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Evaluation of lamb and calf responses to Rift Valley fever MP-12 vaccination

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ABSTRACT

Rift Valley fever (RVF) is an important viral disease of animals and humans in Africa and the Middle East that is transmitted by mosquitoes. The disease is of concern to international agricultural and public health communities. The RVFV MP-12 strain has been the most safety tested attenuated vaccine strain; thus it is being considered as a potential vaccine for the US national veterinary stockpile. This study was designed to establish safety protocols for large animal research with virulent RVF viruses, establish a target host immune response baseline using RVF MP-12 strain, and independently evaluate this strain as a potential US emergency response vaccine. Ten, approximately four month-old lambs and calves were vaccinated with RVF MP-12 strain; two additional animals per species provided negative control specimens. The animals were monitored for clinical and immune response, fever, and viremia. Two animals per species were sacrificed on 2, 3, 4, 10 and 28 days post infection and full necropsies were performed for histopathological examination. No clinical or febrile responses were observed in this study. The onset and titer of the immune response is discussed. There was no significant histopathology in the lambs; however, 6 out of 10 vaccinated calves had multifocal, random areas of hepatocellular degeneration and necrosis. RVF MP12 antigen was detected in these areas of necrosis by immunohistochemistry in one calf. This study provides independent and baseline information on the RVF MP-12 attenuated vaccination in vaccine relevant age target species and indicates the importance of performing safety testing on vaccine relevant aged target animals.

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1. Introduction

Rift Valley fever (RVF) is a high consequence, trans-boundary agricultural and zoonotic disease caused by a Phlebovirus of the Bunyaviridae family. It is classified as Category A pathogen and an overlap select agent by Center for Disease Control and Prevention and United States Department of Agriculture. The RVF virus was first identified in Great Rift Valley of Kenya in 1931, where it affected thousands of small ruminants causing abortion in ewes and mortality in newborn lambs (Bird et al., 2009). So far major epidemics of RVF virus have been reported in Egypt 1977 (Imam et al., 1979), Kenya 1997–1998 (Woods

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et al., 2002), Saudi Arabia 2000–2001 (Madani et al., 2003), Yemen 2000–2001 (Abdo-Salem et al., 2011), and more recently in Kenya, Tanzania and Somalia 2006–2007 (Nguku et al., 2010), Madagascar 2008 (Jeanmaire et al., 2011), South Africa 2008 (Archer et al., 2011) and Mauritania 2010 (El Mamy et al., 2011). Rift Valley fever is a mosquito-borne disease and mainly affects ruminants (goats, sheep, cattle and camels) and humans. In ruminants, particularly sheep, the RVF virus infection is characterized by high rate of abortion, high mortality rate (~70%) in young animals and significant mortality (20–30%) in adult animals (Bird et al., 2009). The virus is transmitted to humans by mosquito bites or by exposure to blood, other body fluids or aerosol from infected animals during slaughter (Gerdes, 2002). Clinical infection in humans is mostly self-limiting and leads to fever, headache, weakness, muscular pain and photophobia. In a very small number of human patients (1–3%) the virus causes hepatitis, retinitis, blindness, encephalitis or a hemorrhagic fever like manifestation (Bird and Nichol, 2012).

The RVF virus is an enveloped, virus containing three genome segments of single-stranded RNA, designated L, M, and S. The L and M segments are negative sense; whereas, the S segment is of ambisense polarity (Elliott and Schmaljohn, 2013). The Large (L) segment encodes the RNA-dependent RNA polymerase used for RNA replication and viral RNA transcription. A complete genome sequence analysis of various strains of RVF virus has revealed very limited genetic diversity in RVF virus across all lineages (Bird et al., 2007; Ikegami, 2012). Additionally, the M segment, which encodes two surface glycoproteins, exhibit a high degree of sequence conservation suggesting that a single vaccine should be able to provide protective immunity against the currently circulating RVF virus strains (Ikegami, 2012). Currently there is no approved human vaccine and the only veterinary vaccine available outside the endemic areas is the RVFV MP-12 strain that has conditional licensure in the US (Hill, 2012). Recent geographical expansion of RVF virus, combined with its wide range of mosquito vectors (Pepin et al., 2010) including mosquito species in North America (Turell et al., 2010), make this virus a growing threat to international agricultural and public communities. Therefore, the development of countermeasures, such as vaccines, in case of an accidental or intentional introduction of RVF virus into the US or other non-endemic countries is needed (Hartley et al., 2011).

