

Mucosal lesions in a sheep infected with the Border Disease Virus (BDV)

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Abstract

A 28-week-old sheep was presented at the animal hospital because of chronic emaciation, anemia and slight diarrhea. Due to poor general condition and bad prognosis the animal was euthanized and submitted for postmortem investigation. Multiple erosions and ulcerations were found in the dorsal region of the tongue, the pharynx, the hard palate, in the esophagus and the ruminal pillars. Histologically, these lesions consisted of necrosuppurative inflammation. The animal was tested positive for pestivirus antigen both by immunohistochemical and by virological examination (cell culture, antigen capture ELISA and RT-PCR). A non-cytopathic Border Disease Virus was identified, and sequencing revealed a virus belonging to the BDV-3 cluster. Based on the macroscopical, histological, immunohistological and virological results this case was diagnosed as Border Disease with mucosal lesions. This is the first report of such a case in Switzerland.

Keywords: Border Disease, sheep, immunohistochemistry, pathology, virology

Schleimhautläsionen bei einem mit dem Border Disease Virus (BDV) infizierten Schaf

Ein 28 Wochen altes Schaf wurde am Tierspital wegen chronischer Auszehrung, Anämie und leichtem Durchfall vorgestellt. Aufgrund des schlechten Allgemeinbefindens und der ungünstigen Prognose wurde das Tier euthanasiert und eine Sektion durchgeführt. Dorsal auf der Zunge, im Pharynx, dem harten Gaumen, im Ösophagus und auf den Pansenpfeilern wurden multiple Erosionen und Ulzerationen beobachtet. Histologisch stellten sich diese Läsionen als eitrig-nekrotisierende Entzündung heraus. Das Schaf war sowohl immunohistochemisch wie auch virologisch positiv für Pestivirusantigen (Zellkultur, antigen capture ELISA und RT-PCR). Ein nicht zytopathogenes Border Disease Virus wurde isoliert und die Sequenzierung liess es der BDV-3 Gruppe zuordnen. Aufgrund der makroskopischen, histologischen, immunhistologischen und virologischen Resultate wurde dieser Fall als Border Disease mit Mucosal Disease-ähnlichen Schleimhautläsionen diagnostiziert. Dies ist der erste Bericht eines solchen Falles in der Schweiz.

Schlüsselwörter: Border Disease, Schaf, Immunohistochemie, Pathologie, Virologie

Introduction

Border disease is caused by a congenital pestivirus infection found in sheep and rarely in goats. This disease was first reported from the border region of England and Wales in 1959 leading to the term Border Disease (Nettleton et al., 1998). The genus pestivirus within the family Flaviviridae includes four species: Bovine Viral Diarrhea Virus (BVDV) 1 and 2, Classical Swine Fever Virus (CSFV) and Border Disease Virus (BDV) (Weiss et al., 1994; Nettleton and Entrican 1995; Nettleton et al., 1998; Kümmerer et al., 2000; Becher et al., 2003). It was suggested to divide the BDV into three subspecies: classical BDV should be termed BDV-1, sheep-derived pestiviruses BDV-2 (17385-like isolates) and BDV-3 (Gifhorn) (Becher et al., 2003). So far the isolates Gifhorn

from Germany and CH-BD1/R1292/01 from Switzerland are the only described members of the genotype BDV-3 (Braun et al., 2002). Until now, at least three more species have been isolated (Becher et al., 2003; Arnal et al., 2004; Schirrmeier et al., 2004; Thabiti et al., 2005; Stalder et al., 2005; Vilcek et al., 2005).

