

## Vertical Transmission of Rift Valley Fever Virus Without Detectable Maternal Viremia

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### Abstract

Rift Valley fever virus (RVFV) is a zoonotic bunyavirus that causes abortions in domesticated ruminants. Sheep breeds exotic to endemic areas are reportedly the most susceptible to RVFV infection. Within the scope of a risk assessment program of The Netherlands, we investigated the susceptibility of a native breed of gestating sheep to RVFV infection. Ewes were infected experimentally during the first, second, or third trimester of gestation. Mortality was high among ewes that developed viremia. Four of 11 inoculated ewes, however, did not develop detectable viremia nor clinical signs and did not seroconvert for immunoglobulin G (IgG) or IgM antibodies. Surprisingly, these ewes were found to contain viral RNA in maternal and fetal organs, and the presence of live virus in fetal organs was demonstrated by virus isolation. We demonstrate that RVFV can be transmitted vertically in the absence of detectable maternal viremia.

**Key Words:** Rift Valley fever virus—Vertical transmission—Antibodies—Domestic animals—Bunyaviridae.

### Introduction

**R**IFT VALLEY FEVER VIRUS (RVFV) is a member of the genus *Phlebovirus*, one of the five genera of the family Bunyaviridae (Elliott 1996). The virus was first identified in 1930 during an outbreak among sheep on a farm in the Great Rift Valley in Kenya. In the years to follow, many serious outbreaks affecting both humans and livestock occurred in several countries of the African continent (Daubney et al. 1931, Findlay and Daubney 1931, Gerdes 2004, Bird et al. 2009). RVFV also emerged on the Arabian Peninsula (Balkhy and Memish 2003) and Madagascar (Mathiot et al. 1984) and was more recently detected on the Archipelago of Comoros and Mayotte, located in the Indian Ocean between Mozambique and Madagascar (Sissoko et al. 2009).

RVFV has a very broad host range, but sheep, goats, and cattle are the main species affected (Easterday 1965, Coetzer 1977, Coetzer 1982). These species play an important role in the epidemiology of the disease by developing sufficiently high viremia to allow transmission to susceptible mosquitoes. The virus is transmitted among livestock by many different mosquito species, predominantly those belonging to the *Aedes* and *Culex* genera (Pepin et al. 2010). Although humans can also become infected via mosquito bites, most human cases are attributed to direct contact with contaminated animal

tissues and bodily fluids. In most cases, human infections are benign and manifest as a transient febrile illness. A small percentage of individuals, however, develop severe complications, such as retinal lesions, meningoencephalitis or hemorrhagic fever (Laughlin et al. 1979, Al-Hazmi et al. 2003, Balkhy and Memish 2003).

There is a concern for incursions of RVFV into Europe because many susceptible species as well as several potential mosquito vectors are present on the European continent (Moutailler et al. 2008). To improve preparedness for a future incursion, it is important to determine susceptibility of native breeds of domesticated ruminants to RVFV infection. Here we report the results obtained from experimental infection of European breed ewes during the first, second, or third trimester of gestation. Viremia was monitored by quantitative real-time PCR (qPCR) and virus isolation on blood samples. Surprisingly, four ewes did not develop detectable viremia and did not seroconvert for immunoglobulin G (IgG) or IgM antibodies. Viral RNA was, however, detected in the organs of these ewes as well as in the organs of their unborn offspring. The presence of live virus was confirmed by isolation of the virus from fetal brain and liver samples. We demonstrate that RVFV can be vertically transmitted without detectable maternal viremia. The presence of live RVFV in gestating ewes that are seemingly healthy and do not contain

detectable levels of antibodies could pose an as yet unrecognized risk for human and animal health.

## Materials and Methods

### Study design

Conventional gestating European breed sheep were purchased from a commercial sheep farm in The Netherlands. Eleven sheep were divided into three groups according to the stage of gestation. Gestation was confirmed by ultrasound techniques. Four sheep were in the first (~34 days), three in the second (~83 days), and four in the third trimester (~132 days) of gestation at the time of the experimental infection. Groups were housed in three separate biosafety level-3 animal isolation units. Surviving animals were euthanized 21–23 days postinfection.