Animal vaccination strategies using both traditional and novel approaches are underway to develop a safe and efficacious vaccine against RVF virus which can be used safely in the target species of all age groups including pregnant animals and neonates (Indran and Ikegami, 2012). A live attenuated vaccine candidate, MP-12, has been produced by U.S Army Medical Research Institute for Infectious Diseases (USAMRIID) by passaging the wild type ZH548 strain 12 times in the presence of the mutagen 5-Fluorouracil (Caplen et al., 1985). This MP-12 vaccine strain is classified as a BSL-2 agent and is excluded from the select agent registry due to previous safety studies that have been performed. Although a number of studies have demonstrated that MP-12 is safe and protective against virulent RVF virus challenge in sheep (Morrill et al., 1991, 1987), cattle (Morrill et al., 1997a,b) and macaques (Morrill and Peters, 2003), none have published a detailed histopathologic and immunopathologic evaluation of the vaccinates. In addition, a single study has shown that MP-12 vaccination of early gestational pregnant ewes (35th and 42nd day of gestation) can induce severe teratogenic effects on the fetuses and low level abortion in pregnant ewes (Hunter et al., 2002). Hence a more detailed study of potential MP-12 pathogenicity in target species (sheep and cattle) is warranted. The objective of this study was to establish safety protocols for large animal research with virulent RVF viruses, establish a target host immune response baseline using RVF MP-12 strain, and further evaluate the virulence and safety of the RVF MP-12 candidate vaccine in target and vaccine age livestock species.

2. Materials and methods

2.1. Virus strain and cell culture

The RVFV MP-12 strain was kindly provided by the US Army Medical Research Institute for Infectious Diseases. The virus was propagated once in normal fetal lung fibroblast (MRC-5) cell cultures with the medium of DMEM with 10% fetal bovine serum (FBS, Atlanta Biologicals). Cells were infected using 0.01 multiplicity of infection and RNA extractions were performed when approximately 80% of the infected cells showed cytopathic effect (CPE).

The inoculum was titered using a standard plaque assay. Briefly, cells were grown in Medium 199 with Earl's salts (M199-E; Sigma, St. Louis, MO; 100 U/ml penicillin, 100 μg/ml streptomycin sulfate) with 10% FBS and incubated at 37 °C. Following adsorption for 1 h at 37 °C, inocula were aspirated and monolayers were overlaid with a 1:1 mixture of 2× M199-E (20% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin sulfate) and 12% methyl cellulose. At 48 h, monolayers were fixed and stained with crystal violet fixative stain (25% formaldehyde, 10% ethanol, 5% acetic acid, 1% crystal violet).

2.2. Animals and experimental design

Twelve, approximately 4 month old lambs and calves were obtained from the Kansas State University (KSU) Animal Science and Industry Department and moved to the Biosecurity Research Institute (BRI, Animal Biosafety level-3 facility at KSU) according to standard guidelines for transport of live animals. The animals were housed 7–14 days for acclimation in the BRI. To ensure the stress of movement and containment did not cause opportunisitic infections, the animals were dewormed and preventatively treated with antibiotics. The animals were given water and food *ad libitum*. Body temperatures were taken daily. After acclimatization and pre-bleeding, ten calves and ten lambs were subcutaneously (SC) inoculated with 1 ml for a total of 2.9×10^6 PFU of RVFV (MP-12 vaccine strain). The remaining two calves and lambs were mock inoculated with cell-culture media in the same fashion. All of the experiments were conducted

under Biosafety Level-3Ag or Animal Biosafety Level-3Ag to practice safety procedures prior to authorization for research with virulent RVFV.

Nasal swabs and blood (EDTA and serum) samples were collected at the -7, -3, 10, 14, 21 or 28 days post-infection (dpi). Body temperatures were also taken on these same days. The mock-infected animals were necropsied at 1 dpi to practice new safety protocols prior to working with infected animals. Two calves and two lambs of the MP-12 infected animals were euthanized and necropsied on 2, 3, 4, 10 and 28 dpi. Brain, liver and spleen were collected fresh for virus isolation and real-time RT-PCR. A complete set of tissues were collected and fixed in 10% formalin. All the formalin fixed tissues were processed, paraffin embedded and stained with hemotoxylin and eosin for histopathologic and immunohistochemical evaluation. All of the animal studies were performed under an approved Institutional Committee for Animal Care and Use animal protocol.

