Received: 20 January 2020 | Revised: 9 March 2020 | Accepted: 16 March 2020

DOI: 10.1111/tbed.13556

ORIGINAL ARTICLE

WILEY

Borna disease outbreak with high mortality in an alpaca herd in a previously unreported endemic area in Germany

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Funding information
The study was in part funded by the Federal Ministry of Education and Research within the Research Network Zoonotic Infections to the 'Zoonotic Bornavirus Consortium' (ZooBoCo; grant no. 01KI1722A to DT, DH, RGU, MB and DR).

Abstract

Borna disease virus 1 (BoDV-1) is the causative agent of Borna disease, an often fatal neurologic condition of domestic mammals, including New World camelids, in endemic areas in Central Europe. Recently, BoDV-1 gained further attention by the confirmation of fatal zoonotic infections in humans. Although Borna disease and BoDV-1 have been described already over the past decades, comprehensive reports of Borna disease outbreaks in domestic animals employing state-of-the-art diagnostic methods are missing. Here, we report a series of BoDV-1 infections in a herd of 27 alpacas (*Vicugna pacos*) in the federal state of Brandenburg, Germany, which resulted in eleven fatalities (41%) within ten months. Clinical courses ranged from sudden death without previous clinical signs to acute or chronic neurologic disease with death occurring after up to six months. All animals that underwent necropsy exhibited a non-suppurative encephalitis. In addition, six apparently healthy seropositive individuals were identified within the herd, suggesting subclinical BoDV-1 infections. In infected animals, BoDV-1 RNA and antigen were mainly restricted to the central nervous system and the eye, and sporadically detectable in large peripheral nerves and neuronal structures in other tissues. Pest control measures on the farm resulted in the collection of a BoDV-1-positive bicoloured white-toothed shrew (*Crocidura leucodon*), while all other trapped small mammals were negative. A phylogeographic analysis of BoDV-1 sequences from the alpacas, the shrew and BoDV-1-positive equine cases from the same region in Brandenburg revealed a previously unreported endemic area of BoDV-1 cluster 4 in North-Western Brandenburg. In conclusion, alpacas appear to be highly susceptible to BoDV-1 infection and display a highly variable clinical picture ranging from peracute death to subclinical forms. In addition to horses and sheep, they can serve as sensitive sentinels used for the identification of endemic areas.

KEYWORDS

alpaca, Borna disease virus 1 (BoDV-1), *Bornaviridae*, *Crocidura leucodon*, encephalitis, reservoir

Schulze and Große contributed equally to this work.

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1 | INTRODUCTION

Borna disease virus 1 (BoDV-1, species *Mammalian 1 orthobornavirus*, family *Bornaviridae*, order *Mononegavirales*) is the causative agent of Borna disease, an often fatal neurologic condition of horses, sheep and other domestic mammals (Dürrwald, Nowotny, Beer, & Kuhn, 2016; Richt & Rott, 2001). Recently, the zoonotic potential of the virus has been demonstrated by molecular and immunohistochemical detection of several BoDV-1-induced fatal encephalitis cases in humans (Coras, Korn, Kuerten, Huttner, & Ensser, 2019; Korn et al., 2018; Liesche et al., 2019; Niller et al., 2020; Rubbenstroth, Schlottau, Schwemmle, Rissland, & Beer, 2019; Schlottau et al., 2018).

The known BoDV-1 reservoir is the bicoloured white-toothed shrew (*Crocidura leucodon*). The virus is endemic in shrew populations in parts of Southern and Eastern Germany, Austria, Liechtenstein and Switzerland (Bourg et al., 2013; Dürrwald, Kolodziejek, Weissenböck, & Nowotny, 2014; Hilbe et al., 2006; Weissenböck et al., 2017). Incidentally, the virus can be transmitted to a broad range of other mammals, including humans, horses, sheep and New World camelids (Caplazi et al., 1999; Dürrwald et al., 2016; Jacobsen et al., 2010; Korn et al., 2018; Schlottau et al., 2018). These species serve as dead-end hosts to the virus, in which it is strictly neurotropic and induces an immune-mediated non-suppurative encephalitis leading to neurologic deficits, including behavioural abnormalities, apathy, somnolence-like conditions, ataxia and central blindness (Caplazi et al., 1999; Dürrwald et al., 2016; Richt & Rott, 2001; Schmidt, 1951). BoDV-1 infection and Borna disease in New World camelids, such as alpacas (*Vicugna pacos*) and llamas (*Lama glama*), have been described sporadically in the past, but comprehensive reports on their susceptibility and role as BoDV-1 hosts are lacking (Altmann, Kronberger, Schüppel, Lippmann, & Altmann, 1976; Jacobsen et al., 2010; Kobera, 2016; Kobera & Pöhle, 2004; Schüppel, Kinne, & Reinacher, 1994).

New World camelids, which are originally native to the Andean plateau (Altiplano) in South America, have gained considerable popularity in private husbandries worldwide since the 1980s. Alpacas are mainly kept for their fine quality fibre, but also as companion and therapy animals and tourist attractions (Gauly, Vaughan, & Cebra, 2018). In Germany, the number of alpacas registered in the 'Llama & Alpaca Registries Europe' (LAREU; https://www.lareu.org/), Europe´s largest New World camelid database, has increased continuously from less than 500 animals in 2008 to nearly 7,000 individuals in 2019 (Christian Kiesling, personal communication).

Here, we describe a Borna disease outbreak with high mortality on an alpaca farm in the federal state of Brandenburg, Germany. The report provides comprehensive data on the course of disease, neuropathology, diagnostic measures, viral tissue distribution and epidemiology.