RVFV strain 35/74 was isolated from a liver of a sheep that died during a RVF outbreak in the Free State province of South Africa in 1974 (Barnard 1979). The challenge inoculum was prepared as previously reported (de Boer et al. 2010). The virus was handled under biosafety level-3 laboratory conditions in class-III biosafety cabinets. The sheep were inoculated via the intraperitoneal route on study day 0, with 1 mL of culture medium containing 10<sup>5</sup> 50% tissue culture infectious doses (TCID<sub>50</sub>) of RVFV. Administration of this dose via the intraperitoneal route previously resulted in 67% mortality in 3-week-old lambs (Kortekaas et al., unpublished).

The experiment was conducted in accordance with the Act on Experimental Animals of The Netherlands regulated by the Ethical Review Committee of the Central Veterinary Institute.

### Diagnostic assays

Plasma samples were collected on study days 0, 1, 2, 3, 4, 7, 9, 11, 14, 16, 18, and 21. Serological analysis on plasma samples was performed using a commercial IgM enzyme-linked immunosorbent assay (ELISA) and a competition ELISA according to the instructions of the manufacturers (BDSL, Ayrshire Scotland, UK). **The ID Screen competition ELISA (ID-VET, Montpellier, France) was used as a confirmation test.** Virus neutralizing antibodies were detected as described (de Boer et al. 2010).

Blood and tissue samples were analyzed for the presence of viral RNA by quantitative TaqMan reverse-transcriptase real-time PCR (RT-qPCR). The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, The Netherlands) was used, and primers, probes, and cycling conditions were used as previously described (Drosten et al. 2002).

Virus isolation from blood was performed on plasma samples. The plasma samples were diluted by four-fold dilution steps in culture medium [CO<sub>2</sub>-independent medium (GIBCO, Carlsbad, CA) supplemented with 100 U/mL penicillin (GIBCO) and 100 µg/mL streptomycin (GIBCO), 2 mM L-glutamine (GIBCO), and 5% fetal calf serum (FCS)]. The plasma samples were then added to 96-well plates containing 40,000 baby hamster kidney (BHK) cells/well. The plates were incubated for 1 h at room temperature, after which the culture medium was replaced. After 5 days, the plates were scored for cytopathic effect.

For isolation of virus from liver or brain, samples were disrupted by vortexing for 15 min in the presence of Zirconia

beads (Biospec, Bartlesville, OK). Debris was pelleted by slow-speed centrifugation, and the cleared supernatant was either incubated with BHK cells or inoculated in suckling mice by intracranial (IC) injection. To this end, 3-day-old suckling mice (BALB/c cAnCrI, Charles River Laboratories, Maas-tricht, The Netherlands) were inoculated with 20 µL of clarified tissue suspension diluted 1:10 in phosphate-buffered saline (PBS) containing 1.4 mg/mL ampicillin. The brains from deceased mice or surviving mice euthanized at the end of the experiment were collected, and, when required, pools of suspensions derived from a litter were used for a second passage. Virus isolation was detected by cytopathic effect on BHK cells and confirmed by either immunoperoxidase monolayer assays (IPMA) using RVFV-specific antibodies or qPCR. When suckling mice were used for virus isolation, mortality was used as a first indication of successful virus isolation and confirmed by PCR and isolation of the virus from the suckling mouse brain.