2.3. Viral RNA extraction and amplification

The total RNA from serum, nasal swabs or tissue samples was extracted using the magnetic-bead capture MagMAX-96 total RNA Isolation kit. Briefly, 130 μl of lysis/binding buffer was added to 50 μl of sample and mixed by shaking in a 96 well plate. Bead mix (20 μl) was then added and the mixture was shaken for 5 min. After four subsequent washes (150 μl), RNA was eluted in 50 μl of elution buffer at 65 °C. The quadruplex real-time reverse transcriptase-polymerase chain reactions (RT-PCR) that detects all three RNA genome segments (Wilson et al., 2013) was used. This assay utilized an MP-12 specific L primer and probe set (signature), the previous published M signature, and a novel S signature with an external RNA amplification control (Drosten et al., 2002; Schroeder et al., 2012; Wilson et al., 2013).

2.4. Virus isolation

Samples testing positive for RVFV MP12 by real-time RT-PCR were tested for live MP-12 RVFV by standard plaque assay on Vero MARU (Middle America Research Unit, Panama) cells (Bando, 1975). Small pieces from the collected tissue were frozen at −80 °C immediately following the necropsy. The samples were thawed and 50–100 mg of tissue was added to 1 ml of cold 199E media with Penicillin/Streptomycin/Fungizone (PSF) and homogenized with plastic soft tissue homogenizer tips (OMNI International, Kennesaw, GA). Homogenized samples were then sonicated (Q700: Qsonica, Newtown, CT, USA) on ice at setting 3 using a microtip for 15 s four times. The samples were clarified by centrifugation (500 g for 3 min at 4 °C) and 100 μl of clarified supernatant in 900 μl 199E with PSF was added to ~80% confluent Vero MARU cells in a T25 flask. Flasks were incubated (37 °C, 5.0% CO_2) with rocking every 15 min for 1 h. After the adsorption step, 5 ml of 199E media with PSF was added to each flask (total 6 mL). Flasks were incubated (37 °C, 5.0% CO_2) and monitored daily for CPE.

Blind passage CPE: Samples testing positive for RVFV MP12 by real-time RT-PCR were also triple blind passaged on Vero MARU cells grown as described above in T-25 culture flasks. For each passage, the serum inocula were adsorbed on Vero monolayers for 1 h at 37 °C, overlaid with M199-E containing 2% FBS, and incubated at 37 °C for 7 days. At 7 dpi, flasks were freeze–thawed and 2 mls of culture were transferred to fresh Vero flasks for the next passage. After three passages, flasks were freeze–thawed and the supernatant was retested for infectious virus by standard plaque assay as above and real-time RT-PCR.

2.5. Serological methods

Serum neutralizing antibody was determined using an 80% plaque-reduction neutralization test (PRNT80) as previously described except using RVF MP-12 strain (Morrill et al., 1991). Sera were tested for RVFV-specific IgG and IgM antibodies using an enzyme immunoassay as previously described (with modifications as described below) (Jansen van Vuren et al., 2007; Ksiazek et al., 1989). Sera for IgG were tested at an initial dilution of 1:300 and sera for IgM were tested at a 1:200 dilution. The cut-off value for assigning a positive IgG result was determined from a panel of five sera from RVFV IgG negative animals calculated in an adjusted OD450 value greater than 3 SD from the mean OD of the negative animals. After binding antigen, wells were blocked with Odyssey Blocking Buffer (LiCor). For detection of IgM, horseradish peroxidase (HRPO)-conjugated rabbit anti-species IgM (AbCam) was used to detect specifically bound antibody. For detection of IgG, HRPO-conjugated Protein G (AbCam) was used in the assay. Colorimetric substrate 2,2′-azinodiethylbenzothiazoline sulfonic acid (ABTS) was added to each well. Plates were incubated in the dark at room temperature for approximately 30 min and the reaction was stopped by the addition of 50 μL of 3 M H_2SO_4. The optical density (OD) was determined at 405 nm. The results were expressed as a signal to noise ratio of the mean OD value of a test result/negative control mean OD.

There were two antigens used in these assays, one was an *Escherichia coli* recombinant RVFV 548 Np protein (plasmid provided by F. Weber, University of Marburg) similar to the antigen used by Jansen van Vuren et al. (2007). The second was an MP-12-infected baby hamster kidney cell lysate antigen, prepared as described previously (Ksiazek et al., 1989) with modifications as described by Smith et al. (2012).