2 | MATERIALS AND METHODS

2.1 | Origin of samples

The samples analysed in this study originated from an alpaca farm in the north-western part of the federal state of Brandenburg, Germany. Since 2010, the farm owned an alpaca herd that was housed on two different premises sized 1 hectare (ha) and 0.6 ha (premises 1 and 2, respectively), which are located approximately 1 km apart. In addition, the farm harboured five goats (*Capra aegagrus hircus*), five wallabies (*Macropus agilis*) and approximately 50 chickens (*Gallus gallus*) on premise 1 and a cat (*Felis silvestris catus*) on premise 2. Before 2010, sheep had been kept on the same grasslands.

At the onset of the outbreak in December 2018, the alpaca herd consisted of 22 animals (eight adult males, M1 to M8; twelve adult females, F1 to F12; and two juvenile males, J1 and J2). Two additional adult stallions (M9 and M10) and one adult mare (F13) were newly introduced during April to May 2019 and two foals (J3 and J4) were born in May and August 2019, respectively.

Serum samples were collected from all alpacas on 2 April, 12 June, 11 and September 2019. At these time points, nasal, conjunctival and oral swabs as well as faecal samples were collected from selected diseased and/or seropositive alpacas. Additional sera and/or cerebrospinal fluid (CSF) samples were available from alpacas M1, M4, M5 and M6 and from three healthy goats. Furthermore, serum samples were collected from two persons, who lived on the farm and had regular contact to the diseased animals.

Necropsy was performed on six euthanized or perished animals (M1 to M6) and tissue samples were collected for histopathological analysis, detection of viral antigen and RNA. Furthermore, archived formalin-fixed paraffin-embedded (FFPE) kidney and liver tissue or brain tissue were available from two additional alpacas from the farm, which had died in January 2015 (A1) and August 2016 (A4), respectively.

Frozen brain tissue from two BoDV-1-positive horses (*Equus caballus*) originating from the same region in North-Western Brandenburg in 2016 and 2019 were used for sequencing of BoDV-1 genomes and phylogenetic analysis.

Non-fixed tissue and serum samples were stored at −80°C or −20°C, respectively, for further analysis.

2.2 | Trapping of small mammals

For pest control measures, small mammals were trapped on the private ground of the farm using snap traps during April to August 2019. Approximately 150 traps were baited with a mixture of peanut butter with oat flakes and bacon and distributed over both premises of the alpaca farm, focussing predominantly on premise 1. Collected carcasses were stored at −20°C and transported to the laboratory on dry ice. Dissection followed a standardized protocol and samples were immediately stored at −20°C until further processing and analysis. Species were identified by analysis of partial cytochrome *b* gene sequences according to a previously described method (Schlegel et al., 2012).

2.3 | Detection of BoDV-1 RNA by RT-qPCR

Nucleic acid from fresh-frozen tissue samples, swabs and/or CSF from alpacas, horses and trapped small mammals was extracted using the Nucleo Mag Vet Kit (Macherey & Nagel, Düren, Germany) and KingFisher™

Flex Purification System (Thermo Fisher Scientific) according to the manufacturer´s instructions, with or without a prior extraction step with Trizol or Trizol LS reagent (Life Technologies). RNA from FFPE tissue was extracted as described elsewhere (Boos, Nobach, Failing, Eickmann, & Herden, 2019). A defined copy number of in vitro-transcribed RNA of the eGFP gene was added to each sample during RNA extraction as an internal control (Hoffmann, Depner, Schirrmeier, & Beer, 2006).

BoDV-1 RNA was detected by two RT-qPCR assays (BoDV-1 mix 1 and mix 6) detecting the BoDV-1 phosphoprotein (P) and matrix protein (M) gene, respectively. Samples from small mammals were additionally analysed by RT-qPCR panBorna mix 7.2, which is designed to detect a broad spectrum of orthobornaviruses. All tests were performed as described previously (Schlottau et al., 2018) and primer sequences are provided in Table S1. Primers and probes targeting the beta actin gene (Toussaint, Sailleau, Breard, Zientara, & De Clercq, 2007) and the eGFP RNA (Hoffmann et al., 2006) were employed to assess the RNA quality and the efficacy of RNA extraction and RT-qPCR, respectively. Results were determined as cycle of quantification (Cq) values. RNA dilutions from a persistently BoDV-2-infected cell culture were used as positive controls and for calibration of Cq values from independent RT-qPCR runs.

2.4 | Metagenome analysis by high-throughput sequencing (HTS) analysis

To identify the potential pathogen, nucleic acids extracted from FFPE tissue from different areas of the central nervous system of animal M1 and from frozen brain samples of animal M2 were analysed by high-throughput sequencing (HTS) using a metagenomics approach. Briefly, extracted RNA was reverse-transcribed using a combination of SuperScript™ IV First-Strand Synthesis System (Invitrogen) and NEBNext® Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs). The cDNA was fragmented to 200 base pairs (bp) length (FFPE material of animal M1) or 550 bp length (frozen material of animal M2) using an M220 Focused-ultrasonicator (Covaris). Afterwards, cDNA libraries were prepared as described previously (Forth et al., 2019; Wylezich, Papa, Beer, & Höper, 2018). During size exclusion, fragments smaller than 400 bp were additionally retained and purified two times with 1.2× Agencourt Ampure XP Beads (Beckman Coulter). Quality control and library quantification were performed following previously described protocols (Wylezich et al., 2018) and the libraries (lib03059-3066) sequenced together on an Ion 540 Chip using an Ion S5 XL instrument (Thermo Fisher Scientific). The data sets containing 2.7–8.5 million reads were analysed using the metagenomics software pipeline RIEMS (Scheuch, Höper, & Beer, 2015).

2.5 | Sequencing of complete and partial BoDV-1 genomes and phylogenetic analysis

Complete or partial BoDV-1 genome sequences from FFPE material of two alpacas (A4, M1) and frozen brain from a BoDV-1-infected shrew were generated by HTS following library preparation as described above. BoDV-1 reads were mapped to a BoDV-1 reference genome (GenBank accession number U04608) using GS Reference Mapper (Newbler version 3.0; Roche).

