa and Asia and restricted to Venezuela in South America. FMDV serotypes and strains are unevenly distributed in different parts of the world with seven geographic pools of FMDV identified (4). Each virus pool has more than one serotype, within which FMDV strains evolve and circulate (5, 6), giving rise to waves of infection and potential for periodic spread of strains beyond their pools of origin (7–9). Recent examples of FMDV strains that have spread widely are O/ME-SA/Ind-2001 (10) and A/ASIA/G-VII (11).

Prophylactic vaccination is widely used to control FMD where the virus is endemic or where incursions are highly likely (3). Vaccination is also an emergency option in response to incursions in FMD-free countries or upsurges of infection in FMD-endemic countries (12). The emergence and spread of antigenic variants within FMDV serotypes can require multiple vaccine strains, as immunity, whether induced by infection or vaccination, is serotype specific and may be weak or incomplete between antigenically divergent strains (13). The expected level of protection conferred by a vaccine is often measured by vaccine matching, an *in vitro* test which compares the seroreactivity of vaccine antisera to the vaccine strains (homologous reactivity) and the field strains (heterologous reactivity). Vaccination-challenge tests in the target species can also be undertaken to provide empirical data for vaccine performance, but wide-scale use of these *in vivo* approaches is often constrained by cost, time and animal welfare considerations.

Countries that are FMD-free take stringent measures to prevent incursions of FMD and ensure preparedness in the event of an outbreak, including provision of vaccine reserves for implementation of emergency vaccination. These strategic reserves mostly take the form of concentrated FMDV antigens frozen above liquid nitrogen, with a long shelf life, that can be rapidly thawed and formulated as ready-to-use vaccines (14). Europe and North America have established multinational vaccine banks of this type and there may be at least 20 national banks worldwide. Along with rapid formulation into final vaccine product, antigen banks have several technical advantages, such as consistency in production and quality assurance (14, 15). However, the antigens maintained in the bank must be carefully and timely selected to provide protection against the most important viral threats, balancing vaccine availability from manufacturers with the costs of carrying unused antigens. Working with FMD reference laboratories and vaccine producers, bank managers assess recent epidemiological events to determine current and future threats posed by circulating viral strains. The FAO World Reference Laboratory for FMD (WRLFMD) previously provided vaccine antigen bank recommendations on a quarterly basis, in which the most common vaccine strains were classified into high, medium, and low priority [see quarterly reports until December 2017 (WRLFMD)]. However, the criteria for determining into which category an antigen was placed were not clearly defined and these recommendations were based on European vaccine producers and threats to FMD-free European countries that may not have been appropriate for countries in other regions.

In this paper, we describe and apply a novel *Prioritization of Antigen Management with International Surveillance Tool* (PRAGMATIST) to assist vaccine bank managers in selecting which FMDV strains are most important to maintain in their vaccine bank. This tool provides a transparent, evidence-based framework to evaluate available vaccine antigens, that can be adapted according to the region at risk.

Methods

Design of PRAGMATIST

The decision-support tool provides a structured framework to assist vaccine bank managers to prioritize vaccine strains that are candidates for inclusion in an antigen bank. The tool combines three relevant parameters from the perspective of an antigen bank manager, namely (1) the relative likelihood of an FMD incursion from different regions of the world (source areas); (2) the prevalence of circulating FMD viral strains in these source areas (lineage distribution) and (3) the expected protection afforded by different FMD vaccines against these circulating FMD strains (vaccine coverage). The level of protection is based on the antigenic relationships defined by

serological vaccine matching studies (1, 16) which could be complemented by direct evidence of protection in the field where these data are available. The lineage distributions are specific to the source regions, whilst the source area scores and vaccine availability will be specific to the country or region at risk. PRAGMATIST was initially developed and is still currently available in MS-Excel (https://www.eufmd.info/pragmatist). However, to improve accessibility and strengthen science-to-policy linkage (17), the tool has been ported to an easy-to-use interactive dashboard, with the application's scope and interface design crafted with structured input from multiple stakeholder groups, including beta testing of the application. The web-platform (www.openfmd.org/dashboard/PRAGMATIST) was developed in R Shiny (18, 19) by further adding extended functionalities using JavaScript and Cascading Style Sheets (CSS).

Source area score (SAS)

The first step in the tool is to assign source area scores (SAS). The source areas correspond to the geographic extent of each endemic virus pool (4), with an additional area encompassing specific countries in North Africa (Morocco, Algeria, Tunisia and Libya). Long-term maintenance of FMD has not been historically documented in North Africa and therefore this region does not constitute an FMD endemic pool. However, recent introductions of diverse FMDV lineages into this region (O/ME-SA Ind-2001d in 2014–2015 (10), A/AFRICA/G-IV in 2017 (20) and O/EA-3 in 2018 and 2021 (21), pose a distinct threat to FMD-free countries in Europe.

The SAS should be populated by the vaccine bank manager (the user) and will be tailored to address the particular risks of FMD introduction into the country or region covered by the antigen bank. The user allocates 100 points among the potential source areas, giving more points to the areas they consider a higher likelihood of being the source of an incursion. A source area can be allocated zero points if it is not considered important. The tool does not prescribe how the SAS should be defined, but expert elicitation can be used, engaging those knowledgeable about transboundary trade and other risk pathways into the target region.

Lineage distribution score (LDS)

The second step in the tool indicates the lineage distribution score (LDS) which specifies the distribution of specific FMDV lineages circulating within each source area. Viral lineages considered most important for transboundary spread are included in PRAGMATIST.

These virus strains are summarized by serotype|topotype|lineage, and for ease are hereafter referred to as lineages. In some instances, lineages are combined together to simplify the use of the tool, such as: (i) O EA-2, O EA-3, O EA-4 and O WA which are grouped as O EA or O WA; (ii) A Africa G-1 and G-IV grouped as A AFRICA; (iii) Asia 1 Sindh-08 and non-specified Asia 1 lineages grouped as Asia 1; (iv) SAT 1 I(NWZ), SAT 1 II(SEZ), SAT 1 III(WZ), and SAT 1 X grouped as SAT 1; and (v) SAT 2 I, SAT 2 II, SAT 2 III, SAT 2 IV, and SAT 2 VII grouped as SAT 2.

To define the LDS, each source area is allocated 100 points which are divided between the different FMDV lineages circulating in that area. The LDS provides an estimate of how often each lineage would be detected if 100 FMDV-infected animals were randomly selected from a source area in the previous year. The default scores set in the tool are based on data generated through FMD regional surveillance activities. These values are discussed and updated at each annual meeting of the WOAH/FAO Reference Laboratory Network (www.foot-and-mouth.org) and reviewed and reported quarterly by the WRLFMD (41). However, these scores can also be modified by the user when required.

Lineage risk score (LRS)

The lineage risk score combines the SAS and LDS, to give an overall risk score (max possible score = 10,000) for each FMDV lineage. The LRS is calculated according to the formula:

$$LRS = \sum_{source\ area\ 1}^{source\ area\ n} (LDS{*}SAS) \quad (1)$$

Vaccine coverage score (VCS)

The vaccine coverage score (VCS) reflects whether a specific FMD vaccine is likely to provide protection against each of the FMDV lineages. Consequently, a VCS is given for each combination of vaccine and viral lineage included in the tool and is calculated as the proportion of field isolates from each particular lineage that antigenically match the vaccine in question.

$$VCS = \left(\frac{Number\ of\ isolates\ that\ match\ vaccine\ strain}{Number\ of\ isolates\ tested} \right) \quad (2)$$

These data are obtained from routine vaccine matching studies that are undertaken by the WRLFMD, where a match between a vaccine and field strain is defined as a one-way relationship value (r_1 value) of greater than or equal to 0.3, determined by a virus neutralization test using monovalent vaccine-specific antisera (1). The VCS can be adjusted by the user if other information exists about the likelihood that a vaccine provides protection based on efficacy or effectiveness

data from *in-vivo* cross-protection or field studies, respectively. For example, cross-protection vaccine-challenge studies may show that a high potency formulation of a vaccine strain may elicit satisfactory protection to a field strain despite a poor match *in-vitro* (13). Details of known studies where results may influence vaccine coverage scores are shown in the Supplementary Data (Supplementary Data Table 1).

Vaccine score (VS)

Finally, the vaccine score (VS) is calculated according to the formula:

$$VS = \sum_{virus\ strain\ 1}^{virus\ strain\ n} (VCS * LRS) \quad (3)$$

The VS is a final score for each vaccine/lineage combination, and combines the risk posed by specific lineages to a particular region (lineage risk score) with the expected protection conferred by the vaccine (vaccine coverage score). Vaccines with the highest scores will therefore be those that provide protection against the most important FMDV threats in the region targeted by the antigen bank.

Application of PRAGMATIST

As working examples, PRAGMATIST was populated with parameters appropriate for vaccine bank managers from three regions: Europe, North America and Australia where the SAS were obtained using a modified Delphi expert elicitation process (22). A questionnaire was administered to experts who were asked to divide 100 points between the potential source areas, with the most points going to the area(s) that posed the highest risk to the countries serviced by each region's vaccine bank. Results from the first round were summarized and discussed, and then the questionnaire was administered again in a final round. Responses were averaged to obtain the final SAS. For the European vaccine bank perspective, experts were country representatives (one per country) attending the European National Reference Laboratories for FMD Workshop in 2017. For the North America and Australia vaccine bank perspectives, experts were participants at a workshop held at the 2018 EuFMD Open Session (23).

The LDS were assigned by regional experts at the 2020 annual meeting of the WOAH/FAO Reference Laboratory Network. Finally, the VCS were populated through analysis of routine vaccine matching test data performed by the WRLFMD between 2011 and 2021, for vaccines produced by commercial vaccine companies and where reagents (vaccine strains, vaccine antisera and field strains) are available at WRLFMD for this testing.

Sensitivity analysis

An optimisation algorithm was used to identify which sources of uncertainty in the tool's input values have the greatest impact on the prioritization of FMD vaccine antigens. There are several underlying assumptions: (i) when a vaccine is selected it reduces the risk of all matched lineages, (ii) the coverage provided by each vaccine is not additive, such that the risk posed by a lineage is only reduced by the amount equal to the highest coverage of the selected vaccines, and (iii) there is no cross-serotype reactivity.

Uncertainty was considered in all three user inputs (SAS, LDS and VCS). For SASs and LDSs, six levels of user-identified confidence were introduced: (i) "none"—chosen when the user has no confidence in the input values, (ii) "low", (iii) "mid-low", (iv) "mid", (v) "mid-high", and (vi) "high". These categories correlate to the weighting on the variance around the input score, with the input drawn from a truncated normal distribution bound between 0 and 100, where the mean is the user stated input, a standard deviation of 1.5 and the weighting of 7.5, 6, 4.5, 3, and 1.5 or no weighting correlating to user confidence, respectively. All scores were scaled between 0 and 100 as per the tool in the non-stochastic form.

VCS uncertainty is influenced by two main factors. First, from the range of r_1 values obtained in the vaccine matching tests when the same vaccine is matched to different examples of a given field strain (where the uncertainty is influenced by inherent variability of the vaccine matching test and antigenic diversity within each viral lineage), and second, from the number of paired tests performed for each vaccine-field strain combination. Stochasticity was therefore introduced in two steps. Step 1: for each vaccine/lineage combination, a beta distribution was fitted to capture the breadth of r_1 values. From each distribution, N r_1 values were sampled, where N defines the number of vaccine/lineage matching tests in the data. From these simulated values the VCS was calculated as above (equation 2). For Step 2, this VCS was then penalized depending on the number of tests that informed this score. Another draw was made from a beta distribution, this time parameterized based on mean and precision in the form.

$$Simulated\ vaccine\ coverage\ score = \beta\left((\alpha * \tau), (\tau * 1 - \alpha)\right) \quad (4)$$

Where α defines the mean value (i.e., the vaccine coverage score drawn in step 1) and τ the weighting reflecting the number of tests performed. There were seven weightings used: $\tau = 2$ if only one test had been conducted, such that the vaccine coverage score was drawn from a uniform distribution {0,1}. $\tau = 4$ when the number of tests were $\geq$1 and $\leq$10. $\tau = 8$ when the number of tests were $\geq$11 and $\leq$20. $\tau = 16$ when the number of tests were $\geq$21 but $\leq$40. $\tau = 24$ when the number of tests were $\geq$41

TABLE 1 Source area scores obtained through expert elicitation for each region.

Source area	Europe	North America	Australia
Pool 1 [Southeast/ Central/ East Asia]	11	30	70
Pool 2 [South Asia]	8	24	10
Pool 3 [West Eurasia and Middle East]	43	20	5
North Africa	23	10	5
Pool 4 [Eastern Africa]	4	4	2
Pool 5 [West/ Central Africa]	5	4	2
Pool 6 [Southern Africa]	3	4	3
Pool 7 [South America]	3	4	3
Total	100	100	100

and $\leq$60. $\tau = 32$ when the number of tests were $\geq$61 but $\leq$80. Finally, $\tau = 40$, when the number of tests were > 80.

Data analysis

Data analysis was performed using R (version 4.1.2) (19).

Results

Source area score

The SASs obtained from the expert elicitation are shown in Table 1. From the European vaccine bank perspective, the experts considered that Pool 3 posed the highest risk as the source of an incursion of FMDV, followed by North Africa, comprising 43 and 23% of the risk, respectively. For North America, Pool 1 was allocated the highest score (30%), with marginally lower values allocated to Pool 2 (24%) and Pool 3 (20%). For Australia, Pool 1 was ascribed a SAS of 70% which was much higher than any of the other potential source areas.

Lineage distribution score and lineage risk score

The LDSs determined by experts that attended the 2020 WOAH/FAO Reference Laboratory Network meeting, are shown in Table 2. The resulting lineage risk scores are given for each vaccine bank perspective in Figure 1. For Europe, O EA or O WA had the highest LRS, for North America, O ME-SA Ind-2001 had the highest LRS, and for Australia, O SEA Mya-98 and O ME-SA Ind-2001 had the highest LRS.

Vaccine coverage score

A summary of the r_1 values resulting from vaccine matching tests performed at the WRLFMD between 2011 and 2021 is shown in the violin plots in Figures 2–4, along with the number of tests performed and resulting VCSs. These VCSs are displayed in the editable summary in the PRAGMATIST. Further details regarding the number of samples collected per year and region are provided in Supplementary Table 2 and Supplementary Figures 1a-d. Only one sample from South America was obtained for vaccine matching.

The number of vaccine matching tests performed per vaccine/lineage combination ranged from a minimum of 1 and maximum of 97 for serotype O, 1 – 82 for serotype A, 63 tests for Asia 1, 26 for SAT 1, 55-56 for SAT 2, and with only 2 tests for SAT 3. Not all vaccines were tested against all lineages (Figures 2–4). These figures display the range of r_1 values that have been observed for each vaccine/lineage combination.

A VCS of 1.0 was reported for 9 vaccine/lineage combinations suggesting a good antigenic match, however, confidence in these results is poor due to the small sample size ($\leq$ 5). For serotype O, the O/ME-SA/PanAsia-2, O/ME-SA/PanAsia, O/ME-SA/Ind-2001, O EA or WA, and O EURO-SA lineages were generally well matched against the vaccines tested (Figure 2). For the O EURO-SA lineage all r_1 values were above 0.3 (VCS = 1.0), however only one vaccine matching test was performed for this lineage against each of the vaccines: O Campos (BI), O1 Manisa (BI MSD) and O/TUR/5/09 (MSD), and therefore confidence in the VCS is low. Additionally, only a small number of vaccine matching tests were performed for the O-Panasia 2 (BI) vaccine strain. The O CATHAY lineage was the least well matched with any of the vaccines tested.

The performance of the serotype A vaccines against the different lineages was generally poor, however vaccine coverage scores were generally higher against the A/ASIA/SEA-97 lineage (Figure 3). Only the A G-VII (BI) vaccine demonstrated matching against the A/ASIA/G-VII lineage from South Asia, with all r_1 values $> = 0.3$ (VCS = 1.00, sample size = 5). No samples from the A/EURO-SA lineage were obtained for vaccine matching.

The Asia1 Shamir (BI MSD) vaccine and SAT3 ZIM 83 (BI) vaccine were poorly matched to Asia 1 (VCS = 0.24) and SAT 3 (VCS = 0.0) field strains, respectively, with only two vaccine matching tests performed for SAT 3. For SAT 1 and SAT 2, variability was observed for each of the vaccines reflecting the variability in field strains, but with over 50% of isolates tested matching (VCS for SAT1 Rho 78 = 0.62, SAT2 ZIM 83 = 0.56, SAT2 Eritrea 98 = 0.82, Figure 4).

TABLE 2 Lineage distribution scores for each source area.

Serotype |Topotype| Lineage	Pool 1 [Southeast/ Central/ East Asia]	Pool 2 [South Asia]	Pool 3 [West Eurasia and Middle East]	North Africa	Pool 4 [Eastern Africa]	Pool 5 [West/ Central Africa]	Pool 6 [Southern Africa]	Pool 7 [South America]
O ME-SA PanAsia-2	-	-	35	-	-	-	-	-
O ME-SA PanAsia	10	-	-	-	-	-	-	-
O SEA Mya-98	33	-	-	-	-	-	-	-
O ME-SA Ind2001	20	80	7	10	-	-	-	-
O EA or O WA	-	-	3	55	55	70	-	-
O EURO-SA	-	-	-	-	-	-	-	80
O CATHAY	10.5	-	-	-	-	-	-	-
A ASIA Sea-97	26	-	-	-	-	-	-	-
A ASIA Iran-05	-	-	27	-	-	-	-	-
A ASIA G-VII	-	16	15	-	-	-	-	-
A AFRICA	-	-	-	25	22	15	-	-
A EURO-SA	-	-	-	-	-	-	-	20
Asia 1	0.5	4	12.5	-	-	-	-	-
SAT 1	-	-	-	-	8	5	27	-
SAT 2	-	-	0.5	10	14	10	57	-
SAT 3	-	-	-	-	1	-	16	-
Total	100	100	100	100	100	100	100	100

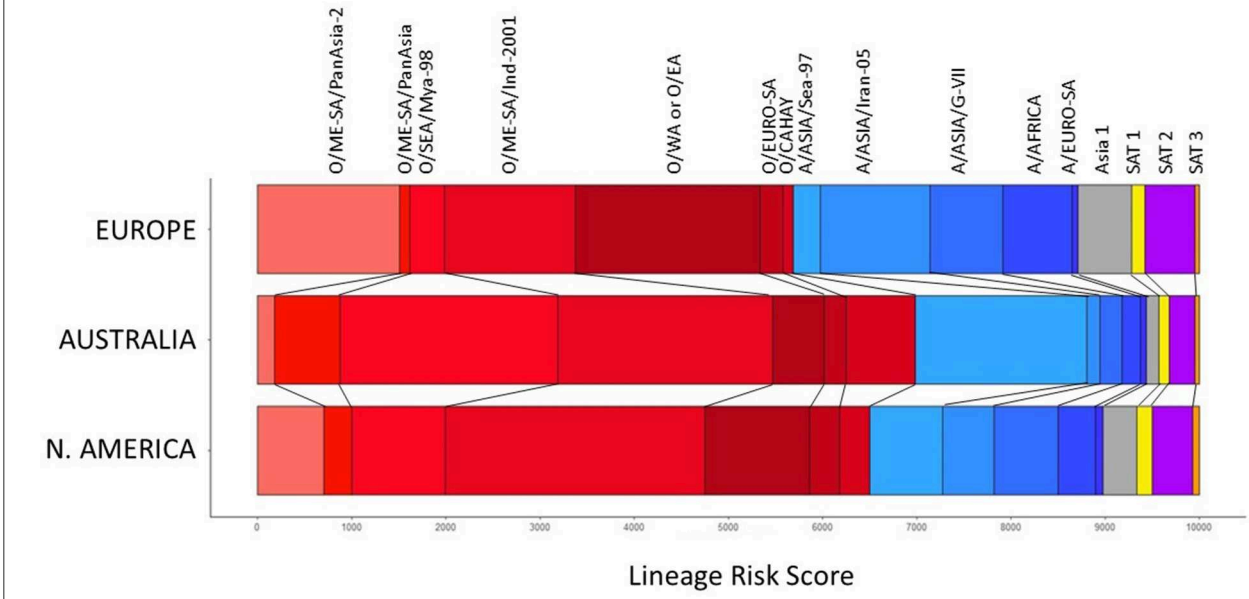

FIGURE 1
Total lineage risk scores per serotype/topotype/lineage for each vaccine bank perspective.

Vaccine scores

Figure 5 summarizes the vaccine scores for each vaccine/lineage combination, for each of the three vaccine bank perspectives. The vaccine scores can be utilized to assist in vaccine selection for each vaccine bank. For example, for serotype O, the O/TUR/5/09 (MSD) vaccine had the highest vaccine score for all three vaccine bank perspectives (Europe,

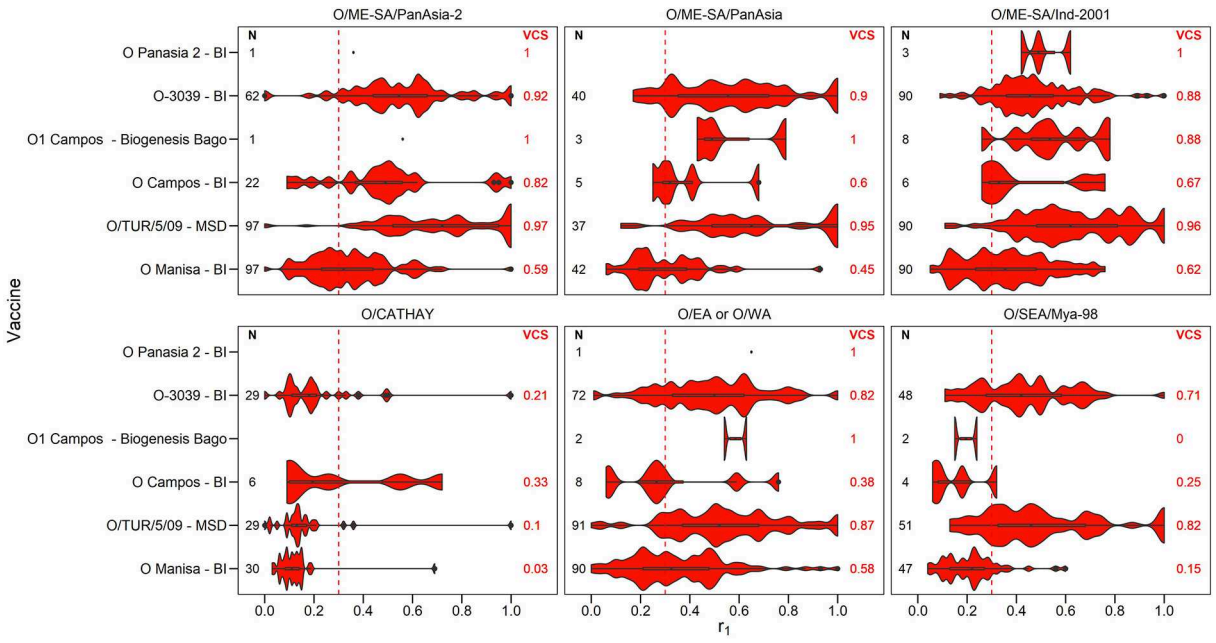

FIGURE 2
Violin plots showing results of vaccine matching tests performed at the WRLFMD between 2011 and 2021, for each vaccine/lineage combination for serotype O. Resulting vaccine coverage scores (VCS) are labeled on the right in red, and the number of tests performed are labeled on the left in black. The red dashed line shows the r_1 cut-off of 0.3 indicative of an effective vaccine match. Values for lineage EURO-SA are not shown as there was only one test performed for each of the vaccines O Campos (BI), O1 Manisa (BI MSD) and O/TUR/5/09 (MSD), with all r_1 values being above 0.3.

North America, and Australia), although the lineage-specific components differ according to the LRSs for each of the three antigen banks. Similar data highlighting the highest priority vaccine antigens and their coverage against the risks posed by different viral lineages are also presented in the figure for other FMD serotypes.

Uncertainty

For the purpose of illustration, the impact of uncertainty in the input values was demonstrated using the European values for the SAS and "mid" levels of uncertainty in the input parameters. Results indicate that identifying which vaccines cover the most risk is largely robust to uncertainty in the input values (Figure 6). Uncertainty in the vaccine coverage score had the greatest impact on the percentage of the total risk covered. This was particularly obvious for vaccines such as O-3039 (BI) that cover a large proportion of the risk. In the simulation of the vaccine coverage score, each vaccine/lineage combination had a wide range of empirical r_1 values underlying the distribution from which the score was drawn from, and then the simulated score was penalized depending on the number of tests. Consequently, uncertainty was compounded for vaccines that protect against multiple lineages. This was also true when considering uncertainty in all three input parameters at the same time [Figure 6 (all)], where the inclusion of uncertainty reduced the estimated percentage risk covered, notably for vaccines that covered a substantial portion of risk. When vaccines do not cover substantial proportions of the risk, the variation in input data for the VCS has little effect. All levels of uncertainty for all regions are shown in Supplementary Data Files 2a–b, 3a–c. In summary, vaccine choice was more tolerant to uncertainty in the SAS and LDS, rather than the VCS.

Discussion

PRAGMATIST provides a transparent and accessible, evidence-based decision support tool to assist FMD vaccine bank managers to determine which vaccine antigens are highest priority for storage. This is achieved through combining the scores for three key criteria: the level of threat posed by different endemic regions (SAS), the prevalence of different FMD viral lineages in those regions (LDS), and the effectiveness of vaccines against those viral lineages, based on *in vitro* vaccine matching testing (VCS). Combining these scores enables vaccine bank managers to select those vaccines that should be most effective against the current threats for that region, based on the available evidence (Table 3).

