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TITTLE: An Outbreak of Abortions, Stillbirths, and Malformations in a Spanish Sheep Flock Associated with a Bovine Viral Diarrhea Virus 2-Contaminated Orf Vaccine

SHORT RUNNING TITTLE: BVDV 2 Contamination in an Orf Vaccine

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Summary

Bovine viral diarrhea virus (BVDV) is a pestivirus that affects both cattle and sheep, causing an array of clinical signs, which include abortions and malformations in the offspring. Manufacturing of modified live virus (MLV) vaccines often includes the use of bovine-derived products, which implies a risk of contamination with viable BVDV. Recently, the circulation of a specific strain of BVDV 2b among Spanish sheep flocks, associated with outbreaks of abortions and malformations, and whose origin was not determined, has been observed. On February 2018 a MLV orf vaccine was applied to a 1600 highly prolific sheep flock in the Northeast of Spain that included 550 pregnant ewes. In May 2018, during the lambing season, an unusual high rate (72.7%) of abortions, stillbirths, congenital malformations and neurological signs in the offspring was observed. It was estimated that about 1000 lambs were lost. Three 1- to -3-day-old affected lambs and a sealed vial of the applied vaccine were studied. Lambs showed variable degrees of central nervous system malformations and presence of pestiviral antigen in the brain. Molecular studies demonstrated the presence of exactly the same BVDV 2b in the tissues of the three lambs and in the orf vaccine, thus pointing to a pestivirus contamination in the applied vaccine as the cause of the outbreak. Interestingly, sequencing at the 5'-untranslated region-(UTR) of the contaminating virus showed a complete match with the virus described in the previously-reported outbreaks in Spain, thus indicating that the same contaminated vaccine could have also played a role in those cases. This communication provides a clear example of the effects of the application of this contaminated product in a sheep flock. The information presented here can be of interest in putative future cases of suspected circulation of this or other BVDV strains in ruminants.

Keyworks: BVDV 2, sheep, abortions, CNS malformations, vaccine contamination

1. Introduction

Bovine viral diarrhea virus species 1 and 2 (BVDV 1 and BVDV 2, or Pestivirus A and Pestivirus B, respectively) are single stranded RNA viruses that belong to the genus *Pestivirus*, family *Flaviviridae*. They infect mainly cattle, although natural and experimental interspecies transmission to pigs, sheep, and other domestic and wild ruminants have been described (Tao et al., 2013; Hill, Reichel, & Brownlie, 2014; Simmonds et al., 2017; Hopkins, Mitchell, Carson, Russell, & Hateley, 2019; Lanyon; Ricci, Bartolini, Morandi, Cuteri, & Preziuso, 2019).

BVDV causes bovine viral diarrhea (BVD) and mucosal disease, which encompass a series of gastrointestinal and hemorrhagic disorders, immunosuppression, and reproductive problems. Indeed, *in utero* infection of the bovine fetus induces embryonic resorptions, stillbirths, and teratogenic effects, which result in central nervous system (CNS) malformations. Furthermore, infected pregnant females may give birth to immunotolerant persistently infected (PI) individuals, which are a permanent source of BVDV (Lanyon et al., 2014; Evans et al., 2019). Similar syndromes in sheep naturally or experimentally infected with BVDV 1 or BVDV 2 have been described (Scherer et al., 2001; Hopkins et al., 2019). In Spain, most of the reported bovine isolates belong to BVDV 1, with three sporadic descriptions of BVDV 2 (Diéguez, Cerviño, & Yus, 2017). Interestingly, the same strain of BVDV 2b has been recently isolated in several Spanish sheep farms associated with outbreaks of a border disease-like syndrome that included abortions and malformations in the offspring (Elvira Partida et al., 2017).

