



First Report on Abortion Caused by *Salmonella enterica* subsp. *enterica* Serovar Enteritidis in Water Buffalo (*Bubalus bubalis*)

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Salmonella enterica subsp. *enterica* Serovar Enteritidis is one of the major pathogens associated with enteric diseases in animals and humans. Thus, due to the importance of *Salmonella* spp. infections for animal production and public health, the aim of the present study was to describe the first detection of *S. enteritidis* in an aborted water buffalo fetus in southern Italy by characterizing the phylogroup profile and the antimicrobial susceptibility of the isolated pathogenic strains. The different clinical manifestations of salmonellosis in animals include diarrhea, abortion, pneumonia, septic arthritis, meningitis, and others, depending on the virulence of the serovars, infectious dose, and host immunity. This study reports the first case of abortion caused by *Salmonella enterica* subsp. *enterica* serovar Enteritidis in water buffalo (*Bubalus bubalis*) in the Campania region, southern Italy. Complete necropsy was performed on the aborted water buffalo fetus under study, and samples and swabs from different organs were collected. Samples were processed by microbiological and molecular analyses to detect bacterial, viral, and protozoarian pathogens possibly responsible for abortion. Whole genome sequencing (WGS) was carried out to further characterize the isolated *S. Enteritidis* strain. Our findings highlight the crucial role of *S. Enteritidis* as a potential abortive agent in water buffalo and its presence should therefore be investigated in cases of bubaline abortion.

Keywords: water buffalo (*Bubalus bubalis*), *Salmonella* Enteritidis, abortion, whole genome sequencing, microbial genomics

INTRODUCTION

Salmonella enterica is one of the most common pathogens responsible for human and animal bacterial intestinal infections. The main subspecies of this species is *enterica*, which includes more than 2,600 serovars (1). *Salmonella enterica* subsp. *enterica* serovars exhibit broadly diverse phenotypes characterized by different infectious patterns, reservoirs, vectors, and host spectrum (2). Despite the high number of *S. enterica* serovars, only one or a few are associated with the majority of cases of illness in mammalian species, with Typhimurium and Dublin predominating in cattle (3–5). These serotypes show different degrees of host adaptation, with *S. Typhimurium* most frequently associated with gastrointestinal disease in young calves (younger than 8 weeks), and

S. Dublin inducing diseases both in young and adult animals (3–5). The severity of the disease is determined by host-pathogen interactions, highly influenced by several factors including the route of infection, the infectious dose, the virulence of the pathogen, natural or acquired host resistance factors, and the possible presence of other pathogens (6, 7).

In water buffalo calves (*Bubalus bubalis*), *Salmonella* induces severe gastrointestinal lesions, diarrhea, hyperthermia, and severe dehydration (6, 8). The major source of infection in water buffalo herds is represented by carrier animals, shedding heavy loads of *Salmonella* through feces (9–11). Other possible sources of infection include contaminated forages and water, as well as rodents, wild winged animals, insects and man (12). The *S. enterica* serovars most frequently isolated from water buffalo are Typhimurium, Muenster and Give (13). Moreover, this pathogen can harbor a variety of virulence factors, with different locations in the genome, such as *Salmonella* pathogenicity islands (SPIs), prophage regions, or virulence plasmids (7). The complex pattern of virulence factors can increase the ability of *Salmonella* to cause systemic infection and consequently to induce abortion in many animal species. Indeed *Salmonella enterica* can infect the fetus by passing through the placental barrier, thus causing abortion, stillbirth, and even pre-term and neonatal infections in animals (14). Abortion due to *Salmonella* spp. has already been described in cattle and small ruminants (15–17), however it has never been recorded in water buffalo. Therefore, the aim of this study was to describe for the first time a case of abortion in water buffalo caused by *S. enterica* subsp. *enterica* serovar Enteritidis.

METHODS

Herd of Origin

The herd where the abortion occurred consists of about 600 animals, grazing outdoor in the corrals with an off-paddock system. It regularly carries out deratting interventions and is fully fenced. The water supply is represented by well water, regularly subjected to microbiological analysis to verify its potability. In the years preceding the abortion, a vaccination protocol was carried out on adult animals exclusively for Rotavirus, Coronavirus and *E. coli* K 99. During the last 2 years only sporadic abortions occurred in the farm, none of which related to *Salmonella* spp., except that reported in the present study. Moreover, in the same period, mortality episodes recorded among calves could not be attributed to *Salmonella* outbreaks, rather to wrong management procedures. The herd is often flied over by many wild birds.