Partial BoDV-1 genomes (positions 20 to 2,291 according to the complete BoDV-1 genome; U04608) from fresh-frozen tissues were determined by Sanger sequencing of overlapping RT-PCR products. Primers used for amplification and sequencing are shown in Table S1. Sequencing was performed by Eurofins (Cologne).

BoDV-1 sequences generated during this study are available from INSDC databases under accession numbers MN937369 to MN937377.

A phylogenetic tree of partial BoDV-1 genomes covering the complete nucleoprotein (N), X protein and P genes (1,824 nucleotides, representing genome positions 54 to 1,877 of BoDV-1 reference genome U04608) was built to include all sequences generated during this study together with GenBank-derived sequences originating from domestic mammals, humans and shrews from the endemic areas in Central Europe. The analysis was performed using neighbor-joining algorithm and Jukes–Cantor distance model in Geneious 11.1.5 software (Biomatters).

2.6 | Detection of BoDV-1-reactive antibodies using an indirect immunofluorescence assay

All sera and CSF samples from alpacas and goats were tested for the presence of bornavirus-reactive antibodies by indirect immunofluorescence assay (iIFA) using a modification of previously described protocols (Schlottau et al., 2018; Zimmermann, Rinder, Kaspers, Staeheli, & Rubbenstroth, 2014). Briefly, confluent overnight culture of either non-infected Vero cells or non-infected Vero cells mixed with 30% Vero cells persistently infected with BoDV-1 isolate Z65-1 (Schlottau et al., 2018) were air-dried for 2.5 hr and subsequently heat-fixed for two hours at 80°C. Twofold dilution series of samples were prepared in Tris-HCl buffer with Tween 20 (T9039; Sigma-Aldrich) and 50 µl of each dilution was added in parallel to the BoDV-1-positive and BoDV-1-negative wells. After incubation for one hour, the plates were washed three times with phosphate-buffered saline (PBS), followed by incubation with goat anti-llama-IgG DyLight488 (Agrisera) or donkey anti-goat-IgG Alexa Fluor 488 conjugate (Jackson Immunoresearch) for another hour. After a final washing cycle, the assays were analysed by fluorescence microscopy. For each serum dilution, BoDV-1-positive and BoDV-1-negative wells were compared. Wells were considered positive, when the expected 30% BoDV-1-positive cells stained markedly brighter than the background staining of uninfected cells in the same well and in the corresponding bornavirus-negative control well. Human serum samples were analysed using a slightly modified procedure as published by Tappe et al. (2019). All tests were performed with a minimal dilution factor of 20. Samples showing no specific signal were assigned a titre of <20.

2.7 | Histopathology and BoDV-1 antigen detection by immunohistochemistry (IHC)

Tissue samples were collected from seven alpacas (A1, A4 and M1 to M6). All tissue samples were immersion-fixed in 4% formaldehyde for up to 48 hr. Formaldehyde-fixed tissues were embedded in paraffin, cut at 1-µm thickness and subsequently stained with haematoxylin and eosin for histological examination.

For immunohistochemical detection of BoDV-1 antigen, sections of FFPE tissue from each animal were dewaxed in xylene, followed by rehydration in descending concentrations of ethanol. After blocking of endogenous peroxidase, slides were incubated with either polyclonal rabbit anti-BoDV-1 P (4,000-fold diluted) or N serum (8,000-fold diluted) overnight at 4°C (Zimmermann et al., 2014). Irrelevant, immunopurified normal rabbit immunoglobulin (Ig) (BioGenex) in PBS at the same dilution served as negative control. Sections were incubated with biotinylated goat anti-rabbit-IgG antibody (200-fold diluted; Vector) for 30 min and subsequently treated with an avidin–biotin–peroxidase complex (ABC; Vector) for 30 min at room temperature. Following exposure to 3,3′-diaminobenzidine tetrahydrochloride (DAB) for eight minutes, slides were counterstained with haematoxylin, dehydrated in ascending concentrations of ethanol, cleared in xylene, and coverslipped.

An alpaca brain without signs of encephalitis originating from a herd without history of BoDV-1 infection served as negative control and a brain of a horse with confirmed Borna disease served as positive control.

3 | RESULTS

3.1 | Identification of BoDV-1 as the cause of fatalities on an alpaca farm in Brandenburg, Germany

A series of fatalities was reported in a herd of totally 27 alpacas from North-Western Brandenburg. Eleven alpacas died during December 2018 to October 2019 following clinical courses ranging from peracute death to chronic neurologic signs (Figure 1; Table 1). Necropsy was performed for six of these animals that had died during March to October 2019.

Due to the lack of a typical clinical presentation reported for the initial cases, the first two investigated cases (M1 and M2) were subjected to metagenomic analysis as part of a study aiming at the identification of unknown viral causes of encephalitis. The analysis led to the detection of BoDV-1 RNA in central nervous system (CNS) samples of both animals. In FFPE tissue from animal M1, the number of reads classified as BoDV-1 ranged from 2,835 to 37,933 (representing 0.1%–0.5% of the total data sets), whereas only up to 324 BoDV-1 reads (0.005% of the data set) were detected in frozen tissues of animal M2. No other pathogens potentially causing the observed clinical signs and microscopic lesions were identified by metagenomic analysis. The diagnosis was confirmed by BoDV-1-specific RT-qPCR and by detection of BoDV-1 N and P antigen by IHC in the brains of both animals as well as for the subsequently necropsied alpacas M3 to M6 (Table 1). Necropsy was not performed for any of the five remaining alpacas that had died during the outbreak (F1 to F4 and J1) and, thus, CNS tissue from these animals was not available for BoDV-1 detection (Table 1).

Five additional alpacas of the farm had died prior to the described outbreak, between January 2015 and January 2018. Archived FFPE brain tissue was available from a subadult stallion (A4) that had died in August 2016 with non-suppurative encephalitis following neurologic disease. Strikingly, detection of BoDV-1 RNA and antigen in this sample confirmed a BoDV-1 infection (Table 1). Furthermore, FFPE liver and kidney tissues were available for stallion A1 that had died in January 2015. IHC staining indicated the presence of bornavirus N and P antigen in renal nerve fibres of this animal (data not shown) but RT-qPCR failed to detect viral RNA. Archived brain tissue was not available for further confirmation of the infection.

