HoBi-like pestivirus experimental infection in pregnant ewes: reproductive disorders and generation of persistently infected lambs

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Abstract

In order to evaluate sheep as experimental model to test the efficacy of HoBi-like pestivirus vaccines for cattle, 10 sheep at different stages of pregnancy (30 or 50 days) were experimentally infected with the Italian prototype isolate Italy-1/10-1. Irrespective of the stage of pregnancy, virus inoculation resulted in reproductive failures, consisting of abortion, stillbirths or birth of weak or persistently infected (PI) lambs. Aborted fetuses, stillborn and dead lambs displayed extensive histopathological changes, consisting of hemorrhages, congestion and mononuclear infiltration in major organs. Pestiviral antigens were detected by immunohistochemistry in most tissues with remarkable signals in lungs and kidneys. PI lambs were constantly viremic, shed the virus through the nasal secretions and feces and, in all cases but one, did not have detectable HoBi-like pestivirus antibodies before the assumption of colostrum. The single seropositive infected lamb showed low-titer viremia and viral shedding that ceased only several weeks after the 3-month observation period. The study proves that sheep are susceptible to the reproduction failures caused by HoBi-like pestivirus infection and can serve as a suitable model for the evaluation of the fetal protection induced by homologous experimental vaccines.

Key words: Sheep; HoBi-like pestivirus; experimental infection; PI lambs.

1. Introduction

Based on the current nomenclature of the International Committee of Taxonomy of Viruses (http://www.virustaxonomyonline.com), the genus *Pestivirus* consists of four recognized species, bovine viral diarrhea virus (BVDV) 1, BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV) (Simmonds et al., 2011). Four additional *Pestivirus* species have been proposed but remain officially unrecognized: i) Pestivirus of giraffe, associated with an outbreak of mucosal-like disease in giraffes in the Nanyuki District of Kenya; ii) Pronghorn virus, isolated from a blind pronghorn antelope in the United State; iii) Bungowannah virus, detected in pigs following an outbreak of stillbirths and neonatal death in Australia, and iv) a group of viruses variously referred to as HoBi-like, BVDV-3, or atypical pestiviruses (Bauermann et al., 2013).

The prototype HoBi-like pestivirus, strain D32/00_'HoBi', was isolated from a batch of fetal bovine serum (FBS) imported from Brazil. HoBi-like viruses contaminating FBS of southern American origin were later detected worldwide. All these viruses were proposed to belong to a new pestivirus species tentatively termed BVDV-3. However, there is no agreement among pestivirologists about this proposal, considering the genetic and antigenic distance of the new viruses from other BVD viruses (Bauermann et al., 2013). Unlike BVDVs, HoBi-like viruses do not appear to be endemic in all continents. In South America, the virus has been associated with reproductive disorders in Brazilian cattle herds, and death of water buffalos as well (Cortez et al., 2006). The first European Hobi-like virus, strain Italy-1/10-1, was isolated from calves with severe respiratory disease in southern Italy (Decaro et al., 2011, 2012c). Additional Hobi-like viruses were associated to abortion in multiparous cows of the same herd (Decaro et al., 2012a) and to respiratory disease in cattle of a neighboring Italian region (Decaro et al., 2013b). In addition, natural infection of cattle with HoBi-like virus resulted in the birth of persistently infected (PI) calves (Decaro et al., 2013a). More recently, outbreaks of

mucosal disease (MD) have been observed in that country (Decaro et al., 2014) and in Brazil (Weber et al., 2014). Evidence of HoBi-like virus in Asia has been also reported. Although no clinical sign was noted, seroconversion to HoBi-like viruses was observed in dairy herds in Thailand and one virus positive calf serum was identified (Kampa et al., 2010). In Bangladesh, HoBi-like viral sequences were detected in samples from animals displaying diarrhea, respiratory distress and/or fever (Haider et al., 2014). Divergent strains were identified more recently in India (Mishra et al., 2014).

