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SHORT PAPER

Borna Disease in an Adult Alpaca Stallion (*Lama pacos*)

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Summary

Borna disease (BD) was diagnosed in a 2-year-old male alpaca with a history of chronic suppressed sexual desire and acute stretching convulsions. Microscopical examination of the central nervous system revealed non-purulent meningoencephalitis with mononuclear perivascular cuffing. The diagnosis was confirmed by immunohistochemistry, in-situ hybridization, polymerase chain reaction (PCR) and sequencing of PCR products and alignment with known Borna disease virus sequences. Serological screening of the herd was performed. This is the first detailed report of naturally occurring BD in alpacas.

Keywords: alpaca; Borna disease; encephalitis; *Lama pacos*

Borna disease (BD) is an endemic, sporadically occurring and usually fatal disorder caused by the highly neurotropic Borna disease virus (BDV) (Richt *et al.*, 2007). The name originates from a devastating epidemic that occurred among cavalry horses during the years 1894–1896 near the town of Borna in Saxony, Germany. BDV is an enveloped virus with a non-segmented, negative-sense, single-stranded (ss) condensed RNA genome and is the sole member of the family Bornaviridae in the order Mononegavirales (Büchen-Osmond, 2003). Clinical BD has only been recognized in the German-speaking part of central Europe, although BDV-specific antibodies have been detected serologically in many countries (Richt *et al.*, 2007). Restricted to the central nervous system (CNS), BD is due to a T-cell-mediated immunopathological event resulting in a non-purulent meningoencephalitis (Stitz *et al.*, 2002). BD predominantly affects horses, other Equidae and sheep, and rarely, other farm, zoo and companion animals (Richt *et al.*, 2007). Reports of new world camelids suffering from BD are rare. One report describes only histological changes in alpacas and lamas during an outbreak in a herd in a zoological garden in the early 1970s (Altmann *et al.*, 1976). The histological results were later confirmed in two of these animals by immunohistochemistry (IHC) with an antibody targeting the BDV nucleoprotein (BDV-N) (Schüppel *et al.*, 1994). The present report describes a case of BD in an alpaca that was investigated by histology and confirmed by IHC, in-situ hybridization (ISH), polymerase chain reaction (PCR) and sequencing of PCR products and alignment of the sequences with known BDV sequences. The remainder of the herd was investigated serologically.

In March 2008, a 2-year-old male alpaca originating from Bavaria, Germany was lent for breeding purposes to a herd in the northern part of Hesse, Germany. During the 2-month stay in this herd the animal showed an unusual lack of sexual desire. The stallion had a 2-day history of illness starting with a sudden onset of stretching convulsions. These convulsions and accompanying frequent mastication were controlled by medication with diazepam.

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Administration of fluid therapy (Ringer's solution), vitamin B and E and selenium supplementation and treatment with antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) led to only a slight clinical improvement. Initially the alpaca was able to stand and eat by itself and the neurological signs were controlled for a 24 h period. However, the animal then became prostrate, but was still able to eat and drink. Shortly afterwards there was a second episode of convulsions and the alpaca died despite further therapy.

The animal was subjected to necropsy examination. The alpaca was in good bodily condition. The only gross findings were accumulation of a small amount of clear serous fluid within body cavities and congestion of the viscera.

Samples of various tissues were fixed in non-buffered 10% formalin and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE). Microscopical examination revealed perivascular cuffing, predominantly consisting of lymphocytes with few histiocytes, throughout the CNS. These cuffs were considered of moderate severity in the olfactory bulb, hippocampus, thalamus and hypothalamus, and were milder in the cerebrum, striatum, pons, brain-stem, cerebellum and medulla oblongata. Additionally, there was focal mild parenchymal encephalitis and activation of glial cells as well as single neuronal necroses and moderate distinct, eosinophilic, BD-specific, intranuclear viral inclusions (Joest–Degen inclusions) (Fig. 1). Multifocal glia nodules and moderate lymphohistiocytic meningitis were noted in the cerebellum. The thoracic spinal cord showed a mild perivascular lymphohistiocytic poliomyelitis. Other organs were microscopically normal.