Ovine, bovine and porcine pestiviruses share the ability to cross the placenta, invade the fetus and set up a persistent infection (pi) in early pregnancy (Carlsson, 1991; Moennig and Plagemann, 1992; Nettleton and Entrican, 1995; Nettleton et al., 1998; Bolin and Grooms, 2004). Ovine fetuses are able to mount an immune response to an antigenic stimulus starting at 60 to 80 days of gestation. Before that time point the ovine fetus can be persistently infected by pestiviruses, and if the fetus survives the infection, the virus can be found in all organs. The

virulence of the strain, the dose of the virus and the developmental stage of the fetus determine the outcome of a fetal infection. Clinical signs of a BDV infection in sheep and goats include abortions, malformations, stillbirths and birth of weak lambs (Carlsson, 1991; Nettleton and Entrican, 1995; Thür et al., 1997; Nettleton et al., 1998; Thür et al., 1998; Braun et al., 2002). The most obvious pathological changes are present in the CNS and in the hair follicles. There is demyelination in the brain, associated with tremor and an increased number of hair follicles leads to hairiness. The resulting clinical symptoms contributed to the name Hairy Shaker as a synonym of Border Disease (Nettleton and Entrican, 1995; Nettleton et al., 1998; Braun et al., 2002).

Between sheep and cattle pestivirus host-cross transmission often occurs, making sheep susceptible for both Border Disease and BVD (Carlsson, 1991; Nettleton et al., 1998). It is widely accepted that within the BVDV and also in the BDV species two biotypes exist. In cell culture the non-cytopathic (ncp) pestiviruses replicate without affecting the viability of the host cells, whereas cytopathic (cp) viruses lead to the death of infected cells (Nettleton and Entrican, 1995; Becher et al., 1996; Nettleton et al., 1998). The cp BVDVs appear to arise mostly by mutation from ncp BVDVs within a persistently infected animal. Mutation can be caused by viral RNA insertion, viral RNA duplication and rearrangements of viral genome and many cp pestiviruses evolved also by insertions of cellular RNA sequences (Weiss et al., 1994; Nettleton and Entrican, 1995; Nettleton et al., 1998). Only a few cp BDV have been described and some authors suggested that the cp BDV strains described have developed from the corresponding non-cp viruses by a RNA recombination process (Becher et al., 1996). However, others state that almost all pestivirus isolates from sheep and goats are ncp in cell culture (Nettleton et al., 1998).

Calves persistently infected with an ncp biotype and subsequently superinfected with a cytopathic biotype, develop Mucosal Disease (MD) with the typical erosions and ulcerations in different hollow organs (Weiss et al., 1994; Nettleton et al., 1998; Kümmerer et al., 2000). In sheep, a similar syndrome resembling Mucosal Disease, associated with BDV, has been published in England and has been commented in a review (Monies and Simpson, 1997; Nettleton et al., 1998; Monies et al., 2004). This Mucosal Disease-like syndrome in sheep appears to occur rarely, and has not yet been described in Switzerland, even though the seroprevalence of pestivirus antibodies in sheep in Switzerland is between 11 to 65% (Thür et al., 1998; Schaller et al., 2000). Here, we report on a sheep with Mucosal Disease like lesions.

Case history

The female lamb presented in this paper was 28 weeks old and belonged to the Swiss White Alpine breed. The lamb was weak from birth on and began to deteriorate with

loss of appetite and slight diarrhea 2 weeks prior to hospitalization. At presentation the animal was in poor condition, and had white mucous membranes. Rectal temperature, heart and breathing rate were normal. Blood analysis showed severe anemia with a hematocrit of 10%, hypoproteinemia (41 g/l), serum urea concentration of 18mmol/l, elevated aspartate aminotransferase (512 U/l) and gamma-glutamate dehydrogenase (169 U/l). Only a temporary improvement was achieved after blood transfusion with 150ml blood from a healthy sheep and as a consequence the lamb was euthanized.

Postmortem examination

Postmortem examination was performed and specimens of several organs (esophagus, tongue, hard palate, pharynx, heart, lung, liver, rumen, spleen, kidney and brain) were fixed in 4% formalin, paraffin-embedded and stained with HE (hematoxylin eosin). Mucosal lesions of different size and shape (erosions and ulcers) were found in the dorsal region of the tongue, the hard palate, the pharynx, in the esophagus and the rumen pillars (Figs. 1 and 2). The fleece, CNS and all other organs were unremarkable macroscopically. The lamb also had decreased body fat deposits and white mucous membranes. At the time of necropsy, no gastric or intestinal parasites were found, either macroscopically nor histologically.



