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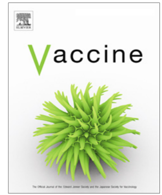

Four-segmented Rift Valley fever virus-based vaccines can be applied safely in ewes during pregnancy

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ABSTRACT

Rift Valley fever virus (RVFV) causes severe and recurrent outbreaks on the African continent and the Arabian Peninsula and continues to expand its habitat. This mosquito-borne virus, belonging to the genus Phlebovirus of the family *Bunyaviridae* contains a tri-segmented negative-strand RNA genome. Previously, we developed four-segmented RVFV (RVFV-4s) variants by splitting the M-genome segment into two M-type segments each encoding one of the structural glycoproteins; Gn or Gc. Vaccination/challenge experiments with mice and lambs subsequently showed that RVFV-4s induces protective immunity against wild-type virus infection after a single administration. To demonstrate the unprecedented safety of RVFV-4s, we here report that the virus does not cause encephalitis after intranasal inoculation of mice. A study with pregnant ewes subsequently revealed that RVFV-4s does not cause viremia and does not cross the ovine placental barrier, as evidenced by the absence of teratogenic effects and virus in the blood and organs of the fetuses. Altogether, these results show that the RVFV-4s vaccine virus can be applied safely in pregnant ewes.

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1. Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne bunyavirus with a negative-strand, three-segmented RNA genome. The large (L) segment encodes the RNA-dependent RNA polymerase. The medium (M) segment encodes a polyprotein precursor that is co-translationally cleaved into the structural glycoproteins Gn and Gc. Additionally, the M-segment encodes two accessory proteins named NSm and "78-kDa" protein. The NSm protein is shown to have an anti-apoptotic function [1], whereas the 78-kDa protein is described to be a major determinant of virus dissemination in mosquitoes [2]. The glycoproteins Gn and Gc are involved in virus entry and fusion with endosomal membranes, respectively. The small (S) segment encodes the nucleocapsid (N) protein in genomic-sense orientation and a non-structural protein named NSs in antigenomic-sense orientation. The NSs protein is the major virulence determinant of RVFV by counteracting host innate immune responses [3–6].

RVFV causes recurrent outbreaks among ruminants and humans on the African continent and has additionally caused outbreaks on the Arabian Peninsula and several islands located off the coast of Southern Africa. The virus has been isolated from over 30 species of mosquitoes, of which some are globally widespread. The latter raises concerns about future incursions into currently unaffected areas [7]. Abortion storms and high fatality rates among new-borns, particularly in sheep herds, are hallmarks of RVFV outbreaks. Humans can be infected via mosquito bite, although most human infections result from contacts with contaminated animal tissues and fluids released during the slaughtering of diseased animals. The majority of infected patients develop flu-like symptoms but occasionally severe to life-threatening disease develops [8].

Vaccination is the most effective countermeasure to control RVF outbreaks. Especially live-attenuated vaccines hold great promise, as these vaccines can be produced cost-effectively and generally provide long-lasting protection after a single vaccination. Until today, two live-attenuated RVF vaccines are commercialized in Africa. The first vaccine, named after its developer, K. C. Smithburn, was developed by attenuating wild-type virus by sequential intracerebral passage in mice [9]. The resulting Smithburn vaccine is highly effective after a single vaccination, but residual virulence prohibits its use in pregnant animals. The second vaccine is based on the Clone 13 virus. Clone 13 was isolated from the blood of a human patient and was found to contain a 70% deletion in the NSs gene [10]. The first experiments with mice demonstrated that Clone 13 is virtually avirulent when administered via the

Abbreviations: RVFV, Rift Valley fever virus; RVFV-4s, four-segmented RVFV; $TCID_{50}$, tissue culture infective dose 50%; FBS, fetal bovine serum.

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intraperitoneal route [11]. In a more recent study however, intranasal administration of a recombinant RVFV lacking the NSs gene to immunocompetent mice resulted in fatal encephalitis, indicating that viruses lacking NSs are not completely avirulent [12]. Nevertheless, the Clone 13 virus was shown to be avirulent and highly immunogenic in several studies involving sheep and cattle [13,14]. Moreover, experiments with pregnant ewes suggested that the virus can be applied safely during gestation [15].