## Results

### RVFV infection during the first trimester of gestation

Four ewes in the first trimester of gestation were inoculated with RVFV strain 35/74. No clinical signs were observed, and fever was only noted in ewe 9605 (Fig. 1A). Viral RNA was detected in the blood of ewes 9605 and 9625 from day 2 until the end of the in life phase (Fig. 1B), and virus was isolated from blood samples obtained on days 3 and 4 (9605) or only on day 3 (9625) (Fig. 2). These ewes developed IgM, IgG, and neutralizing antibodies (data not shown). Postmortem examination in both cases showed firm lungs and remnants of fetal membranes and cotyledons. Histological examination of livers, kidneys, and lungs of the ewes did not reveal specific lesions. A multifocal parasitic pneumonia and peribronchiolar inflammatory cell infiltration were present in the lungs, and a moderate follicular activation was observed in lymph nodes and spleen. A focal tissue necrosis was found in the cotyledons of ewe 9625. Viral RNA was detected in liver and brain samples of the ewes (Fig. 3A, B), and fetal membranes and remnants of cotyledons were also positive for viral RNA (data not shown).

Neither viral RNA nor live virus was detected in the blood of ewes 9639 and 9635 throughout the experiment. ELISA and virus neutralization tests demonstrated that these ewes did not seroconvert for RVFV antibodies. Liver and brain samples were, however, positive for viral RNA at the end of the experiment (Fig. 3A, B). Postmortem analysis of ewe 9639 revealed two live fetuses and no abnormalities. Examination of ewe 9635 revealed firm lungs, no abnormalities in the liver, a diffuse tubular calcification in the kidneys, and two live fetuses. Viral RNA was detected in the livers and/or brains of the fetuses (Fig. 3C, D), but no virus was isolated from these tissues.

### RVFV infection during the second trimester of gestation

Three ewes (nos. 9627, 9631, and 1160) in the second trimester of gestation were inoculated with RVFV. In two of the ewes, a mild fever (rectal temperature >40°C) was observed on 2 days (Fig. 1C). Blood samples from ewes 9627 and 1160 contained viral RNA (Fig. 1D), and virus was isolated from