Figure 1: Multiple erosions and ulcerations of different shape and size in the mucosa of the pharynx (arrowhead).

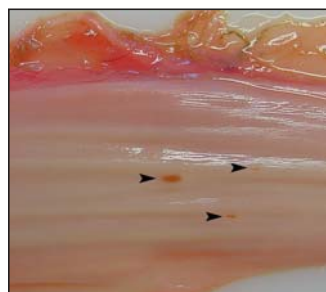


Figure 2: Focal, round to elongated ulceration in the esophagus (arrowhead).

Histology

Histologically, the mucosa of the tongue, palate, pharynx, esophagus and of rumen pillars showed erosions and ulcerations with superficially necrotic, diffusely distributed debris, admixed with moderate to large amounts of degenerating neutrophils. Depending on the localization

this inflammatory process either infiltrated the mucosa superficially or broke through the basal lamina (Fig. 3). No inflammatory lesions or myelin deficiency were visible in the brain sections. In summary, a moderate to severe, multifocal, necrosuppurative, erosive and ulcerative glossitis, stomatitis, pharyngitis, esophagitis and rumenitis were diagnosed.

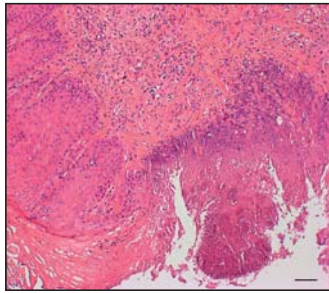


Figure 3: Severe focal extensive necrosuppurative esophagitis. HE. Bar = 75 µm.

Immunohistochemistry

Immunohistochemistry was performed on cryostat sections of skin, tongue and thyroid gland. Tissues for cryostat sections were snap frozen in liquid nitrogen and sections of 6µm were mounted on positively charged glass slides (SuperFrost®Plus, Menzel GmbH & Co KG, Braunschweig, Germany). After pretreatment the incubation with one of the following four antibodies was undertaken for 1 hour at 37 °C. The antibodies Ca3/34-C42 (dilution 1:100; specificity: E2/gp55 and Erns/gp48, Dr. Bommeli AG, NBWU 102, Bern, Switzerland) and C42 (dilution 1:400; specificity: gp48, Prof. V. Moennig, Institute of Virology, Hannover, Germany) are BVDV specific (Moennig et al., 1987; Bolin et al., 1998). The antibodies C16 (dilution 1:100; specificity: NS2–3/nsp125/80, Dr.

Bommeli AG, NBWU 102, Bern, Switzerland) and 15c5 (dilution 1: 1000; specificity: gp48, Dr. E. Dubovi, Cornell University, Ithaca, New York, USA) on the other hand are pestivirus specific antibodies (Moennig et al., 1987; Bolin et al., 1998; Haines et al., 1992). As secondary antibody a peroxidase labeled anti-mouse antibody (DAKO®, EnVision, K4001, Zug, Switzerland) was applied for 30 min. at RT (room temperature) and the chromogen AEC (Aminoethyl Carbazole, Aminoethyl Carbazole Substrate Kit, Zymed Laboratories, Lot No. 80642120, Inc. San Francisco, USA) was applied. Between each step the slides were thoroughly rinsed with PBS (Phosphate Buffer Solution, pH8).

Immunohistochemically, cryostat sections of skin, tongue and thyroid gland of the lamb were positive with the pestivirus specific antibodies, C16 and 15c5, but were negative with the BVDV specific antibodies, Ca3 and C42. In the tongue, mostly the basal epithelial cells, the tunica media cells of the larger arteries and veins and some fibrocytes were positive (Fig. 4). Basal cells in the epidermis, the blood vessel walls and some fibrocytes as well as hair root sheath cells of the hair shafts showed positive coloration. In the thyroid gland follicle epithelial cells as well as the tunica media cells of blood vessels stained positive for pestivirus antigen.