2.6. Detection of MP-12 antigen by immunohistochemistry

MP-12 was detected by immunohistochemistry in formalin-fixed, paraffin embedded tissues as described previously (Drolet et al., 2012). Briefly, blocked tissue sections were incubated with rabbit anti-RVFV nucleocapsid protein antibody, followed by a biotinylated anti-rabbit secondary antibody, avidin-biotin complexes (Vector Laboratories, Burlingame, CA, USA) and detected with Vector Red chromogen substrate (Vector). Tissues were counterstained with Gill's hematoxylin (Vector) and examined for virus-positive red staining by light microscopy. Tissues from uninfected lambs and calves served as negative controls and were stained identically to experimental samples.

3. Results

3.1. Lamb study

The lambs were febrile during the acclimation period and were treated with antibiotics. Unfortunately delaying the experiment was not possible due to scheduling but the animal temperatures were normal at the time of inoculation. None of the animals had a significant increase in temperature post-inoculation. All of the lambs that were infected with MP-12 remaining past 4 dpi developed serum antibodies to RVFV (Fig. 1). Plaque reduction neutralization 80 (PRNT80) greater than 40 (USAMRIID minimum neutralization titer for human IND vaccine) was obtained in 50% of the remaining 4 lambs with an average titer of 160 (Tables 1A and 1B). Remaining lambs at 21 dpi had PRNT80 titers of 320-1280. None of the serum or tissue samples were positive by real-time RT-PCR. On necropsy, one control (Animal no 1054) and 3 vaccinated animals (Animal no 1049, 1021 and 1101) had mild to moderate bronchopneumonia characterized by red, firm consolidation of cranioventral lung lobes. The bronchopneumonia observed in these lambs was likely attributed to the stress of movement and containment. Histologically, the only significant finding was moderate to severe lymphocytic aggregation in the gall bladder submucosa of the vaccinated animals. The lymphoid aggregates were regularly forming secondary lymphoid follicles characterized by the presence of a germinal center (Fig. 3A). In the non-vaccinated control animals, only small numbers of lymphocytes were seen in the gall bladder submucosa without any follicle formation (Fig. 3B). Other incidental findings were mild intestinal coccidiosis, mild periportal lymphoplasmacytic hepatitis and mild lymphocytic infiltration in trachea and pancreatic duct. No viral antigen was detected in liver, spleen or brain tissues of MP-12 infected lambs.

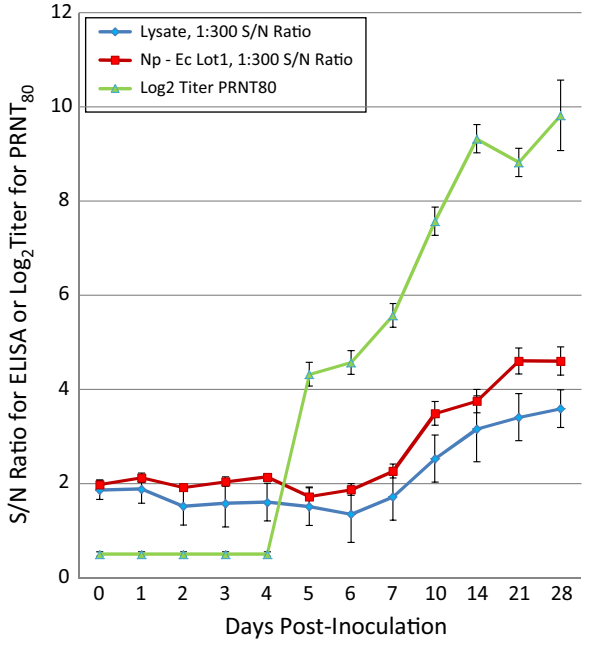

Fig. 1. Indirect ELISA and signal to noise (*S*/*N*) ratio and Log_2 SN titer for 3–4 month-old lambs infected with RVFV MP-12 strain. Error bars indicate standard deviation.

Table 1A
Detection of IgM in sera of lambs and calves by ELISA after infection with MP-12 RVFV – number of positive animals by day.