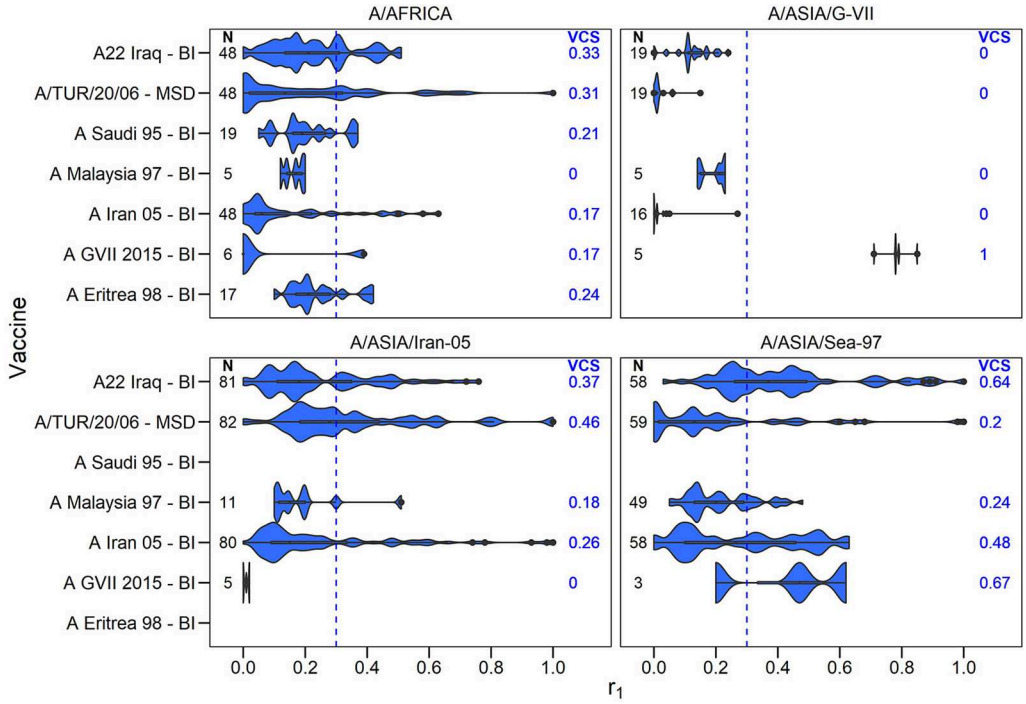

FIGURE 3
Violin plots showing results of vaccine matching tests performed at the WRLFMD between 2011 and 2021, for each vaccine/lineage combination for serotype A. Resulting vaccine coverage scores are labeled on the right in blue, and the number of tests performed are labeled on the left in black. Blue dashed line shows the cut off of 0.3 r_1 indicative of an effective vaccine match.

PRAGMATIST is a simple-to-use tool which is provided with pre-populated values for LDS and VCS, based on expert opinion from the WOAH/FAO FMD Reference Laboratory Network and vaccine matching data from the WRLFMD, respectively. However, the user has complete control to adjust these inputs to accommodate local knowledge and up-to-date epidemiological information.

The outputs from the tool are tailored for different geographical perspectives by the user who inputs a SAS that addresses the likelihood an FMD incursion will originate from different geographical regions. These threats might vary according to the level and complexity of inter-regional connectivity (such as those epidemiological factors associated with geographic proximity, animal movements, plus legal and illegal trade of livestock and animal products, cultural and religious practices), the weight of infection in the source area (e.g. susceptible population sizes, incidence of infection) and the effectiveness of cross-border risk mitigation measures (24, 25). These parameters are difficult to quantify precisely due to their dynamic nature, the multiplicity of determinants and circumstances, the chance nature of transmission opportunities and the many gaps in required information. Therefore, for PRAGMATIST, assessment based on expert knowledge has been used to estimate the SAS, which was deemed appropriate given the expert elicitation process used, the participants involved, and that uncertainty in the SAS had a smaller effect on the outcome compared to the LDS and VCS. Several tools are available to perform more structured, qualitative or quantitative assessments of exotic animal disease incursion risk (26–29). Notably, Condoleo *et al* (30) used the progress of countries along the FMD Progressive Control Pathway (PCP-FMD) (31) to rank the FMD hazard that they pose. Additionally, The European Commission for the Control of Foot and Mouth Disease's (EuFMD) risk monitoring tool (32) combines the disease status, transmission pathways and inter-country connections to provide a rapid assessment of which countries pose the greatest incursion risk for FMD and similar transboundary animal (FAST) diseases. In the future, these tools could inform or link with PRAGMATIST to provide improved justification for SAS values (Table 3).

The LDS requires information on the relative prevalence of serotypes and viral lineages in each viral pool. Knowledge of this is incomplete, due to under-reporting and continuous viral evolution leading to the emergence of new strains. Like other highly contagious diseases, FMD incidence is often cyclical, associated with opportunities for virus spread and the waxing and waning of population immunity (8, 9). Additionally, it is likely that there may be inherent characteristics of particular viral lineages that facilitate their

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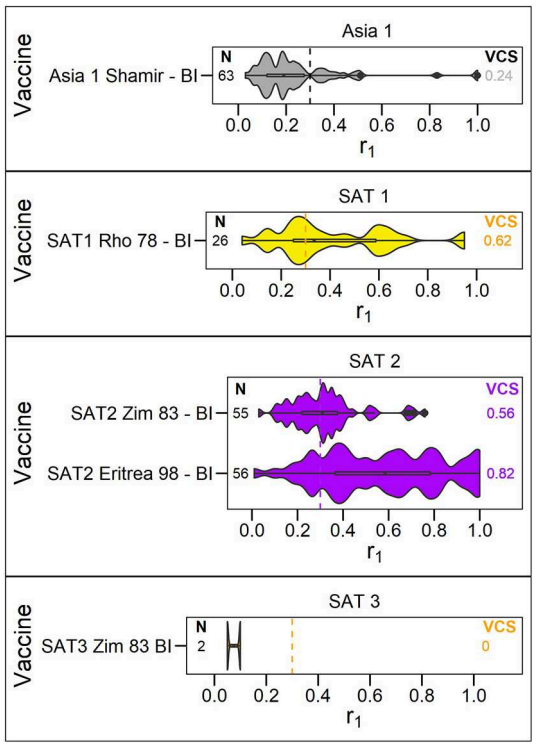

FIGURE 4
Violin plots showing results of vaccine matching tests performed at the WRLFMD between 2011 and 2021, for each vaccine/lineage combination for serotypes Asia 1, and SAT 1–3. Resulting vaccine coverage scores are labeled on the right, and the number of tests performed are labeled on the left in black. Gray dashed line shows the cut off of 0.3 r_1 indicative of an effective vaccine match.

transmissibility. These factors are not considered in this tool, but an ability to transfer between geographical "virus pools" could be a warning sign that a strain poses a greater threat of incursion. For simplicity, PRAGMATIST currently combines the risks associated with certain FMD viral lineages together in the LDS for example those from East and West Africa. Although the African endemic pools provide a low contribution to the SASs in the worked examples in the paper, future development of the tool will inevitably consider the antigenic diversity that exists across the African FMDV serotypes and the suitability of vaccines to provide protection against these lineages.

The lineage risk score provides an overall score taking into consideration the relative prevalence of each viral lineage in each virus pool, and the risk of an incursion of that lineage. For all three vaccine bank perspectives the risk from Asia 1, SAT 1, SAT 2 and SAT 3 was less than $1/5^{th}$ of the total lineage risk, with the majority coming from serotype A and O lineages. Indeed, these two serotypes are the most prevalent, with the widest known geographical distribution. Individual lineages scored differently between the vaccine bank perspectives, as expected, due to the threat of circulating viral lineages in each region. For example, O/SEA/Mya-98 and A/Asia/SEA-97 scored highly from the Australian perspective, reflecting their prominence in pool 1 which is considered the most highly connected source of risk for FMD for Australia, while the score was lower from the European perspective. From the North American perspective, O/ME-SA/Ind-2001 had the largest lineage risk score, reflecting its circulation in pools 1, 2 and 3 as well as in North Africa, all of which are considered important source areas for North America.

The default VCSs included in the tool are based on routine *in vitro* vaccine matching tests performed by the WRLFMD. In calculating these VCSs, previously unpublished vaccine matching data from the WRLFMD from tests performed between 2011 and 21 has been collated for the first time, comprising 2,150 individual data points for field strain/vaccine pairs (1207 for serotype O, 741 for serotype A, 63 for serotype Asia 1, 26 for serotype SAT, 111 for serotype SAT 2 and 2 for serotype SAT 3, respectively). These vaccine matching results help to select antigenically appropriate vaccine strains, and the data presented in this report highlight where individual vaccines are consistently well-matched against field isolates. These data also reveal where the available vaccines indicate the potential for poor protection, where most of the r_1 values are below 0.3, such as for the O/CATHAY topotype. Indeed, these data can identify where there may be gaps in antigenic vaccine coverage, for example, poor matching data for the emerging A/ASIA/G-VII lineage led to the recent development of new specific vaccine strains to cover the spread of this lineage in the Middle East (11, 33, 34). The data reveal that vaccine matching test results can vary substantially for different isolates within the same lineage. It is uncertain the extent to which this variability is attributable to the low repeatability of vaccine matching tests (35) vs. inherent antigenic differences between the isolates themselves. Analysis for temporal trends in the variability of vaccine matching results might reveal evidence for change accumulating through evolution. In the current version of PRAGMATIST, as mentioned above, certain FMD viral topotypes/lineages are grouped together, such as the O/EA/1-4 and O/WA topotypes, and SAT 2 topotypes, with the resulting VCS based on this grouping. Indeed, grouping topotypes/lineages differently, or not at all, would result in differing VCSs, however, the number of vaccine matching tests performed for each grouping would decrease, potentially reducing confidence in these scores.

PRAGMATIST users should apply caution if only a few matching tests have been performed, which was the case for several lineage-vaccine combinations in our study. Ideally, for a given serotype, all available vaccines should be tested against all circulating lineages, using many original isolates. However, availability of field isolates and vaccine strains at the WRLFMD limits the amount of possible testing combinations. For some field strains, isolates from

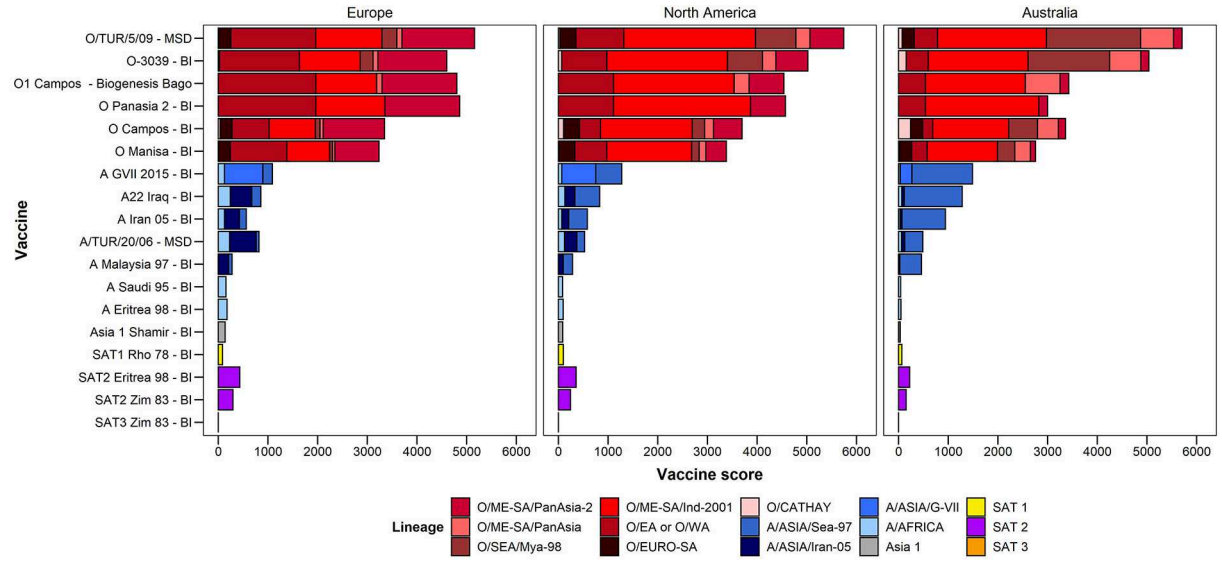

FIGURE 5
Vaccine scores for each vaccine–lineage combination.

multiple sources are available, whilst for others, only a single isolate may have been submitted for testing, despite efforts made to facilitate submission of samples from under-represented regions.

The VCS can be fully edited by the user to accommodate additional vaccine matching data generated locally for vaccine strains not already included in the tool. It is important to realize that antigenic match is not the only consideration regarding vaccination performance. Therefore, additional measures of vaccine performance could be considered, such as data from *in vivo* experiments or field vaccine evaluation studies, which are influenced by other important variables such as vaccine potency, vaccination regime and/or the weight of the infectious challenge (36, 37). The Supplementary Data displays results of published experimental *in vivo* studies that could be used to modify the VCS. Additionally, where vaccine matching data are not available, it is possible that alternative methods of measuring antigenic differences relevant to protection could be utilized, such as antigenic cartography (38) or sequence-based approaches (39).

PRAGMATIST relies on inputs provided by the user, the WOAH/FAO FMD Reference Laboratory Network and the WRLFMD for the SAS, LDS and VCS, respectively. The impact of uncertainty in these estimations (for SAS and LDS) or test variability (VCS) was assessed using sensitivity analyses. The introduction of uncertainty in the VCS resulted in a higher likelihood of change to the final vaccine scores, and therefore the final ranking of vaccine priority, in contrast to SAS and LDS which were more tolerant to a range of plausible input values without affecting the prioritization of the vaccine antigens. These findings demonstrated the importance of accommodating variability in vaccine matching and uncertainty where gaps in data exist into PRAGMATIST and motivate further effort to increase vaccine matching testing or access to data where possible, to improve confidence in these results, and to define the true profiles (distribution shape) for the VCS.

Ultimately, the vaccine score combines the LRS with the VCS, such that the highest scoring vaccines are those with the best antigenic match to the most prevalent lineages circulating in the highest risk source regions. However, when using the tool to assist with vaccine selection, the vaccine bank manager should also consider the diversity (breadth) of protection afforded by different vaccines and the need to choose a portfolio of complementary rather than overly redundant vaccine strains. Thus, if a vaccine provides a reliable match against a particular lineage (VCS close to 1 with many vaccine matching tests performed), adding additional vaccines to the vaccine bank will not provide additional protection against the risk from that specific lineage. For example, storage of O-3039 (BI) in addition to O/TUR/5/09 (MSD) would not provide additional protection against the risk from O/ME-SA/PanAsia-2, as these vaccines both have a high VCS, and a high number of vaccine matching tests were performed for these combinations. Therefore, it is not recommended to simply select the highest-scoring vaccines as these may provide redundant protection. However, the need for multiple vaccines is more obvious for serotype A due to the greater antigenic diversity within this serotype (33, 40). It should be noted that a low score for some vaccines may reflect a lack of vaccine matching

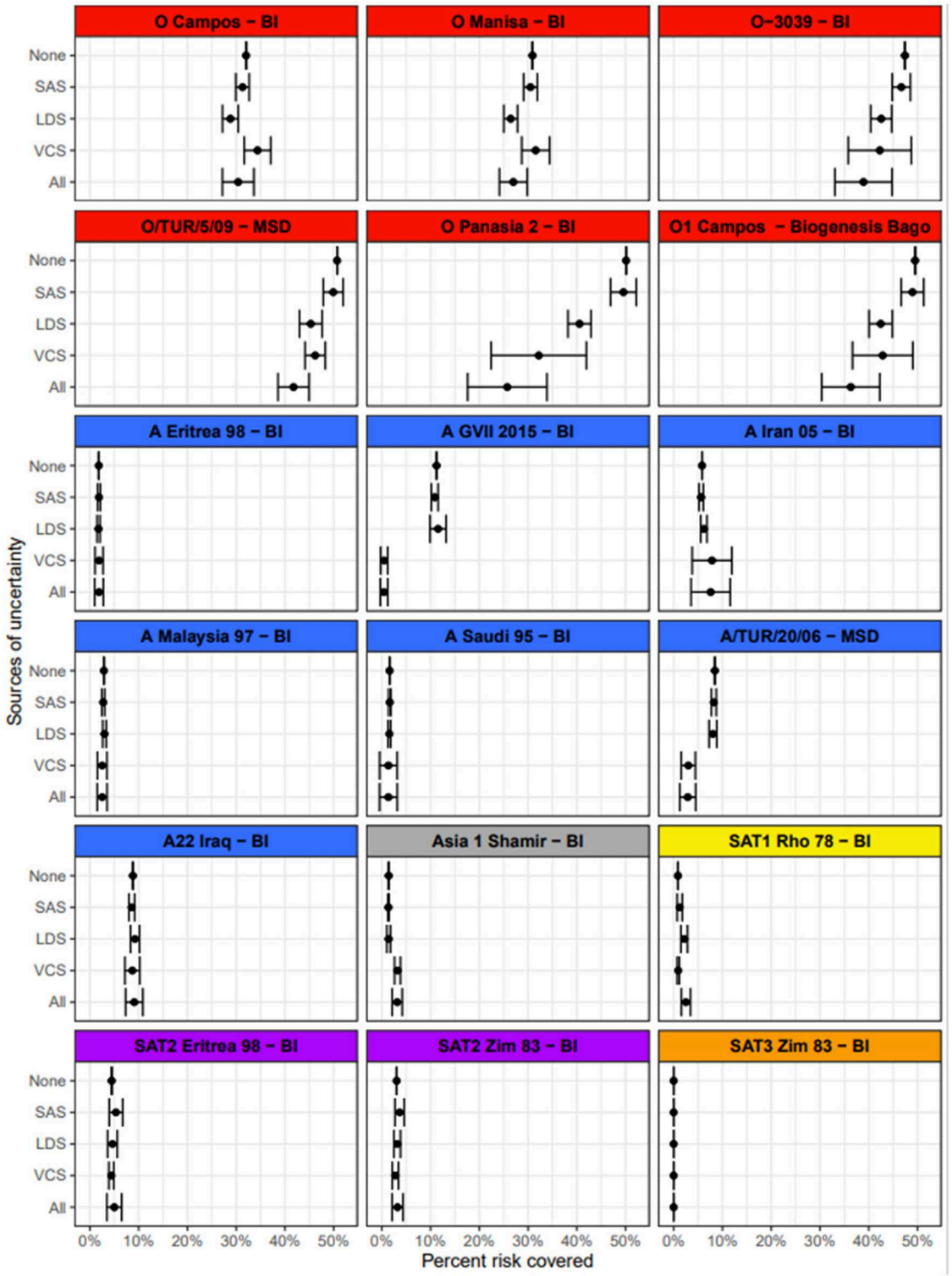

FIGURE 6
The mean and standard deviation for the percentage risk coverage out of the total risk needing to be covered, for each vaccine. The sources of variance are broken down; none indicates how the tool would work as it is, in the absence of any stochasticity. SAS is uncertainty in the source area scores (set to "mid" here), with no variation introduced from other inputs. LDS is uncertainty in the lineage distribution scores (set to "mid" here), with no variation introduced from other inputs. VCS is variation in the vaccine coverage scores based on the breadth of r_1 values from vaccine matching tests and the number of tests performed, with no variation introduced from other inputs. All indicates uncertainty in all parameters, using a "mid" level of uncertainty in the LDS and SAS.

TABLE 3 Using PRAGMATIST.

PRAGMATIST parameter	How to complete each parameter of PRAGMATIST	Considerations/limitations	Potential modifications that can be made by the user
Source area score (SAS)	The user allocates 100 points among the different FMD endemic source areas according to the risk of FMD introduction into the target country/region. This can be informed by expert elicitation.	Expert opinion may differ depending on their knowledge of relevant factors, such as transboundary trade, risk pathways and farm management practices. This parameter is difficult to quantify accurately, due to the number of determinants, the ever-changing situation and gaps in the information required.	Source areas could be tailored to accommodate a different spectrum of countries. Specific scores could be informed by local knowledge or qualitative/ quantitative risk-assessment tools.
Lineage distribution score (LDS)	Each source area is allocated 100 points which are divided between the different FMDV lineages circulating in that area (i.e., relative frequency of these lineages if 100 FMD infected animals were to be randomly sampled). Default scores are based on data generated through FMD regional surveillance activities, updated at each annual WOAH/FAO Reference Laboratory Network meeting (www.foot-and-mouth.org).	Up to date knowledge of circulating viral lineages in each source area is required. Continued viral evolution and emergence of new strains with novel antigenic phenotypes. A lack of disease reporting in some areas may mean that some viral lineages are under-reported. Detailed molecular epidemiological data may not be made widely available/communicated. The grouping of viral lineages as presented (e.g., grouping O EA & O WA) may not represent the diversity of FMDV lineages present in an important source area (for example, sparse surveillance of some of the African endemic pools currently constrains the level granularity that can be achieved).	Expert elicitation by other methods. Grouping of viral lineages can be separated/changed when new data becomes available.
Vaccine coverage score (VCS)	The vaccine coverage score is calculated as the proportion of field isolates from each lineage that antigenically match the vaccine in question (r_1 value of ≥ 0.3). Default scores are based on routine vaccine matching studies undertaken by the WRLFMD.	The r_1 values may lack precision due to incomplete repeatability and reproducibility of neutralization tests. Vaccine matching data does not provide a guarantee that protection will be afforded against a particular lineage, as various factors may affect vaccine efficacy. Not all vaccines are tested against all lineages, and the number of vaccine matching tests performed for some vaccine/lineage combinations may be low. Uncertainty in the vaccine coverage score has the greatest impact on the percentage of the total risk covered and is more obvious for vaccines that cover more risk. Vaccine matching results can vary for different isolates within the same lineage. The grouping of viral lineages as presented may not be appropriate (for example, grouping O EA and O WA together).	Include vaccine matching data from alternative laboratories. The score may be adjusted based on additional information: • Inclusion of vaccine matching data for additional vaccine strains (if local data are available), • Vaccine efficacy data from *in-vivo* cross-protection vaccine-challenge experiments, • Data from field vaccine evaluation studies, • Alternative indicators of protection from e.g., sequence-based approaches, • Vaccine batch-specific data, • Data derived from studies of the performance of polyvalent vaccines. Sub-divide vaccine matching data by lineage geographically or chronologically.
Vaccine score	The vaccine score combines the lineage risk score with the vaccine coverage score. Vaccines with the highest scores will be those that provide protection against the most important FMDV threats.	Choosing only vaccines with the highest scores may provide redundant protection. A low score may indicate a lack of vaccine matching data, rather than a lack of protection.	Sharing arrangements between different vaccine banks may allow for synergistic vaccine selection based on complementary choices.

testing rather than a lack of protection. For example, the O Panasia 2 (BI) vaccine only had vaccine matching results available for 3 of the 7 serotype O lineages (and only testing a maximum of 3 isolates), and thus any possible protection that may exist against the 4 untested lineages was not included in its final score. Other considerations that are not considered in PRAGMATIST but are likely to be important for vaccine antigen choice include the potency at which the vaccine can/will be provided, contractual arrangements with specific vaccine manufacturers, existing stock and expiration dates, and financial considerations. Finally, as described, the PRAGMATIST output is intended to inform vaccine selection given the current viral and incursion risks. However, the user could also parameterise the tool considering anticipated future risks, perhaps eventually applying bioinformatics to predict novel antigenic phenotypes of emerging strains and the protection conferred by current vaccine antigens.

In conclusion, vaccine bank holdings may be crucial to enable a swift and effective response to an incursion of FMD into a free country. Considering the complexity of different FMD vaccine antigens that are produced by different suppliers, PRAGMATIST was developed to support vaccine bank managers in this critical decision-making process, which is likely to have different outcomes depending on the geographical location. Due to the ever-changing dynamics of FMD virus circulation in endemic areas the tool should be updated on a regular basis to reflect the current situation and best data available. The focus of this paper was antigen bank management, and therefore the worked examples included vaccines from vaccine manufacturers that offer well-established antigen bank services. However, by making PRAGMATIST freely accessible in a dedicated, code-based, and highly customisable web-based dashboard, the tool is able to evolve and adapt to user needs, providing, for example, an option to add circulating strains as they are detected, or vaccines as they are developed, or to accommodate specific user's interests. Further, it is foreseen that a similar framework could incorporate heterologous serological data collected testing antisera to specific vaccine batches against regional virus threats. This would take account of both antigenic match and batch-specific vaccine potency in selecting FMD vaccines for preventative and emergency vaccination strategies in FMD endemic countries. Further efforts are also needed to increase the pool of useful matching data by closing surveillance gaps, sharing of material and inter-laboratory standardization of testing.

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 authors.

Author contributions

MM, DK, AL, and MH designed the tool and/or the study. AL, MM, KP, and MH compiled the raw data. BA, JP, DH, and JC performed the data analysis for the manuscript and prepared the figures. AD, UM, and PM co-designed and developed the R Shiny interactive dashboard. BA, AL, MM, DK, AD, DP, KS, FR, JC, and JP were involved in preparation of the manuscript. All authors read, contributed, and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships

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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.2022.1029075/full#supplementary-material

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EDITED BY
Wilna Vosloo,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

REVIEWED BY
Suresh H. Basagoudanavar,
Indian Veterinary Research Institute (IVRI), India
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International Centre for Foot and Mouth Disease (ICAR), India
Eva Perez,
The Pirbright Institute, United Kingdom

*CORRESPONDENCE
Michiel M. Harmsen
✉ michiel.harmsen@wur.nl

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Mapping of foot-and-mouth disease virus antigenic sites recognized by single-domain antibodies reveals different 146S particle specific sites and particle flexibility

Michiel M. Harmsen[1]*, Haozhou Li[2,3], Shiqi Sun[3], Wim H. M. van der Poel[1,2] and Aldo Dekker[1]

[1]Wageningen Bioveterinary Research, Wageningen University & Research, Lelystad, Netherlands, [2]Laboratory of Virology, Wageningen University and Research, Wageningen, Netherlands, [3]State Key Laboratory of Veterinary Etiological Biology, National Foot and Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China

Vaccination with intact (146S) foot-and-mouth disease virus (FMDV) particles is used to control FMD. However, 146S particles easily dissociate into stable pentameric 12S particles which are less immunogenic. We earlier isolated several single-domain antibody fragments (VHHs) that specifically bind either 146S or 12S particles. These particle-specific VHHs are excellent tools for vaccine quality control. In this study we mapped the antigenic sites recognized by these VHHs by competition ELISAs, virus neutralization, and trypsin sensitivity of epitopes. We included two previously described monoclonal antibodies (mAbs) that are either 12S specific (mAb 13A6) or 146S specific (mAb 9). Although both are 12S specific, the VHH M3F and mAb 13A6 were found to bind independent antigenic sites. M3F recognized a non-neutralizing and trypsin insensitive site whereas mAb 13A6 recognized the trypsin sensitive VP2 N-terminus. The Asia1 146S-specific site was trypsin sensitive, neutralizing and also recognized by the VHH M8F, suggesting it involves the VP1 GH-loop. The type A 146S-specific VHHs recognized two independent antigenic sites that are both also neutralizing but trypsin insensitive. The major site was further mapped by cross-linking mass spectrometry (XL-MS) of two broadly strain reactive 146S-specific VHHs complexed to FMDV. The epitopes were located close to the 2-fold and 3-fold symmetry axes of the icosahedral virus 3D structure, mainly on VP2 and VP3, overlapping the earlier identified mAb 9 site. Since the epitopes were located on a single 12S pentamer, the 146S specificity cannot be explained by the epitope being split due to 12S pentamer dissociation. In an earlier study the cryo-EM structure of the 146S-specific VHH M170 complexed to type O FMDV was resolved. The 146S specificity was reported to be caused by an altered conformation of this epitope in 12S and 146S particles. This mechanism probably also explains the 146S-specific binding by the two type A VHHs mapped by XL-MS since their epitopes overlapped with the epitope recognized by M170. Surprisingly,

residues internal in the 146S quaternary structure were also cross-linked to VHH. This probably reflects particle flexibility in solution. Molecular studies of virus-antibody interactions help to further optimize vaccines and improve their quality control.