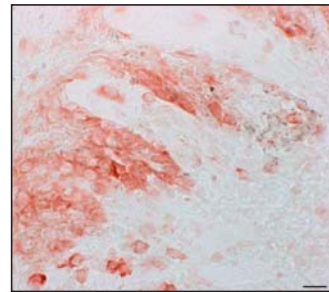


Figure 4: Immunohistochemical detection of pestivirus antigen in the tongue. Monoclonal antibody: C16, peroxidase-labeled polymer, EnVision-method. Bar = 50 µm.

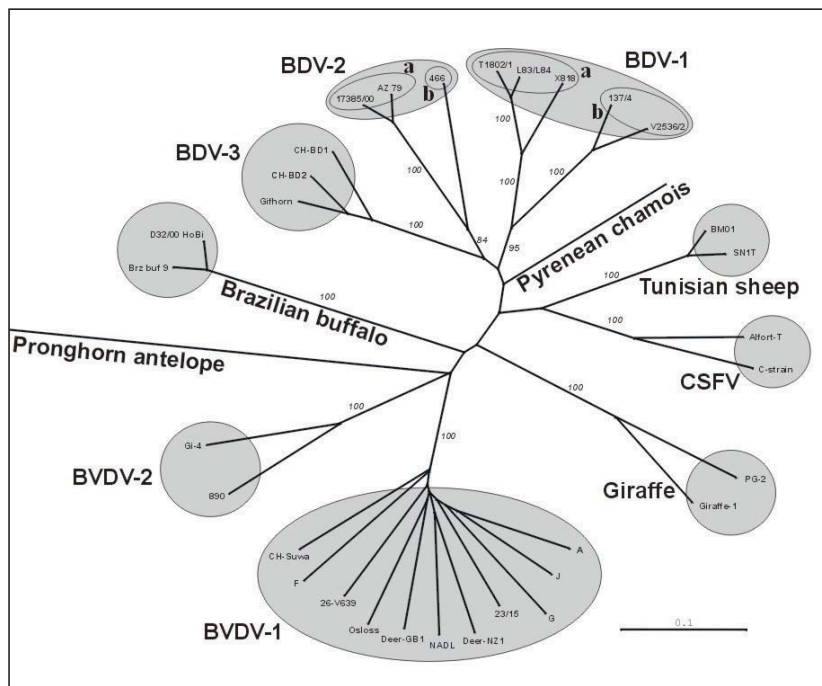


Figure 5: Phylogenetic tree illustrating the genetic relationship of pestivirus strains constructed from the complete N^{pro} coding sequences. The genetic analysis was computed using the programs included in the GCG software package as PILEUP for the multiple sequence alignment and PAUP for calculating the distances by the Kimura 2-parameter method and to construct the tree according to the neighbor-joining method. Branch numbers (in italics) indicate the percentage of 1000 bootstrap replicates. Branch lengths are proportional to genetic distances.

Virus isolation and sequencing

Virus isolation from tongue and thyroid gland was accomplished in cultured goat synovial membrane cells (Thür et al., 1997; Hilbe et al., 2007), passaged once and incubated for 5 days. As positive controls NADL (BVDV 1) and 890 (BVDV 2) strains were used. RNA isolation was performed using the TRIzol reagent (Invitrogen, Basel, Switzerland). One step RT-PCR was carried out using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Hombrechtikon, Switzerland). The primers used recognize BVDV as well as BDV strains (Becher et al., 1997).

The PCR products were run on agarose gels (SeaKem GTG Agarose, FMC BioProducts, Rockland, USA) using standard procedures. Appropriate bands were cut out and purified using the QIAEX II Gel Extraction Kit (QIAGEN, Hombrechtikon, Switzerland). Purified DNA was cloned using the TOPO TA Cloning Kit (Invitrogen, Basel, Switzerland) and minipreps were prepared with the Wizard Plus Minipreps DNA Purification System (Promega, Walisellen, Switzerland). DNA was sequenced using Perkin Elmer Dye Terminator DNA Sequencing methods (Perkin Elmer, Foster City, CA, USA) and examined on an ABIprism 310 or Applied Biosystems Model 373A (Perkin Elmer, Foster City, CA, USA) according to the manufacturer's protocols. Sequences were analyzed using the Wisconsin Package Version 10.1. (Genetics Computer Group (GCG), Madison, Wisconsin, USA). An ncp pestivirus was isolated from the tongue and thyroid gland. Sequencing of the 5'UTR and the N^{pro} genome regions revealed that the pestivirus belonged phylogenetically to the BDV species (CH-BD2) and the cluster 3 (Fig. 5).