3.2 | Course of Borna disease outbreak

The farm possesses two separate grasslands (premises 1 and 2) located approximately 1 km apart. Usually, the adult stallions were kept on premise 1 and the mares and foals on premise 2. However, from 30 September 2018 on, the two groups had switched premises for 3 months. The first fatality of the current outbreak occurred on premise 1 on 27 December. An adult mare (F1) was euthanized after showing non-specific clinical signs, such as anorexia and apathy,

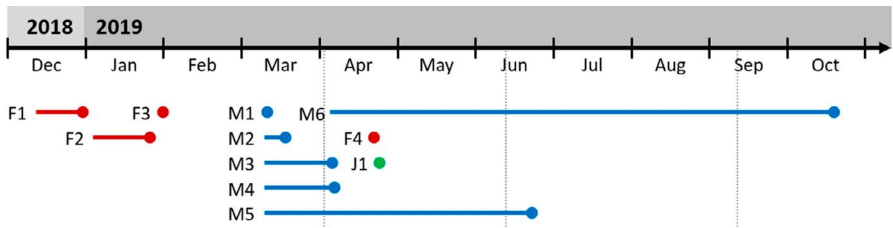


FIGURE 1 Course of disease outbreak on the BoDV-1-affected alpaca farm. Coloured horizontal lines and dots represent duration of disease and time point of death, respectively, of individual animals. Grey dotted vertical lines represent the time points of blood sampling of the complete herd. F = adult female (red), M = adult male (blue) and J = juvenile (green)

TABLE 1 Overview of all fatalities of alpacas reported on the farm from 2010 to 2019

Animal	Sex	Age	Beginning of disease	Death	Duration of disease (days)	Clinical course and signs	BoDV-1 detection [a]
A1	m	3 y.	Unknown	6 January 2015	/	Emaciation, massive endoparasitosis	(pos)[b]
A2	w	/	Unknown	17 February 2016	/	Unknown	/
A3	m	/	Unknown	March 2016	/	Unknown	/
A4	m	1 y.	Unknown	18 August 2016	/	Cachexia, neurologic signs	pos
A5	w	/	Unknown	3 January 2018	/	Unknown	/
F1	f	12 y.	11 December 2018	28 December 2018	17	Reduced general condition, inactivity	/
F2	f	6 y.	2 January 2019	23 January 2019	21	Ataxia, hoisted lips and nostrils, dysphagia, anorexia, weakness	/
F3	f	4 y.	29 January 2019	29 January 2019	0	Sudden death, dyspnoea	/
M1	m	2 y.	9 March 2019	9 March 2019	0	Sudden death	pos
M2	m	9 y.	9 March 2019	16 March 2019	7	Depression, recumbency, weakness	pos
M3	m	7 y.	9 March 2019	3 April 2019	25	Ataxia, head shaking, hypersensitivity, weakness	pos
M4	m	6 y.	9 March 2019	4 April 2019	26	Apathy, ataxia, tremor of the head, later seizures	pos
F4	f	4 y.	20 April 2019	20 April 2019	0	sudden death	/
J1	m	1 y.	22 April 2019	22 April 2019	0	Sudden death	/
M5	m	8 y.	9 March 2019	19 June 2019	102	Ataxia, lack of coordination, opisthotonus, torticollis, head shaking, food material impacted in the cheek, polydipsia, progressing apathy	pos
M6	m	6 y.	4 April 2019	14 October 2019	193	Circle movements, depression, head shaking and severe apathy after six months	pos

Abbreviations: (/), information or sample not available; A, archived; F/f, female; J, juvenile; M/m, male; pos, positive, y., year(s).

[a]Confirmation of BoDV-1 infection by detection of BoDV-1 RNA by RT-qPCR and bornavirus antigen via immunohistochemistry (IHC) from brain tissue.

[b]Only FFPE kidney and liver tissues were available for animal A1. BoDV-1 antigen was detectable by IHC in renal nerve fibres, but detection of viral RNA by RT-qPCR failed.

for 2 weeks (Figure 1; Table 1). Thereafter, the housing of the two groups was reversed again on 1 January 2019 and the adult stallions were transferred back to premise 1. Two further mares (F2 and F3), now located on premise 2, died on 23 and 29 January, respectively. Animal F2 had suffered from neurologic disease for three weeks, whereas F3 died peracutely without showing prior clinical signs (Figure 1; Table 1).

On 9 March, two days after immunization of all alpacas with an inactivated clostridia toxoid vaccine, an adult stallion (M1) on premise 1 died suddenly without previous signs of disease. On the same day, four further stallions were reported to exhibit behavioural changes and neurologic signs (Figure 1; Table 1). These four animals died or were euthanized on 16 March (M2), 3 April (M3), 4 April (M4) and 19 June (M5). An additional adult stallion (M6) on premise 1 showed behavioural changes and slight apathy starting on 4 April. After five months of mild disease, its condition markedly exacerbated during October, requiring euthanasia on 14 October. Furthermore, two sudden fatalities occurred in an adult mare (F4) and a male foal (J1) on premise 2 on 20 and 22 April, respectively (Figure 1; Table 1). The remaining 16 alpacas (M7 to M10, F5 to F13, J2 to J4) as well as all goats, wallabies, chickens and the cat remained apparently healthy throughout the observation period.

3.3 | Clinical signs exhibited by diseased alpacas

Diseased animals during this outbreak exhibited a broad spectrum of clinical signs and varying courses of disease, ranging from sudden fatalities (animals F3, F4, M1, J1) to death after several weeks to months of disease (animals F1, F2, M2 to M6; Table 1).