Lambs were found to be susceptible to HoBi-like experimental infection showing respiratory disease and virus shedding (Decaro et al., 2012b). However, considering that efficacy of BVDV vaccines is evaluated in terms of fetal protection after infection of pregnant cows, with the aim to support sheep as an experimental model for HoBi-like pestivirus pathogenesis and vaccination studies, ewes at different ages of pregnancy were experimentally infected and the outcome of the infections are presented in this manuscript.

2. Materials and methods

2.1. Virus

HoBi-like strain Italy-1/10-1 was isolated from the lungs of a 6-month-old calf belonging to a cattle herd affected by respiratory disease in southern Italy (Decaro et al., 2011). For virus isolation the lung sample was homogenized in Dulbecco's minimal essential medium (D-MEM) containing antibiotics (penicillin 5000 IU/ml, streptomycin 2500 μg/ml, amphotericin B 10 μg/ml). After centrifugation at 3000 x *g* for 15 min, the supernatant was used to inoculate confluent monolayers of Madin Darby bovine kidney (MDBK) cells supplemented with 5% of gamma-irradiated fetal bovine serum (FBS), which was free of pestivirus antibodies and RNA. Viral growth was monitored by an immunofluorescence (IF) assay using a BVDV monoclonal antibody and a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma

Aldrich srl, Milan, Italy). The 10th passage on MDBK cells having a titer of $10^{6.00}$ $TCID_{50}$ ml^{-1} was tested for contaminant viruses (coronaviruses, herpesviruses, respiratory syncytial viruses, parainfluenza viruses, adenoviruses) and mycoplasmas by means of standardized methods as previously described (Decaro et al., 2008) and stored at -70°C in 5-ml aliquots.

2.2. Experimental study

The experimental study was performed according to the European animal health and well-being regulations and was authorized by the Italian Ministry of Health. A total of 13 two-three-years-old Merino sheep were used for the experiment. All animals were housed at the Isolation Unit of the Animal Hospital of the Faculty of Veterinary Medicine of Bari (Italy) and had tested negative for the presence of BVDV RNA in the blood by nested PCR assays (Decaro et al., 2012e) and for pestivirus antibodies in the sera by the Bovine Virus Diarrhoea Virus (BVDV-Ab) SVANOVIR™ ELISA test (Svanova Biotech AB, Uppsala, Sweden) and virus neutralization (VN) using BVDV-1, BVDV-2 and HoBi-like pestivirus (Ståhl et al., 2007). Estrus was induced by subcutaneous administration of PGF2a (Wildeus et al., 2000) in order to synchronize breed dates. Natural breeding was accomplished by introduction of a ram for a two-day period. Thirty days after mating the ewes were submitted to ultrasound examination to confirm the successful fecundation and randomly distributed in three groups that were housed in separate pens and blindly managed and monitored by different attendants. Ewes of groups A (n = 5) and B (n= 5) were oronasally administered 5 ml of the challenge virus at 30 and 50 days of pregnancy, respectively, whereas the remaining three animals (group C), housed in a separate box, served as controls by receiving by the same route 5 ml of the cryolysate of uninfected MDBK cells.

To evaluate the successful infection with HoBi-like pestivirus, based on previous findings (Decaro et al., 2012b), EDTA-blood samples were taken at days post-infection (dpi) 2, 6, 9, 12,

15, 19, 23, 26, 30, 37, 42, 49, 60. Sera were also collected at 2-week intervals for pestivirus serology. Infected and control ewes were subjected to ultrasound examination at two-week intervals to monitor the course of pregnancy. In the case of abortion or stillbirth, tissue samples were collected from several organs (placenta, brain, lung, liver, spleen, kidney) for virological examinations. Lambs that were born alive were bled for detection of pestivirus antibodies prior to ingestion of colostrum. When surviving, lambs were monitored for three months at the clinical and virological (presence of HoBi-like pestivirus RNA) levels by collecting EDTA-blood, nasal and rectal swabs at 2-week intervals.