Anatomopathological and Microbiological Examinations

The study was carried out on an aborted fetus delivered by a water buffalo heifer bred in a herd located in the Campania region, southern Italy. The abortion occurred at the fifth month of gestation. Complete necropsy was performed within 24 h after death by veterinarians from the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM), Salerno, Italy. When the abdominal cavity was opened the subject exhibited severe abdominal and moderate pleural serohematic effusions, with the presence of mild pericardial serohematic fluid. Samples and swabs

from the celomatic organs were collected according to standard protocols (18). Samples of abomasum, lungs, liver, spleen, heart, and kidneys were collected and investigated for the presence of the pathogens most frequently associated with abortion in water buffalo, including Bovine herpesvirus 1 (BHV-1), Bovine viral diarrhea virus (BVDV), *Brucella* spp., *Campylobacter fetus*, *Listeria* spp., *Salmonella* spp., Bovine herpesvirus 4 (BHV-4), *Chlamydia abortus*, *Coxiella burnetii*, *Neospora caninum*, *Toxoplasma gondii*, and *Leptospira* spp. (19). Feces sampled from the heifer were tested for the presence of *Salmonella* spp. by microbiological method (20). **Table 1** reports the investigated abortive agents for each tissue sample by cultural and/or molecular methods (20, 21). Due to its small dimensions, spleen was homogenized and entirely used for the bacteriological research only of *Brucella* spp., while the presence of *Salmonella* was investigated on the liver.

Microbial Identification

Salmonella enterica isolation and identification were performed according to UNI EN ISO 6579-1:2020 (22). The isolated *S. Enteritidis* was serotyped according to the Kaufmann-White scheme (23). Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method. The antibiotics tested were nalidixic acid, ampicillin, cefotaxime, ciprofloxacin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulphonamide, tetracycline, sulfamethoxazole/trimethoprim, colistin, amoxicillin-clavulanic acid, enrofloxacin, cephalothin, and ceftazidime.

WGS-Based Characterization

The isolated *S. Enteritidis* was processed for genomic DNA isolation by using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration was evaluated using high-sensitivity Qubit™ fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Approximately 150 ng of genomic DNA were used for whole genome sequencing. A 400 bp fragment DNA library was prepared using the Ion Xpress Fragment Library kit (Life Technologies, Carlsbad, CA, USA) following manufacturer's instructions. Sequencing reaction was carried out on an Ion Gene Studio S5 platform (ThermoFisher Scientific, Waltham, MA, USA). A coverage of approximately 30X was calculated. The raw sequencing reads were analyzed using FastQC, and low-quality sequences were removed by using PRINSEQ lite. High-quality sequences were assembled using SPAdes (24) version 3.15.0. Sequences assembly produced 46 contigs for a total length of 4,682,282 bp, with a mean contig length of 102,386 nucleotides (maximum contig length: 454,631 nucleotides, and minimum contig length: 537 nucleotides) and an L50 contig value of 9, with an N50 length value of 197,095. Contigs were aligned with the reference sequence NZ_CP075122.1 using ABACAS version 1.3.1. The obtained sequence of the bacterial genome was deposited in GenBank with accession number CP088146.1 and was analyzed with the bioinformatic tools available on the Center for Genomic Epidemiology (CGE) website <https://www.genomicepidemiology.org>.

TABLE 1 | Investigated abortive agents for each tested organ.

Matrix	Pathogen	Test
Lungs	Bovine herpesvirus 1 (BHV-1), Bovine herpesvirus 4 (BHV-4), <i>Listeria</i> spp., <i>Chlamydomphila abortus</i> , <i>Coxiella burnetii</i>	PCR
Liver	Bovine herpesvirus 1 (BHV-1), <i>Campylobacter fetus</i> , <i>Listeria</i> spp., <i>Chlamydomphila abortus</i> , <i>Coxiella burnetii</i>	
Spleen	Bovine viral diarrhea virus (BVDV), Bovine herpesvirus 4 (BHV-4)	
Abomasum content	<i>Campylobacter fetus</i> , <i>Listeria</i> spp., <i>Chlamydomphila abortus</i> , <i>Coxiella burnetii</i>	
Heart	<i>Neospora caninum</i> , <i>Toxoplasma gondii</i>	
Kidney	<i>Leptospira</i> spp.	
Lungs	<i>Brucella</i> spp., <i>Listeria</i> spp.	Bacteriological
Liver	<i>Salmonella</i> spp.	
Spleen	<i>Brucella</i> spp.	
Abomasum content	<i>Brucella</i> spp., <i>Campylobacter fetus</i> , <i>Listeria</i> spp.	

RESULTS

Cultural and biochemical investigations identified the pathogen isolated from the analyzed fetus as *Salmonella enterica* subsp. *enterica* serovar Enteritidis 9,12:g.m:-. The other investigated bacterial, viral and protozoarian pathogens were not detected. No other possible cause of abortion was identified. Feces sampled from the heifer resulted negative for the presence of *Salmonella* spp. when processed by microbiological method. The isolated strain exhibited resistance to the class of aminoglycosides when challenged with the Kirby-Bauer disk diffusion test.