Modified live virus (MLV) vaccines are sometimes manufactured using bovine-derived products, such as fetal bovine serum, which is a frequent component of the culture mediums (Makoschey, van Gelder, Keijsers, & Goovaerts, 2003; Kozasa et al., 2011; Monteiro et al., 2018). This serum may contain viable BVDV if obtained from a PI fetus (Bolin, Matthews, & Ridpath, 1991; Erickson, Bolin, & Landgraf, 1991), which implies a risk of contamination of the finished vaccine and related disease when applied to susceptible species, especially to pregnant females (Wensvoort & Terpstra, 1988; Barkema et al., 2001; Palomares, Marley, Givens, Gallardo, & Brock, 2013; Fox et al., 2019).

The use of BVDV-contaminated vaccines may also induce unapparent PI individuals that promote circulation of previously unreported BVDV strains in a given geographical area. Thus, it is important to report these cases and the characteristics of the contaminating viral agent to determine whether outbreaks of new BVDV strains have a spontaneous or an iatrogenic origin. In this report, we describe a case of abortions and malformations in lambs associated with a MLV orf vaccine contaminated with a strain of BVDV 2b.

2. Materials and methods

2.1. Case history

The case occurred in a 1600, highly prolific (>2 lambs/ewe and lambing season), meat-focused, Lacaune sheep flock, located in the Northeast of Spain. Five-hundred-and-fifty ewes were mated on December 2017. The flock had reported recurrent outbreaks of orf in lambs and ewes in the past, thus all the animals, including the 550 pregnant ewes, were vaccinated subcutaneously with 0.5 mL of a MLV orf vaccine (Overvac EC, Laboratorios Ovejero, Spain; batch No. 141J002A) on February 18th, 2018.

On April 12th, 2018 a rapid alert was released by the Spanish Agency of Medicines and Medical Devices (AEMPS) warning about a likely contamination of this product with pestivirus and asking for a preventative withdrawal from the market (AEMPS, 2018a; AEMPS, 2018b). During the pregnancy, and especially at the beginning of the lambing season (May 2018), the farmer reported an unusual high rate of reproductive failure (400/550; 72.7%) that included abortions, resorptions, and/or stillbirths, and clinical signs such as tremors, ataxia, and prostration in the lambs that were born alive (Supplementary Video S1). The farmer estimated that more than 1000 lambs were lost, either directly or by humane euthanasia.

An abortion diagnostic panel was carried out. Microbiological cultures from vaginal swabs and placentas of 8 aborted sheep were performed using non-selective and selective media for *Salmonella spp*., *Campylobacter spp*., and *Brucella spp.* and smears from these samples were studied by Stamp staining for *Chlamydia (C.) abortus* and *Coxiella (C.) burnetti* inclusions visualization. PCR with primers for *C. abortus, C. burnetti, Toxoplama (T.) gondii*, and pestivirus were performed in placental and fetal

samples of the 8 animals. Finally, sera from maternal blood of 2 of these aborted sheep were analyzed by ELISA to detect antibodies against *C. abortus*, *C. burnetti*, *N. caninum*, and pestivirus. On June 7th, 2018, three 1- to 3-day-old lambs (Nos. 1-3) and a sealed vial of the vaccine were submitted for further testing.

2.2. Macroscopic examination, histopathology, and immunohistochemistry

Necropsies of the three lambs were carried out and tissue samples from different organs were fixed in 10% neutral-buffered formalin for 48h and processed routinely for staining with hematoxylin and eosin (HE) and luxol fast blue (only in CNS). Formalin-fixed, paraffin-embedded tissue sections from brain, thyroid gland, lung, oral mucosa, and aural skin were submitted to immunohistochemistry (IHC) for pestiviral antigen detection. The panpestiviral 15c5 antibody (dilution 1:10000; E. Dubovi, Cornell University, USA) targeting the glycoprotein gp48 (E^{rns}) was used as described (Hilbe et al., 2007).