2.3. Histopathology and immunohistochemistry

Samples for histopathology and immunohistochemistry (IHC) were collected from major organs (brain, thymus, lung, liver, kidney, spleen, placenta) of aborted fetuses, stillborn and dead lambs. Samples were either fixed in formalin followed by embedding in paraffin and sectioning or were snap-frozen in liquid nitrogen-cooled isopentane and stored at -70°C. Three-micrometer sections were obtained, which were stained with hematoxylin-eosin (H&E) or Perls' Prussian blue (PB) for histological examination or treated with an anti-NS3 monoclonal antibody for IHC (Decaro et al., 2011).

2.4. Antibody detection

Pestivirus antibodies were detected using ELISA and VN tests. The SVANOVIR™ ELISA kit was employed following the manufacturer's instructions and using the horseradish peroxidase (HRP) conjugated anti-bovine IgG monoclonal antibodies provided with the kit. This assay had been proven to react with antibodies against HoBi-like pestivirus in cattle and sheep, albeit the optical density (OD) values obtained were only slightly higher than the cut-off value (OD = 0.200) (Decaro et al., 2011, 2012b). In addition, considering that the kit does not

provide conjugated anti-ovine IgG monoclonal antibodies, commercial HRP conjugated anti-ovine IgG purified antibodies (Sigma-Aldrich srl, Milan, Italy) were used at the dilutions currently employed in our laboratory in other ELISA tests for those species (Decaro et al., 2012b, 2012d).

The VN assay was carried out as described by Ståhl et al. (Ståhl et al., 2007), with minor modifications. Briefly, 100 $TCID_{50}$ of isolate Italy-83/10cp (Decaro et al., 2012a) were mixed with serial two-fold dilutions of the tested sera and after an incubation for 45 min at +37°C, the mixtures were inoculated on MDBK cells developed in 96-well plates. Plates were read after four days of incubation at +37°C in the presence of 5% CO_2 and results were expressed as the reciprocal of the highest serum dilution able to inhibit the appearance of cytopathic effect in inoculated cells. VN tests were performed in four replicates with the final titers being calculated with the Reed Muench method.

Seroconversion occurred in the presence of ELISA OD values and VN antibody titers higher than 0.200 and 1:2, respectively.

2.5. Virus detection

A TaqMan assay specific for HoBi-like pestiviruses (Liu et al., 2008) was used for detection and quantification of Italy-1/10-1 RNA in clinical samples. Viral RNA was extracted from nasal and rectal swabs using the QIAamp® Viral RNA Mini Kit (Qiagen S.p.A., Milan, Italy), whereas RNA extraction from the WBC pellets was obtained using the QIAamp® RNA Blood Mini Kit (Qiagen S.p.A.). HoBi-like RNA copy numbers were calculated on the basis of the standard curves generated by 10-fold dilutions of a synthetic RNA obtained by in-vitro transcription of a plasmid containing the 5' UTR of the isolated strain. Reverse transcription was carried out using GeneAmp® RNA PCR (Applied Biosystems, Applera Italia, Monza, Italy), following the manufacturer's recommendations. The quantitative assay targeting the 5' UTR was conducted in a