Due to its high efficacy and safety record, we recently evaluated Clone 13 as an emergency vaccine for use in Europe. To this end, safety experiments were performed according to the regulations described in the European Pharmacopeia [16]. These experiments confirmed that Clone 13 is safe for young lambs, even after a repeated overdose and administration via different routes, and that the virus does not disseminate to the environment. However, inoculation of ewes with an overdose during the first or second trimester of gestation demonstrated that Clone 13 can vertically transmit to the ovine fetus [16]. Vertical transmission was associated with stillbirths and fetal malformations when the virus was administered during the first trimester of gestation, the period in which the fetus is most susceptible to RVFV-mediated teratogenic effects. From this, we concluded that the Clone 13 vaccine virus is safe for non-pregnant sheep, but that the vaccine virus should not be applied during gestation [16].

Recently, we developed a novel experimental live-attenuated RVF vaccine by splitting the M genome segment into two segments encoding either Gn or Gc [17,18]. Remarkably, the resulting four-segmented RVFV (RVFV-4s) was shown to be avirulent in mice even in the presence of an intact NSs gene [17]. To optimize safety, a RVFV-4s lacking NSs expression was constructed and used for further studies. Studies with young lambs demonstrated that RVFV-4s does not induce viremia, yet elicits a protective immune response after a single administration [19].

To further elaborate on the safety profile of the RVFV-4s vaccine, we here show that RVFV-4s virus is harmless to mice when administered intranasally, whereas Clone 13 inoculated mice uniformly succumb to the infection. Administration of a high dose to ewes during the first trimester of gestation subsequently showed that RVFV-4s does not induce viremia and is unable to cross the ovine placental barrier, again in contrast to the Clone 13 virus [16]. These results show that the RVFV-4s vaccine virus can be applied safely, even during the most sensitive period of gestation.

2. Material and methods

2.1. Ethics statement

The animal experiment was conducted in accordance with the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081).

2.2. Cells and viruses

The RVFV-4s variant used in this study is based on the Clone 13 genetic background and was constructed as described previously [17,18]. The Clone 13 virus was kindly provided by Michèle Bouloy of Institut Pasteur. Both RVFV-4s and Clone 13 were amplified on Vero E6 cells which were grown in minimal essential medium supplemented with Earle's salts (Life Technologies), 5% FBS, 1% L-glutamine and 1% antibiotic/antimycotic (Invitrogen). Titers were determined by incubating Vero E6 cells with serial dilutions of the viruses for three days followed by fixation with 4% paraformaldehyde and immunostaining with the anti-Gn mAb 4-39-cc, as previously described [20,21].

2.3. Intranasal inoculation of mice

Thirty, 6 weeks-old female BALB/cAnCrl mice (Charles River Laboratories) were divided into three groups of 10 mice and kept in filter top cages in biosafety level-2 (BSL-2) animal facilities. After 6 days of acclimatization, the mice were infected intranasally (0.01 ml/nostril) with either $10^{3.6}$ $TCID_{50}$ of Clone 13 (group 1), or with $10^{4.9}$ $TCID_{50}$ of RVFV-4s (group 2) or with complete culture medium (group 3). The bodyweights of the mice were measured daily. At two weeks post challenge, all remaining mice were euthanized and liver and brain samples were collected for analysis with a RVFV-specific quantitative reverse-transcription PCR (qRT-PCR) as described previously [17].

2.4. Pregnant ewe study

At a conventional sheep farm in the Netherlands, the oestrus of 20 Swifter ewes, between 1 and 1.5 years of age, was synchronised using progesterone sponges. The ewes were subsequently allowed to mate with Swifter rams. Forty-three days later, all ewes were screened for pregnancy using ultrasound. Ten pregnant ewes, which serologically tested negative for Chlamydophila abortus, Border disease virus, Q fever and RVFV, were moved to a BSL-2 animal facility. Ewes were allowed to acclimatize for one week and were subsequently vaccinated. The European Pharmacopeia prescribes that the safety of veterinary vaccines should be evaluated by applying at least a 10-fold overdose [https://www.edqm.eu/en/european-Pharmacopeia-9th-edition]. As we have previously demonstrated that a single intramuscular vaccination with a dose of 10^5 $TCID_{50}$ provides sterile immunity [19], we here applied a 100-fold overdose ($10^{7.1}$ $TCID_{50}$) via the same route. However, as most sheep vaccines are applied via the subcutaneous route, which was also shown to be effective in our previous study, some farmers may prefer to use this vaccination route. Therefore, to comply with the European Pharmacopeia, we applied the vaccine via both the intramuscular and subcutaneous routes, in 1 ml complete culture medium.