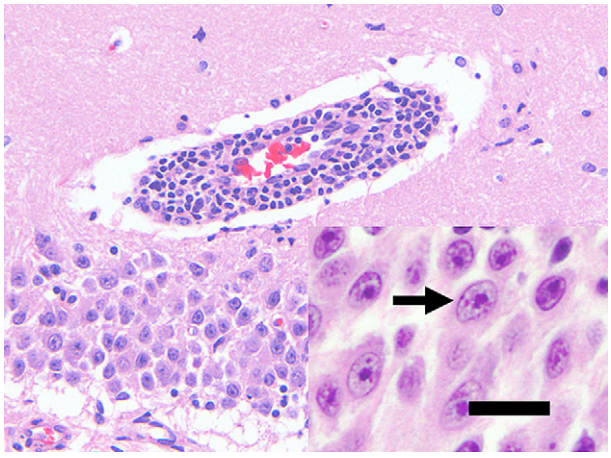

Fig. 1. Lymphocytic perivascular cuffing in the hippocampal region. HE. Bar, 50 μm. Inset: eosinophilic intranuclear inclusions (arrow). HE. Bar, 15 μm.

For IHC, antibodies (Table 1) specific for BDV-N, BDV phosphoprotein (BDV-P), BDV matrix protein (BDV-M), BDV glycoprotein (BDV-GP) and glial fibrillary acidic protein (GFAP) were applied. The avidin–biotin complex (ABC) method was used for detection of antibody binding (Herden *et al.*, 2000; Werner-Keiss *et al.*, 2008). The immunohistochemical investigation revealed moderate and diffuse nuclear and cytoplasmic labelling of neurons throughout the brain, mainly in the hippocampal and hypothalamic region, by antibodies specific for BDV-N and BDV-P (Fig. 2A, B). The antibody specific for BDV-M produced mild and mainly cytoplasmic labelling of neurons throughout the brain. The antibody directed against BDV-GP revealed minimal neuronal cytoplasmic labelling. GFAP immunolabelling revealed a slight increase in the number of astrocytes in the histologically affected areas.

IHC was also used to rule-out infection with rabies virus, porcine herpesvirus 1 (Aujeszky's disease) and *Listeria monocytogenes* (Schwab *et al.*, 2007) and infection with bluetongue virus serotype 8 was ruled out by PCR (testing performed by the method of the Friedrich-Löffler Institute).

For ISH studies, several digoxigenin (DIG)-labelled RNA probes targeting mRNA sequences of BDV-N, BDV-P, BDV-GP and BDV polymerase (BDV-L) were applied (Table 2). The hybridization was performed with a detection system consisting of an anti-DIG antibody conjugated to alkaline phosphatase and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Gaedke *et al.*, 1997; Werner-Keiss *et al.*, 2008). The mRNA probes for BDV-N, BDV-P and BDV-GP revealed similar results to the IHC (Fig. 2C, D). The probe targeting BDV-L-mRNA exhibited moderate labelling of neuronal nuclei throughout the brain.

For sequence analysis of the alpaca BDV, RNA was isolated from frozen brain tissue using the TRIzol® method followed by RNA purification (RNeasy™ Mini Kit, Qiagen, Hilden, Germany) and DNA digestion (Qiagen) as described by Schaudien *et al.* (2007). A standard PCR of BDV-specific cDNA was performed using six primer pairs (Kolodziejek *et al.*, 2005) to amplify the genome region of the outer reading frame (ORF) I, II and x1. Sequences were aligned with CodonCode™ Aligner-Software (LI-COR, Inc., Lincoln, USA) and analysed by the basic local alignment search tool (BLAST). Sequence analysis (GQ861449) of the three ORFs of the BDV–RNA obtained from the alpaca brain revealed 99% homology to isolates of the endemic cluster Bavaria I (Kolodziejek *et al.*, 2005; GenBank accession numbers AY374521, AY374532 and AF158629–AF158631).

Table 1
Immunohistochemical procedures

Primary antibody	*Type and specificity*	*Dilution*	*Buffer*	*Secondary antibody*
BDV-N	Monoclonal, mouse anti-BDV-N (Bo18)	1 in 500	PBS with 1% bovine serum albumin	Goat anti-mouse
BDV-GP	Polyclonal, rabbit anti-BDV-GP	1 in 800	PBS with 20% normal sheep serum	Goat anti-rabbit
BDV-M	Polyclonal, rabbit anti-BDV-M	1 in 200	PBS with 20% normal sheep serum	Goat anti-rabbit
BDV-P	Polyclonal, rabbit anti-BDV-P	1 in 2,000	PBS with 1% bovine serum albumin	Goat anti-rabbit
GFAP	Polyclonal, rabbit anti-bovine	1 in 1,000	PBS with 20% normal sheep serum	Goat anti-rabbit

PBS, phosphate buffered saline.