◀ F1

◀ F2

◀ F3

VERTICAL TRANSMISSION RIFT VALLEY FEVER VIRUS

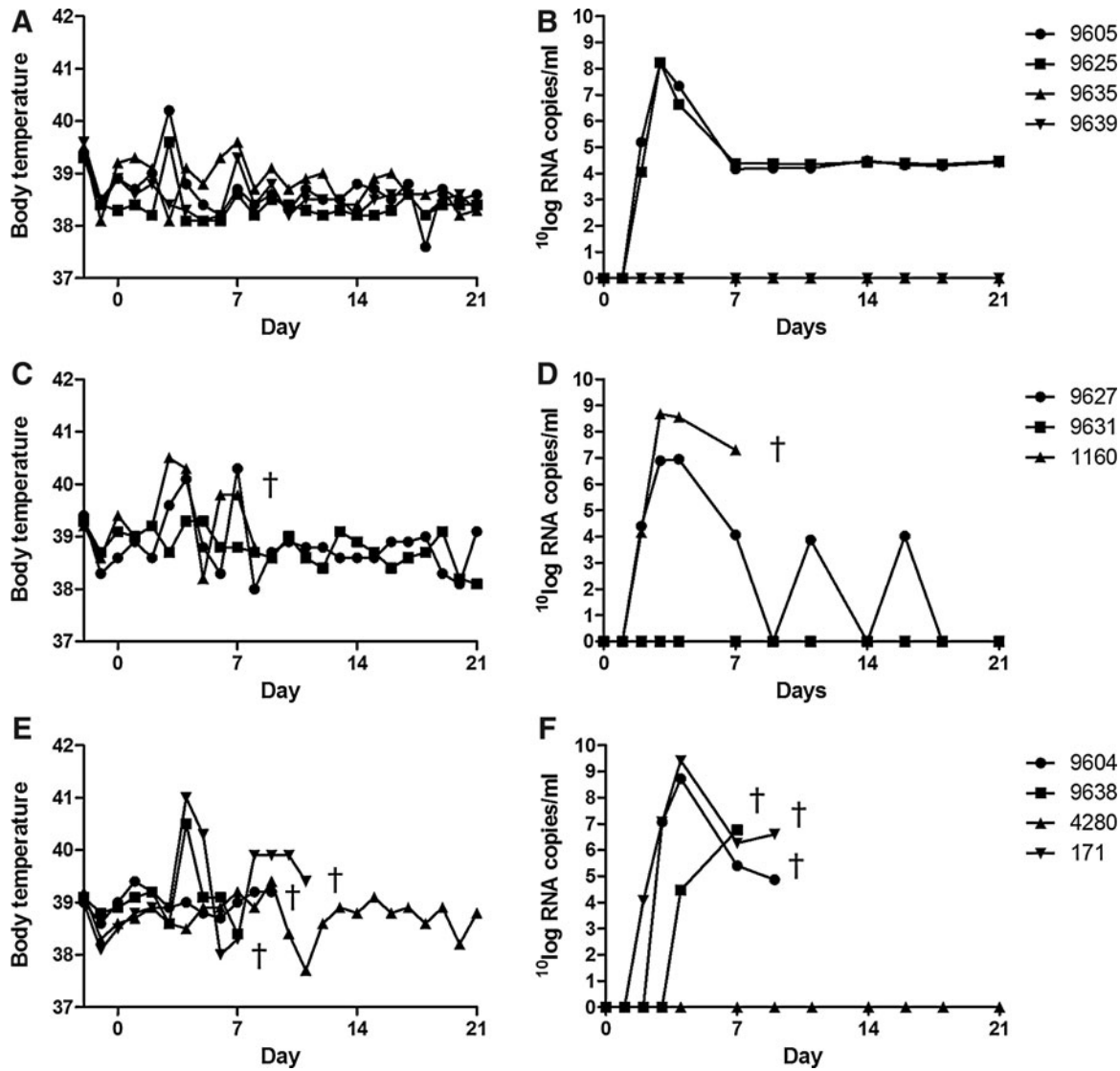


FIG. 1. Detection of fever and viral RNA in ewes infected with Rift Valley fever virus (RVFV). (A, C, E) Rectal body temperatures (°C) were determined daily until 21 days postchallenge. Fever was defined as a body temperature above 40°C. (B, D, F) Viral RNA in EDTA blood samples was detected by qPCR.