During the entire course of the experiment, the animals were monitored daily for general health and signs of abortion. EDTA blood samples were collected daily during the first 11 days post inoculation and weekly starting from day 14 post inoculation. Serum samples were collected weekly during the entire course of the experiment. At 28 days post vaccination (78 days of gestation) the ewes were euthanized and their organs and fetuses were examined macroscopically. From all fetuses, EDTA blood was collected as well as samples from the brain, kidney, lung, liver, spleen and placenta. From the ewes; the liver, spleen, the intramuscular inoculation site and its draining prescapular lymph node were collected. All tissues and plasma samples were tested for the presence of RVFV-4s RNA by qRT-PCR. Serum samples from the ewes were tested for the presence of N-specific antibodies using the ID Screen® Rift Valley Fever Competition ELISA (ID-VET, Montpellier, France) and for the presence of neutralizing antibodies using a virus neutralization test (VNT) as described below.

2.5. Virus neutralization test

Virus neutralization titers were determined using a VNT based on a RVFV-4s variant expressing eGFP. Briefly, threefold serial dilutions of sera were made in micro titer plates and mixed with a fixed amount of $RVFV\text{-}LMMS_{eGFP}$ (~200 $TCID_{50}$) [17]. After a 1.5 h incubation period, BHK-21 cells were added to the serum-virus mixtures. After an incubation period of 2 days at 37 °C and 5% CO_2, eGFP expression was evaluated with an EVOS-FL microscope. VNT titers were calculated using the Spearman-Kärber algorithm [22,23].