50 µl-reaction mixture containing 25 µl of IQ™ Supermix (Bio-Rad Laboratories Srl), 600 nM of primers T134-F (5'-GACTAGTGGTGGCAGTGAGC-3') and T220-R (5'-GAGGCATTCCTTGATGCGTC-3'), 200 nM of probe T155r-P (6FAM– 5'-ACTCGGGGCTTCGGTGATCCAGGG-3'-BHQ1) and 20 µl of c-DNA. The thermal profile consisted of activation of iTaq DNA polymerase at 95° C for 10 min, followed by 45 cycles of denaturation at 95° C for 15 s and annealing-extension at 60° C for 1 min. To confirm the successful infection of the experimental animals and rule out concurrent infections with other pestiviruses, a nested PCR (nPCR) assay recently developed for detection and characterization of pestiviruses was employed (Decaro et al., 2012e). This assay is able to type correctly all pestiviruses infecting cattle by using virus-specific nPCR primers, whereas BDV and CSFV are detected only by the first-round amplification but do not react with nPCR oligonucleotides. RT-PCR (first-round amplification) was carried out using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies, Invitrogen, Milan, Italy) and the following thermal protocol: reverse transcription at 50 °C for 30 min, inactivation of *Superscript II RT* at 94 °C for 2 min, 45 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min, with a final extension at 68 °C for 10 min. The PCR products were detected by electrophoresis through a 1.5% agarose gel and visualization under UV light after bromide ethidium staining. Nested PCR was performed using AmpliTaq Gold (Applera Italia, Monza, Italy) The reaction was carried out in a total volume of 50 µl containing PCR buffer 1X (KCl 50 mM, Tris-HCl 10 mM, pH 8,3), $MgCl_2$ 2 mM, 200 µM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 1 µmol $l^{-1}$of the RT-PCR reverse primer and of each internal species-specific primer, 1 U of AmpliTaq Gold and 5 µl of a 1:100 dilution in distilled water of the primary PCR product. The thermal conditions consisted of activation of AmpliTaq Gold polymerase at 94°C for 10 min and 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and polymerization at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were detected as for the first-round amplification.

3. Results

3.1. Outcome of the experimental infection of pregnant ewes

Sheep of the control group remained uninfected through the pregnancy and delivered lambs that were in good health conditions for the entire monitoring period. Group A and B ewes were successfully infected by HoBi-like pestivirus, displaying low-titer viremia from dpi 6 to 30 and 6 to 26, respectively. As already noted (Decaro et al., 2012b), commercial ELISA did not find HoBi-like pestivirus antibodies in any infected animals. However, by using VN assay with the homologous virus, all infected sheep were found to seroconvert already at dpi 14 post-infection, with antibody titers that peaked between dpi 112 and 133 (median VN titers of 1:2048 and 1:1024 in group A and B animals, respectively) and remained high over the entire observation period.

The outcome of the HoBi-like pestivirus infection in pregnant ewes is summarized in Table 1. The three ewes of the control group delivered healthy lambs that remained pestivirus antibody- and virus-negative for the entire monitoring period. One sheep of treatment group A aborted at 41 dpi, another one displayed stillbirth at 119 dpi and a third sheep delivered two lambs, one (#771-1) born dead and the other one (#771-2) alive but showing neurological disorders similar to those observed in BDV PI animals. These were initially represented by rhythmic contractions of the muscles of the hindlegs and back, which were later replaced by fine trembling of the head, ears, and tail. All aborted fetuses and stillborn animals tested positive for HoBi-like pestivirus RNA in multiple organs. The remaining ewes infected at 30 days of pregnancy gave birth to apparently healthy lambs that remained alive during the monitoring period, although they had smaller sizes and body weights with respect to lambs born to control ewes. By ELISA and VN assays carried out on serum samples collected before the assumption of colostrum, all group A lambs born alive but one (#770)

tested negative for HoBi-like pestivirus antibodies, whereas real-time PCR detected the viral RNA in their blood and body fluid, thus proving that they were PI animals. Lamb #770 remained HoBi-like pestivirus viremic and seropositive even after the end of the observation period (six month of age).

As for ewes of treatment group B, which were infected at 50 days of pregnancy, two animals aborted at 69 and 79 dpi, respectively, another sheep displayed stillbirth (two lambs) at 97 dpi and a fourth animal gave birth to a healthy lamb. The remaining group B ewe delivered one stillborn animal and one weak lamb that died the day after birth. All aborted fetuses and only one of the two stillborn lambs turned out positive for HoBi-like pestivirus. Of the lambs born alive, that dying at 1 day of age tested negative for pestivirus antibodies and positive for HoBi-like pestivirus RNA; in contrast, the healthy lamb was seropositive and virus negative.