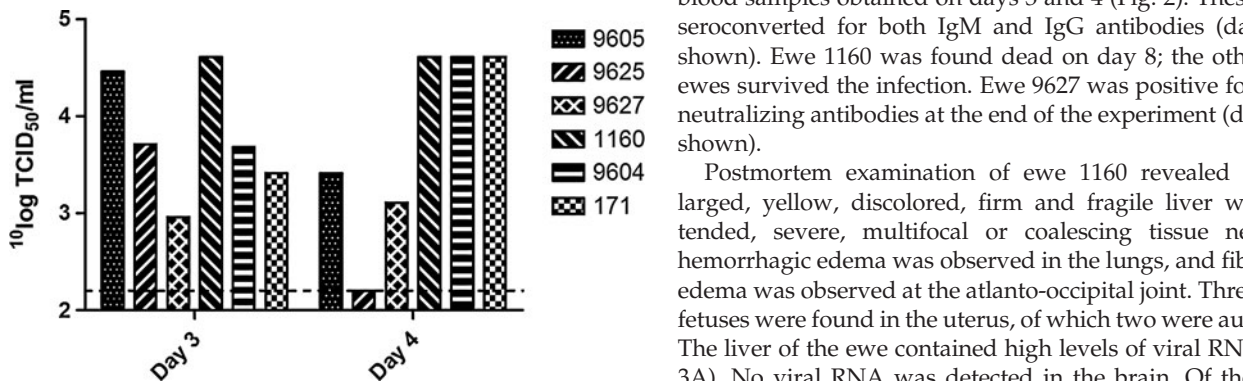
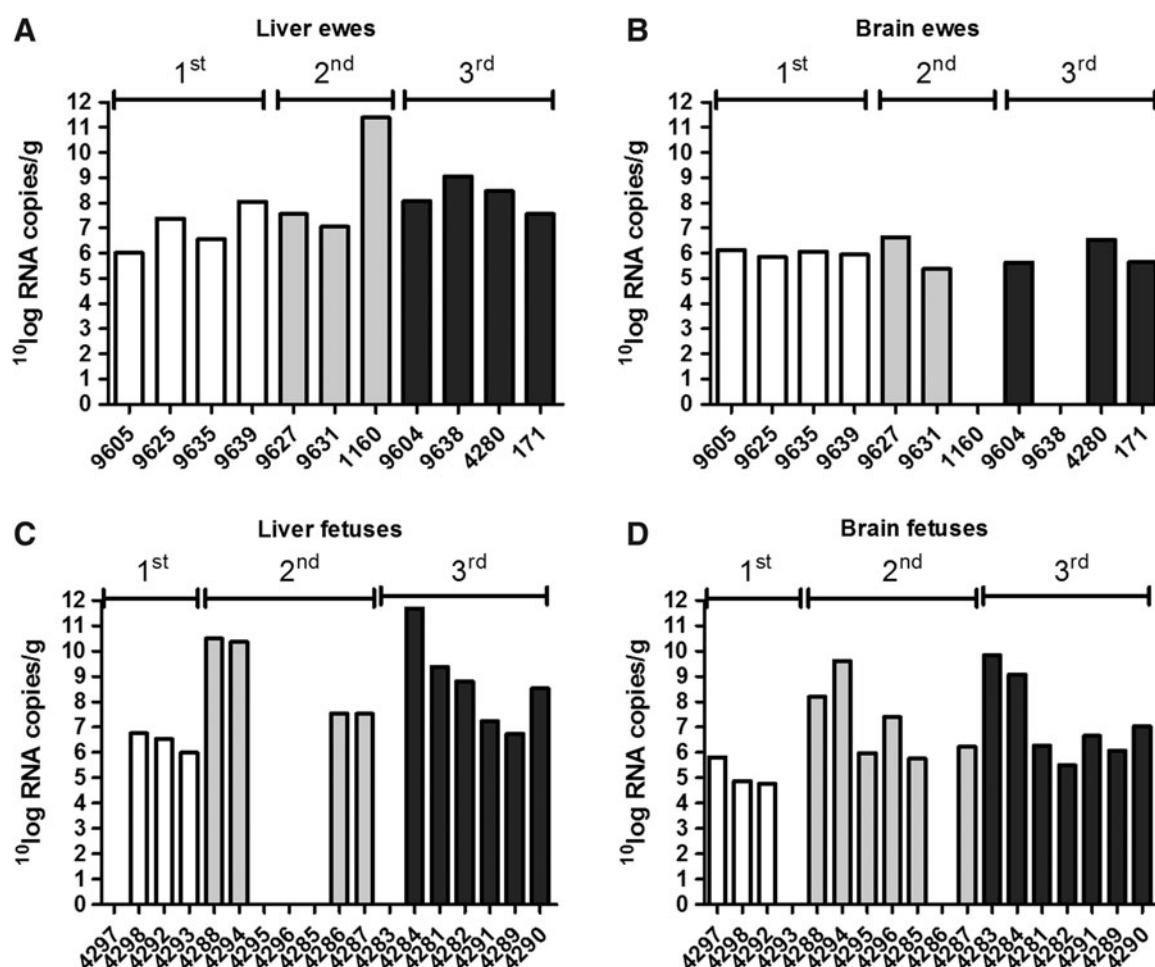


FIG. 2. Virus isolation from plasma samples. Virus isolations were performed on plasma samples. Only samples positive in virus isolation are depicted. The detection limit [ $10^{2.20}$  50% tissue culture infectious doses (TCID<sub>50</sub>/mL)] is indicated by a dashed line.

blood samples obtained on days 3 and 4 (Fig. 2). These ewes seroconverted for both IgM and IgG antibodies (data not shown). Ewe 1160 was found dead on day 8; the other two ewes survived the infection. Ewe 9627 was positive for virus neutralizing antibodies at the end of the experiment (data not shown).