KEYWORDS

neutralizing antibody, epitope, XL-MS, nanobody, VHH, ELISA, foot-and-mouth disease virus (FMDV)

Introduction

Foot-and-mouth disease (FMD) affects cloven-hoofed animals, causing vesicular lesions at the feet, mouth and udders of lactating animals. The direct economic losses are high, due to loss of milk-production, growth and draft power. Indirect costs due to loss in trade and other restrictions also impact countries where the disease occurs (1). The causative agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* within the *Picornaviridae* family. FMD is mostly controlled by vaccination. The 7 serotypes of FMDV (O, A, C, Asia1, SAT1, SAT2, and SAT3) by definition lack cross-protection, but within serotypes cross-protection can also be limited. FMD vaccine is mainly produced as inactivated authentic virus capsids adjuvanted in an oil emulsion. However, many novel vaccines are being developed based on virus-like particles (VLPs) that lack the RNA genome, similar to natural empty capsids that sediment in sucrose gradients at 75S (2, 3). For an adequate immune response it is essential that the capsids represent intact (146S) virions. When capsids are heated or kept at a low pH, they disintegrate into stable 12S pentamers that have strongly reduced immunogenicity (4, 5). A monoclonal antibody (mAb 9) was earlier shown to be suitable for FMD vaccine quality control due to its specificity for 146S particles in ELISA (6). This mAb 9 showed high specificity for strain A10/HOL/1/42. We later isolated single-domain antibodies (VHHs) that recognize 146S or 12S particles which can be used in vaccine quality control. The 146S particle specific VHHs M170F and M332F are strictly serotype specific and recognize particular serotype O or Asia1 FMDV strains, respectively, while the 12S particle specific VHH M3F is broadly reactive to many FMDV strains of serotypes O, A, C, and Asia1, although it did not bind SAT2 (5, 7). We have recently isolated 10 FMDV serotype A specific VHHs that consistently showed high specificity for 146S particles (8). Two of these VHHs, M691F and M702F, demonstrated remarkable broad strain specificity when compared to other 146S-specific VHHs. Remarkably, M691F did not recognize 75S particles or VLPs while M702F did bind such empty capsids. Thus, M691F was strictly 146S specific while M702F was specific for both full and empty intact capsids. Since the binding to empty capsids is often unknown we refer to both such specificities as 146S specific in this study. Thus, 146S specificity implies inefficient binding of 12S particles as compared to 146S. Here we study the epitope specificity of the serotype A and Asia1 146S-specific VHHs.

The icosahedral FMDV capsid comprises 60 copies of four capsid proteins, viral protein (VP)1 to VP4. One copy of each VP associates into a protomer, five protomers associate to a pentameric (12S) structure arranged around a 5-fold symmetry axis, and 12 pentamers form the full capsid. The capsid outer surface is formed by VP1, VP2 and VP3 while VP4 is located internally. Upon capsid dissociation VP4 dissociates from 12S particles. The association of 12S pentamers in a 146S particle results in additional 2-fold and 3-fold symmetry axes. FMDV targets host cell receptors, including integrins and heparan sulfate. Protective antibodies can block such virus-receptor interaction to neutralize the virus but other mechanisms also exist (9, 10). Based on sequence analysis of mAb resistant (MAR) viruses, five neutralizing sites have been described for serotype O. Site 1, located at the GH loop and carboxy terminus of VP1, includes VP1–138, 144, 148, 154, and 208. The critical residues of site 2 are VP2–70, 71, 72, 73, 75, 77, 78, 131, and 188. The residues in site 3 include VP1–43, 44, and 45 at the BC loop of VP1 near the 5-fold axis. Site 4 is located at VP3 (VP3–56 and 58), and site 5 has critical residue 149 at the VP1 GH-loop (11–16). These sites identified in serotype O are mostly similar in serotypes A, C (9, 17–21) and Asia1 (22). Antibody competition experiments confirmed such grouping into 5 sites (9), although sites 2 and 4 were found to interact (15). The site recognized by 146S-specific mAb 9 was critically dependent on mutation of VP3-70 although VP3-139 could also be involved (21). Site 1 is a linear epitope located on the VP1 GH-loop, which contains the RGD motif that interacts with integrins (23), whereas all further sites are conformational. Antibody binding to site 1 is sensitive to trypsin treatment of the virions, which is known to cleave the GH loop (23). In addition to these neutralizing sites, mAbs that bind to non-neutralizing sites were identified, including mAb 13A6, which are broadly reactive against FMDV strains from all 7 serotypes and binds a linear peptide representing the N-terminal 15 amino acids of VP2 that is sensitive to trypsin treatment (24, 25).

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TABLE 1 FMDV serotype, strain, and particle specificity of previously-isolated VHHs used.

VHH[a]	FMDV serotype specificity	Binding in ELISA to FMDV strains[b]			FMDV particle specificity[c]	Serotype O antigenic site[d]	References
		A/TUR	A24Cru	Asia1 Shamir			
M8F	O, A, Asia1, C	Y	Y	Y	12S and 146S	I	(26)
M3F	O, A, Asia1, C	Y	Y	Y	12S	II	(26)
M23F	O	N	N	N	12S and 146S	III	(26)
M220F	O, A, Asia1, C	Y	Y	Y	12S and 146S	IV	(26)
M663F	O, A, Asia1, C	Y	Y	Y	12S	ND[e]	(8)
M680F	O, A, Asia1, C	Y	Y	Y	12S	ND	(8)
M665F	O, A, Asia1	Y	Y	Y	12S	ND	(8)
M684F	O, A, Asia1, C	Y	Y	Y	12S	ND	(8)
M675F	A	Y	Y	N	12S and 146S	ND	(8)
M643F	A	Y	N	N	(12S and) 146S	ND	(8)
M652F	A, C	Y	Y	N	(12S and) 146S	ND	(8)
M659F	A	Y	N	N	146S	ND	(8)
M702F	A	Y	Y	N	146S	ND	(8)
M691F	A	Y	Y	N	146S	ND	(8)
M703F	A	Y	Y	N	146S	ND	(8)
M669F	A	Y	N	N	146S	ND	(8)
M676F	A	Y	N	N	146S	ND	(8)
M677F	A	Y	N	N	146S	ND	(8)
M678F	A	Y	N	N	146S	ND	(8)
M686F	A	Y	N	N	146S	ND	(8)
M688F	A	Y	N	N	146S	ND	(8)
M661F	A, Asia1	Y	Y	Y	(12S and) 146S	ND	(8)
M651F	A	Y	Y	N	(12S and) 146S	ND	(8)
M679F	A	Y	N	N	12S and 146S	ND	(8)
M326F	A	N	Y	N	12S and 146S	ND	(8)
M655F	A	N	Y	N	12S and 146S	ND	(8)
M662F	A	N	Y	N	12S and 146S	ND	(8)
M332F	Asia1	N	N	Y	146S	ND	(7, 8)
M658F	Asia1	N	N	Y	146S	ND	(8)
M685F	Asia1	N	N	Y	12S and 146S	ND	(8)
M98F	Asia1	N	N	Y	12S and 146S	ND	(7, 8)

[a]The 14 VHHs of the 7 CDR3 groups comprising the 12 VHHs that bind specifically to 146S of serotype A or Asia 1 strains are color-coded by their CDR3 group, as done earlier (8). Thus, VHHs with the same color belong to the same CDR3 group while VHHs in black all belong to different CDR3 groups.

[b]The A450 values in ELISA obtained earlier (8) were used to determine binding to the indicated FMDV strains, taking A450 = 0.5 as cutoff for binding (Y) or no binding (N).

[c]FMDV particle specificity was determined earlier (8). An EC ratio in ELISA that was > 10 times higher using 146S as compared to 12S was considered 146S-specific binding. Some VHHs showed 146S specificity only using particular strains and are therefore indicated as (12S and) 146S.

[d]Sites I to IV as initially identified in serotype O FMDV (26).

[e]ND, not determined.

A set of VHHs binding to serotype O, including VHHs M3F, M8F, M23F, and M220F were earlier mapped into four separate epitope bins indicated by roman numerals I to IV (26). Most of these four sites could not be linked to the earlier described sites 1 to 5 identified by MAR mutant analysis, although M8F binds a linear epitope on the VP1 GH-loop, similar to site 1 binding mAbs. Site III is specific for serotype O whereas sites I, II and IV are present on other serotypes as well (Table 1). M170F competed with VHHs mapped to sites I and III. The epitopes recognized by M8 and M170 were recently determined based on the cryogenic electron microscopy (cryo-EM) structure of these VHHs complexed to FMDV (10). We refer to these VHHs without the suffix F since they were produced in Escherichia coli without the long hinge region, which is present in our yeast-produced VHHs (27). M8 was found to bind predominantly the VP1 GH-loop, in addition to some VP3 residues, while M170 bound mainly to a region on VP3 close to the 3-fold axis. It was proposed that the 146S-specific binding of M170 relies on binding to VP3 residues which assume a different structure in 12S particles as compared to 146S particles, most importantly D71 and V73 in the BC loop and E131 and K134 in the EF loop. Alternatively, it was suggested that 146S specificity could also rely on antibody binding to an epitope that is present on two adjacent pentamers, on both sides of the 2-fold axis, that is separated into two halves upon dissociation into 12S particles (6), although firm proof for this hypothesis is lacking.

In this study we used different approaches to map the antigenic sites recognized by especially the serotype A 146S-specific VHHs earlier isolated, but also the 12S-specific VHH M3F and Asia1 146S-specific VHHs M332F and M658F. These VHHs were analyzed in competition ELISAs, virus neutralization tests and binding to trypsin-treated FMDV antigen. We further mapped the antigenic sites of two type A broadly strain reactive and 146S-specific VHHs, M691F, and M702F (8), using cross-linking mass spectrometry (XL-MS).

Materials and methods

Viruses and viral antigens

Production of FMDV antigens of strains A/TUR/14/98 (A/TUR), A24/Cruzeiro/BRA/55 (A24Cru), A10/HOL/1/42, O1/BFS1860/UK/67 and Asia1/Shamir/ISR/89 (Asia1 Shamir) was done as described earlier (8). Briefly, FMDV was amplified in BHK-21 cells grown in suspension in industrial-size bioreactors or 850-cm^2 roller bottles. FMDV present in the clarified supernatant was inactivated with 10 mM binary ethylenimine and concentrated using polyethylene glycol-6000 precipitation, resulting in crude antigen. Purification of 146S particles by sucrose density gradient (SDG) was done as described earlier (8). Trypsin-treated antigen (TTA) was prepared as described previously (23). A peptide representing the 22 N-terminal amino acid residues of VP2 (DKKTEETTLLEDRILTTRNGHT) derived from O1/Manisa/TUR/69 (Genbank acc. no. AY593823) appended with a C-terminal cysteine residue for conjugation to BSA (VP2-22-BSA) was produced by GenScript Corporation (Piscataway, NJ, USA).

MAbs and VHHs

The 146S specific mAb 22.9 (6) isolated at our institute against strain A10/HOL/1/42 was called mAb 3.9 in a previous publication, and binds the same antigenic site as mAb 3.32 (21). We refer to them as mAb 9 and mAb 32 in this paper. The non-neutralizing mAb 13A6 was earlier isolated against strain SAT1/ZIM/89 (24). The origin of the VHHs used and their FMDV specificity are shown in Table 1. They were produced in baker's yeast using plasmid pRL188, purified by immobilized-metal affinity chromatography and biotinylated as described recently (27). VHHs produced in yeast using pRL188 are indicated by the suffix F. Only the VHH M655F was found to be N-glycosylated (8). It was deglycosylated by treatment with endoglycosidase H (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

Virus neutralization test

VHH concentrations required for neutralization of FMDV A/TUR, A24Cru and Asia1 Shamir were determined as described previously (26) using 100 (30-300) tissue culture infective doses required to infect 50% of the wells ($TCID_{50}$) incubated with duplicate serial two-fold dilution series of VHHs for 1 h. The non-neutralized virus was then detected by adding IBRS-2 cells and 2 days later the plates were read macroscopically after staining the monolayers with amido black. VHH neutralization was calculated using the Spearman-Kärber method (28, 29) and expressed as VHH concentration in the VHH/virus mixture which neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point (VNT_{50}). Most VHH dilution series started at 1 mg/ml. However, the M220F VHH dilution series started at 0.05 mg/ml since it was produced in baker's yeast at low level.

ELISA for evaluation of VHH binding to FMDV antigens

The procedures for ELISAs have been described (7, 27). High binding 96-well polystyrene plates (Greiner, Solingen, Germany) were coated with 100 µl/well of 0.5 µg/ml unlabeled

VHH in 50 mM $NaHCO_3$ buffer (pH 9.6) overnight at 4°C. Plates were incubated at room temperature (RT) with 100 µl/well of a 2-fold dilution series over 12 wells starting at 1 µg/ml 146S of suitable FMDV antigens. Bound FMDV antigens were detected by subsequent sequential incubation with 100 µl/well 0.25 µg/ml of biotinylated VHH and 0.5 µg/ml of a streptavidin horse radish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories Inc., USA). Bound HRP was detected by staining with 3,3',5,5' tetramethylbenzidine. The color reaction was stopped by addition of 0.5 M sulfuric acid (50 µl per well) and the absorbance at 450 nm (A450) was measured using a Multiskan Ascent spectrophotometer (Thermo Labsystems, Finland). A four-parameter logistic curve was fitted to absorbance and FMDV concentrations using the SOFTmax Pro 2.2.1 program (Molecular Devices) and was used to interpolate the Effective Concentration (EC) resulting in a specific A450 value for each VHH or antibody. VHHs or antibodies that did not reach the A450 value defining the EC value were given an EC value of the highest FMDV concentration used (1 µg/ml).

Binding to a BSA-conjugated VP2 peptide was analyzed by coating ELISA plates with 4 µg/ml VP2-22-BSA that were subsequently incubated with 0.5 µg/ml biotinylated VHH or mAb and streptavidin-HRP conjugate as described above. Controls included plates coated with 4 µg/ml BSA or 2.5 µg/ml of crude FMDV antigens of strains A/TUR, A24Cru or Asia1 Shamir.

ELISA for VHH binning

The ability of VHHs or mAbs to bind independent antigenic sites of FMDV was studied by blocking/competition double-antibody sandwich (DAS) ELISA using biotinylated VHHs and mAb 9 or mAb 32. ELISAs were performed using 0.5 µg/ml unlabeled VHH for coating and subsequent capture of crude FMDV antigens (1 µg/ml 146S), that contained about 20% 12S particles, in addition to 146S. The same VHH as used for coating was used in the next step as biotinylated VHH. For mAb 9 and mAb 32, plates were coated with unlabeled VHH M691F. Initially the optimal concentration of biotinylated VHH or mAb for competition was determined by titration of biotinylated VHH or mAb without competition, as described above for determining EC values. A biotinylated VHH or mAb concentration was selected that provided about 80% of the maximal absorbance value observed with the highest VHH or mAb concentration analyzed. In the final experiment, plates containing VHH-captured FMDV were first incubated with the unlabeled VHH or mAb (5 µg/ml) in 90 µl/well for 30 min (blocking step). Then, without washing plates, 10 µl biotinylated VHH or mAb in the predetermined concentration was added and incubated for another 30 min (competition step). A control without antigen and a control without biotinylated VHH or mAb were included. Bound biotinylated VHH was detected by incubation with 0.5 µg/ml streptavidin-HRP conjugate. Bound mAbs were detected with 2,000-fold diluted rabbit anti-mouse immunoglobulin HRP conjugate (RaM-HRP). The % inhibition of antigen binding due to a competing VHH or mAb was calculated as 100–100* ([A450 with competing VHH or mAb] – [A450 without Ag coating]) / ([A450 without competing VHH or mAb – A450 without Ag coating]).

Octet Red96 affinity measurements

The Octet Red96 System (Sartorius, USA) was used for affinity measurement based on Biolayer Interferometry. An assay temperature of 30 °C was used. PBS containing 0.05% Tween-20 was used as kinetics buffer in all steps of each assay. High precision streptavidin (SAX)-sensors (Sartorius) were hydrated and subsequently loaded with biotinylated M678F (2 µg/ml) for 300 s, with A/TUR 146S particles (2 µg/ml) for 900 s and kinetics buffer for 300 s (baseline step). The concentrations of FMDV particles and VHHs were optimized for affinity measurements prior to the experiments. Then association of serial dilutions of unlabeled VHHs was done for 300 s and finally dissociation for 1,800 s. A reference sensor without unlabeled VHH was included to correct for baseline drift.

The on-rate (k_a) and off-rate (k_d) were determined by global fitting of the association and dissociation phases of a series of unlabeled VHH concentrations. The mathematical model used assumes a 1:1 stoichiometry, fitting only one VHH in solution binding to one binding site on the surface. The equilibrium dissociation constant (K_D), a measure for affinity, was then calculated as the ratio of k_d and k_a. The Octet Analysis Studio v12.2 software (Sartorius) was used for data analysis.

XL-MS analysis

XL-MS relies on cross-linking of protein complexes with a homobifunctional disuccinimidyl suberate (DSS) linker followed by digestion of the cross-linked complex with 5 proteases and identification of cross-linked peptides by MS analysis (30). The use of an equimolar mixture of deuterated and hydrogenated DSS cross-linker facilitates identification by MS. By tandem MS-MS the specific amino acid residues that are cross-linked can be identified. The NHS groups on the DSS bifunctional reagent only react with positively charged amino groups or OH groups, which are present on the protein N-terminus and side chains of amino acids Lys, Arg, His, Tyr, Thr and Ser (31).

XL-MS analysis was done at Coval X (Zurich, Switzerland) using SDG purified A24Cru 146S particles complexed with either M691F or M702F VHH. Strain A24Cru was used for this

A

inhibition of biotinylated VHH binding (%)

Biotinylated VHH	Unlabeled VHH	M3F	M663F	M680F	M665F	M684F	M675F	M220F	M643F	M652F	M659F	M702F	M691F	M703F	M669F	M676F	M677F	M678F	M661F	M651F	M8F	M686F	M688F	M679F	Trypsin Sensitive Binding	VNT_{50} (mg/ml)	Number of Serotype A Strains Recognized	Antigenic Site
M3F		94	14	74	-13	19	1	6	4	3	0	6	-2	19	-13	-4	0	-8	13	4	4	-2	1	-7	N	>1	15	II
M663F		102	90	100	4	36	10	13	-4	0	-10	4	7	30	-5	-2	0	0	8	6	4	5	8	-6	N	>1	15	
M680F		99	66	96	17	4	17	2	1	8	9	23	-7	6	-6	-1	4	0	-6	-5	1	-13	-6	-10	N	>1	14	
M665F		110	104	104	98	88	49	13	-26	20	6	-38	1	21	18	41	29	42	-53	-7	-40	22	23	18	N	>1	1	
M684F		101	99	101	64	86	73	23	-3	-3	-3	-14	-13	-12	-1	17	7	11	25	24	13	12	14	21	N	>1	13	
M675F		8	1	10	5	6	86	1	8	8	-3	6	4	13	-3	-3	9	0	25	2	-3	5	-1	9	N	>1	13	
M220F		1	-1	16	-6	17	45	94	-3	2	-7	0	2	19	5	2	3	4	18	-3	5	10	10	4	N	>0.05	11	IV
M643F		7	2	1	8	2	9	6	87	85	24	36	42	42	23	18	34	30	6	32	0	10	12	14	N	0.0056	7	
M652F		4	-3	1	0	0	8	-3	72	95	44	58	62	57	52	29	58	46	18	12	24	6	3	8	N	0.00071	10	
M659F		3	7	-11	14	-1	-2	8	98	100	94	100	101	100	99	82	100	97	60	59	64	24	33	15	N	0.045	8	
M702F		0	1	-1	-1	2	-1	-3	86	98	74	95	98	96	90	60	95	76	18	49	67	7	11	3	N	0.00016	8	
M691F		-4	-5	11	-2	-1	10	-1	42	79	25	63	98	96	82	31	48	21	87	14	-5	3	12	9	N	0.00030	14	
M703F		11	16	13	4	9	13	17	80	96	70	93	101	100	98	68	97	86	98	17	49	25	30	12	N	0.00027	6	
M669F		-5	-1	-15	2	4	-7	-8	56	84	44	72	100	99	95	50	96	84	92	-1	12	13	15	4	N	0.00050	1	
M676F		9	15	-19	7	9	-21	14	95	98	94	97	100	99	100	97	100	92	100	21	81	49	60	30	N	0.011	1	
M677F		-6	-1	-3	9	3	2	1	84	98	74	90	99	100	98	80	99	89	97	5	14	15	30	3	N	>1	2	
M678F		-8	-4	-6	8	6	1	-7	80	96	67	93	101	100	100	37	101	93	94	17	9	14	19	-1	N	0.13	2	
M661F		4	4	-2	6	2	7	3	2	5	5	6	10	16	8	8	14	-5	94	8	-2	0	-1	5	N	0.71	11	
M651F		10	2	-8	2	-4	6	3	36	30	13	44	3	-8	0	-8	4	18	93	76	51	6	2	1	N	0.022	10	I
M8F		5	-3	8	-9	11	2	3	5	-7	5	4	0	3	-9	4	-9	4	4	4	94	5	4	29	Y	0.0095	15	
M686F		5	1	-11	16	10	16	-8	28	22	26	22	7	12	21	21	41	23	50	22	90	94	93	98	N	0.18	2	III
M688F		13	8	-9	13	2	5	0	26	22	18	24	12	12	21	18	40	26	45	29	88	89	91	95	N	0.13	2	
M679F		9	-1	-6	13	3	1	1	4	6	7	9	7	-4	5	7	8	6	10	4	69	41	45	87	N	0.13	2	

B

inhibition of biotinylated VHH binding (%)

Biotinylated VHH	Unlabeled VHH	M3F	M663F	M680F	M675F	M220F	M691F	M703F	M661F	M662F	M651F	M8F	M655F	M326F	Trypsin Sensitive Binding	VNT_{50} (mg/ml)	Number of Serotype A Strains Recognized	Antigenic Site
M3F		95	38	77	-7	1	-7	-5	-3	10	8	6	6	-6	N	>1	15	II
M663F		100	90	100	4	15	-1	10	21	16	26	18	10	6	N	>1	15	
M680F		99	65	95	-4	-15	-9	-3	2	13	13	0	7	-2	N	>1	14	
M675F		-4	4	-6	76	-6	-14	-7	47	11	19	-13	-2	4	N	>1	13	
M220F		-15	9	10	43	94	12	8	32	17	22	23	26	21	N	>0.05	11	IV
M691F		-11	-2	5	4	-7	99	75	49	41	23	-8	10	10	N	0.0010	14	V
M703F		-14	5	0	10	5	99	94	68	63	28	8	31	18	N	0.0060	6	
M661F		12	12	-6	0	7	2	0	86	58	91	5	2	2	N	>1	11	
M662F		16	-3	8	7	9	24	8	44	87	96	16	2	6	N	0.71	3	
M651F		14	-2	-4	-7	-2	-2	-7	9	14	37	3	5	6	N	0.00071	10	I
M8F		-9	-5	10	22	14	11	4	22	23	82	99	97	97	Y	>1	15	
M655F		16	7	-4	-10	1	3	-12	8	12	11	2	86	90	N	0.0010	3	
M326F		5	5	3	-3	3	-4	-2	17	8	11	-1	98	83	N	>1	1	

C

inhibition of biotinylated VHH or mAb binding (%)

Biotinylated VHH or mAb	Unlabeled VHH or mAb	M3F	M663F	M680F	M684F	M661F	M675F	M220F	mAb 13A6	M691F	M703F	mAb 9	mAb 32	M8F	Antigenic Site
M3F		92	38	91	18	12	13	-7	-5	-4	-6	-4	12	-2	II
M663F		93	95	102	41	25	17	9	17	3	-15	18	-7	-9	
M680F		98	70	98	18	20	9	8	10	-2	1	-7	-4	7	
M684F		92	98	102	81	16	6	14	8	5	-2	6	22	-5	
M661F		41	32	35	26	82	51	31	7	34	3	6	3	22	
M675F		11	7	-17	-3	29	94	12	1	-5	-7	-3	0	12	
M220F		7	12	4	8	9	5	63	4	19	-7	0	1	2	IV
mAb13A6		-26	15	-6	-10	16	31	70	92	4	-4	18	12	31	
M691F		-12	-4	12	-7	-4	5	-10	-1	76	89	40	20	3	V
M703F		7	2	4	3	-6	-10	-1	-11	99	94	99	98	-1	
mAb 9		-12	8	4	-15	-7	-8	4	ND	101	93	ND	ND	8	
mAb 32		5	3	5	-1	10	-3	4	ND	100	94	ND	ND	12	
M8F		0	-1	-2	-7	-6	0	-5	-5	4	0	-13	-12	73	I

D

inhibition of biotinylated VHH binding (%)

Biotinylated VHH	Unlabeled VHH	M3F	M220F	M8F	M332F	M658F	M661F	M685F	M98F	Trypsin Sensitive Binding	VNT_{50} (mg/ml)	Antigenic Site
M3F		95	17	5	4	11	5	10	6	N	>1	II
M220F		-7	93	0	-9	-9	1	1	-1	N	>0.05	IV
M8F		18	36	97	78	76	-40	98	5	Y	>1	I
M332F		0	-3	-5	96	98	2	-1	11	Y	>1	
M658F		1	-8	-3	93	96	35	-3	18	Y	0.016	
M661F		-5	6	-10	76	73	78	99	100	N	>1	
M685F		1	6	0	2	-13	-3	66	97	N	0.032	
M98F		4	6	-1	17	8	28	74	97	N	1.8	

FIGURE 1
Epitope mapping of FMDV binding VHHs and mAbs by competition ELISAs using FMDV strains A/TUR **(A)**, A24Cru **(B)**, A10/HOL/1/42 **(C)**, and Asia1 Shamir **(D)**. A red-blue coloring is used to visualize differences in percentage inhibition of biotinylated VHH binding. The 14 VHHs of the 7 CDR3 groups comprising the 12 VHHs that bind specifically to 146S of serotype A or Asia1 strains are color-coded by their CDR3 group. VNT titres and binding to trypsin sensitive epitopes of VHHs is also indicated **(A, B, D)**. The number of serotype A strains recognized in ELISA by serotype A binding VHHs **(A, B)** was derived from ELISA data obtained earlier (8) using 15 serotype A strains assuming an absorbance > 0.5

(Continued)

FIGURE 1 (Continued)
indicative of binding. VHH binding is either sensitive to trypsin treatment of FMDV (Y) or not (N). Competition of mAb 9 and mAb 32 by unlabeled mAbs **(C)** could not be determined (ND) due to using RaM-HRP for mAb detection. However, mAb 13A6 was used in biotinylated form, enabling detection of competition with unlabeled mAb. Roman numerals indicate VHH epitope bins.

purpose because it produces a high amount of 75S particles in addition to 146S particles and thus could also be used for analyzing VHH binding to 75S particles. Prior to XL-MS analysis, the A24Cru 146S particles were subjected to digestion with five proteases and MS analysis (see below) without complexing with VHH and cross-linking to determine whether sufficient peptides can be identified that cover the complete FMDV capsid sequence. Such peptide mass fingerprints were made for VP1, VP2, and VP3 but not for VP4, which is internal in the FMDV 3D structure and thus not accessible to antibodies. For both VHHs 10 µl 0.5 mg/ml 146S particles were mixed with 10 µl 0.5 mg/ml VHH in PBS and 2 µl N,N-dimethylformamide containing 2 mg/ml disuccinimidyl suberate (DSS) cross-linker. The DSS consisted of an equimolar mixture of two forms that contain either 12 hydrogen (H12) or 12 deuterium (D12) atoms but were otherwise chemically identical. After incubation for 3 h at room temperature (RT) cross-linking was quenched with 20 mM ammonium bicarbonate and proteins were reduced with 50 mM dithiothreitol for 1 h at 37°C and subsequently alkylated with 100 mM iodoacetamide for 1 h at RT. Then five different aliquots of the reduced/alkylated sample were separately incubated with five different proteases (all Promega, Madison, WI, USA), overnight at 37°C (trypsin, elastase and ASP-N), 25°C (chymotrypsin) or 70°C (thermolysin). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was then performed. Peptides were separated on a C18 PepMapRSLC column at a flow rate of 300 nl/min ramping a gradient from 2 to 40% mobile phase B (water/acetonitrile/formic acid, 20:80:0.1) using an Ultimate 3000-RSLC system. Peptides in the range of m/z 350–1,700 were analyzed by LC-MS/MS using a Thermo LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, Rockford, IL, USA). MS data were analyzed using xQuest V2.0 (32) and Stavrox V3.6 software (33) using a database of the VHH sequences and a sequence for A24Cru.