3.2. Post-mortem, histopathological and immunohistochemical findings in aborted fetuses, stillborn and dead lambs

Aborted fetuses, stillborn and dead lambs displayed smaller sizes with respect to their ages, rough hair coats, especially on the neck and back, and arthrogryposis of all legs (Fig. 1A). Major internal organs were affected by extensive congestion and hemorrhage.

At histopathology, placental tissues (when available) were diffusely deteriorated with hemorrhages and hemosiderin accumulation. Brains of aborted fetuses and stillborn lambs were partially autolytic with meninges showing hyperemia and hemorrhage and, in some cases, also perivascular mononuclear infiltrates. Lymphoid tissues (thymus and spleen) were markedly congested with two stillbirths displaying multifocal granulomatous splenitis.

Moderate to severe enlargement of the alveolar capillaries and mononuclear infiltration of the alveolar walls were the prominent lesions affecting lungs, while livers showed extensive hemorrhages, sinusoid dilatation, multifocal mononuclear infiltration and accumulation of

hemosiderin and/or biliary pigments. Kidneys were affected by diffuse interstitial hemorrhages, dilatation of glomerular and vasal capillaries (Fig. 1B) and (in one stillbirth) moderate mononuclear infiltration.

By IHC, pestiviral antigens were detected in most collected tissues, with positive cells being frequently detected in the alveolar and bronchial epithelia of lungs (Fig. 1C), and glomerular and tubular epithelia of kidneys (Fig. 1D).

3.3. HoBi-like RNA distribution in tissues of aborted fetuses, stillborn and dead lambs

Aborted fetuses showed the highest virus amounts in the lungs (median titers of 1.46×10^5 RNA copies μl^{-1} of template), which were followed by spleen (1.03×10^5 RNA copies), liver (1.02×10^5 RNA copies), brain (9.02×10^3 RNA copies), and placenta (1.30×10^3 RNA copies) (Fig. 2). In stillbirths, brain was the tissue displaying the highest viral RNA titers, with 2.17×10^5 median RNA copies μl^{-1} of template, whereas the lamb dead at one day of age had the greatest amount of virus in the lungs (3.71×10^7 HoBi-like RNA copies μl^{-1} of template). In this animal, all tissues were found to have very high virus amount (Fig. 2).

3.4. HoBi-like RNA viremia and shedding in PI lambs

The two surviving virus positive and seronegative lambs (#765, #771-2) displayed high-titer HoBi-like pestivirus viremia at the day of birth and remained viremic for the three-month observation period, peaking at day 30 (median titers of 1.85×10^7 RNA copies μl^{-1} of template). Shedding through the nasal and rectal routes occurred at high titers as well over the three months (Fig. 3). Soon after its birth (before colostrum assumption), lamb #770 that was viremic and seropositive had VN HoBi-like antibody titers of 1:1024, which did not undergo significant fluctuations during the 3-month observation period and persisted at the same levels even at six month of ages. In this animal, which analogously to sheep tested

constantly negative by antibody ELISA test, only faecal shedding was comparable to that of other PI lambs, while viremia and nasal shedding occurred at very low titers (Fig. 3). In addition, at about 5 months of age the virus was no longer detected in any sample collected from the lamb (data not shown).

4. Discussion

The outcome of BVDV infection depends on the age, immunological and physiological conditions of the infected cattle, as well as on the virulence of the pestiviral strain. The effects of BVDV infection on reproduction vary according to the stage of gestation at the time of infection. Exposure of naive cattle to the virus at or near the time of breeding may induce reduced pregnancy rates due to decreased conception rates and early embryonic death. Abortion is most common when infection occurs in the first trimester but may occur at any time during pregnancy. Infection of the fetus at 2-3 months of gestation may result in the birth of PI calves. These animals can be weak at birth but as well as clinically normal, but they represent the main source of the virus in the herd, shedding BVDV through their body secretions. Fetuses that are infected after acquiring the immunocompetence may develop congenital defects or be born completely healthy (usually seropositive and virus negative) (Grooms, 2004).