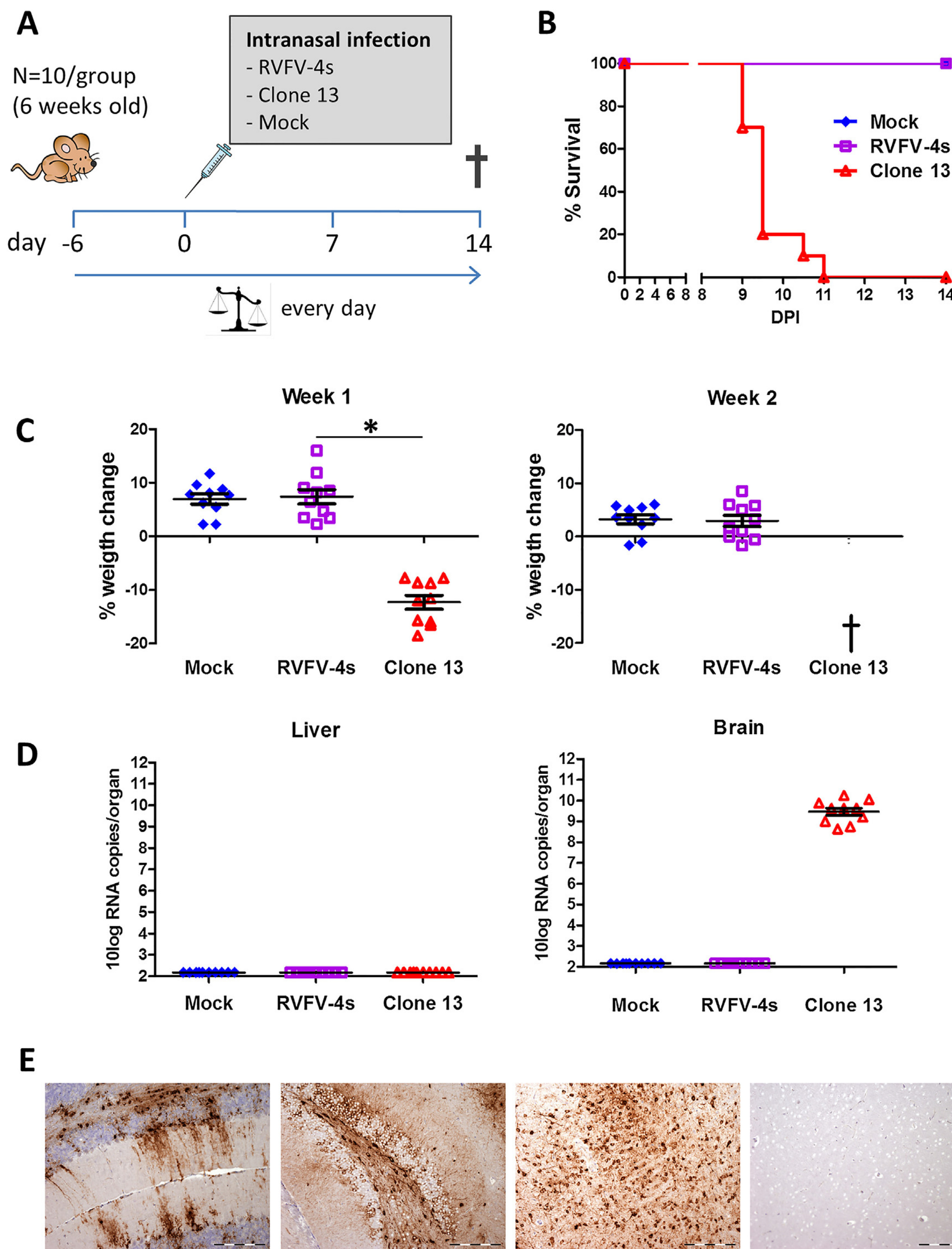

Fig. 1. Intranasal inoculation of mice with RVFV-4s or Clone 13. (A) Schematic presentation of the experimental setup. (B) Survival curve of Clone 13 and RVFV-4s infected BALB/c mice. (C) % Weight change in the first and second week post infection. The asterisk indicates a statistical difference. (D) Viral RNA copies in liver and brain homogenates obtained from mice that succumbed to the infection and from mice euthanized at the end of the experiment. (E) Immunohistochemical detection of the RVFV Gn protein in the cerebellum, hippocampus and cerebral cortex in the brain of a mouse intranasally infected with Clone 13. At the right, the immunohistochemical staining of the brain of a mock infected mouse. Antibody 4-D4, bar = 200 µm.

2.6. Preparation of organ sample homogenates

Organ sample homogenates were prepared using the ULTRA-TURRAX system in combination with DT-20 tubes (IKA, Staufen, Germany). Briefly, 5 ml culture medium was added to 0.5 g of tissue. Samples were homogenized for 40s and cell debris was removed by slow-speed centrifugation. The resulting cleared homogenates were evaluated for the presence of RVFV specific RNA using qRT-PCR as previously described [17].

2.7. Histology and Immunohistochemistry

Tissue samples were fixed in 10% phosphate buffered formalin for at least 48 h followed by routine processing into paraffin blocks.