Animals suffering from sudden fatalities were usually reported as clinically healthy until few hours before death. Animals with acute-to-chronic courses of disease exhibited varying degrees of neurologic signs and behavioural abnormalities, such as polydipsia, hoisted lips and nostrils, dysphagia, incoordination, ataxia, circle movements, opisthotonus, torticollis, tremor, seizures and paralysis,

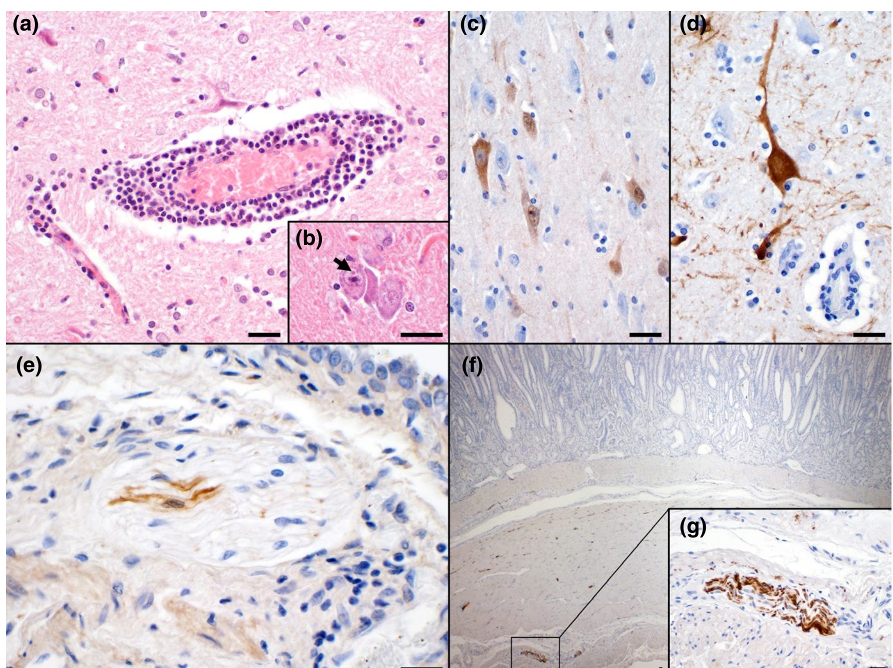


FIGURE 2 BoDV-1 antigen detection in the nervous system of alpacas. (a) A moderate to severe, chronic, multifocal, non-suppurative encephalitis with lymphocytic perivascular cuffing and (b) occasionally distinct, eosinophilic, intranuclear viral inclusions (Joest–Degen inclusion bodies, arrow) was diagnosed (animals M2 and M5, respectively; HE staining). (c) BoDV-1 phosphoprotein and (d) BoDV-1 nucleoprotein were immunohistochemically detected in neurons and axons of the cerebrum (M4). In peripheral organs such as (e) the nasal mucosa of animal M3 and (f,g) the intestine of animal M5. BoDV-1 nucleoprotein was localized in peripheral nerve fibres. Scale bar: 20 µm

but also non-specific signs, such as apathy, anorexia and progressive weight loss. Some animals showed phases of temporary recovery with subsequent exacerbation of the disease.

Two stallions (M4 and M5) had been hospitalized at the Clinic for Ruminants and Swine of the Freie Universität Berlin and were therefore clinically examined in more detail. Animal M4 had been submitted to the clinic on 9 March, when after exhibiting prominent clinical signs. The animal was weak, showed ataxia and progressively diminishing feed intake. Within the following 3 weeks, it developed head tremor and seizures, particularly during handling. Clinical chemistry did not reveal alterations, but differential blood count revealed a left-shift. May–Grünwald–Giemsa-stained blood smears revealed characteristic haemoplasma-like organisms in the cytoplasm or attached to the cell wall of erythrocytes. The organisms were identified as *Mycoplasma haemolamae* by PCR. Antibiotic treatment was initiated with procaine penicillin (40,000 IU/kg daily) because of initially suspected clostridial enterotoxemia and later changed to oxytetracycline (three applications of 20 mg/kg at three-day intervals) after confirmation of *M. haemolamae* infection, but neither treatment resulted in clinical improvement. Following a single dexamethasone dose (2 mg/kg), the animal became considerably more active for three days but its condition progressively exacerbated thereafter, requiring euthanasia on 4 April.

Starting on 9 March, stallion M5 showed stereotypic behaviour, dysphagia and polydipsia for about one week. Thereafter, the behavioural changes subsided but the animal progressively lost weight and became less active. Neurologic signs, including opisthotonus, torticollis, head tremor and ataxia, considerably exacerbated from the beginning of June on. Clinical chemistry and haematology did not reveal marked alterations. The alpaca was admitted to the clinic on 17 June and euthanized two days later.

3.4 | Microscopic lesions in BoDV-1-infected alpacas

Necropsy of the seven confirmed BoDV-1-positive alpacas (A4 and M1 to M6) revealed no relevant macroscopic lesions, but a chronic, multifocal, non-suppurative meningoencephalitis with lymphocytic perivascular cuffing (Figure 2a) and occasional intranuclear Joest–Degen inclusion bodies (Figure 2b) was diagnosed histologically in all animals. The severity of the inflammation ranged from mild (A4, M5, M6) to moderate or severe (M1 to M4). No other significant lesions were identified in any of the examined tissues.

3.5 | BoDV-1 tissue distribution

The tissue distribution of BoDV-1 RNA was determined by semi-quantitative RT-qPCR of tissue samples available for animals M1 to M6 (Figure 3). Highest levels were usually detectable in hippocampus, olfactory bulb and brain stem, followed by cerebellum, spinal cord, optic nerve and retina. Only in animal M6, viral RNA was not found widely distributed in the CNS but restricted mainly to olfactory bulb, hippocampus and eye (Figure 3). Low-to-moderate RNA levels were sporadically detected in peripheral nerves, nasal mucosa and/or salivary glands of alpacas M3, M5 and M6 (Figure 3). Viral RNA was found widely distributed in the organs of animal M5, including lacrimal glands, adrenal gland, gastrointestinal tract and urinary bladder, but it was not detectable in any other peripheral site tested for M1 to M4 and M6 (Figure 3).