2.3. Molecular analyses in tissues

Ultra-frozen (-80°C) tissue samples from the same organs chosen for IHC were used for viral nucleic acid detection of ruminant pestiviruses as described and performed in our Swiss reference laboratory for ruminant pestiviruses (Braun et al., 2018). Briefly, tissue samples were divided into small pieces, and homogenized using the QIAshredder from the QIAamp RNeasy blood mini Kit (Qiagen, Hombrechtikon, Switzerland) for isolation of total RNA. Quantitative reverse transcription PCR (RT-qPCR) was performed with probes for BVDV 1 and BVDV 2 using the QuantiTect Probe One Step RT-PCR Kit (Qiagen). Reactions were run with primers in the 5'-untranslated region (-UTR), and the amount of viral RNA in the samples was expressed in cycle threshold (Ct) values. A Ct value of ≤ 32 was considered as positive, a value of >32 up to <45 as weakly positive, and a value of ≥45 as negative. For positive and weakly positive samples, a classic RT-PCR was done, again with primers targeting the 5'-UTR. In all cases where a band in the agarose gel was obtained, the fragment was sequenced by Sanger method and the data analyzed as described (Stalder et al., 2016). In addition, for selected samples, a fragment in the NS3/4A region (1149 nt) was sequenced by the Sanger method for further confirmation of the identity of the strains.

2.4. Molecular analyses in the vaccine

The vaccine was reconstituted in 5 mL of the provided solvent as indicated by the manufacturer. The reconstituted product was homogenized and three aliquots of 200 µL were used for pestiviral RNA detection and 5'-UTR sequencing as described for the tissues. Virus isolation and propagation studies were done on primary bovine turbinate (BT) cells prepared in house and on the MDBK cell line. These cells and the serum used to propagate them were free of BVDV and antibodies against BVDV (Lussi, Sauter, & Schweizer, 2018). Two subsequent cellular passages were done. For the first one, 300 µL of the reconstituted vaccine suspension was added to the cells in a test tube together with 2 mL of medium, and incubated at 37°C in a humidified 5% CO_2-atmosphere for 5 days. For the second passage, 300 µL of the supernatant was added to fresh cells with 2 mL of fresh medium. Supernatants of both passages were analyzed for the presence of pestiviral RNA and antigen. For antigen detection, 50 µL of both cell culture supernatants together with 100 µL fresh medium were added per well to the corresponding cell types in 96-well plates (in duplicates), incubated for 4-5 days prior to fixation, and stained with immunoperoxidase (IPO) using a polyclonal swine-α-BVDV hyperimmune serum as primary antibody prepared at the Institute of Veterinary Virology, University of Bern (Kaiser, Nebel, Schupbach-Regula, Zanoni, & Schweizer, 2017). For pestiviral RNA detection, 200 µL of the supernatant of each passage were used for RNA isolation using the NucleoMag® VET Viral RNA/DNA isolation kit (Macherey-Nagel GmbH, Oensingen, Switzerland) on the Thermo Scientific KingFisher Flex purification system™ followed by RT-qPCR and 5'-UTR or NS3/4A sequencing as described for the tissues.

3. Results and discussion

A severe outbreak of embryonic loss, abortions, stillbirths, and congenital CNS malformations occurred in a Lacaune sheep flock and prompted to research on its etiology. As a first approach, an abortion diagnostic panel on placentas, fetal organs, and maternal blood was carried out. All microbiological cultures tested negative, and neither *C. abortus* nor *C. burnetti* inclusions were observed in the smears. PCR from fetal organs and placentas were positive for pestivirus, only. ELISA tests on maternal blood showed positive antibody titers for *C. abortus* (2/2 samples), *C. burnetti* (1/2), *N. caninum* (2/2),

and pestivirus (2/2). However, serology of individually aborted animals is of little value as the presence of serum antibodies indicates contact rather than infection, and may also be influenced by vaccination (i.e. in the case of *C. abortus*) (Holler, 2012). Furthermore, no nucleic acids from *C. abortus*, *C. burnetti*, or *N. caninum* were detected in the fetal organs/placental tissues, thus the panel pointed to pestivirus as the most likely contributor to the clinicopathologic picture observed. On top of that, the Spanish authorities had already warned about the possibility of pestivirus contamination in the applied vaccine (AEMPS, 2018a; AEMPS, 2018b), and therefore further studies were performed to explore that possibility.