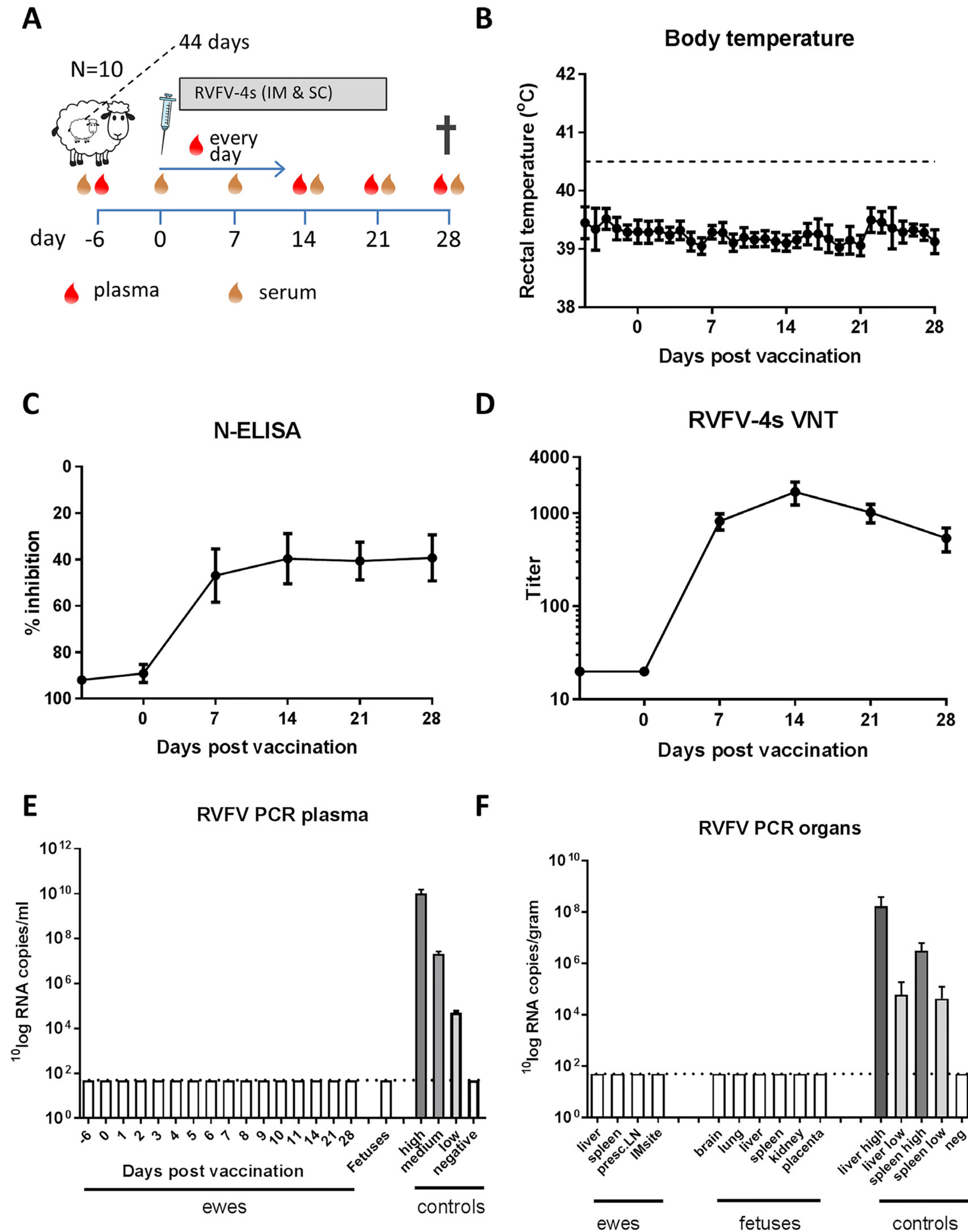

Fig. 2. Inoculation of ewes with RVFV-4s at 50 days gestation. (A) Schematic presentation of the experiment. Ten 50-day-pregnant ewes were inoculated via the intramuscular (IM) and subcutaneously (SC) route with $10^{7.1}$ $TCID_{50}$ of RVFV-4s at day 0. Serum and plasma samples were collected at the indicated time points. At 4 weeks post infection ewes and fetuses were euthanized and evaluated for abnormalities. (B) Average body temperatures and SDs of the ewes during the course of the experiment. (C) Detection of anti-N antibodies in weekly obtained sera by ELISA. Titers are expressed as percentage competition (% S/N). (D) RVFV neutralizing antibody response. Weekly obtained sera were evaluated for the presence of RVFV neutralizing antibodies by a RVFV-4s based VNT. Error bars represent SD. (E) Monitoring of viremia by qRT-PCR. Plasma samples of the ewes and fetuses were analysed by qRT-PCR for the presence of RVFV-specific RNA at the indicated days post-challenge. The dashed line indicates the limit of detection. Positive and negative control plasma samples were obtained from a previous study. (F) Viral RNA levels detected by qRT-PCR in liver, spleen, prescapular lymph node and the intramuscular inoculation site samples of the ewes and in brain, lung, liver, spleen, kidney and placenta samples of the fetuses. Positive and negative control tissue samples were obtained from a previous study.

Four µm thick sections were cut and collected on silane-coated glass slides. After drying for 24 h, sections were stained with haematoxylin-eosin stain (HE) or immunostained for detection of RVFV antigen. For the immunostaining, sections were immersed for 30 min in methanol/H_2O_2 to block endogenous peroxidase and autoclaved for 15 min in citrate buffer (pH 6) to unmask the Gn epitope for the monoclonal antibody 4-D4. Mouse Envision peroxidase and DAB+ chromogen (Dakopatts, Denmark) was used as a secondary antibody, respectively substrate, according the manufacturers' instructions.

2.8. Statistical analysis

Data were statistically analysed using the Mann-Whitney test in GraphPad Prism. P-values <0.05 were taken as significant.