Immunohistochemical analysis confirmed BoDV-1 antigen in the brains of all investigated animals. Viral antigen was predominantly

Tissue	Animal:	M1	M2	M3	M4	M5	M6
	Day of death:	March 9	March 16	April 3	April 4	June 19	Oct 14
Brain	Olfactory bulb	13.3	19.2	11.2	13.0	13.1	25.0
	Hippocampus	18.2	17.1	12.0	11.5	12.6	24.0
	Brain stem	13.4	21.1	14.0	14.2	13.7	neg
	Hypophysis					24.9	neg
	Cerebellum	15.1		14.7	18.2	18.4	neg
Spinal cord	Spinal cord (cervical)	14.0		15.4	15.8	13.9	neg
	Spinal cord (thoracal)			23.8	21.9	22.1	neg
	Spinal cord (lumbal)			23.2	23.2	24.9	neg
Eye	Optic nerve (right)			15.1	16.1	18.6	28.2
	Optic nerve (left)		25.1	15.3	19.2	21.2	26.4
	Retina (right)			21.1	28.0	22.8	21.0
	Retina (left)			23.8	25.7	30.1	22.8
Peripheral nerves	Trigeminal nerve & ganglion (right)					17.1	neg
	Trigeminal nerve & ganglion (left)					20.5	27.4
	Facial nerve (right)			26.2	33.3	26.4	neg
	Facial nerve (left)			28.9	neg	26.9	neg
	Brachial plexus (right)			31.2	neg	25.6	neg
	Brachial plexus (left)			neg	31.9	28.2	neg
	Sciatic nerve (right)			neg	neg	31.3	neg
	Sciatic nerve (left)			neg	neg	29.6	neg
Nose & glands	Nasal muscosa (right)			25.5	neg	27.0	neg
	Nasal muscosa (left)			27.0	neg	28.7	neg
	Lacrimal gland (right)					25.8	26.0
	Lacrimal gland (left)					28.9	neg
	Parotid gland (right)					29.3	neg
	Parotid gland (left)					24.6	neg
Other organs	Kidney			neg	neg	neg	neg
	Liver	neg		neg	neg	neg	neg
	Spleen			neg	neg	neg	neg
	Lung			neg	neg	neg	neg
	Heart, septum			neg	neg	neg	neg
	Heart, sinoatrial node			neg	neg	24.0	neg
	Adrenal gland (right)			neg	neg	32.0	neg
	Adrenal gland (left)			neg		25.2	neg
	Stomach			neg	neg	28.2	neg
	Jejunum	neg	neg	neg	neg	27.0	neg
	Colon	neg	neg	neg	neg	28.5	neg
	Urinary bladder					27.3	neg

FIGURE 3 Tissue distribution of BoDV-1 RNA in deceased alpacas. BoDV-1 RNA in six alpacas with available tissue samples (M1 to M6) was detected using semi-quantitative RT-qPCR. Results are presented as minimal cycle of quantification (Cq) value of two RT-qPCR assays (BoDV-1 mix 1 and mix 6) used in parallel. Low Cq values (dark green shadings) represent high viral RNA loads. neg = no BoDV-1 RNA detectable (Cq value > 37)

identified in neurons (Figure 2c and d). In consistence with the RT-qPCR results, BoDV-1 antigen was occasionally detected in peripheral nerves of the nasal mucosa of animals such as M3 (Figure 2e) and in neuronal structures within several organs of animal M5, including the gastrointestinal tract (Figure 2f and g).

Despite the presence of BoDV-1 infection in brain, eye and occasionally nasal mucosa, viral RNA was not detectable in CSF or nasal swabs collected from BoDV-1-positive animals and only very low levels were detected in two conjunctival swabs (Table 2).

3.6 | Screening for bornavirus-reactive antibodies in animals and humans

Serum samples were collected from all alpacas on 2 April, 12 June and 11 September 2019 for detection of BoDV-1-reactive antibodies by iIFA (Table 3). Additional sera and/or CSF samples were available from the diseased alpacas M1 and M4 to M6 (Figure 4).

The four stallions that exhibited a subacute or chronic course of disease (M3 to M6) developed moderate-to-high levels of

bornavirus-reactive antibodies (titres 320–1,600; Table 3). Animal M4 remained seronegative for at least nine days after the onset of disease and reached moderate titres (160–320) from day 16–26 of disease (Figure 4). A CSF sample collected during euthanasia at day 26 showed a similar titre of 400. Animal M5 was first sampled three weeks after the onset of disease, when it already showed high levels of bornavirus-reactive antibodies (titre 1,600). High antibody titres were also detected in three subsequent serum samples from M5 collected during the last week before death (titres 800–1,600) and in a CSF sample (titre 1,280) collected during euthanasia after more than three months of disease (Figure 4). Animal M6 had been tested negative two days before onset of disease and was first tested positive 10 weeks later, reaching a titre of 640 after five months of disease. Notably, a serum sample collected at the day of euthanasia tested negative (Figure 4), while a CSF sample from the same time point was weakly positive (titre 80).

Stallion M1, which had died peracutely, tested likewise positive (titre 320) in a single available serum sample collected at necropsy (data not shown), whereas bornavirus-reactive antibodies were not detectable in sera from animals F4 and J1 collected three weeks before their sudden deaths (Table 3).

Strikingly, seroconversion was observed also for three stallions and three mares that stayed apparently healthy throughout the study period (M7, M8, M10, F6, F8 and F11), reaching maximal titres of 80 to 1,280. Stallion M10 was newly introduced into the farm in May 2019 and first tested positive in September 2019 (Table 3). All remaining alpacas, including the newborn foal J3 originating from the weakly seropositive mare F6, remained seronegative (Table 3). Likewise, bornavirus-reactive antibodies were not detectable in sera collected from three healthy goats on 2 April and from two healthy humans living on the farm (data not shown).