The serological investigation of the herd was carried out with samples from 18 animals by an indirect immunofluorescence assay (Herzog and Rott, 1980), but all sera tested were negative for antibodies directed against BDV.

Naturally occurring BD typically occurs in horses and sheep in endemic areas of central Europe. Occasionally, other farm animals, zoo or companion animals can become infected and develop clinical disease. Only two cases of BD in alpacas have been reported. In one of these, BDV infection was confirmed only by IHC using the monoclonal antibody Bo18 specific for BDV-N (Schüppel *et al*., 1994). Expression of other viral proteins and RNAs have not been analysed in alpacas or other mammalian species other than horses and sheep. In should be noted that, albeit rarely, natural mutations in the BDV-N may occur and might mask the diagnosis of natural BDV infection (Richt *et al*., 1997; Herden *et al*., 1999). Moreover, the recently described avian BDV isolates have a homology of only up to 70% compared with mammalian strains of BDV (Honkavouri *et al*., 2008; Kistler *et al*., 2008). In these cases, only the antibody specific for BDV-P can recognize avian BDV (Rinder *et al*., 2009; unpublished observations). These findings indicate the need for a better understanding of the occurrence and pathogenesis of natural BDV infections in non-equine species.

In the two alpacas reported with BD, inflammation was present throughout the brain (Altmann *et al*., 1976), but in a further case no information regarding pathological changes is given and only the sequence of the BDV is reported (Dürrwald *et al*., 2006). The distribution of inflammatory lesions in the present

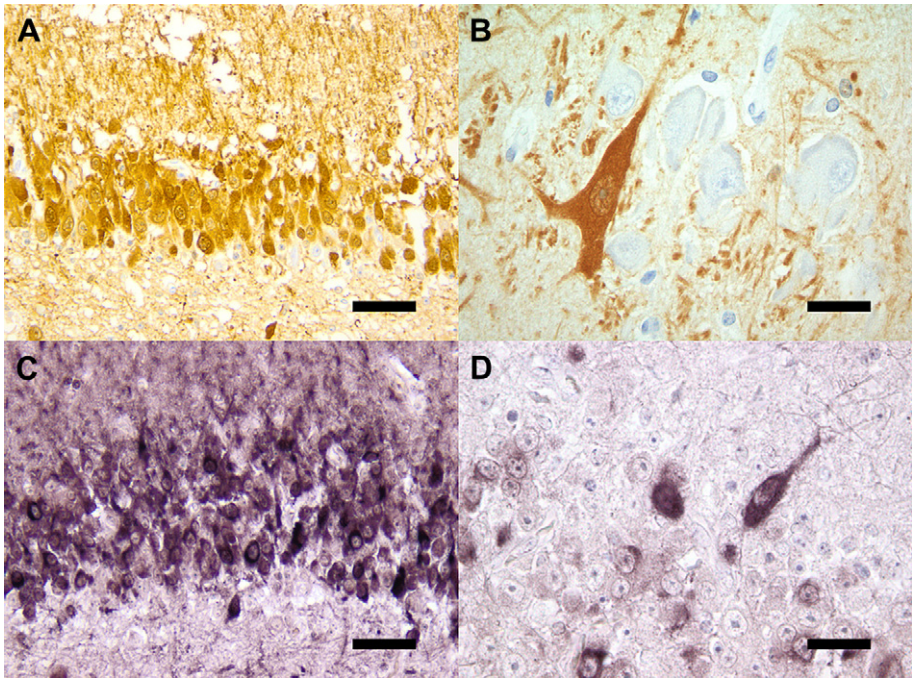

Fig. 2. Diffuse nuclear and cytoplasmic labelling for BDV-N expression in neurons in (A) the hippocampal region and (B) the hypothalamic region. IHC. Bar, 50 µm (A), 25 µm (B). (C) Diffuse nuclear and cytoplasmic labelling for BDV-P expression in neurons in the hippocampal region. ISH. Bar, 50 µm. (D) Diffuse nuclear and cytoplasmic labelling for BDV-N expression in neurons in the hypothalamic region. ISH. Bar, 25 µm.