The three lambs presented different degrees of similar changes in the CNS (Figure 1; Supplementary Figures S1-2). Grossly, hydrocephalus (lamb No. 2), severe hydranencephaly with almost no remaining cortical parenchyma in the rostral areas and meningeal collapse (lambs Nos. 1 and 3), together with markedly reduced cerebellar sizes (lambs Nos. 2 and 3) were observed. Histologically, the three animals presented variable combinations of atrophy and dysgenesis in the cortex. Cerebellar sections demonstrated irregular and poorly-developed folia (Figure 2a). There were rarefaction, spongiosis, and hypomyelination in the white matter, and rare meningeal infiltrates of mononuclear inflammatory cells. Those lesions suggested *in utero* pestiviral infection, and the lesion variability among the three animals pointed to infection of fetal CNS during different times of prenatal development, since tissue susceptibility varies according to fetal age (Cantile, 2016).

Pestiviral antigen was detected at least in one tissue per animal by IHC. Brain (neurons and endothelial cells) showed the most consistent results across the three lambs, with positive signal obtained in lamb No. 1, and mildly positive signals in lambs Nos. 2 and 3 (Figure 2b; Supplementary Figures S3-4). Indeed, the degree and location of pestiviral antigen detection in brain tissue vary according to the moment of pregnancy and the extent and severity of microscopic lesions (Hewicker-Trauwein & Trautwein, 1994). Furthermore, the following tissues were also positive: thyroid gland (endothelial cells, fibrocytes, and follicular epithelial cells; lambs Nos. 1 and 2), lung (endothelial cells; lamb No. 1), oral mucosa (epithelial and endothelial cells; lambs Nos. 1 and 3), and aural skin (keratinocytes, endothelial cells, and chondrocytes; lambs Nos. 1 and 2).

RT-qPCR for ruminant pestiviruses was positive or weakly positive with the BVDV 2 probe in various samples from all three animals (Table 1). The 5'-UTR could be sequenced at least in one tissue per animal and confirmed the presence of an identical nucleotide sequence belonging to the same species of BVDV 2b. Interestingly, positive results and successful sequencing were obtained in brain samples from the three animals, which along with the lesions described and the successful antigen detection pointed towards the selective neurotropism of this BVDV 2b strain in sheep and to an active BVDV 2 infection in the three lambs.

To investigate whether pestiviral RNA and infectious pestiviruses were present in the orf vaccine, a fresh, sealed vial of the product was analyzed by RT-qPCR and virus isolation. A first RT-qPCR in the 5'-UTR, run directly from an aliquot of the reconstituted vaccine, yielded a negative result. To confirm this result, the direct RT-qPCR analysis of the reconstituted vaccine was repeated. Two new, fresh 200 µL aliquots from the vaccine suspension were prepared and the RT-qPCR was run in duplicate. This time, both samples yielded weakly positive results for the BVDV 2 probe, with Ct values of 36.9 and 37.4, respectively. Re-amplification of the newly isolated RNA generated an amount of product that was sufficient for subsequent sequencing. These sequences yielded a complete match to the ones obtained in tissues from the three animals. Subsequently, the presence of infectious virus was evaluated by viral propagation in two cell lines (i.e., BT and MDBK). Analysis by IPO staining of both passages showed positive result for pestivirus antigen. Controls in the absence of added vaccine samples remained negative in all the passages. RT-qPCR analyses yielded positive results for BVDV 2, with Ct values around 21 and 20 for BT cells, and 29 and 20 for MDBK cells in passages 1 and 2, respectively. All internal positive and negative controls showed the expected results. Sequencing in the 5'-UTR of both passages on either cell type yielded positive results, with all four samples displaying identical nucleotide sequences belonging to BVDV 2b species. Furthermore, this nucleotide sequence was again the same as in the tissues of the three lambs studied and as found in the reconstituted vaccine. To further confirm the identity of the viruses in the vaccine and in the fetuses, we further sequenced a fragment of 1149 nt in length in the NS3/NS4A region of the viral genome in the brain of lambs 1 and 2 and from the supernatants of the first passages of the vaccine on BT and MDBK