3D structure analysis

The A24Cru strain was sequenced again using earlier described methods (8). The protein sequence of the P1 region was identical to an earlier obtained sequence (accession number AY593768), except for VP2 residue 82 which was lysine instead of glutamic acid and VP2 residue 86 which was asparagine instead of aspartic acid (result not shown). The FMDV residues cross-linked to a VHH were mapped on the 3D structure of FMDV A22/IRQ/24/64 (A22IRQ; PDB: 4GH4) using PyMOL 2.5.2 (Schrodinger, Portland, USA). The protein sequence of A22IRQ has 90% amino acid sequence identity to A24Cru. To define an epitope, amino acid residues were selected that were closest to each other on the 3D structure.

Results

Epitope binning of VHHs

We mapped the antigenic sites of 30 VHHs binding to serotype A or Asia 1 FMDV by competition of biotinylated VHH with unlabeled VHH in DAS-ELISA. For competition ELISA we used FMDV strains A/TUR, A24Cru, A10/HOL/1/42 and Asia1 Shamir (Figures 1A–D). We earlier determined (5, 7, 8) the specificity of the VHHs for these strains as well as the particle specificity, which revealed 12 VHHs to be 146S specific (Table 1). Color-coding was used to indicate the seven complementarity-determining region (CDR)3 groups that contain the 12 VHHs that bind specifically to 146S of serotype A or Asia1 strains, as done earlier (8). CDR3 is the most variable region of immunoglobulin domains and most important for determining antigen binding specificity. VHHs of the same CDR3 group are most likely derived from the same B-cell but have diverged due to somatic hypermutation. VHHs M643F and M652F were not strictly 146S specific, although M652F showed high 146S specificity for C1/Detmold/FRG/60. They belong to the same (purple) CDR3 group as 146S specific VHHs M659F and M702F. VHHs M3F, M8F, and M220F were earlier found to recognize three independent antigenic sites of serotype O strain O1/Manisa/TUR/69 (26) that were indicated by roman numerals II, I, and IV, respectively (Table 1). Since these 3 VHHs cross react to serotype A and Asia1 strains, they were included in the current epitope mapping. M3F was earlier shown to be highly 12S-specific (5). We further included mAb 13A6 in this analysis since it binds to a known epitope at the VP2 N-terminus and was also found to be 12S-specific (see below).

Epitope binning of strain A/TUR (Figure 1A) was done using all 23 out of the 30 VHHs that recognize this strain. A subset of these VHHs was included in epitope binning using strain A24Cru (Figure 1B), together with A24Cru specific VHHs M326F, M655F and M662F. Furthermore, a subset of these VHHs was used in epitope binning using strain A10/HOL/1/42 (Figure 1C) that was also recognized by 146S-specific mAb 9 and mAb 32. VHHs M3F, M8F and M220F recognize three independent antigenic sites (II, I, and IV, respectively) on the three serotype A strains (Figures 1A–C), as observed earlier for type O strain O1/Manisa/TUR/69 (26). Site II was recognized by 4 further VHHs, although competition was sometimes

non-reciprocal (Figures 1A–C). M675F showed non-reciprocal competition with M220F (site IV) when using A/TUR and A24Cru (Figures 1A, B) but not when using A10/HOL/1/42 (Figure 1C). VHHs M651F, M655F and M326F were mapped to site I since they efficiently inhibited biotinylated M8F binding to A24Cru, although competition was non-reciprocal (Figure 1B). However, the 146S-specific VHHs M686F and M688F, as well as 12S binding M679F were unable to compete with biotinylated M8F using A/TUR, although M8F could compete with the biotinylated versions of these VHHs (Figure 1A). We therefore mapped these 3 VHHs to an independent site (III). The remaining 8 146S-specific VHHs and M643F and M652F from the purple CDR3 group all mapped to the separate site V (Figure 1A). The non-146S specific VHHs M643F and M652F were less efficiently competed by the eight highly 146S-specific VHHs. M661F showed strong competition with many VHHs of site I and site V when used in unlabeled form but was not competed at all by any VHH when used as biotinylated VHH. Therefore, it was not grouped into an epitope bin. M8F of site I showed similar non-reciprocal competition of several site V VHHs. Notably, M661F and M8F showed 53% and 40% negative inhibition, respectively, of biotinylated M665F binding (Figure 1A). Furthermore, the two 146S-specific VHHs of site III, M686F and M688F, also showed competition with many VHHs of site V, although the percentage inhibition was lower. The 146S-specific VHHs, M691F and M703F, that bind A10/HOL/1/42 were found to compete with the earlier isolated 146S-specific mAb 9 as well as mAb 32 that was later found to bind the same antigenic site as mAb 9 (Figure 1C). MAb 13A6 showed nonreciprocal competition with M220F (site IV) only but did not compete with VHHs mapped to site II (Figure 1C) despite its similar FMDV strain and particle recognition (see below).

VHHs M3F, M8F, and M220F also recognize three independent antigenic sites (II, I and IV, respectively) on strain Asia1 Shamir (Figure 1D). The 146S-specific VHHs M332F and M658F showed non-reciprocal competition with M8F and were thus grouped to site I. The remaining Asia1 Shamir binding VHHs were difficult to map. M661F again showed non-reciprocal competition with many VHHs and 40% negative inhibition of M8F binding.

Virus neutralization and FMDV strain recognition of VHHs

Epitopes were further characterized by VNT titers (Figures 1A, B, D) and broadness of serotype A strain ($n = 15$) recognition (Figures 1A, B). VHHs that recognized sites II and IV as well as M675F that sometimes competed with M220F of site IV consistently did not neutralize FMDV while they displayed broad recognition of serotype A strains (11 to 15) with exception of M665F that bound only one serotype A strain. Nine out of 10 VHHs recognizing site V were neutralizing. Neutralizing titers were generally lower using strain A/TUR than A24Cru and also lower using VHHs that recognized more serotype A strains. Among the eight Asia1 Shamir binding VHHs, only three VHHs showed virus neutralization, including M658F which recognized antigenic site I and 2 VHHs that were difficult to map (Figure 1D).

Binding to trypsin sensitive epitopes and VP2 peptide recognition of VHHs

To further characterize the epitopes, we also determined the binding to trypsin sensitive epitopes for all 30 VHHs by incubation of dilution series of untreated and trypsin-treated antigen (TTA) in ELISA (Supplementary Figure 1). VHHs were considered binding to trypsin sensitive epitopes (Figures 1A, B, D) if the FMDV antigen concentration resulting in an absorbance value of 0.2 in dilution series of antigens ($EC_{0.2}$) was at least tenfold higher in trypsin-treated samples as compared to untreated samples. Using this criterium only VHHs M8F, M332F, and M658F showed trypsin sensitive binding (Figures 1A, B, D). This is consistent with the classification of M332F and M658F to antigenic site I for Asia1 Shamir but not consistent with the classification of M651F, M655F, and M326F to antigenic site I for A24Cru.

Since only the Asia1 Shamir 146S-specific VHHs M332F and M658F fell into the same epitope bin as M8F, we titrated both untreated FMDV antigen consisting mostly of 146S and heated antigen (12S) in the M8F ELISA. M8F showed considerable 146S specificity for strain Asia1 Shamir (Supplementary Figure 2C) although not as high as M332F (Supplementary Figure 2M). Consistent with earlier results (8) 146S specificity for serotype A or O strains was much lower (Supplementary Figures 2A, B, D). MAb 13A6 showed a high 12S specificity for 4 FMDV strains from serotypes A, O and Asia 1 that was comparable to M3F (Supplementary Figures 2E–L).

None of the VHHs bound in ELISA to a peptide representing the N-terminus of VP2 conjugated to BSA (Supplementary Figure 3A) although they did bind at least one of the FMDV strains used also for epitope binning (Supplementary Figures 3C–E). However, mAb 13A6 was able to bind this peptide as it reacted well with VP2-22-BSA but not with BSA (Supplementary Figures 3A, B).

The above classification of antigenic sites is summarized in Table 2.

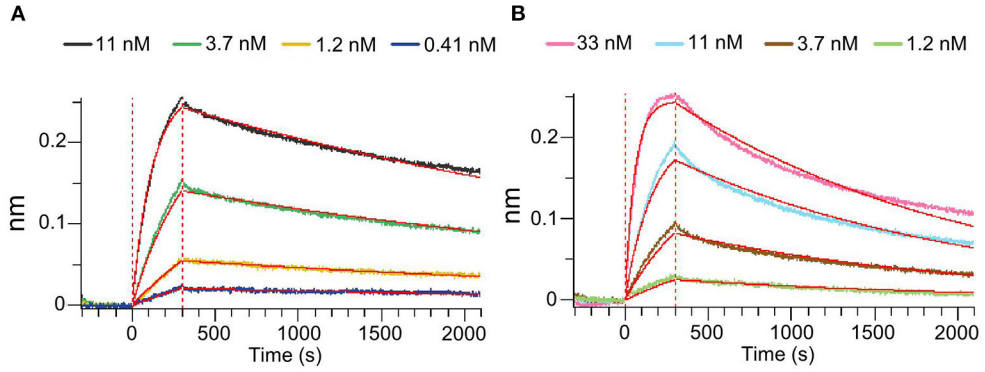
Affinity of VHHs M691F and M702F

The VHHs M691F and M702F that bound site V were selected for further study because of their high specificity for intact particles and broad strain recognition. Their affinity for FMDV strain A/TUR was determined by biolayer

TABLE 2 Characteristics of antigenic sites recognized by VHHs.

Antigenic site[a]	FMDV serotype specificity[b]	Prototype VHHs for different FMDV serotypes			Antigenic site identified using mAbs	FMDV particle specificity[c]	Neutralizing[e]	Trypsin sensitive epitope[f]
		O	A	Asia1				
I	Low (M8)	M8F	M8F	M8F, M332F	1	(12S and) 146S[d]	Yes	Yes
II	Low	M3F	M3F	M3F	unknown	12S	No	No
III	High	M23F	M686F, M688F	-	unknown	(12S and) 146S	Yes	No
IV	Low	M220F	M220F	M220F	unknown	12S and 146S	No	No
V	High	-	M691F, M702F	-	2 and/or 4	146S	Yes	No

[a]Sites I to IV were initially identified in serotype O FMDV (26); site V, this work.
[b]Low, reactivity with at least 2 serotypes; High, reactivity with strains from 1 serotype only.
[c]See Table 1.
[d]M8F and M332F specifically bind 146S particles of Asia 1 Shamir, whereas M8F recognizes 12S of serotype O and A strains.
[e]Neutralizing at least one of the strains tested.
[f]At least one of the VHHs mapped to this site binds to a trypsin sensitive part of the epitope.



C

VHH	Affinity constant K_D (nM)	Association rate k_a (1/Ms)	Dissociation rate k_d (1/s)
M691F	0.33	7.4×10^5	2.4×10^{-4}
M702F	1.1	5.0×10^5	5.5×10^{-4}

FIGURE 2
Affinity binding curves and deduced affinity constants of VHHs. Using Biolayer Interferometry on an Octet Red96 system, association and dissociation rates were determined by tight multivalent capturing of FMDV A/TUR on optical streptavidin sensors that were loaded with biotinylated M678F VHH. The FMDV-bound sensors were incubated with specific concentrations of M691F **(A)** or M702F **(B)** to allow association at time = 0 s. After 300 s the sensors were then moved to VHH-free solution and allowed to dissociate over a time interval. Curve fitting using a 1:1 interaction model (red lines) allows for the affinity constant (K_D) to be measured for each VHH **(C)**.

interferometry using an Octet Red96 biosensor. Both VHHs bound 146S particles with high affinity as shown by the low K_D values of 0.33 nM for M691F and 1.1 nM for M702F (Figure 2).

Epitope mapping by XL-MS

We started the XL-MS analysis using A24Cru 146S particles without complexation to VHH. All three VPs had a high sequence coverage in the peptide mass fingerprints (Supplementary Figure 4) that varied from 96.7 to 98.6% (Supplementary Table 1). Only the DSS reactive residues VP1-K210, VP3-S203, and VP3-S205 were not represented in the peptide mass fingerprints (Supplementary Figure 4).

XL-MS analysis of M691F and M702F complexed to 146S particles identified several A24Cru amino acid residues cross-linked to a VHH (Table 3). M702F was cross-linked to residues in VP2 and VP3 whereas M691F was cross-linked to residues

TABLE 3 Cross-linked amino acid residues (X) of FMDV A24Cru 146S particles complexed with M702F or M691F VHHs identified by XL-MS analysis.

Cross-Linked residue A24Cru	Corresponding Amino acid Residue		146S Particle surface exposed[b]	Amino acid residues of VHHs cross-linked (IMGT position)[c]																			
				M702F													M691F						
				CDR1 (27–38)				FR2 (39–55)	CDR2 (56–65)		CDR3 (105–117)					CDR1 (27–38)		FR2 (39–55)	CDR2 (56–65)	CDR3 (105–117)			
	A22IRQ	O1K[a]		S26	S29	S31	Y33	R55	S59	T62	S109	T111	S113	R114	Y117	S26	T34	T55	S61	K105	T111	T116	Y117
VP1																							
S141	T141	V141	Yes													X	X				X		X
R143		L144	Yes																			X	
S149	P149	V150	Yes																			X	
VP2																							
S97			Yes															X					
Y100			Yes												X						X		
R102			Internal					X	X						X		X			X			
T134	P134	K134	Yes				X							X	X								
R135			Yes												X								
T141			Internal	X							X												
H145			Internal								X												
H209			Inner surface																	X		X	
VP3																							
T53			Internal						X	X													
K61		V61	Yes						X	X													
Y63			Yes						X														
T65	V65		Yes							X													
T66			Yes						X														

(Continued)

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TABLE 3 (Continued)

Cross-Linked residue A24Cru	Corresponding Amino acid Residue		146S Particle surface exposed[b]	Amino acid residues of VHHs cross-linked (IMGT position)[c]																			
				M702F												M691F							
				CDR1 (27–38)				FR2 (39–55)	CDR2 (56–65)		CDR3 (105–117)					CDR1 (27–38)		FR2 (39–55)	CDR2 (56–65)	CDR3 (105–117)			
	A22IRQ	O1K[a]		S26	S29	S31	Y33	R55	S59	T62	S109	T111	S113	R114	Y117	S26	T34	T55	S61	K105	T111	T116	Y117
T68			Yes																	X	X		
R72			Yes																			X	
K76		Q76	Yes						X														
S80			Internal	X																			
H85			Yes													X							
T112			Yes										X										
T115			2-fold axis						X														
S117		A117	Internal						X						X								
R120			2-fold axis						X														
Y121			Internal							X													
Y170		(Y169)	Yes																X				
T191		(T190)	2-fold axis	X	X	X		X															
H192		(H191)	Yes			X	X																
T199		A198	Yes													X							
K208		(K207)	Inner surface																		X		

[a]Due to an insertion at VP3 position 142 of O1K as compared to serotype A strains residues between parentheses are identical in O1K and A24Cru. RGD is position 145-147 in O1K and 144-146 in A22IRQ.

[b]Residues are classified as exposed at the outer surface of the 146S particle (yes), exposed at the inner surface, exposed at the 2-fold axis of a 12S pentamer only, or internal.

[c]The IMGT system (34) was used to define the three CDRs often involved in antigen binding, the second framework region (FR2) of VHHs, and VHH numbering.

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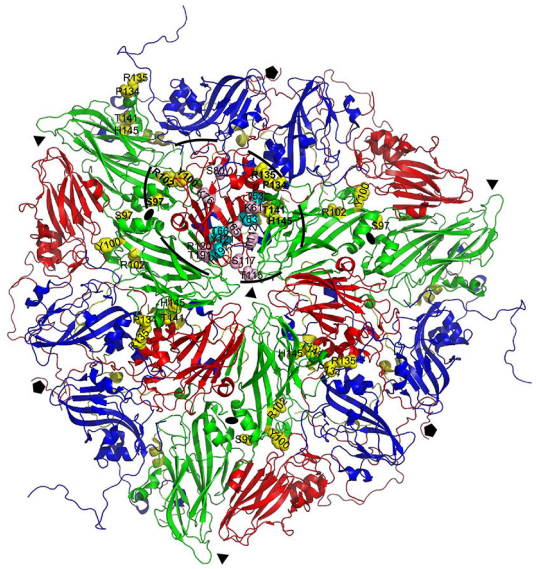

FIGURE 3
Location of VP2 residues cross-linked to M702F relative to VP3 cross-linked residues. Cross-linked residues are mapped onto a cartoon presentation of the A22IRQ structure (PDB: 4GH4). Six protomers that originate from three different 12S pentamers surrounding the 3-fold symmetry axis are shown. M702F cross-linked residues are shown for VP3 of the protomer on top and the 4 VP2 molecules closest to this VP3 molecule. The VP2 residues closest to the cross-linked VP3 residues (bold) most likely form the same antigenic site (dashed oval). VP1, blue; VP2, green; VP3, red; VP4, yellow. Cross-linked residues are shown with side chains as yellow (VP2) or cyan/pink (VP3) spheres. Symmetry axes: 5-fold, pentagon; 3-fold, triangle; 2-fold, oval.

in VP1, VP2, and VP3. We mapped the cross-linked residues onto the 3D structure of the A22IRQ 146S particle. Residues VP1-141 and 149, VP2-134 and VP3-65 are different in A22IRQ compared to A24Cru (Table 3). We further refer to these residues based on the corresponding residues in A22IRQ. The residues cross-linked to M702F were visualized in cyan or pink for a single VP3 molecule on the A22IRQ 3D structure showing six protomers around the 3-fold axis (Figure 3). Most cross-linked residues were located close to the 3-fold symmetry axis where three 12S pentamers associate. All seven residues cross-linked to VP2 were marked in yellow on 4 of the 6 VP2 molecules surrounding the VP3 molecule with marked cross-links (Figure 3). Residues P134, R135, T141, and H145 located on the VP2 molecule of an adjacent protomer shown at the right of the VP3 molecule with visualized 16 cross-linked residues were clearly closest to these VP3 residues. However, S97, Y100, and R102 shown on the right on this same VP2 molecule were located far away from these marked VP3 residues, whereas these same 3 residues of the VP2 molecule of the same (top left) protomer were closest to these VP3 residues and thus most likely part of the same M702F antigenic site.

Some VHH residues were complexed to different FMDV residues. This was especially the case for M702F residue S59, that was cross-linked to 9 different FMDV residues, but also S26, T62, and Y117. The contact residues for all these four M702F residues are spread over a large area covering both VP2 and VP3 that exceeds twice the 11.4 Å length of the DSS spacer arm (Supplementary Figure 5).

The putative M702F antigenic site was located on a single 12S pentamer, close to the 2-fold symmetry axis that separates two pentamers (Figure 4A). VP3-K61, Y63, V65, T66, K76, T112, and VP2-Y100, P134, R135 are surface exposed when looking at a pentamer from a top view (Figure 4B), although in case of VP2-Y100 only the OH group is exposed. VP3-T115, R120, T191, H192, and VP2-S97 are surface exposed when looking at a pentamer from a side view toward the 2-fold symmetry axis (Figure 4C). However, VP3-T115, R120, and T191 are not surface exposed in a 146S particle when this 2-fold symmetry axis is blocked by a protomer of an adjacent 12S pentamer, whereas VP3-H192 and VP2-S97 are also surface accessible in 146S particles (Figure 4D). Surprisingly, cross-linked residues VP2-R102, T141, H145 and VP3-T53, S80, S117, Y121 are not surface accessible but internal in a 12S pentamer. However, these residues are mostly located close to surface accessible cross-linked residues (Figure 4A): VP2-R102 is close to VP2-Y100; VP2-T141, VP2-H145, and VP3-T53 are located close to VP2-P134 and R135. Similarly, VP3-S117 and Y121 are located close to surface exposed VP3-V65, T66, and H192. VP3-S80 is buried under the C-terminus of VP1. The surface accessibility of cross-linked residues is summarized in Table 3.

The M691F antigenic site was also mapped onto the A22IRQ 3D structure. However, since cross links were observed with the highly flexible VP1 GH-loop, which is disorded and thus invisible in the A22IRQ structure, we introduced the O1 Kaufbeuren (O1K) GH-loop that could be resolved by X-ray crystallography due to reducing the disulfide bond involving C134 at the base of the GH loop (35). The putative M691F antigenic site was also located on a single protomer of a 12S pentamer (Figure 5A), on a similar position as the M702F antigenic site (cf. Figure 4A).

Cross-linked residues VP2-H209 and VP3-K208 are surface accessible only at the inner surface of 12S pentamer (Figure 5B) which is not accessible in a full 146S particle. VP1-V141, L144, V150, and VP3-T68, R72, H85, Y170, and T199 are surface exposed in a 146S particle, without being blocked by an adjacent pentamer (Figures 5C, D). VP2-Y100 was also cross-linked to M702F and surface accessible without artificial introduction of the O1K GH-loop (Figure 4B) but is hidden by the reduced O1K GH-loop (Figure 5C) which is known to lie flat on the virion surface, as opposed the natural GH loop that stands up from the surface (35). VP2-R102 was also cross-linked to M702F. It is internal, but close to surface accessible residue Y100. Although VP2-H209 is only accessible from the 12S pentamer inner

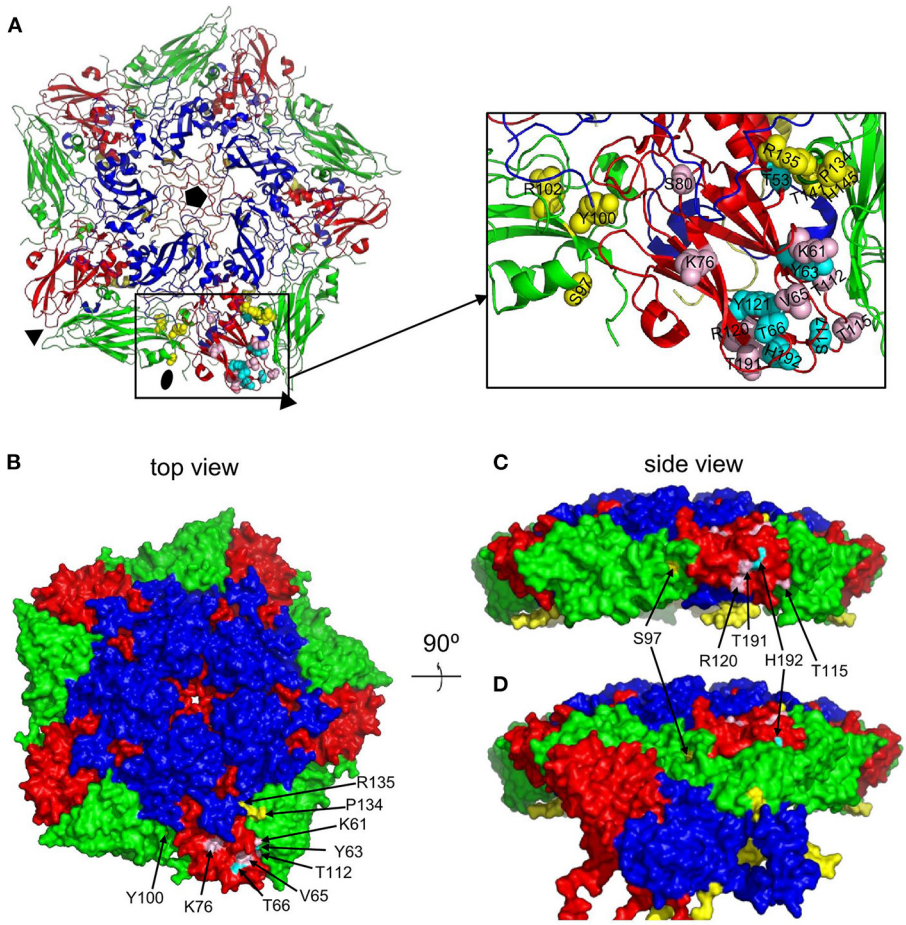

FIGURE 4
Surface accessibility of M702F cross-linked residues mapped on a 12S pentamer of the A22IRQ structure (PDB: 4GH4). The M702F cross-linked residues forming a single antigenic site on VP3 of the protomer at the bottom and the relevant VP2 residues adjacent to this VP3 molecule are shown as cartoon graph **(A)**. Cross-linked residues are shown with side chains as yellow (VP2) or cyan/pink (VP3) spheres. The surface accessible area of a pentamer is shown from a top view **(B)** or side view **(C, D)**. The nine residues that are surface accessible even when 12S pentamers associate are indicated by arrows **(B)**. Cross-linked residues surface exposed at the 2-fold symmetry axis are shown by a side view of a single pentamer **(C)** or this same pentamer with a protomer of an adjacent 12S pentamer that associates at the 2-fold symmetry axis in a 146S particle **(D)**. Among the 5 cross-linked residues at this 2-fold symmetry axis (arrows), R120, T191 and T115 are only surface accessible in a 12S pentamer **(C)** but not a 146S particles **(D)**. VP1, blue; VP2, green; VP3, red; VP4, yellow. Fivefold symmetry axis, pentagon; 3-fold symmetry axis, triangle; 2-fold symmetry axis, oval.

surface (Figure 5B), its localization close to VP2-R102 and Y100, which were cross-linked to both M691F and M702F, suggests that its cross-linking is not an artifact. The surface accessibility of residues cross-linked to M691F is summarized in Table 3.

Residues VP3-E70 and K139 that were mutated in mAb 9 resistant mutants (21) are also located close to the 2-fold symmetry axis (Figure 5). E70, which is most important for mAb 9 binding, is close to M691F cross-linked residues VP3-T68 and R72, which are part of the VP3 BC-loop.