Table 2
Design of BDV-specific RNA probes for ISH

Probe target proteins	*Length*	*Position on BDV-genome*[*]
BDV-N	342 bp	141–482
BDV-P	222 bp	1,621–1,842
BDV-L 459	337 bp	4,571–4,907
BDV-GP-N	315 bp	2,594–2,909

L 459, polymerase; GP-N, glycoprotein N-terminal segment; bp, base pairs.
[*]Accession number AJ311522.

case was comparable with changes described in naturally infected horses. The dominance of lymphocytes within the infiltrates and the presence of only mild gliosis, as well as the absence of plasma cells, indicates an acute to subacute stage of infection. Sequence analyses of the present alpaca field virus revealed almost complete homology to the endemic cluster Bavaria I, which is the most common BDV isolate in the region from which the alpaca originated (Kolodziejek *et al.*, 2005). This finding suggested that the alpaca had already been infected in Bavaria before being transported to the second herd and that the incubation time of the infection was at least 2 months. In the herd from which the alpaca originated, a case of naturally occurring BD had been diagnosed in 2001 (Herzog, personal communication). However, the present case (born in December 2005) was not in this herd at that time. The precise incubation period for natural BD infection is unknown, but possibly ranges from 2 to several months (Schmidt, 1952).

The behavioural abnormalities in this animal, reflected in a decreased sexual desire, were possibly due to viral infection and inflammation in the hypothalamic region. This region integrates autonomic response and endocrine function with behaviour and regulates, among others, reproduction through hormonal control of mating (Kandel *et al.*, 2000). A concentration of inflammatory lesions in the hypothalamus has been reported in rats infected with a particular strain of BDV, leading to an obesity syndrome (Herden *et al.*, 2000). Even without inflammatory reaction, BDV may disturb neuronal plasticity by interference with molecular signalling pathways, leading to behavioural changes as seen in experimentally infected gerbils and other animals used as models (Gonzalez-Dunia *et al.*, 2005). In addition, inflammatory lesions in the hypothalamus may have exacerbated other pre-existing disturbances. From the expression profile of BDV proteins and RNAs and the sequence analysis of the alpaca BDV, no significant alterations in comparison with the cluster Bavaria I or the closely related strain RW98 were found. On the one hand, this cannot rule-out changes in other parts of the viral genome such as the viral polymerase as described for adaptation processes from rats to mice (Ackermann *et al.*, 2007a,b). On the other hand, alpacas might react differently or display a different susceptibility to natural BDV infection than horses. This was already shown for experimental BDV infection of tupaias, which only exhibited changes in social behaviour and no neurological signs (Sprankel *et al.*, 1978). Additional clinical signs in the alpaca consisted of inability to stand on its own, which may reflect movement disorders typical of equids and sheep, although proclivity for head pressing, another typical sign in equids and sheep, was not observed (Grabner and Fischer, 1991; Solbrig *et al.*, 1995; Richt *et al.*, 2007).

By IHC and ISH, proteins and mRNA of BDV-N and BDV-P were detected successfully in the cytoplasm and nuclei of brain cells, mainly neurons. The distribution within the brain as well as the intracellular localization was similar to that described in horses infected with BDV and in experimentally infected rats (Herden *et al.*, 1999; Werner-Keiss *et al.*, 2008). Additionally, the expression of BDV-GP mRNA and protein were tightly restricted, indicating comparable viral strategies to control its gene expression as described in other animals (Werner-Keiss *et al.*, 2008). This might enable BDV to establish persistence in other mammalian species.

The 99% homology of the alpaca virus with BDV-strains from the Bavaria I-cluster underline that BDV genetic clusters are independent of the animal species and correspond more to territorial origin (Kolodziejek *et al.*, 2005). This implies the presence of natural reservoirs as demonstrated in shrews (*Crocidura leucodon*; Hilbe *et al.*, 2006). These animals are distributed from central Europe eastward to the Caspian Sea with the exception of southern France, the Iberian Peninsula and the islands of the Mediterranean Sea (Raese and Yahnke, 2004). They also belong to the original fauna of Bavaria, with a variable density of population (Kraft, 2008). However, to date BDV-infected shrews from Bavaria have not been reported. The reservoir hypothesis could be further substantiated by the fact that no BDV-specific serum antibodies were detected in the other animals from the herd in the north of Hesse, indicating no alpaca-to-alpaca transmission as assumed for the infection of horses (Staeheli *et al.*, 2000; Dürrwald *et al.*, 2006; Richt *et al.*, 2007).

In conclusion, BDV infection should be considered as a differential diagnosis for non-purulent inflammation of the CNS in alpacas, especially in regions endemic for BD such as the German-speaking countries or alpacas originating from any of these areas.

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