Postmortem examination of ewe 1160 revealed an enlarged, yellow, discolored, firm and fragile liver with extended, severe, multifocal or coalescing tissue necrosis; hemorrhagic edema was observed in the lungs, and fibrinous edema was observed at the atlanto-occipital joint. Three dead fetuses were found in the uterus, of which two were autolytic. The liver of the ewe contained high levels of viral RNA (Fig. 3A). No viral RNA was detected in the brain. Of the three fetuses, one was PCR positive in the brain and liver; the other two fetuses were either positive in the brain or liver (Fig. 3C, D). No virus was isolated from these fetuses.

Viral RNA was detected in the blood of ewe 9627 from day 2 to day 16, but this ewe did not show any clinical signs.



**FIG. 3.** Detection of viral RNA in livers and brains of ewes and fetuses. Gestating ewes in the first, second, or third trimester of gestation were inoculated with Rift Valley fever virus (RVFV) strain 35/74. Liver and brain samples were collected from deceased or euthanized ewes and their fetuses. The presence of viral RNA was determined by qPCR. No fetuses were recovered from ewes #9605 and #9625. Ewe #9635 carried fetuses #4297 and #4298; ewe 9639 carried fetuses #4292 and #4293; ewe #9627 carried fetuses #4288 and #4294; ewe #9631 carried fetuses #4295 and #4296; ewe #1160 carried fetuses #4285, #4286 and #4287; ewe #9604 carried fetuses #4283 and #4284; ewe #9638 carried fetuses #4281 and #4282; ewe #4280 carried fetus #4291; ewe #171 carried fetuses #4289 and #4290.

Postmortem analysis revealed two autolytic lambs and focal necrosis was seen in the placentae. Both liver and brain samples of the ewe contained viral RNA (Fig. 3A, B). Despite the presence of high levels of viral RNA in fetal liver and brain samples (Fig. 3C, D), virus was not isolated from these samples.

Ewe 9631 did not develop detectable viremia nor fever or other clinical signs of infection, and RVFV antibodies were neither detected by ELISA nor by virus neutralization test. Postmortem analysis at the end of the experimental period revealed multiple foci in the caudal lung lobe and two living fetuses. Viral RNA was detected in the liver and brain of the ewe (Fig. 3A, B). No viral RNA was detected in the livers of the fetuses, but brain samples were positive for viral RNA (Fig. 3D, nos. 4295 and 4296). Virus was isolated by inoculation of BHK cells with a brain sample of fetus no. 4296 (Fig. 3D), and virus isolation was confirmed by qPCR on passaged supernatant. This result demonstrates that RVFV can be vertically transmitted without preceding detectable viremia.

*RVFV infection during the third trimester of gestation*

Four ewes were inoculated with RVFV during the third trimester of gestation. In two of the ewes, a mild fever (rectal temperature >40°C) was observed for 1–2 days (Fig. 1E). Viral RNA was detected in the blood by PCR in three ewes (Fig. 1F), and these ewes had to be euthanized due to severe clinical signs on days 7 (ewe 9638), 9 (ewe 9604), and 10 (ewe 171), respectively. Virus was isolated from plasma samples obtained on days 3 and 4 from ewes 171 and 9604 (Fig. 2). These ewes seroconverted for both IgM and IgG antibodies (data not shown). Neither live virus nor viral RNA was detected in the blood of ewe 4280, and this ewe did not seroconvert for IgG, IgM, or neutralizing antibodies (data not shown). Although viral RNA was detected in the blood of ewe 9638 on days 4 and 7, no virus was isolated from these samples and no antibodies were detected.

Gross pathological examination of ewe 9638 revealed an enlarged, yellow, discolored, and firm liver, small foci on the lungs, and fatty, yellow, discolored kidneys. The ewe



**VERTICAL TRANSMISSION RIFT VALLEY FEVER VIRUS**

contained two dead fetuses. The major histological finding in the ewe was a multifocal necrotizing hepatitis. In one of the fetuses, focal hemorrhages were present in liver tissue and edema was observed in lymph nodes. Viral RNA was detected by qPCR in the liver but not in the brain of the ewe and in both livers and brains of the fetuses (Fig. 3). Vertical transmission was confirmed by isolation of virus from one of the fetal livers, revealing a titer of  $10^{5.61}$  TCID<sub>50</sub>/mL.