HoBi-like viruses are an emerging group of novel pestiviruses that are being increasingly reported in different parts of the world as responsible for clinical conditions commonly associated with BVDV infection (Cortez et al., 2006). These included respiratory distress (Cortez et al., 2006; Decaro et al., 2011), abortion (Bauermann et al., 2013, Decaro et al., 2012a), birth of PI calves (Decaro et al., 2013a), and occurrence of mucosal disease (Decaro et al., 2014; Weber et al., 2014).

Experimental infections of non-pregnant calves and lambs with HoBi-like pestiviruses caused only moderate respiratory distress, which appeared along with viremia and intermittent shedding through the nasal and fecal routes (Decaro et al., 2012b). In the present study, ewes at different ages of pregnancy were infected with an isolate of the novel pestivirus species in order to assess whether the sheep is a suitable model to evaluate the efficacy of vaccines against HoBi-like viruses. The two gestational periods were selected in order to maximize the effects of pestivirus infection on the ovine pregnancy; in the case of BDV infection, these effects have been reported to be more evident before the development of immunocompetence, which is usually reached before 60 days of pregnancy (Nettleton et al., 1998). All but one infected ewes underwent reproductive failures that included abortion (n = 3), stillbirth (n = 4) or generation of PI lambs (n = 4). Similar results were obtained from cows experimentally infected with HoBi-like pestivirus at about 70 days of gestation. Of the seven inoculated pregnant cows, one aborted at 8 month of gestation, whereas the other six animals gave birth to PI calves (Bauermann et al., 2014).

In pestiviral infections, generation of PI animals is due to transplacental infection of fetuses before the onset of immune competence, which in the ovine species usually occurs between approximately 60 and 85 days of gestation. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and a high frequency of fetal death is common. When fetuses infected at this stage of pregnancy survive, they become tolerant of the virus and are born with a persistent viremia in the absence of any immune response. PI calves and lambs are the main source of virus shedding in the environment, thus contributing to perpetuate pestiviral infections in the herds/flocks. PI animals can be healthy or present several disorders, including lack of growth or weight gain, fleece/hair alterations, as well as neurological, skeletal disorders (Grooms, 2004; Nettleton et al., 1998).

Clinical and virological findings observed in a HoBi-like PI calf were recently reported (Decaro et al., 2013a). This animal displayed stunted growth, ruffled hair and concurrent fungal and protozoan infections, and developed MD, a fatal form of pestivirus infection caused by superinfection by a cytopathic virus in cattle harboring a noncytopathic strain.

HoBi-like pestivirus PI lambs generated in the present study were of smaller size in comparison with those of the control group, with one animal showing neurological disorders that were typical of border disease, a clinical syndrome caused by BDV in its natural host, i.e., sheep. This was in agreement with previous observations that sheep infected with BVDV-1 or BVDV-2 display clinical forms resembling to border disease (Carlsson, 1991; Scherer et al., 2001), although the "hairy shaker" syndrome was observed in lambs born to ewes exposed to BVDV-1 (Carlsson, 1991) but not in those infected with BVDV-2 (Scherer et al., 2001). A striking finding in our study was the birth of a PI lamb that displayed low viral titers in all body fluids and in the blood, having at the same time HoBi-like pestivirus antibodies. It has already reported that PI calves may develop an immune response against the homologous virus that results from changes in BVDV quasispecies sequences that arise as the PI animals mature. Few amino acid changes in the E2 protein, the major antigenic determinant of pestiviruses, were able to induce VN antibodies in PI animals (Collins et al., 1999; Collen et al., 2000). Although no sequence analysis was conducted in the present study, a similar mechanism could also explain the presence of VN antibodies in a PI lamb, considering that the animal was tested before colostrum assumption and remained highly seropositive at an age when the maternal immunity should have declined. According to this scenario, mounting of the immune response may have led to control the viral replication, thus accounting for the less extent and duration of viremia and virus shedding with respect to the other PI animals.