Days post infection	Lambs	Calves
0	0% (0/12)	0% (0/12)
4	17% (1/6)	0% (0/6)
5	100% (4/4)	75% (3/4)
6	100% (4/4)	100% (4/4)
10	100% (4/4)	100% (4/4)
21	100% (4/4)	100% (4/4)

3.2. Calf study

No significant temperatures were noted during the study. All of the calves that were infected with MP-12 and lived past 4 dpi developed serum antibodies to RVFV (Fig. 2). The plaque reduction neutralization 80 (PRNT80) greater than 40 was obtained in 75% of the remaining 4 calves after day 4 (Tables 1A and 1B). All of the calves had a greater than 40 titer by 6 dpi. The real-time RT-PCR of calf serum samples only detected one suspect positive at 3 dpi. This sample had *Ct* values of greater than 35 and was not consistent in the three replicates. Only one of the replicates had *Ct* values for L and M segment. The S segment was not detected in any of the replicate samples. Grossly, 5 out of 10 calves had moderate bronchopneumonia characterized by red, firm consolidation of cranioventral lung lobes. The rest of the tissues from all the calves were grossly normal. Microscopically, the lesions were primarily seen in liver, gall bladder, lung and trachea (Summarized in Table 2). Multifocal, minimal to moderate, random, 0.3–0.7 mm in diameter, areas of hepatocellular necrosis were noted only in the vaccinated calves necropsied on 2, 3 4 and 10 dpi of the experiment (Fig. 3C, Table 2). These areas contained moderate numbers of degenerate and intact neutrophils and macrophages (Fig. 3D). The calves necropsied on 3 dpi had the most severe lesions. Mild periportal lymphoplasmacytic infiltration was seen in the liver of both the vaccinated and non-vaccinated calves. Mild to moderate lymphoplasmacytic infiltration and occasional lymphoid follicle formation was also noted in the gall bladder submucosa of both the vaccinated and non-vaccinated calves. Other incidental microscopic findings noted in both vaccinated and non-vaccinated animals included minimal to moderate suppurative bronchopneumonia in 5 calves,

Table 1B
Detection of neutralizing antibody in sera of lambs and calves by the $PRNT_{80}$ assay after infection with MP-12 RVFV – number of positive animals by day with titer ≥40.

Days post infection	Lambs	Calves
0	0% (0/12)	0% (0/12)
4	33% (2/6)	0% (0/6)
5	75% (3/4)	25% (1/4)
6	100% (4/4)	75% (3/4)
10	100% (4/4)	100% (4/4)
21	100% (4/4)	100% (4/4)

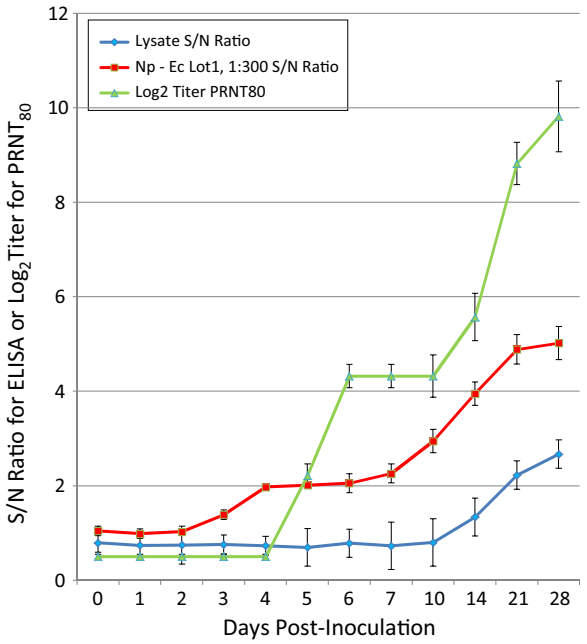

Fig. 2. Indirect ELISA and signal to noise (*S*/*N*) ratio and Log_2 SN titer for 3–4 month-old calves infected with RVFV MP-12 strain. Error bars indicate standard deviation.

fibrinosuppurative tracheitis in 6 calves and minimal to moderate lymphoplasmacytic infiltration in tracheal submucosa of all the calves (Table 2). Viral antigen was detected by immunohistochemistry in the liver of one calf (#485) and was associated with necrotic lesions (Fig. 3E and F). No viral antigen was detected in calf spleen or brain tissues. Tissue samples from five of the calves in this study were found weakly positive ($Ct \geq 30$) for RVF viral RNA by real-time RT-PCR including liver, spleen, lymph node and brain tissues (Table 3). However, these positive tissue samples were negative for virus isolation after triple blind passage in Vero MARU cells.