Comparison of epitopes identified by XL-MS to M170 epitope identified by cryo-EM

The epitope recognized by M170 on type O FMDV as resolved by cryo-EM of M170/FMDV complex (10) was visualized on a single protomer, including the C-terminal 20 amino acids of VP1 from an adjacent protomer, since M170 interacts with two residues in this region (Figures 6A, B). For comparison the epitopes recognized by M702F and M691F as

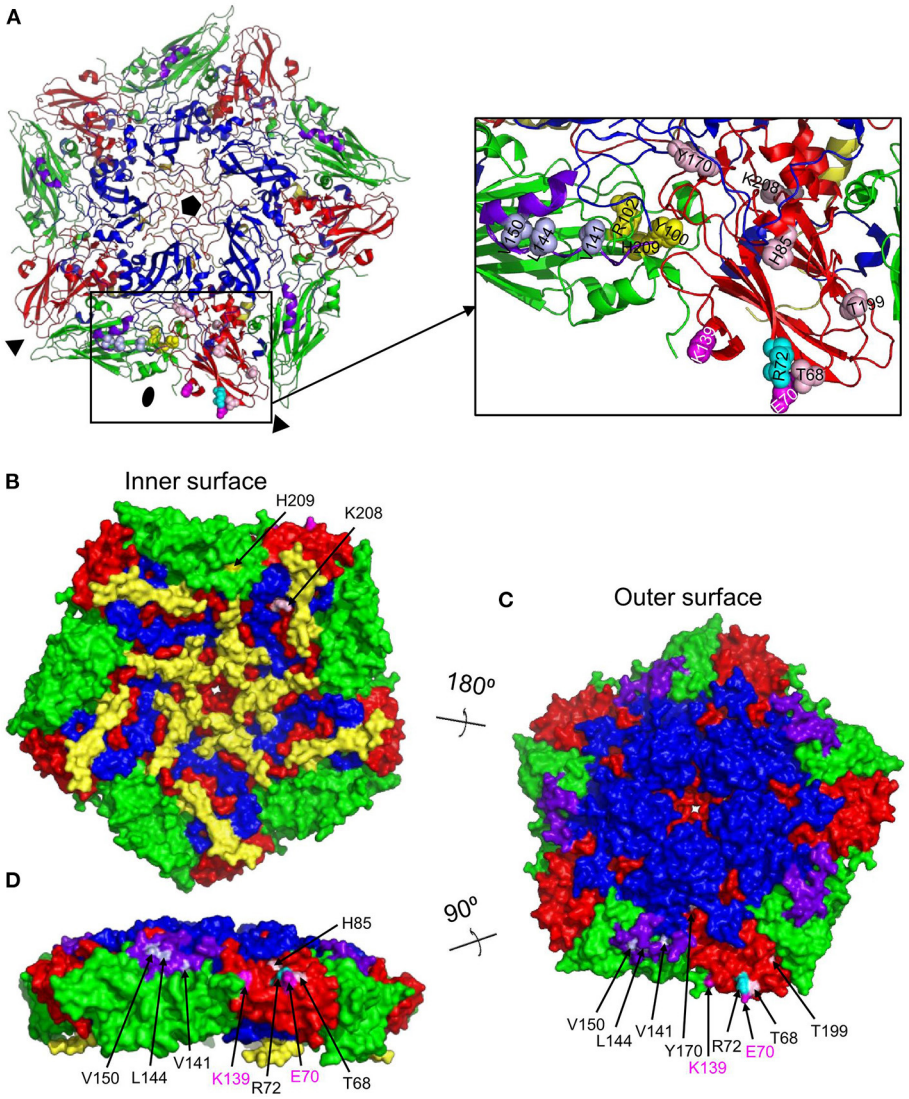

FIGURE 5
Surface accessibility of M691F cross-linked residues mapped on a 12S pentamer of the A22IRQ structure (PDB: 4GH4) with superimposed GH loops of reduced O1K (PDB: 1FOD). The M691F cross-linked residues forming a single antigenic site on the protomer at the bottom is shown as a cartoon graph **(A)**. Side chains of cross-linked residues are shown as light blue (VP1) yellow (VP2) or cyan/pink (VP3) spheres. The surface accessible area of a pentamer **(B, C)** is shown for the inner surface **(B)**, outer surface **(C)** or from a side view looking at the 2-fold symmetry axis **(D)**. The 8 residues that are surface accessible are indicated by arrows **(B–D)**. VP1, blue; VP1 GH-loop, purple; VP2, green; VP3, red; VP4, yellow. The side chains of VP3-K139 and VP3-E70 that are mutated in mAb 9 escape mutants are indicated in magenta spheres. Fivefold symmetry axis, pentagon; 3-fold symmetry axis, triangle; 2-fold symmetry axis, oval.

identified by XL-MS were visualized in an identical manner on an A22IRQ protomer, including part of VP2 of an adjacent protomer in case of M702F, since cross-links presumably occur to this region (Figures 6C, E). The M170 footprint is located mostly on VP3 and includes two residues on the VP1 C-terminus of an adjacent protomer. Superposition of the M170/FMDV cryo-EM structure and the A22IRQ structure visualizing the cross-links to M702F or M691F reveals M170 in the middle of the residues cross-linked to M702F or M691F (Figures 6D, F). Note that the GH loop of O1K superimposed on this structure visualizing the M691F cross-links is in a "down" conformation and thus more distant to the other cross-linked residues as when this loop was in an "up" conformation, similar as observed when complexed to M8 (10). Taken together, this superposition confirms that the sites recognized by M170, M702F and M691F largely overlap. Several residues of the M170 epitope, such as D71 and V73 in the BC loop, and E131 and K134 in the EF loop, lie in the region with the largest conformational changes between 12S and 146S (Figure 6A). This region is likely also part of the M691F and M702F epitopes, explaining their 146S specificity.

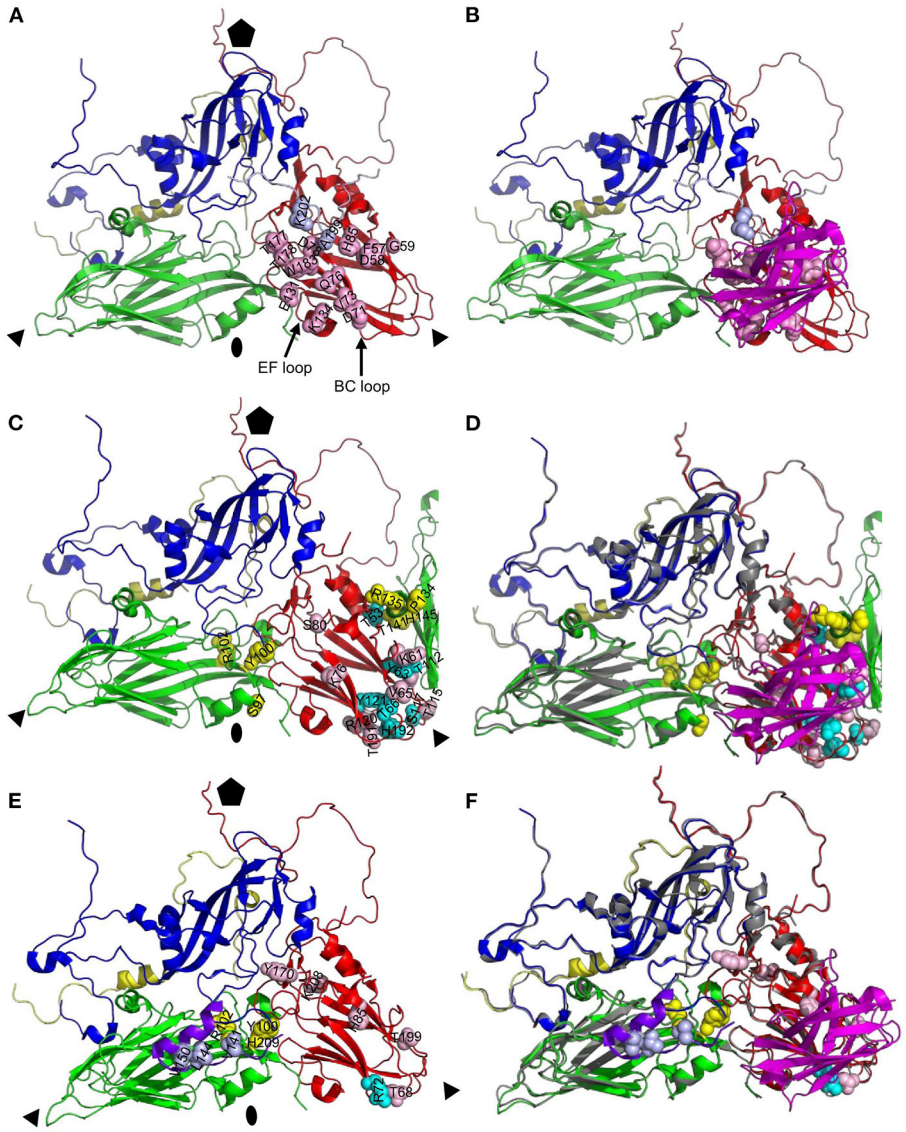

FIGURE 6
Comparison of antigenic site of VHH M170 determined by cryo-EM and M702F and M691F determined by XL-MS. Protomers of O/BY/CHA/2010 [PDB: 7DST; **(A, B)**] or A22IRQ [PDB: 4GH4; **(C–F)**] are shown as cartoon with footprints of M170 **(A, B)**, M702F **(C, D)** or M691F **(E, F)** shown by indicating the side chains of residues within 4 Å of the VHH **(A, B)** or cross-linked to the VHH **(C–F)** as light blue (VP1), yellow (VP2), or cyan/pink (VP3) spheres. VPs and VHHs are shown as cartoon: VP1, blue; VP1 of O1K GH-loop **(E, F)**, purple; VP2, green; VP3, red; VP4, yellow; M170, magenta. The C-terminal 20 amino acids of VP1 from an adjacent protomer are shown as light blue cartoon to visualize the M170 footprint **(A, B)**. Part of VP2 from an adjacent protomer is shown to visualize the M702F cross-linked residues **(C, D)**. An alignment of the structures of A22IRQ (VP coloring as done throughout this paper) with M702F or M691F cross-linked residues and O/BY/CHA/2010 (gray) complexed to M170 is shown **(D, F)**. Fivefold symmetry axis, pentagon; 3-fold symmetry axis, triangle; 2-fold symmetry axis, oval.

Discussion

The antigenic sites of type A and Asia1 FMDV binding VHHs were characterized by competition ELISA, virus neutralization tests, trypsin sensitivity of the epitope, broadness of the serotype A recognition and, for 2 VHHs, XL-MS. In competition ELISAs some VHHs showed non-reciprocal competition, which complicates antigenic site mapping. In case of M8F this could be due to VHH binding causing structural changes in the FMDV capsid, since M8 binding is known to decrease the virion stability and stimulate viral uncoating (10). Such effects can also explain the negative inhibition observed with some VHHs, especially M665F (Figure 1A), which shows improved binding to FMDV antigen complexed with a surplus

of M8F. Such cooperative binding is more often observed, for example with tetanus toxin binding antibodies (36). M661F, which also shows cooperative binding of M665F and non-reciprocal inhibition of several 146S-specific VHHs (Figures 1A, B, D), possibly also causes virion structural changes. The non-reciprocal competition of several 146S-specific VHHs by M643F and M652F is consistent with the binding of 12S particles by these latter VHHs since the antigen used in the competition ELISAs was not purified by SDG and thus contained 12S in addition to 146S. Non-reciprocal competition can also be caused by differences in antibody affinity. The high affinity of M691F (K_D= 0.33 nM) probably explains some of the non-reciprocal competition seen with M691F, which shows less than 50% inhibition by VHHs M659F, M676F, M677F, M678F, and mAb 9 while inhibiting the binding of these 5 VHHs/mAb for >98% (Figures 1A, C). Taking the above considerations into account, we mapped the type A VHHs into sites I, II, IV and V and type Asia1 VHHs into sites I, II and IV. In this mapping we also considered the broadness of FMDV strain recognition, virus neutralization and trypsin sensitivity of the epitope recognized by the VHHs (Table 2). However, it should be noted that some VHHs, such as M675F and M661F, could not be mapped into these antigenic sites due to different maps obtained with different FMDV strains.

The 12S-specific non-neutralizing VHH M3F was found to bind a different antigenic site than mAb 13A6. Furthermore, M3F did not bind to a trypsin sensitive epitope and did not bind a peptide representing the VP2 N-terminus. Freiberg et al. (24) were surprised to find that mAb 13A6 is suitable for detection of FMDV antigens in ELISA since it is specific for the VP2 N-terminus, which is hidden internally in a full capsid. We have shown in this study that mAb 13A6 shows considerable preference for 12S particles. Thus, the exposure of the VP2 N-terminus in 12S particles but not 146S particles explains the particle specific binding by mAb 13A6. Most likely the antigens used by Freiberg et al. (24) also contained some 12S. A 12S-specific mAb (23KF-1) that binds strains from all six serotypes except SAT2 was identified earlier (37). Possibly the epitope recognized by M3F is similar to the epitope recognized by mAb 23KF-1 as M3F also does not bind SAT2 strains while binding strains from serotypes O, A, C and Asia1 (7). Taken together, at least two independent 12S specific antigenic sites exist, one of them being the trypsin sensitive VP2 N-terminus while the other site is not trypsin sensitive and located in an unknown position.

Most type A 146S-specific VHHs bound site V whereas M686F and M688F bound a separate site, that was given roman numeral III, similar to the site identified in type O by M23F (26), since M23F is highly strain specific, although we do not know whether these sites in type O and A cover a similar epitope. Thus, 146S specificity of type A binding VHHs relies on at least two separate antigenic sites. The two serotype Asia1 146S-specific VHHs, M332F and M658F, presumably recognize site I based on competition with biotinylated M8 and binding to trypsin-sensitive epitopes. Based on the previously mapped M8

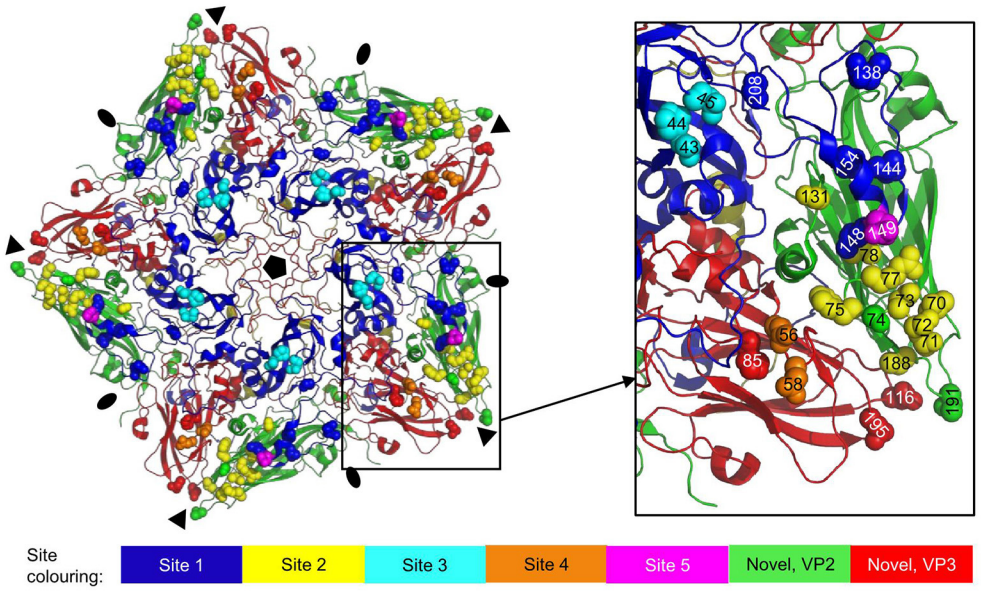

FIGURE 7
Antigenic sites 1-5 as earlier identified by sequence analysis of mAb escape mutants mapped onto the 3D structure of an O1K (PDB: 1FOD) pentamer. VPs are shown as cartoon: VP1, blue; VP2, green; VP3, red; VP4, yellow. Side chains of residues defining the antigenic sites are shown as spheres in different colors for sites 1-5 and in case of the novel site (16, 38) also VP2 and VP3 residues. Fivefold symmetry axis, pentagon; 3-fold symmetry axis, triangle; 2-fold symmetry axis, oval.

epitope (10), site I encompasses predominantly the VP1 GH-loop, and is thus similar to site 1 identified in type O using conventional mAbs (12, 13) which is identical to site A in type A FMDV (9, 19). We mapped the type O antigenic sites 1-5 onto the O1K 3D structure (Figure 7). Antibodies binding site 1 generally recognize both 12S and 146S particles, which was also observed for M8 using type A and O strains (8) and is consistent with this site being a linear epitope (23, 26). However, site 1 of type Asia1 is conformational (39), explaining the 146S-specific binding of M8F to strain Asia1 Shamir that we observed here. Several 146S specific mAbs binding Asia1 Shamir were earlier reported to bind two independent antigenic sites as assessed by competition ELISAs and isolation of MAR mutants (40), that were not sequenced, and thus cannot be coupled to known antigenic sites.

The 146S-specific VHHs M691F and M702F both recognized antigenic site V. Their epitopes were further mapped by XL-MS. Several VHH residues were cross-linked to different FMDV residues that are located at a distance that far exceeds the 11.4 Å length of the spacer arm of the DSS cross-linker used. This could be due to the VHH-FMDV complexes adopting different conformations. Such flexibility was earlier reported for complexes of FMDV with VHHs M8 and M170 (10) or integrin receptors (41). FMDV structural flexibility probably also explains the cross-linking to residues that are not surface exposed in the capsid 3D structure. Structural flexibility is more often reported for other picornaviruses than FMDV. Poliovirus undergoes a global conformational change upon receptor binding, characterized by a 4% expansion. These 135S particles can adopt further different conformations (42). Cross-linked FMDV residues are not necessarily making contact with the VHH. Due to the use of a chemical cross-linker with a relatively long 11.4 Å spacer arm, residues that are quite far apart can be cross-linked (43). Such effects can at least partly explain the relative large area covered by the M691F or M702F footprints identified by cross-linked residues as compared to the M170 epitope resolved by cryo-EM (Figure 6). Furthermore, the M691F residues cross-linked to the VP1 GH-loop are probably closer to the VP3 cross-linked residues than displayed in Figures 5A, 6E since here the reduced O1K GH-loop was used, which lies flat on the virion surface. Nevertheless, the footprints of M691F and M702F clearly overlapped with the M170 epitope, and included cross-links to the VP3 BC-loop that showed a different conformation in 12S and 146S particles, explaining the 146S-specific binding of M170 (10). Most notably, we believe all cross-links observed are located on a single pentamer without crossing the 2-fold symmetry axis, on an adjacent pentamer (Figure 3), because no cross links were found to VP2 residues close to the 2-fold axis opposing the VP3 cross-linked residues of an adjacent 12S pentamer. Thus, 146S specific binding of M691F and M702F is unlikely caused by dissociation of the epitope due to dissociation of 146S- into 12S particles and more likely due to altered structures of the epitopes due to 146S particle dissociation, similar to M170. This most likely also applies to mAb 9. The bovine mAb R55 neutralizing A/WH/09 was recently described (44). The cryo-EM structure of the FMDV-R55 complex showed R55 binding to two adjacent pentamers close to the 3-fold axis. The cross pentamer binding was strongly dependent on VP3-E70 and caused virus neutralization due to prevention of virus dissociation and genome release. The binding of mAb R55 to 12S particles was not previously reported.

The site recognized by 146S-specific VHHs M170, M691F, and M702F overlaps with antigenic sites 2 and 4, which lie close together (Figure 7) and are known to interact (15). In addition to sites 1-5, a novel neutralizing site was reported for type O, encompassing VP2-74 and 191 (16) and VP3-85, 116 and 195 (38), that is conserved in type A (17). This site is also located close to sites 2 and 4, and close to the 3-fold axis (Figure 7). The 146S-specific binding of mAbs is often not thoroughly discussed in literature on FMDV antigenic sites even when such data are present (9, 22). Two mAbs against type A FMDV that recognize the same antigenic site were specific for 146S particles and had escape mutations at VP1-H201 (mAb 2PE4) or VP3-T178 (mAb 2PD11) (18). Due to insertions/deletions between types A and O, these residues correspond to VP1-202 and VP3-177 in type O, which are contact residues for M170 (Figure 6D). MAb C8 and mAb C9 against type O recognize independent antigenic sites and were specific for 146S particles (45). They were later (13) found to have escape mutations at VP2-72 (C9) and VP1-43 and 44 (C8), which corresponds to sites 2 and 3, respectively (Figure 7). MAb S11B that binds type O site 3 also appears to be 146S specific (46). Thus, for type O, also two independent antigenic sites exist that are 146S specific, as we observed also for type A. The 146S-specific binding by mAbs against site 3 is surprising since it is distant from the 2-fold symmetry axis (Figure 7).

M702F was cross-linked to VP3 residues T115, R120 and T191 at the 2-fold symmetry axis surface that is accessible in a 12S particle, but not in a 146S particle. Holes in the viral capsid have earlier been observed at the 2-fold axis of other picornaviruses, including poliovirus 135S particles (42) and acidified Seneca Valley virus, which resulted in a major reconfiguration of the pentameric capsid assemblies, resembling a potential uncoated intermediate (47). The cross-linking of an 146S-specific VHH to residues that are hidden at the 2-fold symmetry axis of rigid 3D models of 146S particles is probably again explained by flexibility of virions in solution, with holes at the 2-fold axis. Such cross-linking to residues buried at the interface of two 12S particles is not observed for M691F. Possibly, the difference in recognition of full and empty particles by M691F and M702F (8) relies on structural differences between these particles at the 2-fold symmetry axis.

Conclusions

We have shown for type A FMDV that both 12S and 146S particle specificity relies on at least two independent antigenic sites. The major 146S specific antigenic site that is also recognized by the VHHs M691F and M702F was located close to the 2-fold and 3-fold symmetry axes on a similar position as the type O 146S specific VHH M170. Since this site was located on a single 12S particle the 146S-specific binding of M691F and M702F is probably also caused by different conformations of 12S and 146S particles, as earlier suggested for M170 (10). The cross-linking of FMDV residues that are not surface exposed in 146S particles (M691F and M702F), or only surface exposed in 12S particles but not in 146S particles (M702F) suggests that FMDV particles are more flexible than suggested by rigid cryo-EM or crystal structures. Much research is done on making FMDV VLPs for use in vaccines. The increased knowledge of particle specificity of VHHs can be used for further improving production of VLPs with enhanced immunogenicity.

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.

Author contributions

AD, MH, and WP designed and coordinated the study. AD and SS performed grant acquisition and financial reporting. HL and MH performed the experiments. The XL-MS study done at Coval X was coordinated by AD and MH. HL, MH, SS, and AD completed data analysis. MH wrote the first version of the manuscript, that was subsequently edited by HL, AD, and WP. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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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.2022.1040802/full#supplementary-material

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EDITED BY
Andres M. Perez,
University of Minnesota Twin Cities,
United States

REVIEWED BY
Theo Knight-Jones,
Tanzania Livestock Research Institute (TALIRI), Tanzania
Gustavo Machado,
North Carolina State University,
United States

*CORRESPONDENCE
Manuel Jose Sanchez-Vazquez
✉ sanchezm@paho.org;
✉ manueljsanchezvazquez@gmail.com

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Advances in the eradication of foot-and-mouth disease in South America: 2011–2020

Alejandro Mauricio Rivera, Manuel Jose Sanchez-Vazquez*, Edviges Maristela Pituco, Lia Puppim Buzanovsky, Monica Martini and Ottorino Cosivi

Pan American Center for Foot-and-Mouth Disease and Veterinary Public Health (PANAFTOSA/VPH), Pan American Health Organization, Regional Office for the Americas of the World Health Organization, Rio de Janeiro, Brazil

For more than 70 years, the countries of South America have been attempting to eliminate foot-and-mouth disease (FMD), but a regional strategy had not been established by all the affected countries until 1988. The Action Plan 1988–2009 of the Hemispheric Program for the Eradication of Foot-and-Mouth Disease (PHEFA 1988–2009) resulted in an FMD-free status in 88.4% of the bovine population of South America. However, countries of the Andean sub-region maintained an FMD endemic. In addition, sporadic outbreaks in vaccinated cattle populations have been reported in countries of the Southern Cone, endangering the disease-free status in these countries. Within this context, the PHEFA 2011–2020 was approved to eliminate FMD from the subcontinent, and this review describes the most important milestones during its execution. FMD in Ecuador and sporadic outbreaks in the Southern Cone sub-region were effectively eliminated. The outbreaks that occurred in Colombia in 2017 and 2018 were successfully controlled. The type C virus was removed from the vaccines in use in most countries, based on a risk assessment. This review also describes the progress made by the countries advancing toward official recognition as FMD-free in all their territories, with Bolivia, Brazil, and Peru leading the progressive suspension of vaccination to achieve FMD-free status without vaccination. Consequently, at the end of PHEFA 2011–2020, Venezuela was, and still is, the only country in the region whose control program has suffered setbacks, and no evidence has suggested that the transmission and infection of the bovine population have been eliminated. At the end of 2020, a new PHEFA Action Plan 2021–2025 was approved with a five-year horizon, to complete the eradication of the disease in the Americas.

KEYWORDS

foot-and-mouth disease, disease elimination, hemispheric program for the eradication of foot-and-mouth disease (PHEFA), foot-and-mouth vaccination, foot-and-mouth control program

Introduction

Foot-and-mouth disease (FMD) was introduced in South and North America in 1879, with two different outcomes. While in North America the sporadic occurrence of outbreaks resulting from imports of animals and products was confronted with an elimination strategy by stamping-out and quarantining, in South America the infection spread to bovine populations in all affected countries (i.e., Argentina, Bolivia, Colombia, Chile, Brazil, Paraguay, Peru, Uruguay, and Venezuela) (1). This spread was enabled by the expansion of extensive bovine livestock farming, colonizing large territories in South America, leading to a very active livestock movement network (1).

The Pan American Center for Foot-and-Mouth Disease, PANAFTOSA-PAHO/WHO established in 1951, which initially focused on laboratory diagnosis, characterization of FMD epidemiological areas, identification of the virus strains, development of vaccines, and delivering training and technical cooperation to the affected countries was a game changer for the control of FMD in South America. In the 1970s, the South American Commission for the Fight against Foot-and-Mouth Disease (COSALFA) was established as the high-level technical and political mechanism to coordinate national plans for the control of FMD in South American countries (1). The COSALFA delegates from each country (both from public and private sectors) meet annually to assess the progress made and address regional issues, such as establishing priorities for PANAFTOSA-PAHO/WHO technical cooperation (e.g., to focus on specific countries), technical recommendations (e.g., those to progress toward FMD status without vaccination or risk assessment), and logistics tools (e.g., vaccine banks). By the end of the 1980s, the Hemispheric Committee for the Eradication of Foot-and-Mouth Disease (COHEFA) was created, and it approved the Hemispheric Program for the Eradication of Foot-and-Mouth Disease (PHEFA), which provides a strategic framework to coordinate the eradication efforts of national plans in the six sub-regions of the American continent (1). The program relied on the extensive knowledge gained on the natural history of the disease and its determinants in South America, being characterized in four sub-regions (Figure 1). Bovine production systems and movement patterns determined the historically observed disease presentation and its dissemination; therefore, PHEFA promoted a control strategy based on reducing the susceptibility of bovine populations to the infection by means of systematic mass vaccination campaigns, together with strict animal movement control and response to outbreaks in all the affected countries of South America (1–3).