Discussion

Mucosal lesions are well known lesions in Mucosal Disease in cattle persistently infected with an ncp BVDV and superinfected with a homologue cp BVDV. In our case the 7-month-old lamb was also infected with a ncp BDV-3 virus. This animal showed mucosal lesions of various size and shape in the dorsal region of the tongue, the hard palate, the pharynx, in the esophagus and the rumen pillars, which led to the diagnosis of BDV infection with mucosal lesions, confirmed by the demonstration of an ncp pestivirus belonging to the BDV-3 cluster. According to literature most BDV isolates are non-cytopathogenic but cp strains have been isolated in sheep dying of Mucosal Disease-like syndrome (Nettleton et al., 1998). On the other hand two cp BDV were isolated from field cases but no signs resembling Mucosal Disease were reported. Still the significance and pathogenicity of cp BDV viruses have to be further elucidated (Becher et al., 1996). In our case a ncp BDV strain was isolated.

It is known that sheep can be infected by BVDV when in contact with persistently infected cattle, and the transmission of BVDV type 1 from bovines to ovines and vice

versa has been shown, so that despite their host-specific-based nomenclature, the pestiviruses can not be regarded as fully host-specific (Carlsson, 1991; Nettleton and Entrican, 1995; Paton et al., 1997; Thür et al., 1997). No BVDV could be demonstrated in our lamb.

In Switzerland a high prevalence of BDV antibodies can be found. In a study by Braun et al. (2002) 41 sheep were examined for the presence of pestiviruses and pestiviral antibodies. 31 animals were seropositive, 6 had borderline values and 4 animals were seronegative. From 8 animals BDV was isolated belonging to the cluster 3 (CH-BD1). 3 of them were seronegative, 3 had borderline values and 2 were seropositive. The source of the infection was thought to be sheep from another flock, because both flocks shared the same pasture. In another study (Schaller et al., 2000), 3866 sheep out of 226 flocks from different breeding associations and 1218 sheep from 15 independent sheep owners were tested for the presence of BDV-antibodies in the serum. Prevalence of antibodies (ELISA) to BDV was 20% in sheep of breeding associations and 65% in those of independent sheep owners.

In persistently BDV infected sheep the distribution of viral antigen was evaluated earlier (Waldvogel et al., 1995) and the following structures were found to be positive: smooth muscle cells and blood vessels of hollow organs, epithelial cells of the alimentary tract and urogenital organs as well as the majority of cells of the thyroid gland, some lymphocytes in lymphoid organs, endocrine cells, neurons and glia cells. This is very much compatible with the viral antigen distribution in persistently BVDV infected calves (Hilbe et al., 2007b). By immunohistological techniques, BVD antigen can be demonstrated in a variety of specific tissue structures in the tongue, thyroid gland and skin biopsies, so that a persistent infection can well be diagnosed (Haines et al., 1992; Thür et al., 1996; Thür et al., 1997; Njaa et al., 2000; Brodersen, 2004; Hilbe et al., 2007a). Positive cells in the skin are basal cells of the epidermis, epithelial cells in the hair root sheaths, tunica media cells of blood vessels, fibrocytes and follicular epithelial cells (Waldvogel et al., 1995; Thür et al., 1996; Braun et al., 2002). Thus, the immunohistochemical pattern of BDV antigen distribution found in the present case strongly points towards a persistent infection.

In summary, this is the first report of a lamb persistently infected with a ncp BDV belonging to the BDV-3 cluster with mucosal lesions diagnosed in Switzerland.

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Received: 28 October 2008

Accepted: 30 January 2009