4. Discussion

The potential for the introduction of RVF into a non-endemic country has spurred the development of new vaccine candidates (Bird and Nichol, 2012). These new vaccines include attenuated, reverse genetics derived and vectored vaccines (Bird and Nichol, 2012; Kortekaas et al., 2012) that are very promising vaccine candidates. The RVFV MP-12 vaccine strain has recently been approved for conditional licensure in the US due to extensive research of the efficacy and safety of this vaccine (Morrill et al., 1991, 1997a,b; Morrill and Peters, 2011). The investigators promoting this vaccine have conducted a majority of this work. In this study, an independent evaluation of the time course of infection of the MP-12 vaccine candidate in two target livestock species was performed, including a complete histopathological and immunohistochemical evaluation. The age of the animals chosen for this study was specific to the age that vaccination would typically be done by livestock owners. The calves and lambs infected with the RVFV MP-12 strain at a higher dose (10^6) than vaccination dose (10^3) recommended by the manufacturer for a previous study, yet still did not develop a detectable viremia. The lack of viremia with RVFV MP-12 strain is consistent with our previous studies in sheep (Miller et al., unpublished data). Previous studies with RVFV MP12 strain virus by others have also shown a lack of viremia in sheep (Morrill et al., 1991) and brief, low titer viremia in pregnant cattle (Morrill et al., 1997a). Unfortunately, in both our studies, the young animals did have background opportunistic infections, although this is not an uncommon situation in a normal vaccination program. A definitive etiology for the opportunistic infection in both

Table 3
Summary of histology/pathology lesions noted with positive result my quadruplex RVF real-time RT-PCR and immunohistochemistry.

Animal #	DPI	Histology+	PCR+	IHC+
435	2	Liver	Liver and spleen	
472	2	Liver		
482	3	Liver	Liver and spleen	
485	3	Liver	Liver	Liver
481	4			
484	4	Liver		
500	10			
510	10	Liver		
483	28		Lymph node and Spleen	
503	28		Brain	

Table 2
Summary of histopathologic lesions in calves after infection with MP-12 RVFV and mock vaccination.

Animal #	DPI	Liver (necrosis/periportal Inflammation)	Gall bladder (submucosal inflammation)	Lung (Pneumonia)	Trachea (suppurative tracheitis/ submucosal inflammation)
456	1	0/0	++	+++	0/+++
460	1	0/++	++	++++	++++/++
435	2	+/+	++	0	0/++
472	2	+/++	++	0	0/++
482	3	+++/+	++	+	+/+++
485	3	+++/+	+++	0	++/+++
481	4	0/+	+++	++	+++/+++
484	4	+/+	+	0	+/+
500	10	0/0	+	0	0/+++
510	10	+/++	–	+++	++/+++
483	28	0/0	+	0	++++/+++
503	28	0/0	+	0	0/+

None = 0, minimal +, Mild ++, Moderate +++, Severe ++++, not evaluated –.