cells. Again, all the sequences were identical in these four samples (GenBank No. MT113951), with the exception of one to three synonymous changes with the base A instead of G at position 919 in the two cell culture supernatants and in the brain of lamb 2, and two ambiguities in the sample of the brain of lamb 2 (A or T instead of an A at position 1008, and an A or G instead of an A at position 1092).

Therefore, BVDV 2b was found in the vaccine vial but, based on the RT-qPCR results in the 5'-UTR, most likely at a rather low level. In consequence, by taking a random aliquot of the reconstituted vaccine, not every sample might contain viral RNA at a sufficient amount to be detected by RT-qPCR, explaining the variable results observed. By contrast, a small number of infectious virus might be sufficient to infect and propagate in cell cultures. Thus, based on successful virus isolation on two different types of cell lines, the vaccine contained viable (i.e. infectious) virus that was able to propagate once it was on cell culture and, most likely, when injected into a live animal. Indeed, the nucleotide sequences in the tissues of the lambs was the same as that in the vaccine, thus pointing to such a vaccine as the sole source of infection and the cause of the massive outbreak that occurred in the flock.

The severity and explosiveness of the outbreak studied may be related to the moment of pregnancy in which the vaccine was applied. Some protocols of ovine immunization recommend applying orf vaccines during pregnancy in order to guarantee passive transfer of antibodies to neonatal lambs through the colostrum (Lacasta et al., 2015). It is a common practice among farmers to have their ewes vaccinated around the last month of pregnancy (120-150 days), when the susceptibility of the developing fetus to pestiviral infection is lowest (Nettleton & Entrican, 1995; Schweizer & Peterhans, 2014). Actually, vaccination (i.e. BVDV 2 inoculation) at that moment would probably have produced apparently healthy animals and a low rate of abortions and malformations. Due to flock management practices in the present case, vaccination was carried out earlier, around the second month (60 days) of pregnancy, when the susceptibility of the fetus to pestiviral infection is highest.

The observed reproductive failure rate resembles that of an experimental BVDV 2 infection carried out in pregnant ewes (Scherer et al., 2001), in which animals were inoculated either at 55-60 days or at 65-70 days of pregnancy, with abortion rates of 77%

and 66.6%, respectively. A third group of ewes was inoculated at 120-125 days, which gave birth to seropositive, virus-negative, healthy lambs. Similarly, another orf vaccine contaminated with pestiviruses, also applied in early pregnancy, led to reproductive failure in 82% of the pregnant goats (Løken, Krogsrud, & Bjerkås, 1991). Moreover, in this last description, virus was also transmitted from PI goats to sheep and cattle, thus demonstrating that further damages in addition to the direct losses in the vaccinated animals may occur.

A BLAST search provided two identical 5'-UTR sequences as the one described herein: One from a strain in Portugal in 2006 (GenBank No. AY944277) and another from Spain in 2015 (GenBank No. KX369602). In the first case, the strain was found in cattle affected by hemorrhagic disease (Barros, Ramos, Pauperio, Thompson, & Fevereiro, 2006). In the Spanish case, it was found in four different sheep flocks located in the Northwest and center of the country and that showed severe outbreaks of abortions and malformations, in which the origin of the infection could not be established (Elvira Partida et al., 2017). The complete nucleotide match in the 5'-UTR and the geographical similarities between those ovine outbreaks and the one described in this communication suggest that they may have had a similar origin, either by direct application of the contaminated vaccine or by introduction of PI animals originated in flocks where the vaccine was used. However, as no sequences outside of the 5'-UTR were reported for these strains, a more detailed comparison including the sequences in the NS3/4A region of the viral genome reported here is not possible. Eiras et al. (2017) described another outbreak of abortions in the Northwest of Spain associated with a BVDV 2d strain in sheep. The origin of the infection was not determined but the authors suggested that the introduction of 4 new rams, one of them being seropositive to pestivirus, could have played a role.