The first PHEFA Action Plan extended from 1988 to 2009, and although it did not eradicate the disease, it made significant progress: approximately 85% of the South American bovine population was recognized as free from FMD, with or without vaccination. Nevertheless, some areas still experienced FMD endemics (1). For example, in the Andean sub-region, regular FMD outbreaks were observed in Ecuador and Venezuela. Furthermore, the FMD-free status of Argentina, Bolivia, Brazil, and Paraguay, was suspended due to the sporadic occurrence of FMD outbreaks from 2002–2006, after the 2000–2001 epidemic (1), which jeopardized their FMD-free status with vaccination. Although the states of the northern region of Brazil, as well as the Amazon and Bolivian valleys exhibited a long period without FMD outbreaks, they had not demonstrated the absence of virus circulation, failing to achieve FMD-free status (1).

Therefore, in 2010, COHEFA approved a new action plan for 2011–2020 to complete the eradication process in South America, as the contribution of the Americas to the Global Foot-and-Mouth Disease Control Strategy, fostered by the Food and Agriculture Organization of the United Nations (FAO)/World Organization for Animal Health (WOAH) launched in 2012 (1, 4, 5). The involved countries seek international recognition of the progress made toward eradication within their territories through the WOAH free status recognition process (6). Recognition of FMD-free status by the WOAH reflects advances made by the countries in their wider capacity to control diseases of livestock and provides a tool to enable access to international markets.

This review aims to describe the key milestones that characterized the implementation of the second PHEFA action plan between 2011 and 2020, namely, FMD elimination in Ecuador, FMD elimination in Paraguay following the 2011 outbreaks, FMD elimination in Colombia after the 2017 and 2018 outbreaks, the epidemiological situation in Venezuela, the evidence to not having experienced a type C outbreak since 2004 allowing the suspension of vaccination against this serotype, progress toward WOAH recognition of FMD-free status in more zones within the continent, and transitioning toward more zones achieving FMD-free without vaccination status. Figure 2 presents a timeline highlighting the key milestones that are addressed in this review.

Elimination of FMD in Ecuador

By the end of the PHEFA Action Plan 1988–2009, Ecuador was experiencing epidemic outbreaks of FMD throughout the country, caused by type O virus, due to the poor implementation of vaccination campaigns and animal movement control (7).

In their phylogenetic analysis of the type O FMD viruses circulating in the Andean sub-region of South America in 2002 and 2008, Malirat et al. (8) characterized 11 different lineages. Virus isolates grouped within lineage 1 were mostly native to Ecuador, and some virus isolates collected from outbreaks that occurred in Colombia and Peru corresponded to virus incursions from Ecuador (8). Within lineage 1, nine subgroups were identified, corresponding to virus isolates collected during

FIGURE 1
PHEFA South American sub-regions.

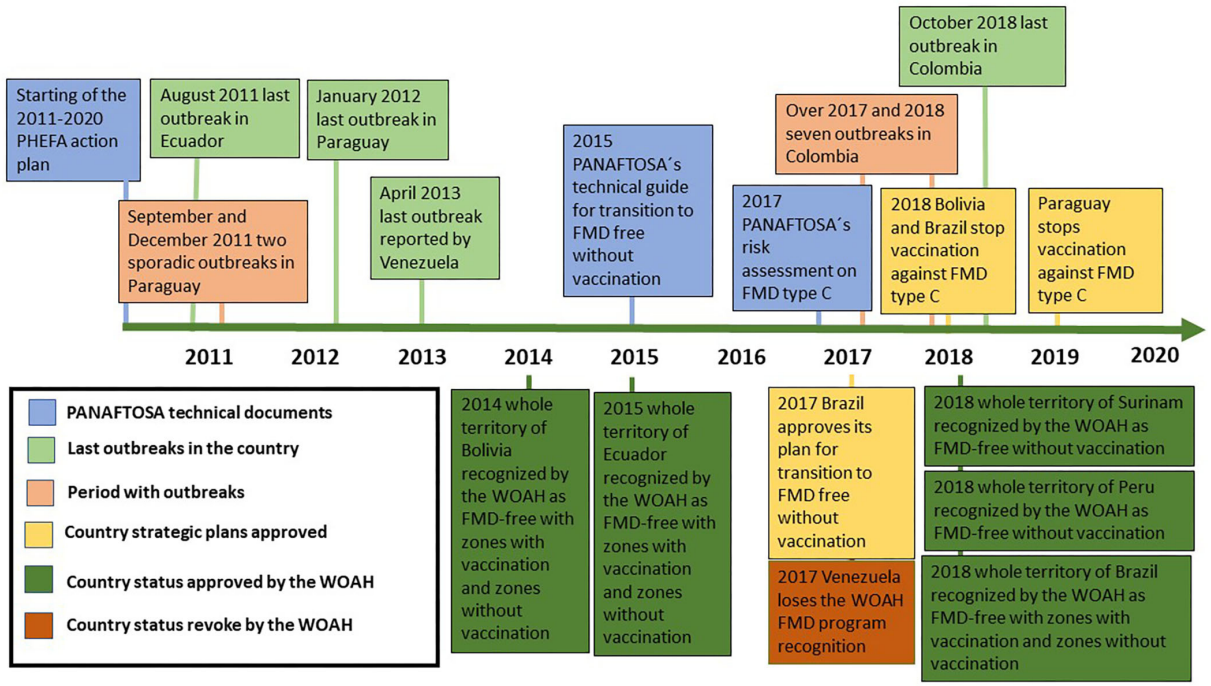

FIGURE 2
Timeline of key events and milestones during the implementation of PHEFA 2011–2020.

outbreaks that occurred in different provinces and years, with divergence values of 6–14% (8). Within this period, no virus isolates belonging to other lineages from other serotype O viruses circulating in the Andean sub-region were documented, suggesting that the occurrence of FMD in Ecuador resulted from endemic virus transmission in its bovine population along with epidemic cycles (8).

The FMD epidemic observed from 2009 to 2010 prompted the response of international cooperation, led by PANAFTOSA-PAHO/WHO, which collaborated with the veterinary authorities of Ecuador in a full review of the FMD control program, which was under the responsibility of a private sector entity. The technical cooperation of PANAFTOSA led to a major change in the management, responsibilities, and control and monitoring mechanisms of the FMD program, complemented with good vaccination practices and farm registry management, which resulted in a rapid decrease in the incidence of FMD. The last five FMD outbreaks in Ecuador were documented in 2011 (7) (Figure 3).

A controversy emerged with *in vivo* and *in vitro* laboratory analyses performed on virus isolates obtained during the 2009 epidemic to predict the effectiveness of commercial vaccines against field virus strains. Maradei et al. (9) reported that *in vitro* vaccine matching studies, carried out by virus neutralization tests (VNTs), suggested a loss of protective response, which was supported by *in vivo* studies using the Protection against Podal Generalization test in cattle.

Duque et al. (7) observed that on the virus isolates from Ecuador in 2010, while the "r" values of the antigenic correlation between the field isolate and the strain of the vaccine were in the low range of the predictive scale for protection, the results of both the Expected Percentage of Protection (EPP) test developed by PANAFTOSA and the Protection against Podal Generalization test showed that protection was satisfactory (90%) in revaccinated animals but not in cattle that received just the first dose (approximately 50% of protection). PANAFTOSA (10) observed that the different methodologies and interpretations of vaccine matching studies explained the discrepancies in laboratory conclusions, but they were not consistent with the epidemiological situation observed in the field, both in the country and in the borders of neighboring countries. Therefore, decisions about changes in the composition of vaccines could not yet be made. As a result, biannual vaccination campaigns were supplemented for all cattle in continental Ecuador with booster vaccination for young animals on at-risk premises located in areas of extensive livestock farming, along with vaccine quality assurance and the implementation of good vaccination practices and effective control of animal movement (11).

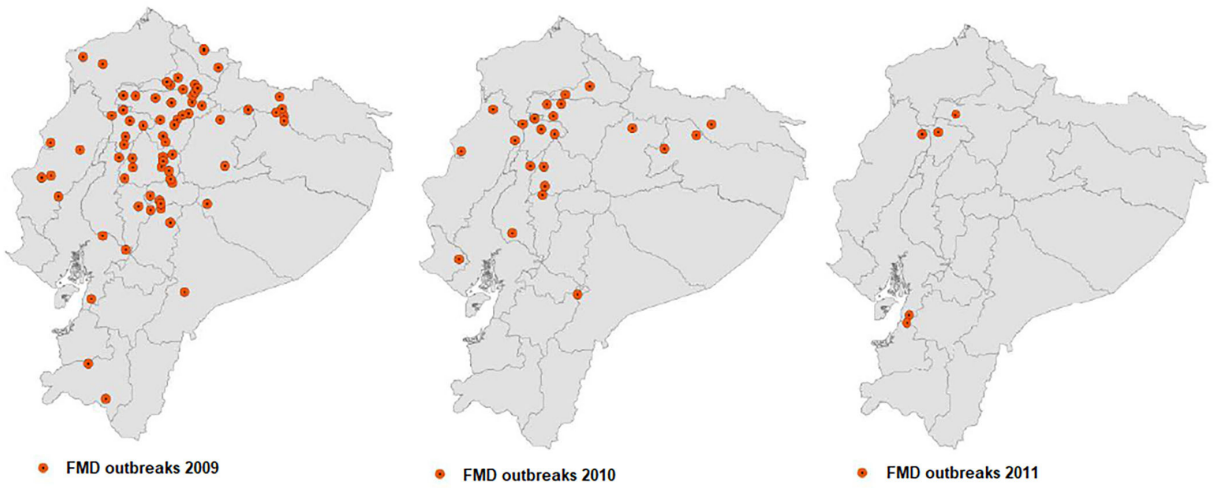

FIGURE 3
Location of the FMD outbreaks in Ecuador: 109 reported in 2009, 42 in 2010, and 5 in 2011.

In August 2011, the last case of FMD was documented, and the continental region of Ecuador achieved in 2015 disease-free status with vaccination, according to the WOAH, upon completion of serological studies showing the absence of virus transmission, while the Galapagos Islands achieved recognition as FMD-free without vaccination. Since then, Ecuador has been implementing serological surveys annually to check the immune status of vaccinated populations and confirm the absence of virus circulation to maintain its FMD-free status (12).

The 2011 FMD outbreaks in Paraguay

At the start of PHEFA 2011–2020, the countries of the Southern Cone sub-region (i.e., Argentina, Bolivia, Chile, Paraguay, and Uruguay) had systematic vaccination programs in place and were recognized as FMD-free (1). However, in the previous period (1), sporadic recurrences of FMD outbreaks caused by the type O virus were observed in border territories of Southern Cone countries already recognized as free from FMD with vaccination, which affected Paraguay (2002 and 2003), Argentina (2003 and 2006), and Brazil (2005) (1).

Malirat et al. (13) described that the type O viruses responsible for these sporadic outbreaks from 2005–2006 in border areas of the Southern Cone countries not only corresponded to the Europe-South America topotype of the FMD virus but also showed a close phylogenetic (>90%) similarity. Therefore, these viruses were grouped within a single lineage with FMD type O viruses isolated during the epidemic outbreaks of 2000 that occurred in Brazil, Argentina, and Uruguay as well as in outbreaks that occurred in Brazil in 1998 and Bolivia in 2000, 2001, and 2003. This lineage was substantially different from that of the other circulating viruses in the Andean sub-region and in other geographic regions of South America.

An FMD outbreak was detected in September 2011 in a bovine herd in the department of San Pedro in central Paraguay. The virus was classified as type O and belonged to the Europe-South America topotype of the FMD virus, and phylogenetic analysis confirmed that it was the same lineage of previous isolations made in the Southern Cone sub-region (14, 15). The outbreak was controlled with measures including a stamping-out policy in addition to emergency vaccination in the control areas without the identification of secondary outbreaks associated with the index case. However, by the end of December 2011, a new infected herd was detected in the periphery of the controlled areas, which led to the reimplementation of sanitary measures for its control and elimination (16, 17). In both outbreaks, evident clinical signs of the disease were observed in young animals. The primary focus of those cases could not be determined, and the investigation showed no relationship between the two outbreaks. Nevertheless, the evidence that the virus acting in these outbreaks belonged to the same lineage of virus O circulating in the Southern Cone, at least since 1998, that had also been isolated in the outbreaks of 2002 and 2003 that had occurred in Paraguay, suggested that viral transmission was maintained in the vaccinated population due to the existence of endemic niches in the territory that were not detected by the surveillance system. Caporale et al. (18) suggested that when the proportion of immunized animals in a population does not reach a minimum value to block virus transmission, the herd immunity level is too high to enable an epidemic occurrence but too low to eliminate virus circulation, resulting in an endemic niche of infection, which is probably clustered in a

particular production system or localized in marginal areas. This endemic niche could sporadically result in an FMD outbreak due to changes in the equilibrium between the virus and the population or when animals of these endemic niches are moved to areas where animals are not vaccinated or have low levels of vaccination coverage.

Following the end of the outbreak in March 2012, a full review of the national FMD program in Paraguay was carried out particularly of the vaccination program, with the support of PANAFTOSA-PAHO/WHO and the member countries of the Permanent Veterinary Committee of the Southern Cone (17). Several deficiencies and gaps detected in the national FMD program were addressed, which included booster vaccination targeted at all bovines under 1 year of age. Thus, as of 2012, the annual vaccination schedule included two general vaccination cycles targeted at all bovines and buffaloes, and one booster cycle for all young animals administered 30 days after the first general cycle. Maradei et al. (15) conducted tests that estimated the protection given by the vaccine strain, O_1 Campos, from the vaccines in use against the virus isolated in Paraguay in 2011 and observed low protection (48.9%) 30 days post-vaccination in estimates from the EPP tests. However, the Podal Generalization tests performed 79 days after vaccination and 79 days after revaccination, showed an estimated protection of 75.0 and 87.5%, respectively. Furthermore, the Reference Laboratory of PANAFTOSA-PAHO/WHO estimated the immune coverage of the O_1 Campos vaccine strain against the strain isolated in Paraguay in 2011 in the EPP test using ELISA-CFL to be 78.99% 30 days after vaccination and 99.70% 30 days after revaccination (14).

Annual FMD surveillance aimed at detecting the disease was also supplemented in 2014 with serological studies to determine the prevalence of post-vaccination antibodies and intended to estimate immune protection in different categories of bovines (19). Since then, these studies have been conducted annually throughout Paraguay to maintain a high immune level in the bovine population. As a result, these studies have improved vaccine coverage, encouraged good vaccination practices, and provided detailed information of the immune status of the population. As of 2012, no new cases of FMD had been detected in Paraguay or in the Southern Cone countries, demonstrating the elimination of infection and endemic niches that caused the sporadic outbreaks observed until 2011 (12).

Outbreak of FMD in Colombia

Since Colombia achieved FMD-free status, it had experienced sporadic transboundary incursions of the FMD virus on the border with Venezuela in 2004 and 2008 and on the border with Ecuador in 2009. In 2011, a small zone in the northwest region of Colombia was recognized as FMD-free without vaccination, while the rest of the country was FMD-free with vaccination, except for a protection zone including parts of the departments bordering Venezuela. Since Ecuador achieved FMD-free status in 2015, the transboundary risk of FMD introduction was limited to the eastern border shared with Venezuela. Moreover, Colombia and Venezuela share a similar bovine husbandry system on both sides of the border.

In June 2017, Colombia reported an FMD outbreak in the department of Arauca, bordering Venezuela. The investigation of this outbreak presumed that the source of infection was the smuggling of infected animals from Venezuela. In the same month, a second outbreak was reported in a mountain area in the department of Cundinamarca, in the center of the country, which affected several small herds. Later, in July, a third outbreak was confirmed in a small bovine herd in the same department, 134 kilometers from the second outbreak (20). The two outbreaks reported in the department of Cundinamarca were suggested to be related to contaminated meat products introduced by Venezuelan immigrants, which might have been used as swill feeding in pigs (20), although no clear evidence supported this hypothesis. The occurrence of FMD that year ended up with the detection of a fourth outbreak in the protection zone, very close to the border with Venezuela, which was also related to the illegal entry of animals from this country. Figure 4 illustrates the distribution of the 2017 and 2018 outbreaks that occurred in Colombia.

The outbreaks that occurred in Arauca and the protection zone were considered to be unrelated to each other or to those that occurred in Cundinamarca; instead, they were considered different virus incursions coming from the same country.

The phylogenetic analysis of virus isolates obtained from the four outbreaks conducted in the WOAH Reference Laboratory of PANAFTOSA-PAHO/WHO confirmed that all of these isolates belonged to lineage 6 of the type O virus, according to the classification of virus genotypes circulating in South America proposed by Malirat et al. (8). Specifically, lineage 6 isolates were identified in FMD outbreaks that occurred both in Venezuela and in bordering departments of Colombia between 2003 and 2009; therefore, the virus isolates of 2017 are consistent with a genotype that has been circulating in the north of the Andean sub-region, at least since 2003.

The outbreaks were controlled according to the standard measures applied by FMD-free countries with vaccination in South America: imposing quarantines and sanitary control zones, applying stamping-out to all animals in the affected herds followed by cleaning and disinfection, increasing surveillance for the detection of new cases, and performing an epidemiological investigation to determine the origin and the relationship between outbreaks. These measures enabled the establishment of a containment zone that included the three outbreaks in the FMD-free zone, which was recognized by WOAH in December 2017, enabling the official FMD-free status to be restored for the rest of the country (20).

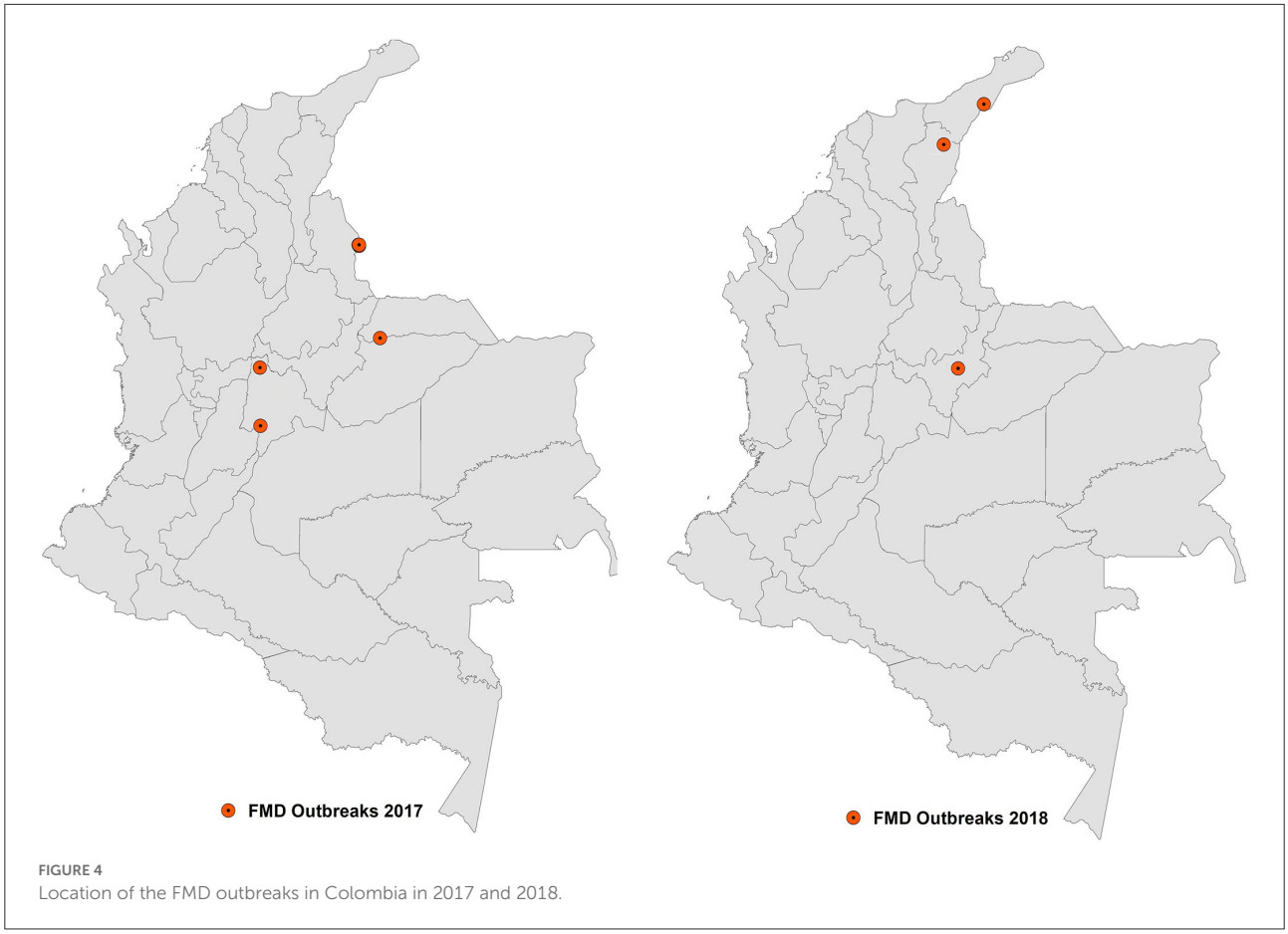

FIGURE 4
Location of the FMD outbreaks in Colombia in 2017 and 2018.

In September 2018, a new outbreak was confirmed in the department of Boyacá in the containment zone caused by the type O virus. This recurrence caused the FMD-free with vaccination status of the country to be suspended. This outbreak could not be associated with a new virus incursion from Venezuela, and it was likely the result of remaining infection transmission within the containment zone. Additionally, in October of the same year, two new outbreaks were detected very close to the border with Venezuela in the departments of El César and La Guajira, both located outside of the containment zone. These two outbreaks were considered to be the result of two incursions of the FMD virus associated with the illegal entry of infected animals from Venezuela (20). Virus isolates obtained in the outbreaks of 2018 belonged to lineage 6, according to the classification proposed by Malirat et al. (8) and showed a high level of homology with isolates from 2017 (21).

The recurrence of the disease led to a full review of the FMD control and prevention strategy in Colombia. The information obtained through population immunity studies was key to the review. Post-vaccination monitoring of the whole bovine population located in the containment zone was carried out to assess its immune status. Although the serological study revealed an overall prevalence of animals protected against FMD type O virus of 78% (95% CI 77.0–80.0%), 5 to 6 months after the last vaccination cycle, small herds of some departments included in the containment zone showed a level of protection significantly lower than expected, which may have led to the establishment of niches facilitating virus transmission after reintroduction. Vaccine matching tests against isolates obtained in 2018 with EPP using the VNT and ELISA-CFL tests performed by the WOAH Reference Laboratory of PANAFTOSA-PAHO/WHO showed that the vaccine provided protection equivalent to 99.90% in a panel of sera obtained 30 days after vaccination and 99.99% in a panel of sera obtained 30 days after revaccination (21). After the second vaccination cycle of bovines conducted in October and November 2018, an additional vaccination cycle was conducted in January and February 2019 for the entire bovine population located in the departments where the FMD outbreaks occurred in 2017 and 2018.

In March and April 2019, four cross-sectional serological surveys were conducted on the bovine population. Three of them covered the entire vaccinated bovine population in the national territory with its official FMD-free status suspended, and one covered the protection zone. Tests to detect virus transmission and assess the apparent prevalence of FMD protective antibodies were performed. The selection of sampling units was carried out in two phases, and samples were randomly distributed and stratified according to the size of the herd. The

three surveys conducted in the territory with official FMD-free status suspended estimated levels of protection against the virus between 83.7 and 95.7% for type O virus and between 80.1 and 94.2% for type A; similar results were observed, regardless of the size of the herd. No evidence of virus transmission was observed (22).

In February 2020, the WOAH Scientific Commission recommended the reestablishment of FMD-free status with vaccination with a subdivision of the area in four FMD-free areas to reduce the impact of new potential virus incursions from Venezuela (22). One booster vaccination cycle targeted at young animals in the two FMD-free zones bordering Venezuela was also suggested, along with a reinforcement of police action to mitigate the risk of illegal movement and entry of animals and livestock products (23).

Control of FMD in Venezuela

Venezuela, Colombia, and Ecuador reported their first FMD outbreaks in 1950, 1951, and 1956, respectively and were affected by type O and type A viruses (24). Between 2001 and 2010, Venezuela exhibited an annual average of 24 FMD outbreaks, ranging from 3–63 outbreaks. As of 2006, the vaccination program was strengthened with a social plan, in which the state covered, free of charge, the vaccination of the bovine population of small stockbreeders, who had been historically excluded from the two annual vaccination cycles, which led to an increase in population immunity (25). The improvement of vaccination coverage in the population was reflected in the reduction in the number of annual outbreaks since 2009, with the last FMD outbreak reported in the state of Barinas in April 2013. Furthermore, the Venezuelan Program for the Control and Eradication of Foot-and-Mouth Disease was validated by the WOAH in 2015 (26). In the same year, a random cross-sectional sampling of herds and bovines was carried out to evaluate the prevalence of post-vaccination antibodies in two states in the southwest of the country, and the sampled population showed a satisfactory level of protection consistent with the vaccination frequency of the control program in the different categories of sampled bovines (19).

However, in 2016, Venezuela reported to COSALFA that the provision of vaccines, particularly those for the social plan, would be restricted due to the critical economic situation of the country that year (27). In 2017, the WOAH withdrew validation of the FMD program from Venezuela (28).

The deterioration of the vaccination program can be observed by comparing the average number of vaccinated bovines by year. In 2015, 15,448,097 bovines (average of the two annual vaccination cycles) were vaccinated during the vaccination cycles, corresponding to a coverage >90% of the bovine population (29), whereas in 2020, an average of only 6,358,255 bovines were reported to have been vaccinated, indicating a coverage <50% (12). However, the absence of reports of FMD outbreaks since 2013 has a high degree of uncertainty, as the surveillance system has experienced increasing limitations that compromise its sensitivity and coverage. Moreover, serological studies have not been conducted to detect virus transmission in vaccinated bovine populations. Since 2021, efforts have been made between the public and private sectors, jointly with international cooperation, to reestablish the control program.

Verifying the elimination of the type C FMD virus

The occurrence of FMD caused by the type C virus in South America was described by Saraiva and López (30), and more recently by Sanchez-Vazquez et al. (31), while its phylogenetic evolution in the subcontinent was described by Paton et al. (32). In 2016, 12 years after the last case of FMD caused by the type C virus, only four countries in South America (Argentina, Bolivia, Brazil, and Paraguay) kept the type C virus in their vaccines in use. Therefore, COSALFA requested PANAFTOSA-PAHO/WHO to carry out a risk assessment, at the regional level, to estimate the risk of serotype C persistence, recommending the applicable risk-management measures.