3.7 | BoDV-1 screening of small mammals trapped on the farm premises

During pest control measures on the farm from April to August 2019, 36 small mammals were trapped by the owner and submitted for analysis. The tested animals included two shrews (a bicoloured white-toothed shrew, *Crocidura leucodon* and a common shrew, *Sorex araneus*; order Soricomorpha) and 34 rodents (11 house mice, *Mus musculus*, 16 yellow-necked mice, *Apodemus flavicollis*, five striped field mice, *Apodemus agrarius*, and two common voles, *Microtus arvalis*; order Rodentia). Brain tissue of these animals was used for viral screening, since high viral loads had been reported in the CNS of BoDV-1-infected shrews (Bourg et al., 2013; Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). BoDV-1 RNA was detected in the brain of the bicoloured white-toothed shrew that had been trapped on 29 May on the grassland of premise 1. In contrast, no bornavirus RNA was detectable in the brain of any of the other small mammals.

3.8 | Phylogeny and geographic distribution of BoDV-1 infections in Brandenburg

Complete or partial BoDV-1 sequences were generated from six alpacas for which brain tissue had been available for analysis

TABLE 2 Analysis of BoDV-1 RNA by RT-qPCR in samples collected *intra vitam* from diseased and/or seropositive alpacas

Animal	Day of sampling	CSF	Nasal swabs	Conjunctival swabs	Oral swab	Faeces
M3	2 April	/	neg/neg	neg/neg	/	neg/neg
M4	4 April	neg/neg	neg/neg	**36.7**/neg	/	/
M5	2 April	/	neg/neg	neg/neg	/	neg/neg
	12 June	neg/neg	neg/neg	neg/neg	/	/
M6	11 September	/	neg/neg	neg/neg	neg/neg	/
	14 October	neg/neg	neg/neg	neg/neg	neg/neg	/
M7	12 June	/	neg/neg	neg/**34.2**	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
M8	12 June	/	neg/neg	neg/neg	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
F6	12 June	/	neg/neg	neg/neg	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
F8	11 September	/	neg/neg	neg/neg	neg/neg	/
F11	11 September	/	neg/neg	neg/neg	neg/neg	/

Note: Results are presented as cycle of quantification (Cq) value determined by two semi-quantitative RT-qPCR assays used in parallel (BoDV-1 mix 1 and mix 6).

Abbreviations: (/), sample not available; CSF, cerebrospinal fluid; neg, negative (Cq value > 37).

Positive results are depicted in bold.

TABLE 3 Bornavirus-reactive antibodies in sera of healthy and diseased alpacas

Animal	Sex	Age	Beginning of disease	Death	BoDV-1 detection[a]	Anti-BoDV-1 iIFA titres[b]		
						2 April	12 June	11 September
M3	m	7 y.	9 March	3 April	pos.	**1,280**	/	/
M4	m	6 y.	9 March	4 April	pos.	**320**	/	/
M5	m	8 y.	9 March	19 June	pos.	**1,600**	**1,600**	/
M6	m	6 y.	4 April	14 October	pos.	<20	**160**	**640**
F4	f	4 y.	20 April	20 April	/	<20	/	/
J1	m	1 y.	22 April	22 April	/	<20	/	/
M7	m	8 y.	-	-	/	**160**	**160**	**160**
M8	m	3 y.	-	-	/	**1,280**	**320**	**320**
M9[c]	m	7 y.	-	-	/	/	<20	<20
M10[c]	m	3 y.	-	-	/	/	<20	**160**
F5	f	10 y.	-	-	/	<20	<20	<20
F6	f	9 y.	-	-	/	**80**	**40**	**160**
F7	f	1 y.	-	-	/	<20	<20	<20
F8	f	9 y.	-	-	/	<20	**80**	**80**
F9	f	6 y.	-	-	/	<20	<20	<20
F10	f	6 y.	-	-	/	<20	<20	<20
F11	f	11 y.	-	-	/	<20	**80**	**320**
F12	f	7 y.	-	-	/	<20	<20	<20
F13[c]	f	3 y.	-	-	/	/	<20	<20
J2	m	1 y.	-	-	/	<20	<20	<20
J3[c]	m	4 mo.	-	-	/	/	<20	<20
J4[c]	f	1 mo.	-	-	/	/	/	<20

Abbreviations: (/), sample not available; f, female; m, male; mo., months; y., years.

Positive results are depicted in bold.

[a]Detection of BoDV-1 RNA by RT-qPCR and bornavirus antigen by immunohistochemistry from brain tissue (if available).

[b]Detection of bornavirus-reactive antibodies by indirect immunofluorescence assay (iIFA). Samples were tested at the lowest dilution of 20-fold. Titres of samples without specific signal were regarded as < 20.

[c]Animals were introduced or born during May to August 2019.