3. Results

3.1. RVFV-4s does not induce disease in mice after intranasal administration

RVF viruses lacking NSs expression, including Clone 13, are virtually avirulent in mice when administered via intraperitoneal route. However, Dodd and co-workers demonstrated that intranasal administration of a recombinant RVFV lacking NSs results in fatal encephalitis [12]. Apparently, virulence of RVFV after intranasal inoculation of mice is independent of NSs expression. Here, we inoculated mice intranasal with RVFV-4s or Clone 13. As a negative control, mice were inoculated with culture medium. As expected, all Clone 13 infected mice developed lethal encephalitis within 11 days post infection (Fig. 1). qRT-PCR analysis of brain and liver samples subsequently revealed high levels of viral RNA in their brains whereas no viral RNA was detected in liver samples. Immunohistochemistry of brain tissue of severely affected Clone 13 infected mice confirmed the presence of viral antigen in neurons and neuronal processes throughout the brain. Interestingly, the RVFV-4s infected mice did not show any symptoms of disease and did not lose weight during the course of the experiment (Fig. 1). Moreover, no viral RNA or viral antigen was detected in liver and brain samples of these mice.

3.2. RVFV-4s is unable to cross the ovine placental barrier

After the unexpected finding that Clone 13 can pass the ovine placental barrier, associated with stillbirths and malformations when administered to ewes at 50 days of gestation [16], we evaluated the safety of RVFV-4s in pregnant ewes at the same time point of pregnancy and with the same dose. To this end, ewes were inoculated with $10^{7.1}$ $TCID_{50}$ of RVFV-4s via intramuscular and subcutaneous route. A schematic representation of the experimental design is presented in Fig. 2A. During the entire experimental period, none of the ewes displayed elevated body temperatures (Fig. 2B) or signs of disease and none of the ewes developed detectable viremia (Fig. 2E). All ewes responded well to vaccination and developed high levels of anti-N antibodies (Fig. 2C) and neutralizing antibodies (Fig. 2D).

At the time of necropsy (28 days post inoculation) no abnormalities were observed in the organs of the ewes that could be attributed to RVFV-4s infection. The ewes carried 17 live and healthy fetuses (3 single lambs and 7 twins) which all were between 17 and 18.5 cm in crown-rump length, which is the expected size of healthy fetuses at this time point of gestation. Analysis of fetal blood and organ samples confirmed the absence of RVFV-4s RNA (Fig. 2F).

4. Discussion

The first live-attenuated RVF vaccine was developed by K.C. Smithburn in the late 1940s by intracerebral passage in mice [9]. The Smithburn vaccine is still commercially available and can provide long-lasting, perhaps lifelong immunity after a single vaccination. However, due to residual virulence, this vaccine should not be used during pregnancy. This is a major drawback, as the ruminant fetus is the most susceptible to RVFV infection. Another live-attenuated experimental vaccine was developed in the 1980s, by passage of RVFV in the presence of the mutagen 5-fluorouracil [24]. This attenuated virus, initially called MVP 12 and later renamed MP-12, was reported to be safe when applied to ewes during the second and third period of gestation [24,25]. Vaccination of ewes during the first trimester of gestation, however, resulted in teratogenic effects that were similar to those observed after vaccination with the Smithburn vaccine [26]. More recently, inoculation of ewes during the first trimester of gestation with MP-12 or a recombinant derivative (arMP-12) did not cause untoward effects in the ewes, although in both groups of four animals, one animal was found to carry a dead fetus at the end of the experiment [27]. The arMP-12 virus was subsequently attenuated further by deletion of NSs or the NSm-coding region. Vaccination of the arMP-12 virus lacking NSs resulted in neutralization responses that were deemed insufficient to warrant further development, whereas a similar virus lacking the NSm-coding region was highly immunogenic in both sheep [27] and calves [28]. Unfortunately, also this virus, named arMP-12ΔNSm21/384, was shown capable of vertical transmission as revealed by the detection of viral RNA in several organ samples collected from a dead, autolyzed fetus [27]. Since even a highly promising recombinant virus lacking both the NSs and NSm coding regions, named DDvax, was found to be capable of causing teratogenic effects [29] it was in hindsight not so surprising to find that Clone 13 can also transmit to the ovine fetus [16].

Despite the clear ability of the live-attenuated (experimental) RVF vaccines listed above to transmit to the ovine fetus, it must be noted that these vaccines are highly efficacious and do not suffer from safety concerns in non-pregnant animals. In contrast to the above listed vaccines, the results of the present work indicate that RVFV-4s does not cross the ovine placental barrier and can be applied safely during gestation.

Conflict of interest statement

P.J. Wichgers Schreur and J. Kortekaas are inventors of WIPO Patent Application WO/2014/189372 "Bunyaviruses with segmented glycoprotein genes and methods for generating these viruses".

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