The risk assessment considered that the infection caused by the type C virus in the vaccinated bovine population might naturally occur *via* 3 routes: the environment, wild animals, and carrier cattle or endemic niches. Additionally, the risk of non-detection by surveillance activities carried out by veterinary services was evaluated (33). The probability of persistence of the type C virus in the environment was considered negligible due to the time elapsed since the last outbreak. The probability of persistence of the type C virus in wild animals was also considered negligible since no role of wild animals as reservoirs of FMD virus had been demonstrated in the South American subcontinent (33).

Because the bovine species has played the main role in maintaining and disseminating FMD in South America, evaluating the risk of release of the type C virus by carrier animals and in endemic niches of infection was of interest. Although several research projects have focused on identifying the role of carrier animals in the dissemination of FMD, it was not possible to determine whether they played a significant role in the transmission of the infection (34), and considering the time elapsed since the last outbreak caused by the type C virus, the risk was considered negligible. Moreover, evaluating the risk of persistence of endemic niches of infection is important, due to the evidence of the occurrence of sporadic FMD outbreaks caused by a genotype of the type O virus in FMD-free countries with vaccination of the Southern Cone sub-region. However, considering that presentation patterns of the type C virus are characterized by a lower prevalence of outbreaks, a more

limited geographic distribution, and different temporal patterns compared to those observed with type O and type A viruses, lower transmissibility than the other virus types is suggested. Additionally, type C virus is likely to have a higher response to systematic vaccination programs, as demonstrated by the absence of recurrence of infection after withdrawal of the vaccine or the removal of the type C virus from vaccines, three to 6 years after the last outbreak. This was the case in Chile (1980), Uruguay (1994), Argentina (1999), Peru (2001), and the state of Santa Catarina in Brazil (2001). Therefore, the probability of endemic niches of infection caused by the type C virus in vaccinated populations was estimated to be negligible.

Finally, the risk of not detecting infection in vaccinated populations was assessed based on the information gathered by both passive and active surveillance systems since 2004. Clear evidence has suggested that the passive surveillance system implemented by the veterinary services in South America reaches all territories with susceptible animal populations and is specialized in attending to all cases with a suspected vesicular disease, which is supplemented with active surveillance actions, mainly regular serological surveys for the detection of virus transmission (33). Therefore, the evidence gathered by the combined passive and active surveillance systems provides high confidence of the condition of being free from infection and suggests that the likelihood of not detecting an infection caused by the type C virus in FMD-free countries with vaccination is negligible. Consequently, PANAFTOSA-PAHO/WHO suggested the suspension of the inclusion of the type C virus in the vaccines and a specific risk mitigation strategy for the stocks of the type C virus in vaccine manufacturing laboratories and diagnostic virology laboratories in the region (33).

The inclusion of the type C virus in the vaccines in use was suspended by Bolivia and Brazil in 2018 and by Paraguay in 2019 (20, 22). Argentina, which had decided to reintroduce the type C virus in the vaccines in 2004 due to the FMD outbreak in the Brazilian Amazon, still maintained this virus type in the vaccines used in the national program through the end of 2021.

Transition to FMD-free status without vaccination from 2011–2020

The PHEFA considers the status recognized by the WOAH as the milestone to achieve disease eradication in the affected countries of South America. In 2010, 56% of herds and 81% of bovines and buffaloes were in countries and zones recognized as FMD-free with vaccination, and only 6.8% of herds and 3.4% of bovines and buffaloes were in countries and zones with FMD-free status without vaccination (4). However, 34% of herds and 15% of the bovine and buffalo population of South America had no official recognition of their FMD status. This latter group with no official recognition included Ecuador and Venezuela, which at that time were experiencing an endemic occurrence of FMD, as well as the departments of the Altiplano, Los Valles y Llanos Orientales of Bolivia, the north and northeast regions of Brazil, departments of the north and northwest of Peru, and Suriname, which had no occurrence of FMD (4).

Bolivia had recorded its last case of FMD in 2007 and had achieved the recognition of two isolated zones as FMD-free with vaccination in the Altiplano y Llanos Orientales. In 2011, the progressive official recognition of the regions of Bolivia began, and in 2012, the departments that made up the Bolivian Altiplano were recognized as FMD-free without vaccination. In 2013, a zone including the regions of Chaco and Los Valles was recognized as FMD-free with vaccination, a status which spread to the rest of the country in 2014 (11, 13). In 2018, Bolivia suspended vaccination in the department of Pando, which was recognized as FMD-free without vaccination the following year. Furthermore, vaccination was suspended in the rest of the departments with FMD-free with vaccination status in 2019, except for the department of Santa Cruz. However, until the end of 2021, no actions had been taken to achieve the official recognition of these departments as FMD-free without vaccination.

In May 2013, Peru achieved the status of being FMD-free with vaccination in a region located in the north of the country bordering Ecuador. This recognized zone was used to serve as a protection zone for the rest of the country, since at that time Ecuador was a country without recognized health status. Concomitantly, Peru completed the requirements to gain the status of FMD-free without vaccination in a zone that included departments of the northeast of the country, where vaccines were no longer used and the last FMD outbreak had occurred in 2004, thus achieving recognition as FMD-free throughout the country (35). In 2017, Peru suspended the use of vaccines in the FMD-free zone located in the north of the country and, in 2018, the whole country was recognized as FMD-free without vaccination (36).

In 2014, Brazil extended its official recognition as FMD-free with vaccination to a zone that included seven states of the northeast region and part of the state of Pará, where the last outbreak had occurred in 2003 (11). In 2018, the whole country was recognized as FMD-free when official recognition was achieved for the states of the northern region, Amazonas, Roraima, Amapá, and part of the state of Pará (36). The last outbreak in that zone had been documented in 2004 (37).

In 2017, Brazil approved the Strategic Plan 2017–2026 of its National Foot-and-Mouth Disease Prevention and Eradication Program, which established a schedule for the transition to the status of FMD-free without vaccination by means of the progressive suspension of the vaccine in the 5 blocks in which the 25 states and the federal district of the country with vaccination had been grouped (38). The goal was to recognize the whole country as FMD-free without vaccination by 2023. In

2019, the use of vaccines was suspended in the states of Paraná, Acre, Rondonia, and a group of municipalities in the states of Amazonas and Mato Grosso to which the state of Rio Grande do Sul was added at the beginning of 2020. In 2021, these zones were recognized as free without vaccination, which along with Santa Catarina (recognized as FMD-free without vaccination since 2007) comprised 20% of the bovine population of the country (23). Consequently, by the end of 2021, 1,945,161 herds (35.9% of the total) and 57,372,953 bovines and buffaloes (15.5% of the total) were in FMD-free countries or zones without vaccination in South America (12).

Suriname, a country that had never recorded an FMD outbreak, achieved all the necessary requirements for recognition as FMD-free without vaccination in 2018 (36).

In 2015, COSALFA confirmed that no new outbreaks of FMD had occurred for 3 years in the South American territories that were FMD-free, and the last stage of the PHEFA began. Thus, COSALFA approved a technical guideline with methodologies that would allow the FMD-free countries with vaccination to evaluate the risks for making a safe transition to FMD-free status without vaccination while reducing the vulnerabilities in their animal defense systems to preserve the FMD-free status (39).

The PHEFA action plan 2021–2025

By the end of the PHEFA Action Plan 2011–2020, the percentage of South American territory officially recognized as FMD-free had increased from 67.6% in 2011 to 95.1% by the end of 2020. Moreover, the herds in FMD-free countries and zones that accounted for 63.7% of those in South America at the beginning of the Action Plan 2011–2010 increased to 98.6%, and the percentage of the bovine and buffalo population in FMD-free countries and zones increased from 84.4 to 95.8%. Nearly 1.4% of the herds and 5% of the bovine population of South America remained without sanitary recognition, including the whole territory of Venezuela in which, although no cases have been reported since 2013, the elimination of virus transmission has not been verified in the vaccinated population (39). Likewise, North America, Central America, and the Caribbean have not documented the occurrence of FMD outbreaks during the whole period as a result of an FMD prevention policy characterized by a high level of protection (40).

By the end of 2020, the risk of FMD was confined to the north of the Andean sub-region, as confirmed by phylogenetic studies conducted by Malirat et al. (8, 13), which found evidence of the circulation of specific lineages of FMD viruses in bovine populations restricted to certain sub-regions of South America, with no historic evidence of their presence in other sub-regions, thus reflecting the high degree of epidemiological independence among sub-regions (40).

Since 2011, more than 9 years have elapsed without new occurrences of FMD in FMD-free countries with vaccination (except for Colombia), compelling these countries to confirm the elimination of the virus in vaccinated populations by suspending vaccination campaigns.

In 2020, at the request of the 13 countries of COSALFA, the representatives of the six sub-regions of the Americas in COHEFA approved the third Action Plan of PHEFA covering the 2021–2025, with the overall purpose of completing the eradication of FMD in South America and strengthening the prevention and response capacity of the veterinary services of the countries in this continent. Such a goal can be achieved with actions aimed at three specific objectives: (a) eradicating the FMD virus in the territory of Venezuela and mitigating the risk in the Northern Andean sub-region, (b) making the transition to the official status of FMD-free without vaccination in the FMD-free countries still using vaccines, and (c) maintaining the status of the FMD-free territories without vaccination (40).

Discussion

In its more than 33 years of execution, PHEFA has provided valuable insights in the efforts to eliminate FMD in South America.

First, PHEFA has had a regional governance mechanism made up of COHEFA and COSALFA, which has allowed not only coordinated actions under a master program that has guided national programs, but also permanent monitoring of progress in the elimination of the disease and a space for discussion and genuine collaboration between the public and private sectors with the support of international cooperation. As a result, the definition and implementation of regional strategies to solve problems and delays found during the execution of the program have been made possible.

Second, the adoption of oil-type vaccines accompanied by quality control of all the series in use, complying with regional and international standards, has allowed national programs to rapidly control outbreaks and eliminate infection, since the vaccination programs reached high coverage, both at the herd and population level.

Third, a pattern of sporadic outbreaks in bovine populations that reached FMD-free status with vaccination revealed the persistence of FMD virus transmission and the presence of endemic niches in vaccinated populations. These niches corresponded to sub-populations with low immunity due to lower coverage and/or bad practices in vaccination campaigns.

Fourth, the introduction of studies to measure post-vaccination immunity, not only in a generalized manner, but also to characterize it through simple indicators such as the size of the farm, the age of the animals, and the identification of geographic clusters, served to identify the failures in vaccination and introduce corrections, both in the frequency of vaccination

and in its application. The review and strengthening of vaccination campaigns, including additional cycles for young animals, as was done in Colombia, Ecuador, and Paraguay, was a decisive strategy for mitigating the risk of transmission and eliminating the infection.

Fifth, the isolation of the active viruses in each outbreak of the disease has allowed a phylogenetic characterization of the different lineages of viruses present in the region, linking them to their area of occurrence, and has revealed that the transmission patterns among the sub-regions of South America (i.e., Southern Cone, Andean area, and Northwest) have been limited, demonstrating a particular segregation of risk in the region.

Finally, the movement of animals, conditional on compliance with the vaccination program, has been strengthened with the improvement of the cadaster and identification of herds supported by computer tools, which ensure centralized and effective control of the movement of each batch of animals, without requiring the individual identification of the animals, except in the case of batches intended for the export of livestock products.

The territories that have taken the step toward withdrawing the vaccine and being recognized by the WOAH as FMD-free without vaccination are contributing to the absence of virus transmission, confirming the elimination of FMD in those areas.

Author contributions

AR led the article conception, writing, and review of this article. MS-V has shared the article conception task with AR and has contributed to the writing and review. EP and LB have contributed to the writing and review. MM and OC has contributed to the review. All authors contributed to the article and approved the submitted version.

Conflict of interest

The 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.

Publisher's note

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EDITED BY
Bouda Vosough Ahmadi,
European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy

REVIEWED BY
Polly Compston,
Royal Veterinary College (RVC), United Kingdom
Gisselle N. Medina,
Agricultural Research Service (USDA), United States
Melissa McLaws,
European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy

*CORRESPONDENCE
Sarsenbay K. Abdrakhmanov
✉ s_abdrakhmanov@mail.ru

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The progressive control of foot-and-mouth disease (FMD) in the Republic of Kazakhstan: Successes and challenges

Akhmetzhan A. Sultanov[1], Samat Tyulegenov[2], Gulzhan N. Yessembekova[2], Maksat A. Berdikulov[3], Yersyn Mukhanbetkaliyev[2], Amina Akhmetzhanova[4], Andres M. Perez[5] and Sarsenbay K. Abdrakhmanov[2]*

[1]Kazakh Research Veterinary Institute, Almaty, Kazakhstan, [2]S. Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan, [3]Republican State Enterprise "National Veterinary Reference Center," Committee of Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan, Astana, Kazakhstan, [4]Kazakh National Agrarian Research University, Almaty, Kazakhstan, [5]Department of Veterinary Population Medicine, University of Minnesota Twin Cities, St. Paul, MN, United States

Foot-and-mouth disease (FMD) has historically caused far-reaching economic losses to many regions worldwide. FMD control has been problematic, and the disease is still prevalent in many West and Central Asia countries. Here, we review the progress made by Kazakhstan in achieving freedom from FMD and discuss some of the challenges associated with maintaining the FMD-free status, as evidenced by the occurrence of an outbreak in 2022. A combination of zoning, movement control, vaccination, and surveillance strategies led to eliminating the disease in the country. However, the circulation of the FMD virus in the region still imposes a risk for Kazakhstan, and coordinated strategies are ultimately needed to support disease elimination. The results presented here may help design effective pathways to progressively eliminate the disease in West and Central Asia while promoting the design and implementation of regional actions to support FMD control.

KEYWORDS

Kazakhstan, foot-and-mouth disease, vaccination, control, epidemiology

Introduction

Foot-and-mouth disease (FMD) is an infectious disease of cloven-hoofed animals caused by the infection with a picornavirus generically referred to as the FMD virus (FMDv). FMD causes far-reaching losses to affected countries (1, 2). Although some regions have made substantial progress toward controlling the disease, most countries have not reached FMD-free status, as described by the World Organization for Animal Health (WOAH).

Much progress has been made since the inception of the West Eurasia Roadmap for FMD Control in 2008, and the 14 countries included in the regional effort have made some level of progress toward the progressive control of the disease (3). Kazakhstan is the only country in the region that has achieved FMD-free status, as recognized by WOAH in 2017. However, FMD is believed to still be present in many countries of the region, which represents a threat to Kazakhstan. For example, Mongolia and China have consistently reported serotype A and O FMD outbreaks to WOAH (https://wahis.woah.org/) almost annually over the last

10 years. In Russia, outbreaks of FMD caused by the O/ME-SA/Ind-2001 virus were first registered in Zabaykalsky Krai, Russia, in 2016 and 2019 and the Orenburg region, close to the border with Kazakhstan in 2021. In 2022, an FMD outbreak was reported in Kazakhstan, resulting in the provisional suspension of the disease-free status of the affected zone. A description of the outbreaks reported in 2022 is available elsewhere (4). Briefly, the outbreak was initially suspected through passive surveillance. In response to the emergency, Kazakhstan initiated a national vaccination campaign, which resulted in the suspension of the FMD-free status without vaccination in the zones that previously had such status. Although the cost that FMD causes to Kazakhstan is unknown, noteworthy, the federal government is responsible for the cost of control and prevention activities and for compensating producers at live market value. The value of 1 kg of beef and 1 kg of live animal is, approximately, USD 5.4 and USD 2.6 per kg, respectively. Thus, culling of, say, 10 cattle of, on average, 350 kg each, will cost USD 9,100 to the federal government in terms of compensation and will represent a loss of approximately USD 9,800 to the affected producer due to the differential price. Those estimates do not include other losses, such as those associated with the genetic and productive value of lost animals, cost of disease control activities (e.g., vaccination and movement restrictions), and loss of export markets.

The remarkable success of Kazakhstan in achieving the FMD-free status in the Western Asia region, followed by the subsequent loss of the status in association with a new FMD incursion, is of interest because it represents an example of the potential opportunities and risks associated with the control of FMD in the region.

The objective of the paper here was to review the evolution of the FMD control program in Kazakhstan and to offer a discussion of the emerging challenges toward eliminating the disease in the country. The results and discussion here will be helpful for the design and implementation of effective FMD control programs in the region.

Demographic features of Kazakhstan

Kazakhstan is the largest land-locked country in the world, resulting in more than 14,000 km of borders with five neighboring countries. Administratively, the country is divided into 14 regions (Figure 1). The agricultural industry is vital for Kazakhstan, with almost 50% of the country's population living in rural areas and approximately one-third of the population directly or indirectly associated with the agricultural sector. Also, ∼75% of all agricultural land is used for grazing, mostly, ruminants susceptible to FMD infection (5).

Environmental conditions and animal production features vary regionally in Kazakhstan.

In Western Kazakhstan, an area rich in large meadows and pastures, animal production tends to be more extensive and most often includes sheep, and horses. Because of the relatively low animal density, extensive conditions, and relative isolation, FMD outbreaks are relatively rare and self-limiting in this region. In Northern Kazakhstan, production is typically relatively intensive, and much of the swine and dairy production in the country is located here. The livestock industry is expanding in this region, primarily because of the interest in exporting dairy products. There are plans to build 52 dairy farms here in the following years, at a rate of 10–15 farms per year, and the federal government has already allocated 3 billion tenges (∼USD 6.5 million) for the construction of some initial dairy farms. Because of the relatively extensive production in Western Kazakhstan and the intentions to develop the industry to target exports from Northern Kazakhstan, the objective of reaching FMD-free status without vaccination was considered for those regions.

In turn, prevailing high-temperature conditions in the foothills of Southern Kazakhstan result in the production of livestock adapted to those conditions, most importantly, small ruminants. In Eastern Kazakhstan, non-irrigated agriculture is relatively standard and beef and dairy cattle farms are rapidly growing mainly to provide the Kazakh internal market with dairy products and beef. Approximately seventy dairy farms and a hundred feedlots operate in Eastern Kazakhstan. Because of those environmental and demographic conditions, Eastern and Southern Kazakhstan are considered at higher risk for FMD than other parts of the country.

Maps depicting the density of cattle, small ruminants, and pigs in Kazakhstan, along with population data, have been provided in Supplementary material.

Epidemiological pattern of FMD in Kazakhstan prior to the achievement of the FMD-free status (1955–2013)

A detailed description of the epidemiological dynamics of FMD infection in Kazakhstan is available elsewhere (6). Briefly, 5,260 serotype O and A FMDv outbreaks were recorded in Kazakhstan between 1955 and 2013. Most (>75%) outbreaks affected cattle. FMD outbreaks were spatiotemporally clustered before 1970, with two seasonal peaks (in spring and fall). Between 1984 and 2013, outbreaks occurred only sporadically and in spring, with clusters associated only with the incursion of specific variants of serotype A FMDv.

The risk for disease incursions into the Southern part of the country became evident when a series of outbreaks, caused by various serotypes and strains, including A SEA 97, A Iran 05, O PanAsia, and O / PanAsia 2, were reported in these zones between 2011 and 2013 (Figure 1). During that period, outbreaks were controlled through a mass vaccination program that resulted in the application of 16.5 million and 6.3 million FMD vaccine doses in cattle and small ruminants, respectively. The vaccine included FMDv strains Asia-1 Shamir, Manisa type 01 and Iraq type A22.

Implementation of the pathway for an FMD control program (2013–2022)

A strategic plan for FMD control was designed according to WOAH recommendations and considering the social, demographic, and epidemiological features of the disease and setting and implemented following order No. 7-1/587 of the

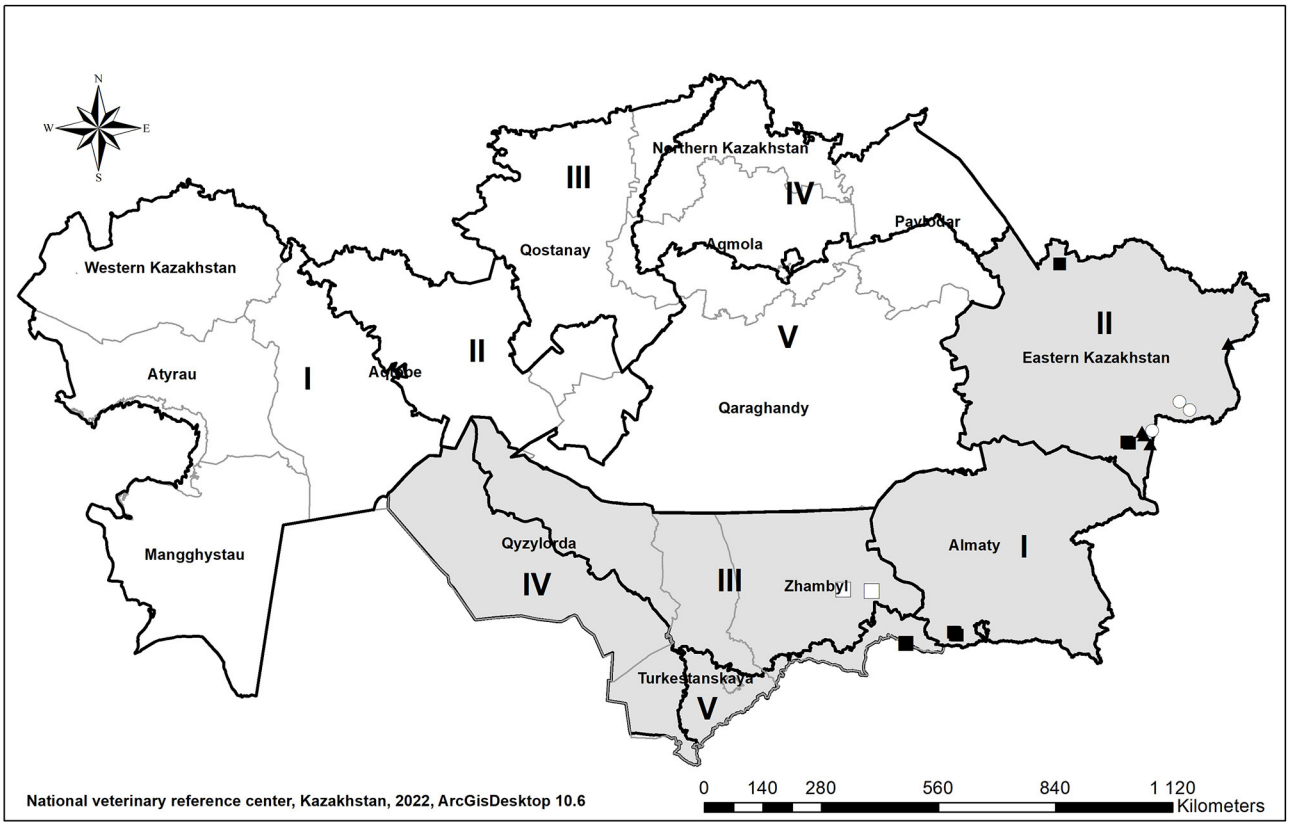

FIGURE 1
The fourteen administrative units of Kazakhstan (thin borders) grouped into ten zones (thick borders designated in Roman, I–V, numerals) according to their foot-and-mouth disease (FMD) status (gray: with vaccination; white: without vaccination), as approved by the World Organization for Animal Health (WOAH) in 2019. FMD outbreaks reported in 2011 (triangles), 2012 (squares), and 2013 (circles) caused by serotype O (black) and serotype A (white) FMD viruses are also indicated.

Minister of Agriculture of the Republic of Kazakhstan, dated June 29, 2015 (7). The plan was based on a combination of zoning, preventive vaccination followed by the serological evaluation of population immunity, and control of movements between zones. Key pillars of the plan also included (a) all costs, including vaccination, laboratory testing, elimination of positive animals, and compensation, were publicly funded; (b) engagement of the entire network of veterinary diagnostic laboratories in the country, the National Reference Center for Veterinary Medicine and the Kazakh Scientific Research Veterinary Institute; (c) implementation of animal identification and movement tracking system; and (d) agreement with neighboring countries to strengthen surveillance and inspection activities at the borders, including mobile checkpoints, the creation of bi-national and multi-national committees to monitor the epidemiological conditions and share information on outbreaks, and joint implementation of surveillance activities. The state compensated farmers for destroying sick and in-contact animals at market value for 1,118,076,416 tenges or 7.6 million U.S. dollars in 2011–2013 only. The strategy was successful in helping Kazakhstan evolve from stage 1 of the Progressive Control Pathway for FMD, PCP-FMD (8) in 2013 to the recognition of the 14 administrative regions of the country as FMD-free in 2017-−9 and 5 with and without vaccination, respectively (3).

The 14 administrative units of Kazakhstan were grouped into 10 zones according to their FMD status, half corresponding to zones with and without vaccination (Figure 1). The five FMD-free zones without vaccination included West Kazakhstan, Atyrau, Mangystau regions and the southwestern part of the Aktobe region (zone I), the north-eastern part of the Aktobe region, the southern part of Kostanay region and the western part of Karaganda region (zone II), the northern and central part of Kostanay region, the western part of North Kazakhstan and Akmola regions (zone III), the central and eastern part of North Kazakhstan region, the northern part of Akmola and Pavlodar regions (zone IV), and the central and eastern part of Karaganda region, the southern part of Akmola and Pavlodar regions (zone V). The five FMD-free zones with vaccination included Almaty (zone I), East Kazakhstan (zone II), part of the Kyzylorda region, the northern part of South Kazakhstan region, the northern and central parts of Zhambyl region (zone III), the southern part of Kyzylorda region and the southwestern part of South Kazakhstan region (zone IV), and the southeastern part of South Kazakhstan region and the southern part of Zhambyl region (zone V).

TABLE 1 Epidemiological features of the last foot-and-mouth disease (FMD) outbreaks reported in Kazakhstan in each control zone and before 2022.

FMD status	Zone	Last FMD incursion prior to 2022			
		Districts affected (year)	Number of outbreaks	Serotype	Number of animals (cattle and small ruminants) culled
Free without vaccination	I	Tinali and Lbischensk Akzhaik (2011)	2	O	4,299
	II	Kobda (1969)	1	O	Not available
	III	FMD has never been recorded, at least since 1955			
	IV	Yereymentau (2010)	1	O	2,025
	V	Zhezkazgan (2007)	1	O	60
Free with vaccination	I	Districts bordering China and Kyrgyzstan in Almaty (2012)	4	O	1,698
	II	Various districts (2011–2013)	14	O and A	18,869
	III	Districts bordering Kyrgyzstan in Zhambyl (2012)	3	A	270
	IV	FMD has never been recorded, at least since 1955			
	V	Kordai (2012)	1	O	270

The decision to maintain the vaccination program in Eastern and Southern Kazakhstan was due to the combination of observed demographic, environmental, and epidemiological conditions, resulting in high FMD risk levels compared to other regions. Specifically, FMD outbreaks have been relatively uncommon in the Northern and Western districts of the country (Table 1). In contrast, results of the epidemiological investigations of outbreaks reported in Southern and Eastern Kazakhstan suggested that they were associated with incursions from neighboring countries. Transmission between countries in the region, including the neighboring countries of China and Russia, and also Mongolia, may be explained by the strong social and economic relations among populations. In Mongolia, FMDV O/ME-SA/Ind-2001 was first identified in March 2015 in the westernmost region of Bayan-Olgii. In 2021, multiple FMD outbreaks caused by the O/ME-SA/Ind-2001 genetic lineage virus were registered, covering 19 of 21 regions of Mongolia, and causing outbreaks among wild Mongolian gazelles (*Procapra gutturosa*) (https://wahis.woah.org/#/in-review/3800?reportId=158431&fromPage=event-dashboard-url). Many Kazakhs live in the Bayan-Ulgiy region of Mongolia and in Russia, maintaining close ties with relatives in Kazakhstan. This situation may result in the introduction of FMD and other diseases through vehicles and contaminated food and supplies. For those reasons, it was perceived that Eastern and Southern Kazakhstan were at the highest risk for FMD introduction.