In any case, it seems that the contaminated orf vaccine could be contributing to the circulation of BVDV 2b among Spanish sheep flocks, especially in those where the vaccine was applied later in pregnancy, in which the clinical signs could remain unnoticed for the farmer at short term. However, long term issues associated with the presence of PI animals and viral circulation might occur in those flocks in the future, and the origin of the infection would remain unclear. Spontaneous circulation of BVDV 2 strains and

related disease in sheep have already been described in other countries (Decaro et al., 2017; Giangaspero & Harasawa, 2004). Actually, in a survey performed in Italy, BVDV 2c was more common among small ruminants than in cattle (Decaro et al., 2017).

Eventually, on 23rd July, 2018, the AEMPS released a new communication (AEMPS, 2018c), which confirmed the contamination and called for a definitive withdrawal of the product from the market. However, they did not provide either the characteristics of the contaminating pestivirus or details about the different outcomes of the vaccine application in the affected sheep flocks. This communication describes the clinicopathologic presentation in a flock vaccinated with that contaminated product, and provides the 5'-UTR and NS3/4A nucleotide sequences of the contaminating virus, which will be useful in order to determine the origin of BVDV 2b circulation in similar outbreaks in the future.

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Ethics statement

Authors confirm that ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Animals used in this study were submitted alive for diagnosis and euthanized for humanitarian reasons upon arrival, following requirements of the Spanish Policy for Animal Protection (RD53/2013) and the European Union Directive 2010/63 on protection of experimental animals.

Conflicts of interest

Authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Brain, Lamb No. 2. Occipital and parietal lobes show thinning of the cortex, loss of cerebral circumvolutions, and hydrocephalus. Cerebellum is markedly reduced in size and cerebellar gyri show disorganization. Bar = 1 cm.

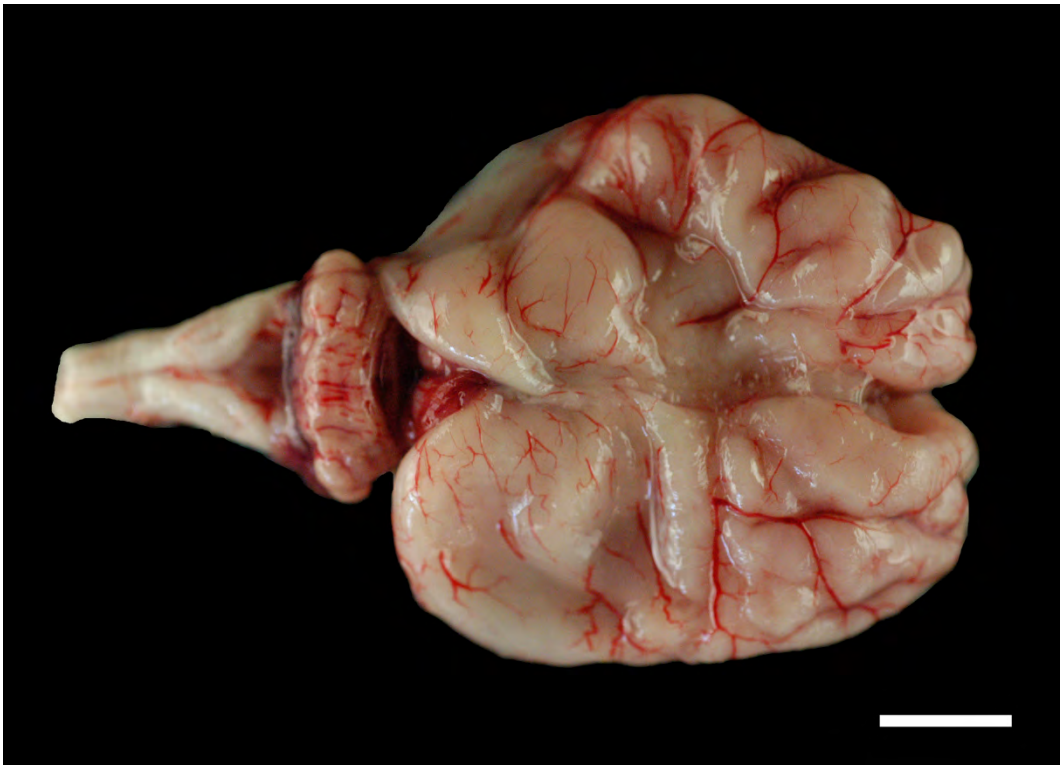
Figure 2. Histological findings and pestiviral antigen detection in the central nervous system of lambs infected by BVDV 2b. **Fig 2A**. Histopathological image, cerebellum, Lamb. No. 3. There is disorganization and atrophy of cerebellar folia. External granular layer is irregular and forms scattered cellular niches within the molecular layer (arrow). Purkinje cells are distributed throughout all the gray matter. There is no discernible inner granular layer. White matter area shows rarefaction and spongiosis (hypomyelination; asterisk). Hematoxylin-eosin stain, bar = 200 µm. **Fig 2B**. Immunohistochemically-stained image, brain, Lamb No. 1. There is punctate, brownish, positive signal within neurons (arrows) and endothelial cells (arrow heads). Panpestiviral 15c5 antibody, bar = 200 µm.