The major finding during pathological examination of ewe 9604 was a moderate-to-severe hepatitis. Also in the fetuses, a multifocal necrotizing hepatitis was present, and diffuse calcification and vascular thrombosis were observed in the placenta. A dead fetus was located in the uterus and another in the birth canal. Viral RNA was detected in both liver and brain samples of the ewe and in one of the fetuses (Fig. 3). The second fetus contained viral RNA only in the brain. Virus was isolated from one of the fetal livers.

The postmortem of ewe 171 revealed tracheal edema and two dead, autolytic fetuses in the uterus. In the liver of the ewe, a moderate, multifocal necrotizing hepatitis was present. Viral RNA was detected in both liver and brain samples of both ewe and fetuses (Fig. 3). Vertical transmission was confirmed by isolation of virus from the fetal liver that contained the highest number of RNA copies ( $10^{8.33}$  RNA copies/gram). Virus isolation on BHK cells was negative, but after IC inoculation of suckling mice  $10^{7.89}$  RNA copies/mL were detected in the supernatant from the pooled brain homogenate. From this sample, virus was successfully isolated on BHK cells, revealing a titer of  $10^{5.52}$  TCID<sub>50</sub>/mL.

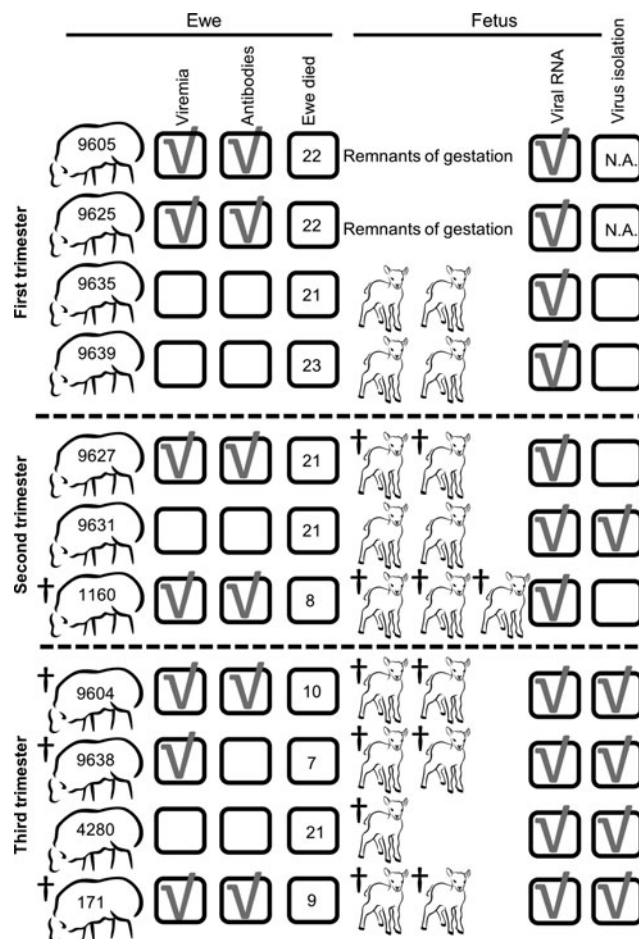
Ewe 4280 did not develop fever or any other clinical sign of infection, and no viremia was detected. ELISA and virus neutralization tests did not reveal RVFV-specific antibodies (data not shown). Postmortem examination revealed a dead fetus in the uterus. In the ewe, a multifocal chronic pleuritis was detected, and few foci with single hepatic cell necrosis were found in the liver. Neither gross pathological nor histological changes were seen in the fetus or the placenta. The detection of viral RNA in the liver and brain confirmed that the ewe was productively infected with RVFV. Viral RNA was also detected in the liver and brain of the fetus, demonstrating that vertical transmission of the virus had occurred (Fig. 3C, D). Virus was not isolated after incubation of BHK cells with supernatant from a pooled liver homogenate, but after IC inoculation of suckling mice,  $10^{6.10}$  RNA copies/gram were detected in the pooled brain homogenate. From this sample, virus was successfully isolated on BHK cells, revealing a titer of  $10^{4.74}$  TCID<sub>50</sub>/mL. Virus isolation was confirmed by cytopathogenic effect on cells as well as qPCR on passaged culture supernatant.