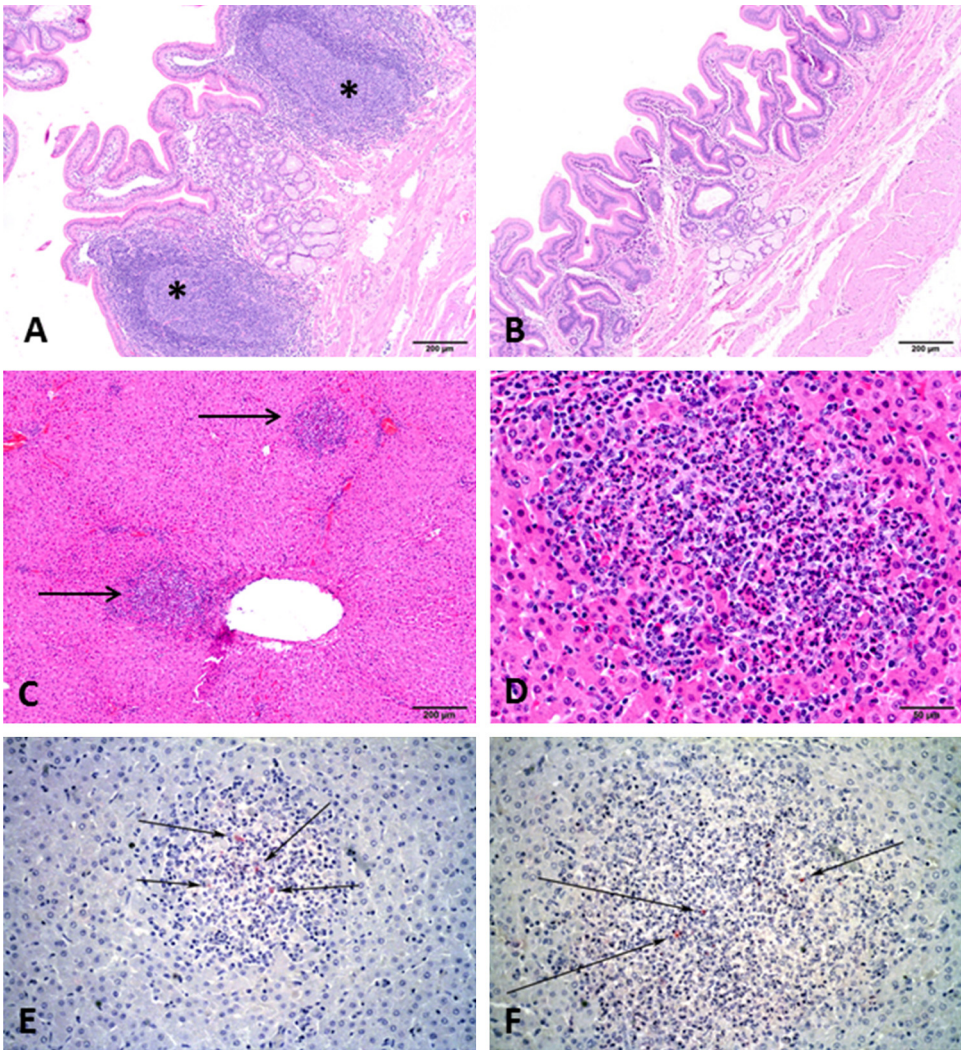

Fig. 3. Histologic and immunohistochemical analysis of MP 12 vaccinated lambs and calves. In the vaccinated lambs, the gall bladder submucosa contained large aggregates of lymphocyte, which were regularly forming secondary follicular structures (asterisk) with a germinal center (A). In the non-vaccinated control animals, the gall bladder submucosa contained only small numbers of lymphocytes (B). Multifocal, random, 0.3–0.7 mm in diameter, areas of liver necrosis (arrows) were seen in 6 calves (C). Necrotic and hypereosinophilic degenerate hepatocytes were mixed with many neutrophils and fewer macrophages (D). RVFV positive immunohistochemical staining was observed in the liver of calf #485 and was associated with necrotic lesions (E and F).

studies could not be determined due to lack of capability to diagnose non-viral pathogens in our BSL-3 laboratory.

Serology revealed all of the lambs and calves developed serum antibodies to RVFV MP-12 strain virus by 5 dpi (Tables 1A and 1B). None of the serum collected from the lambs had detectable RVF MP-12 viral RNA. Of the calf serum samples collected, only one had a questionable detection of RVF MP-12 viral RNA at 3 dpi, but this was neither consistent among the replicates nor confirmed by subsequent single gene target real-time RT-PCR. No pathological lesions attributable to the RVFV infections were observed in the lambs at any of the time points, and no viral RNA was detected in any of the lamb tissue samples by real-time RT-PCR. In the calves, however, there was detectable viral RNA in the tissues of five of the animals (Table 3). Three of these were from animals also found to have liver necrosis, which was only noted in the RVFV MP-12 infected calves. One of these also had detectable RVFV antigen associated with the liver lesion (Fig. 3E and F). Important to note is that infectious virus was not isolated from any of the RVF viral RNA positive tissues. Because there was an underlying opportunistic infection in these calves, these findings are not conclusive, but the results are consistent with high dose infection with RVFV MP-12 producing minor liver necrosis in vaccine age calves. It is quite possible that an underlying opportunistic infection compromised the immune system and predisposed the calves to pathogenic potential of RVFV MP-12 vaccine; however, a similar opportunistic infection (bronchopneumonia) was noted in lambs but with no associated liver necrosis. This observation emphasizes the importance of performing safety testing of veterinary vaccines on target species at the ages and health conditions where they are to be used. This study

provides independent and baseline information on RVF attenuated vaccination in vaccine relevant age target species.

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