Whole Genome Sequencing of the isolated *S. Enteritidis* strain allowed to perform a molecular characterization of the pathogen under study. Specifically, this strain exhibited the MLST profile type 11 and harbored two plasmids, IncFIB(S) and IncFII(S). Moreover, the molecular characterization confirmed the antigenic profile obtained by the Kaufmann-White scheme and resistance to aminoglycoside identified by microbiological methods. The strain under study displayed the presence of 148 genes coding for virulence factors (**Supplementary Table 1**). Most of these virulence genes were involved in mechanisms responsible for fimbrial and non-fimbrial adherence determinants and secretion systems. The characterized strain also exhibited the presence of several *Salmonella* Pathogenicity Islands (SPIs), including SPI-1, SPI-2, SPI-3, SPI-5, SPI-9, SPI-10, SPI-12, SPI-13, and SPI-14. The pathogen finder tool indicated that the analyzed strain was predicted to be a possible human pathogen with a probability of 94% based on the presence of the identified virulence genes.

DISCUSSION

This report describes for the first time a case of abortion associated with *S. Enteritidis* in water buffalo. Abortion is a major concern in water buffalo dairy farms, with potentially significant economic losses. Causes of abortion for this animal species, as well as for bovine, can be either infectious or non-infectious. Among the infectious causes of abortion, in addition to frequently described pathogens, such as *Brucella* spp., Bovine Viral Diarrhea Virus, Bovine Herpesvirus 1, *N. caninum*, *Toxoplasma gondii*, *Campylobacter fetus*, *C. burnetii*, *Chlamydia* spp. and *Leptospira*, other less common pathogens, such as

some *Salmonella* serovars can also be included (16, 17, 25). In particular, in cattle, *S. Dublin* has been described as a pathogen responsible for abortion (17, 25, 26).

In water buffalo herds *Salmonella* Enteritidis is a matter of concern since it can cause serious economic losses in livestock and is a zoonotic agent responsible for foodborne illness (8, 27). Indeed, the pathogens either causing reproductive disorders and infectious diseases in water buffaloes and/or transmitted through their products pose a serious risk to the overall livestock production because they affect animal health status and milk production. In water buffalo, *Salmonella* is a major pathogen because it induces calf diarrhea leading to early-age calf-mortality (28) and can be transmitted through animal products thus contaminating the food chain. *Salmonella* can enter and survive in the farm environment for long periods of time. The presence of this pathogen is widespread in water buffalo herds globally (13, 29–32) and its prevalence can be up to 26% in farm environment, as reported by a recent study (32). In farm animals information on the circulating pathogens and, in case of abortion, the correct identification of the etiological cause, could lessen the impact of the related pathologies.

The *S. Enteritidis* strain under study exhibited a high number of genes coding for virulence factors. Consistently with the abortion as the outcome of the infection, this strain harbored several virulence factors known to be responsible for the ability of some *Salmonella* strains to escape the host immune system, overcome the intestinal wall and gain systemic circulation, such as Type I fimbriae and genes located on diverse SPIs (33, 34). This strain was instead lacking some other virulence factors also known to be implicated in the systemic infection of *Salmonella*, such as those associated with the capsule. This evidence suggests that the pathogenesis of this microorganism is complex and likely modulated by a high number of genes, many of which have not yet been fully elucidated in regard to their role in the course of the infection. Moreover, the host-pathogen interaction also plays a fundamental role in the outcome of the infection, with particular regard to the animal species considered in this study. Our data suggest that in water buffalo *S. Enteritidis* might exhibit a particular tropism for the reproductive apparatus based on the evidence that the heifer under study showed the absence of gastroenteric disease either before or after abortion, as well as the absence of the bacterium in her feces. Finally, although

Salmonella spp. is frequently characterized by multi-antibiotic resistance (35), the strain under study exhibited only resistance to aminoglycoside, while was susceptible to all the other tested antibiotics. In general, the analysis of bacteria from food animals, foods, and clinically ill humans with the subsequent evaluation of antimicrobial resistance trends throughout the food production and supply chain is a primary requirement for an integrated and efficient surveillance program.

For the first time we have shown that *S. Enteritidis* causes abortion in water buffalo. This confirms the health risk posed by *Salmonella enterica* to farm animals and represents a further loss for the water buffalo marketing sector. In light of these findings, considering the zoonotic potential of *Salmonella enterica* and its ability to act as a potential abortive agent in water buffalo, its presence should always be investigated in cases of bubaline abortion. More research is needed to understand why few *Salmonella* serovars are responsible for the majority of animal diseases and demonstrate such unique reservoirs and pathogenesis. With a better understanding of serovar specificity and pathogenicity, effective strategies could be set up to control *Salmonella* in livestock farms.

DATA AVAILABILITY STATEMENT

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

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ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the experimental Zooprophyllactic institutes (IIZZSSMM) are official laboratories designed by the Italian Ministry of Health that are involved in epidemiological surveillance, animal health, food, and feed safety and diagnostics. For this reason, ethics approval was deemed unnecessary in agreement with institutional policy and national regulations. Written informed consent for participation was not obtained from the owners because the study was conducted during institutional activities.

AUTHOR CONTRIBUTIONS

LD'A, RP, DA, MR, GB, and GG drafted the manuscript. LD, GB, and GG conceded and revised the study. DA, RP, and MR conducted the microbiological and molecular analyses. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2022.894154/full#supplementary-material>

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