A schematic representation of the combined results is depicted in Figure 4.

F4 ▶

**Discussion**

In the current study, experimental infection of gestating ewes resulted in clinical signs that were either severe or unapparent. Of the total of 11 ewes, seven developed viremia, of which four either succumbed to the infection or were euthanized due to severe morbidity. The three surviving ewes maintained viral RNA levels in the blood, suggestive of persistent infection (Fig. 1). Persistent infection of European breed sheep was recently reported by Busquets et al., who



**FIG. 4.** Overview of results obtained from experimental Rift Valley fever virus (RVFV) infection of gestating ewes during the first, second, or third trimester of gestation. Viremia boxes are checked when virus was detected by both qPCR and virus isolation. Antibody boxes are checked when immunoglobulin G (IgG) and/or IgM antibodies were detected by enzyme-linked immunosorbent assay (ELISA). The days when ewes succumbed to the infection or were euthanized are indicated. Ewes and fetuses that did not survive the infection are marked by crosses. Viral RNA boxes are checked when RNA was detected by qPCR in liver and/or brain. Virus isolation boxes are checked when virus was isolated from fetal brain or liver samples. N.A., Not applicable.

suggested that transient immune suppression could be at the basis of this phenomenon (Busquets et al. 2010). It is important to study the underlying mechanism of RVFV persistence in ruminants further and to establish the potential risk for human and animal health.

It was surprising to find that four ewes did not develop any clinical sign or detectable viremia, a finding that does not seem to correlate with the stage of gestation. These ewes were diagnosed as negative by three commercial ELISAs as well as by virus neutralization tests. Even though a low virus dose was used to inoculate the sheep, the detection of viral RNA in livers and brains of these ewes demonstrated that infection was productive. Seven fetuses were carried by the serologically negative ewes, all of which were positive for viral RNA in the liver and/or the brain. Virus was isolated from the liver

of one of these fetuses, which, together with its sibling, did not survive the infection. Two fetuses carried by a ewe in the second trimester were alive at the end of the experiment, and virus was isolated from the brain from one of these fetuses. Thus, virus was successfully isolated from the liver of a deceased fetus and the brain of a live fetus, both carried by an ewe that did not develop detectable viremia upon experimental infection.

In the current work, we selected the intraperitoneal route of inoculation because this was successfully applied in early studies on RVFV pathology (Findlay 1932, Findlay et al. 1936, Easterday et al. 1962a, b). Because lack of detectable viremia was not reported in these previous studies, we first assumed that our finding was explained by the low inoculated virus dose. We recently found, however, that intraperitoneal inoculation of lambs with a dose of  $10^5$  TCID<sub>50</sub> similarly did not result in detectable viremia in one out of six inoculated animals (Kortekaas et al. 2012), suggesting that our finding is correlated with the inoculation route rather than the virus dose.

### Conclusions

To our knowledge, our work represents the first description of RVFV vertical transmission without detectable maternal viremia. Because the fetuses that survived the infection were not brought to full term, the relevance of this finding with respect to the epidemiology of the disease remains speculative at this point. The results reported here are obtained from experimental infection of a limited number of animals and therefore do not warrant any generalizations. Our findings nevertheless call for further study on cryptic RVFV infections and persistence of the virus both under experimental conditions and in the field using combined classical and molecular diagnostic methods.

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### Author Disclosure Statement

No competing financial interests exist.

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