Table 1. Presence of BVDV 2b in tissues from three lambs with central nervous system malformations. Results of RT-qPCR / sequencing at the 5´-UTR are shown for the five different tissues studied in each lamb.

	Lamb No. 1	**Lamb No. 2**	**Lamb No. 3**
Brain	pos / seq	wpos / seq	wpos / seq
Thyroid	wpos / seq	neg / nd	wpos / no seq
Lung	wpos / seq	wpos / seq	wpos / nd
Oral mucosa	wpos / no seq	neg / nd	wpos / nd
Aural skin	wpos / nd	neg / nd	neg /nd

pos: Positive in RT-qPCR (Ct≤ 32)
wpos: Weakly positive in RT-qPCR (Ct >32 & <45)
neg: Negative in RT-qPCR (Ct≥45)

seq: 5´-UTR could be sequenced as BVDV 2b
no seq: 5´UTR could not be sequenced
nd: Sequencing not done (i.e. negative result in pre-sequencing, classic RT-PCR)



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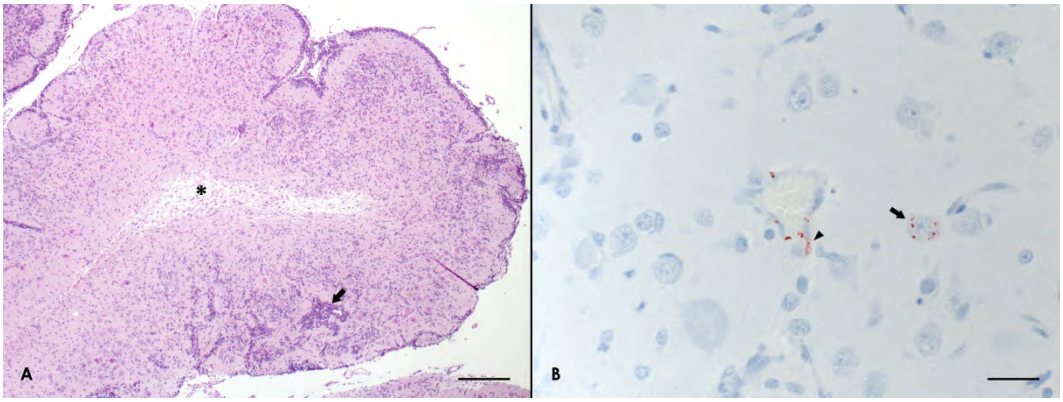

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