Additionally, as described above, Southern and Eastern Kazakhstan are densely populated with small ruminants, and their products supply the internal market; thus, there was an intention to actively mitigate that risk through preventive vaccination. In turn, production in the Western region of the country is extensive, with little opportunity for disease transmission, and there is an intention and motivation to create appropriate conditions for exporting in the Northern region. For those reasons, FMD vaccination has been maintained in Southern and Eastern regions to serve as a buffer for the rest of the country, whereas it has been banned in Northern and Western parts. Consequently, Kazakhstan also becomes a major buffer between Eastern and Central Asia, and Russia and Eastern Europe, preventing the spread of FMD into free regions.

In coordination with WOAH's sub-regional office, which was established in Astana, Kazakhstan, in 2013, and to support the recognition of the FMD-free status, Kazakhstan requested WOAH to conduct Performance of Veterinary Services (PVS) evaluation missions in 2016 and 2018, which helped to identify strengths and areas for improvement with the final objective of strengthening the ability of the veterinary services to implement the measures required to control and prevent the introduction of the disease. Areas identified as key for the success of the program and in light of the results of the PVS were the structure of the veterinary service, including control, supervisory bodies, and executive bodies, a developed network of accredited veterinary laboratories, and the allocation of a national budget to support preventive measures. Additionally, simulation exercises were conducted in Karaganda and West Kazakhstan regions with the support of international experts and representatives from WOAH subregional office to improve the effectiveness of early detection and control activities.

Vaccination, active surveillance, and evaluation of immunity to support the FMD-free status

The FMD vaccination campaign is supervised by the Minister of Agriculture of the Republic of Kazakhstan through order No. 7-1/587, which regulates the provisions of subparagraph 6, Article 8 of the Law of the Republic of Kazakhstan No. 339 ("On Veterinary Medicine") approved in July 2002. These regulations align with the list of selected animal diseases prevention, diagnosis, and control, which is conducted at the expense of national funds, approved by

order of the Minister of Agriculture of the Republic of Kazakhstan No. 7-1/559, dated October 30, 2014. Because of the extensive borders of Kazakhstan with countries in which the disease is present or suspected and in response to the 2011–2013 epidemic, since 2014, a mass vaccination campaign for cattle, small ruminants, and pigs has been implemented using a trivalent (A, O, and Asia-1) purified vaccine produced by the FFE "Shchelkovo Biocombine" and FGBI "ARRIAH" (Russia) with the activity of at least 6PD50 for each valency in a dose. The FMD mass vaccination campaign covers all susceptible animals in the five FMD-free zones where vaccination is practiced, representing approximately 14.7, 67.5, and 1.6% of the total number of cattle, small ruminants, and pigs of the country, respectively. Adult (>18-month-old) animals are vaccinated twice per year, in spring (April–May) and fall (September-October), whereas young (3–18 month-old) animals are vaccinated every 3 months.

In order to quantify the efficacy and coverage of the vaccination campaign, as well as the quality of the vaccine, post-vaccination monitoring was conducted annually among susceptible animals (cattle, small ruminants, and pigs) using an ELISA test and including at least 1% of the estimated population of vaccinated animals.

The level of immunity raised against all FMD serotypes and across the five FMD with vaccination regions was >80% after 2013 and >90% after 2015, which was considered sufficient to prevent FMD spread. In contrast, the immunity levels were substantially low (and for some serotypes and regions, <80%) prior to 2014, which may explain, at least in part, the occurrence of 15 FMD outbreaks in the region from 2011 through 2013. The number of tested animals (cattle, small ruminants, pigs) ranged from 135,900 in 2016 to 332,400 in 2017 (Supplementary material).

Additionally, screening for non-structural protein (NSP) antibodies was conducted using an ELISA test for young (3–12 month-old) livestock. The number of tested animals was calculated using a two-stage random sampling design and following guidelines provided in Chapter 1.4.4. of WOAH Terrestrial Code (9). The selection of units was implemented by the consecutive and random identification of villages, herds, and animals to sample. The total number of blood serum samples, which was stratified per zone both in the regions with and without vaccination, was 109,192 in 2016 (69,352 in the zones with vaccination, of which 36 were NSP positive, and 39,840 in the zones without vaccination); 54,138 in 2017 (14,658 in the zones with vaccination, of which 18 were NSP-positive and 39,480 in the zones without vaccination); 48,928 in 2018 (11,920 in the zones with vaccination, of which 23 were NSP-positive, and 37,008 in the zones without vaccination); 44,450 in 2019 (19,929 in the zones with vaccination, of which 13 were NSP positive and 24,519 in the zones without vaccination). Probang samples were collected from NSP-positive animals and tested by PCR; all animals tested negative. No NSP-positive result was found in animals sampled in the zones without vaccination.

Discussion: Challenges and opportunities

FMD control is critically important to support the development of countries. FMD impact on countries' economies is associated with a combination of (1) direct losses due to reduced production and changes in herd structure; and (2) indirect losses caused by costs of FMD control, poor access to markets and limited use of improved production technologies. Although we failed to identify accurate and current estimates of the impact of FMD in Kazakhstan, it was estimated in 2013 that the annual median FMD impact on Asian countries (excluding China and India) is approximately USD 1.3 billion, considering production losses and vaccination costs only (2). Although the nature of the relationships between reaching FMD-free status and trade is difficult to measure, it is worth to note that the volume of exported meat and meat products in 2017, when Kazakhstan first reached the FMD-free status in 14 administrative regions, was 4,154 tons, whereas by 2022, this value was 27,402 tons, representing a 6.6 fold increase (Kazakhstan government, unpublished data).

A key component of Kazakhstan's success in controlling FMD may have been the allocation of sufficient financial and human resources to support the plan. As described elsewhere, the financial resources allocated to Asian veterinary services have been inadequate, impairing the effectiveness of FMD control and elimination in the region (10, 11). Despite the remarkable success of Kazakhstan in establishing an effective FMD control program, becoming the first country in the region to be recognized as disease-free by WOAH, there is still a high risk for disease incursions, as evidenced by the occurrence of an FMD outbreak in January 2022, in the FMD-free zone (V) where vaccination is not practiced. The epidemic resulted in the suspension of Kazakhstan's FMD-free status. Further investigation of the incursion revealed that the outbreak was caused by a strain that had been previously identified in neighboring countries, demonstrating the need for regional policy and actions intended to secure the free status of neighboring countries and to prevent the transboundary spread of the disease (4). Such need is not unique to the Central Asia region. Strengthening veterinary services, political will and cooperation, technical expertise, and human resources to achieve compliance with controls are also key components of FMD control, as identified in South East Asia and South America (12, 13).

The country's investment to support the control has been key to engage producers. Given that vaccine and vaccination costs were covered by the country and delivered entirely free of charge to producers, and that outreach activities were organized to engage farmers, there was strong support from producers to the FMD control campaign. Implementation of an accurate traceability system supported by the country to monitor and control animal movements was also critically important. Another relevant incentive for the private sector was the investment in technology for producers to enhance livestock productivity and reach international markets. Those actions led to a bilateral agreement with China, which required FMD-free status for trade, but that considered the regionalization plan proposed and implemented by Kazakhstan (Kazakhstan government, unpublished data).

Results here demonstrate, the opportunity to succeed in the implementation of a PCP-FMD in the West Asia region, as suggested by Kazakhstan's achievements in obtaining support from producers for the implementation of the control plan, and recognition as a disease-free country, and the need for ongoing active monitoring of the disease status in-country, and also, advancing in strategies coordinated among countries in West and

Central Asia toward the ultimate goal of eliminating FMD in the region.

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 authors.

Author contributions

AS: secured the data, planned much of the study, conceived the study, supervised activities, and wrote some of the paper. AP: collaborated with the design of the paper and wrote some of the paper. ST, GY, MB, YM, and AA: collected and organized data and wrote some of the paper. SA: conceived the study, supervised activities, and wrote some of the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

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EDITED BY
Alejandra Victoria Capozzo,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Rajeev Ranjan,
ICAR-National Institute on Foot and Mouth Disease, India
Ehab M. El-Nahas,
Benha University, Egypt

*CORRESPONDENCE
Nicholas A. Lyons
✉ nl222@cantab.net

†PRESENT ADDRESS
Nicholas A. Lyons,
Animal and Plant Heath Agency, Surrey, United Kingdom

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Comparison of vaccination schedules for foot-and-mouth disease among cattle and sheep in Mongolia

Gerelmaa Ulziibat[1], Eran Raizman[2], Amarsanaa Lkhagvasuren[1], Chris J. M. Bartels[1], Orgikhbayar Oyun-Erdene[3], Bodisaikhan Khishgee[4], Clare Browning[5], Donald P. King[5], Anna B. Ludi[5] and Nicholas A. Lyons[2*†]

[1]Food and Agriculture Organization of the United Nations, Ulaanbaatar, Mongolia, [2]Food and Agriculture Organization of the United Nations, Regional Office for Europe and Central Asia, Budapest, Hungary, [3]Provincial Veterinary Service (PVS), Bayan-Undur soum, Orkhon, Mongolia, [4]General Authority for Veterinary Services, Ulaanbaatar, Mongolia, [5]The Pirbright Institute, Woking, United Kingdom

Vaccines are a critical tool for the control strategy for foot-and-mouth disease (FMD) in Mongolia where sporadic outbreaks regularly occur. A two-dose primary vaccination course is recommended for most commercial vaccines though this can be logistically challenging to deliver among nomadic pastoralist systems which predominate in the country. Although there is evidence that very high potency vaccines can provide prolonged duration of immunity, this has not been demonstrated under field conditions using commercially available vaccines. This study compared neutralizing titres to a O/ME-SA/Panasia strain over a 6-month period following either a two-dose primary course or a single double-dose vaccination among Mongolian sheep and cattle using a 6.0 PD_{50} vaccine. Titers were not significantly different between groups except in sheep at six-months post vaccination when the single double-dose group had significantly lower titers. These results indicate the single double-dose regimen may be a cost-effective approach for vaccination campaigns supporting FMD control in Mongolia.

KEYWORDS

vaccine, foot-and-mouth disease, vaccine schedules, immunogenicity, nomadic

1. Introduction

Vaccines are extensively used in the control of foot-and-mouth disease (FMD), a disease of cloven-hooved livestock endemic through large parts of Africa and Asia. Mongolia is a vast, landlocked country with a long history of nomadic pastoralism (1). Herders dominate the rural economic landscape, depending on livestock production for their livelihoods. The low population density and nomadic lifestyle isolate many rural communities which can create problems for the delivery of veterinary services and vaccination (2). Regular incursions of FMD virus occur with a large impact among herders and cost to the government for control. In 2017, the cost of vaccination was estimated at approximately 60% of the total costs from reaction and expenditure and equivalent to US$4.3 million (3).

Post-vaccination monitoring is required to ensure vaccines are appropriate and effective (4). A previous study in Mongolia evaluated the immunogenicity of imported FMD vaccines in cattle, sheep and camels against high-risk strains for the region (5). This indicated that the current vaccines were well suited, although a two-dose primary course was required

to avoid a rapid decrease in titers, and an oil-based adjuvant had a superior performance over an aqueous equivalent. A two-dose primary course is generally recommended for FMD vaccines, typically given 1 month apart followed by boosters every 4–6 months (6, 7). However, the extensive nature of the nomadic production system of Mongolian herders creates logistical difficulties in delivering a two-dose primary course frustrating disease control efforts.

A previous study under experimental conditions demonstrated that a single dose of a very high potency vaccine (>40 PD_{50}) with an oil (Montanide® ISA 25) adjuvant maintained high neutralizing titers over a 6-month period in sheep to the A22 Iraq vaccine strain (8). However, to the authors' knowledge this approach has not been used or evaluated under field conditions using a commercially available vaccine. This study aimed to compare the relative immunogenicities of a single injection of double the volume dose of a 6 PD_{50} vaccine with a conventional two-dose primary course among Mongolian cattle and sheep.

2. Methods

2.1. Study design

Studies were performed among cattle and sheep with eligible animals randomly selected from the farm for the study. Animals were assigned to one of three groups: single double-dose, two-dose and unvaccinated. The single double-dose (from here referred to as "double dose") group were given a single injection of double the recommended volume of vaccine (2 ml in sheep, 4 ml in cattle). The two-dose group were given two single doses (1 ml in sheep, 2 ml in cattle) 14 days apart as per the manufacturer's recommendation. Unvaccinated controls received no intervention but were sampled on the same dates. Animals in each study were kept in the same group and had unique ear tag numbers to facilitate follow up vaccination and sampling. Serum samples were taken from all animals at first vaccination (0 dpv), 14 dpv, 56 dpv, 112 dpv, and 180 dpv with the unvaccinated controls sampled on the same day.

2.2. Farm and animal selection

Study herds were in Orkhon Aimag (Province), selected based on having no history of FMD, likely compliance with the study protocol, convenience in being close to Ulaanbaatar to facilitate repeat visits, and with no history of using FMD vaccine. Separate herds were used for the cattle and sheep studies. Animals were eligible for recruitment if between 4 and 18 months of age at the start of the study with no recent history of poor health. All animals were local Mongolian breeds. Before enrolment in the study, all animals were serologically negative to non-structural protein (NSP) antibodies. Animals were provided with ear tag identification at first vaccination with the first animals assigned to the two-dose group until the required number were reached, followed by assignment to the double dose group, then the controls.

2.3. Vaccine

The vaccine was commercially available (ARRIAH, Vladimir, Russia), contained strains from the O/ME-SA/PanAsia and A/ASIA/Sea-97 lineages, NSP purified, over 6 PD_{50} per dose, and adjuvanted with Montanide® ISA 25. This was the same product as the previous study (5) but a different batch (number 120819, produced in August 2019) and delivered intramuscularly in the mid-cervical region.

2.4. Sampling and serology

Cattle and sheep were blood sampled through the caudal tail and jugular veins, respectively. Samples were kept on ice while transported to the laboratory where sera were separated and stored at −20°C prior to testing. All sera were tested for NSP antibodies using a commercially available ELISA kit (ID Screen® FMD NSP Competition, ID Vet) at the State Central Veterinary Laboratory, Ulaanbaatar. No animals had received FMD vaccine previously, so NSP antibody negative animals were assumed negative for structural protein antibodies. Serum samples from 0 dpv were also tested using a serotype O solid-phase competitive ELISA (IZSLER, Brescia, Italy). Virus neutralization tests (VNT) were performed at the FAO World Reference Laboratory for FMD, Pirbright, UK as previously described (9). To reduce costs, VNT was only performed from 14 dpv onwards. Neutralizing titres were measured using the same field strain from the O/ME-SA/PanAsia lineage (O/MOG/13/2017) as the previous study (5), selected to provide a more conservative estimate than the previously used serotype A strain which was associated with higher titres.

2.5. Sample size calculation

The sample size was based on non-inferiority between the two protocols using previously reported titres at 56 dpv among sheep receiving a two-dose primary course (5). The non-inferiority margin was a 2-fold dilution, equivalent to $\log_{10}0.3$. Assuming a 5% loss to follow up, 32 animals were required (16 per group). Using data from the same study, the statistical power at 180dpv was 67%. The sample size was therefore inflated to 20 per group which was feasible for the selected farm and had a more acceptable power of 76%. Two unvaccinated controls were included as disease sentinels as recommended in the FAO-OIE guidelines for small-scale immunogenicity studies (4). All calculations were performed using the ssi module in Stata 14.2 (10). Although the sample size was possible in sheep, due to cost it was only possible to use half the number of cattle although due to the lower standard deviation the power at 180 dpv was acceptable at 76%.

2.6. Data analysis

Age and sex data were compared between groups using non-parametric Wilcoxan rank sum and Fisher exact tests respectively. VNT data were analyzed using multivariable interval regression,

accounting for left and right censoring of neutralizing titres as described previously (5). Separate models were created for cattle and sheep, both including dosing group (two-dose vs. double-dose) and sampling time post vaccination as categorical variables. To estimate differences in titres at different sampling points, dpv was included as an interaction term in the model. Robust standard errors were estimated to allow for correlation of observations at the individual animal level. All analysis was done in Stata 14.2 (StataCorp LP, Texas, USA).

2.7. Ethical approval

Ethical approval for the study was granted through order 01/720 dated 15th June 2020, of the Director in General Authority for Veterinary Services, Mongolia.

3. Results

Twenty-two cattle and 42 sheep were used for the studies. The first injection was administered on the 15th June 2020 in both groups and species. In both the cattle and sheep double-dose groups, a single animal died during the study period at 137 and 173 dpv respectively. The reason for death in the cattle group was unknown and no post-mortem examination was performed whilst the sheep was predated by a wolf. One sheep in the two-dose group was successfully treated with parenteral antibiotics for an eye infection at 14 dpv and was retained in the study and given the second dose as per protocol. No local reactions at the injection sites were observed in any of the animals.

Cattle in the double-dose group tended to be older than the two-dose group. This was also the case in the sheep study, with the double-dose group also tending to have more females (Table 1). No animals showed clinical signs consistent with FMD during the study period and all samples were negative for NSP antibodies. Samples at 0 dpv were also negative to structural protein antibodies to serotype O using a solid phase competition ELISA (see Supplementary material).

Amongst cattle, the neutralizing titres in the double-dose group were higher at all sampling points compared to the two-dose group, although there was no statistical evidence of a difference between groups (Table 2, Figure 1). A possible anamnestic response was observed in the two-dose group with higher titres observed at 56 compared to 14 dpv when the second dose was administered. Otherwise, titres were similar throughout the study period.

In sheep, titres in the two-dose group were higher at all sampling points and the multivariable model indicated a significant difference between groups (Table 2). This effect was greatest at 180 days when titres for the double-dose group were significantly lower than the protective cut-off established in cattle (Figure 1). A possible anamnestic response was observed in both groups when comparing titres at 14 and 56 dpv (Figure 1).

4. Discussion

The results of this study indicated that neutralizing antibody titres to the O/ME-SA/Panasia strain after a single injection of a double-dose FMD vaccine were not significantly different to those elicited after a two-dose primary course delivered 14 days apart in cattle. In sheep, titres in the double-dose group were lower at 180 dpv and significantly below the protective cut-off established in cattle (11). There was some statistical evidence that ages and sex varied between the groups; however, the margin of difference was small and unlikely to invalidate the results of the study.

FMD vaccines are typically formulated into standard (3 PD_{50}) and higher (6 PD_{50}) potency types based on the number of 50% protective doses contained in each dose (12). The vaccine used in this study is advertised as being over 6 PD_{50}, although the quantity of antigen is not stated. Very high potency (e.g., >10 PD_{50}) vaccines have potential application for emergency reactive campaigns in an FMD-free setting due to the rapid onset of immunity supported by numerous studies under experimental conditions (13–15). However, consideration for longer duration of titres in a non-free setting has received less attention. One study quantified homologous neutralizing titres up to 6 months after a single dose of >40 PD_{50} vaccine in sheep (8). The results of that study indicated titres were maintained at "nearly peak" for up to 6 months in sheep with a Montanide® ISA 206 oil adjuvant, although there was a gradual decline using the Montanide® ISA 25 adjuvant as used in the current study.

TABLE 1 Descriptive data for animals randomly assigned to groups in a study (not including unvaccinated controls[a]), comparing two vaccination schedules for foot-and-mouth disease among cattle and sheep in Mongolia, 2020.

Variable	Species	Category	Two-dose	Double-dose	*P*-value
Age (months)	Cattle	-	Mean: 12.0 Median: 12.0, Range: 11–13	Mean: 13.0 Median: 13.0 Range: 11–14	0.014
	Sheep	-	Mean: 13.1 Median: 14.0 Range: 12–14	Mean: 13.9 Median: 14.0 Range: 12–16	0.011
Sex	Cattle	Female	6 (54.6)	6 (54.6)	0.99
		Male	5 (45.4)	5 (45.4)	
	Sheep	Female	9 (45.0)	17 (85)	0.019
		Male	11 (55.0)	3 (15)	

For sex, column percentages are presented in parentheses. [a] Cattle: two controls were both 12 months of age, one female and one male. Sheep: two controls were both 14 months of age, one female and one male.

TABLE 2 Multivariable interval regression model comparing the impact of two foot-and-mouth disease vaccination schedules (double dose at day 0, and two dose primary course at days 0 and 14) and sampling time post vaccination on the titres against O/ME-SA/PanAsia, Mongolia, 2020.

Variable	Category	Coefficient	SE (Robust)	95%CI	*P*-value
Cattle					
Vaccination schedule	Two-dose	Baseline	-	-	-
	Double dose	0.15	0.14	−0.13, 0.44	0.28
Sampling (days post vaccination)	14	Baseline	-	-	-
	56	0.090	0.098	−0.10, 0.28	0.36
	112	0.092	0.091	−0.087, 0.27	0.31
	180	−0.035	0.13	−0.030, 0.23	0.79
Constant	-	2.0	0.096	1.8, 2.2	<0.0001
Sheep					
Vaccination schedule	Two-dose	Baseline	-	-	-
	Double-dose	−0.33	0.13	−0.59, −0.079	0.010
Sampling (days post vaccination)	14	Baseline	-	-	-
	56	0.26	0.082	0.10, 0.43	0.002
	112	0.18	0.089	0.0080, 0.36	0.040
	180	−0.052	0.096	−0.24, 0.14	0.59
Constant	-	2.3	0.13	2.1, 2.6	<0.0001

CI, confidence interval.

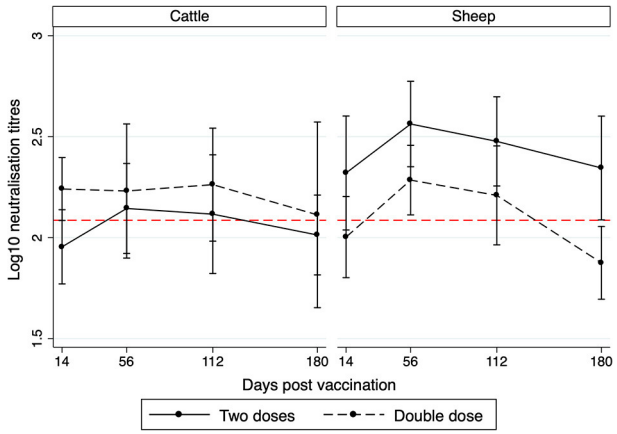

FIGURE 1
Post-vaccination neutralizing titers in cattle and sheep against O/ME-SA/PanAsia following either a two-dose primary course at days 0 and 14, or with a double dose administered at day 0. Sampling time was included in the model as an interaction term with dosing group, Mongolia, 2020. Data points represent model estimates ±95% CI. The horizontal dashed red line represents a titer that correlates with protection in 95% of cattle experimentally challenged with homologous strains from the serotype O lineage from the same laboratory (11). A similar estimate for sheep is not available in the published literature.

The study was limited by not measuring titres to a strain from the serotype A lineage present in the vaccine due to limited resources. Serotype O was preferred since this is more commonly reported in Mongolia, and due to higher titres against the A strain in the previous study meaning that an O strain would likely provide a more conservative evaluation (5). Before any changes in vaccination policy are implemented, it would be prudent to measure the titres against a relevant serotype A strain.

Based on titres at 14 and 56 dpv, a possible anamnestic response to the second dose in the two-dose group was observed in cattle. This contrasts with sheep where there appeared to be increases between these sampling days in both groups. In the previous study using the same vaccine and neutralizing strains, no increase in titer was observed between day 14 and 56 after a single dose of vaccine in either species (5). "Late responders" in sheep have been reported previously with increases in titres occurring up to 3 months after a single dose of FMD vaccine adjuvanted with different oil adjuvants (16, 17). Such a prolonged response was not observed in the current study with an apparent decline at 112 dpv although there was no statistical evidence to support this observation and there were no samples taken at the 3-month timepoint to allow direct comparison.

In conclusion, these results indicate similar titres between groups of cattle given a double-dose or two-dose primary course of FMD vaccine over a 6 month period although in sheep the former was significantly lower at 180 dpv. Administering a double-dose may avoid the logistical difficulty of delivering a second dose to extensive and pastoralists production systems although further studies including an economic assessment comparing the two approaches in cattle and sheep would be worthwhile. Caution should be taken when extrapolating these results to other FMD vaccines with different potencies and strains for which bespoke studies are required.

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.

Ethics statement

The animal study was reviewed and approved by the Director in General Authority for Veterinary Services, Mongolia. Written informed consent for participation was not obtained from the owners because this was obtained verbally with herders.

Author contributions

GU contributed to the study design, led the fieldwork, and collected the data. ER conceived the study and contributed to the study design. DK and ALu contributed to the study design and interpretation of the results. CBa and ALk contributed to the analysis and interpretation of the results. BK and OO-E facilitated the field work. CBr led the laboratory work and contributed to the interpretation of the results. NL led the study design, performed the analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

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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.2023.990043/full#supplementary-material

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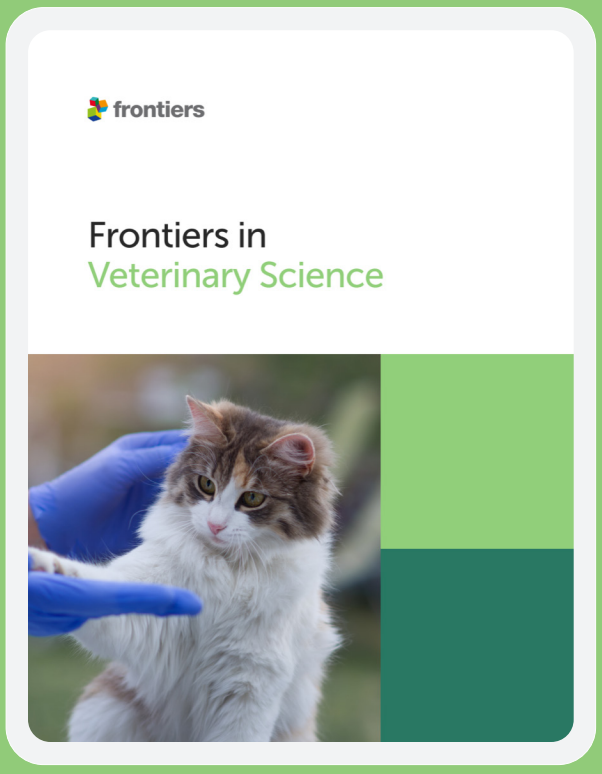

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