The goal of current BVDV vaccines is to induce fetal protection in pregnant cows, thus preventing the creation of PI calves that act as reservoirs of the virus (Newcomer et al., 2015).

However, experimental studies involving cattle to evaluate the fetal protection of pestivirus vaccines require great efforts as for handling a high number of large-size animals, adopting adequate biosecurity measures and spending much budget for purchasing and feeding cattle. Presently, the role of sheep in the epidemiology of HoBi-like pestiviruses is not known. Considering that they are susceptible to HoBi-like pestivirus infection and clinical forms in both non-pregnant (Decaro et al., 2012b) and pregnant (this study) animals, extensive epidemiological surveys are needed in flocks of endemic areas to assess the circulation of the novel pestiviruses.

Evaluation of the tissue distribution of HoBi-like pestivirus RNA revealed that maximal titers are reached in the lungs and brains of aborted fetuses and stillbirths (Fig. 2), whereas in PI animals high viral amounts were detected not only in the blood, but also in nasal and fecal specimens (Fig. 3). Even considering the small number of animals that were tested, these findings may have diagnostic implications if transferred to the bovine species. In fact, in abortion storms induced by HoBi-like pestivirus, the expulsion of the fetus may occur several weeks after infection of the cows, leading to progressive degradation of viral RNA. Thus, the exact knowledge of the tissues harboring the highest amounts of virus may prevent misdiagnosing this infection. In addition, the discrete viral loads detected in nasal secretions and feces of PI animals may be helpful whenever blood collection is cumbersome, which is common when bleeding calves and bulls.

As previously observed (Decaro et al., 2012b), commercial ELISA, based on BVDV-1 antigens, did not detect HoBi-like pestivirus antibodies in sheep after intranasal inoculation, which again poses severe concerns about the ability of BVDV surveillance programs to efficiently identify animals that had been exposed to infection caused by this novel group of viruses. To date, VN is the gold standard for detection of cattle that are positive for HoBi-like pestivirus antibodies, but the test is cumbersome and labor-intensive. Therefore, the development of

specific serological assays, which can be run with high sample throughput, would be beneficial to evaluate the real circulation of HoBi-like pestivirus among cattle herds of different parts of the world.

Conflict of interest statement

None.

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Figure legends.

Fig. 1. Gross (panel A), histopathological (panels B, C) and immunohistochemical (panel D) findings in aborted fetuses, stillborn and dead lambs induced by experimental infection of pregnant ewes. **A.** Stillbirth (#768-1): fetus carried to term with kyphosis, arthrogryposis, "camel legs" and incomplete development of the fleece. **B.** Abortion (#024): kidney section showing dilatation of glomerular and vasal capillaries, interstitial hemorrhages and putative hemosiderin accumulation (HE staining, 10×). **C.** Weak lamb (#768-2): pestiviral antigens (arrows) detected in a kidney section (IHC with monoclonal antibody, 40×). **D.** Stillbirth (#771-1): pestiviral antigens (brown stained) detected in a lung section (IHC with monoclonal antibody, 20×).

Fig. 2. Logarithmic distribution of the RNA amounts of HoBi-like pestivirus RNA in tissues of aborted fetuses, stillborn and dead lambs induced by experimental infection of pregnant ewes. Values are expressed as RNA copy numbers per µl of template. Each dot represents a sample.

Fig. 3. Viremia (**A**), nasal (**B**) and fecal (**C**) shedding in PI lambs generated after experimental infection of pregnant ewes inoculated with HoBi-like strain Italy-1/10-1. Animals were monitored for 90 days after birth. Viral RNA titers as determined by real-time RT-PCR are expressed as copy numbers per µl of template.