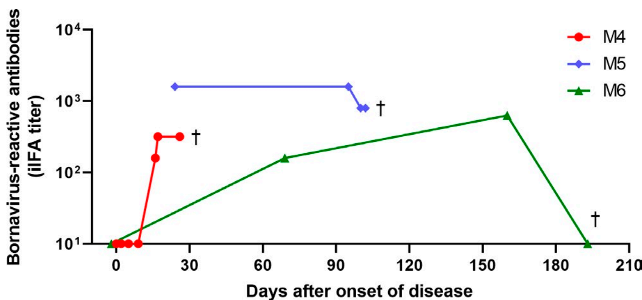
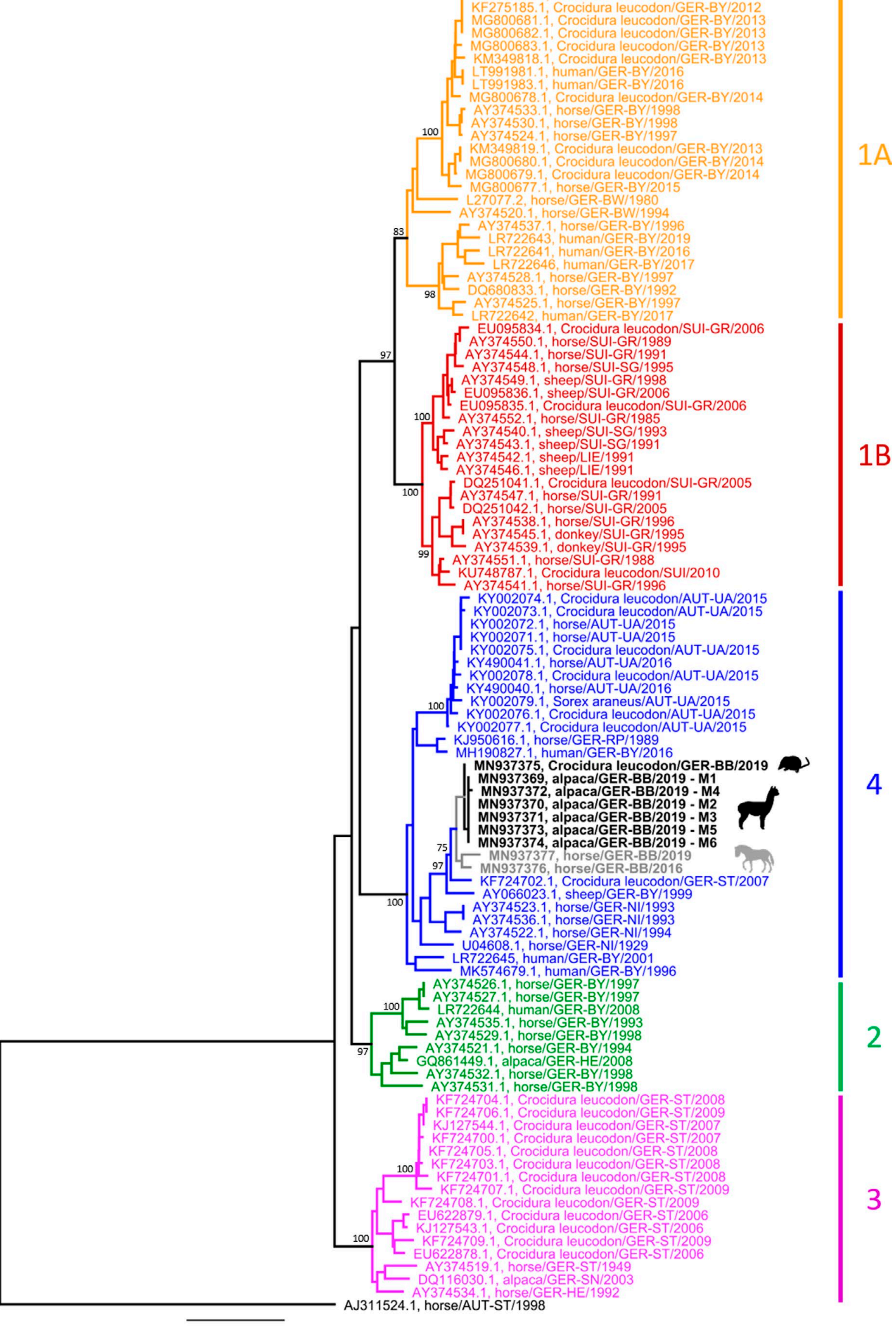


FIGURE 4 Course of bornavirus-reactive antibody titres in three BoDV-1-infected alpacas (M4 to M6). BoDV-1-reactive antibodies were determined by indirect immunofluorescence assays (iIFA). The lower limit of the y-axis represents the detection limit of the assay. †The last sample collected from each animal represents the day of death

(M1 to M6) and from the BoDV-1-positive shrew trapped on the farm. The seven sequences were almost identical to each other, with only up to two nucleotide alterations within the 2,237 nucleotide positions shared by all sequences (99.91%–100% pairwise nucleotide sequence identity). The complete viral genomes available from alpaca M1 (MN937369) and from the BoDV-1-infected shrew (MN937375) differed at six nucleotide positions (99.93% nucleotide sequence identity; data not shown). Phylogenetic analysis revealed the sequences to belong to BoDV-1 cluster 4 (Figure 5). Strikingly, the most closely related sequences originated from recently analysed samples from two BoDV-1-infected horses from North-Western Brandenburg in 2016 and 2019 (MN937376 and MN937377, respectively; Figures 5 and 6), which shared 99.0%–99.5% nucleotide sequence identity with the sequences from the alpaca farm.

Due to the high degree of RNA degradation, HTS from the FFPE brain tissue of animal A4 revealed only a few short reads that were nearly identical to the sequences from the other alpacas and the shrew (data not shown).



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KF275185.1, Crocidura leucodon/GER-BY/2012
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MG800682.1, Crocidura leucodon/GER-BY/2013
MG800683.1, Crocidura leucodon/GER-BY/2013
KM349818.1, Crocidura leucodon/GER-BY/2013
LT991981.1, human/GER-BY/2016
LT991983.1, human/GER-BY/2016
MG800678.1, Crocidura leucodon/GER-BY/2014
AY374533.1, horse/GER-BY/1998
AY374530.1, horse/GER-BY/1998
AY374524.1, horse/GER-BY/1997
KM349819.1, Crocidura leucodon/GER-BY/2013
MG800680.1, Crocidura leucodon/GER-BY/2014
MG800679.1, Crocidura leucodon/GER-BY/2014
MG800677.1, horse/GER-BY/2015
L27077.2, horse/GER-BW/1980
AY374520.1, horse/GER-BW/1994
AY374537.1, horse/GER-BY/1996
LR722643, human/GER-BY/2019
LR722641, human/GER-BY/2016
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AY374528.1, horse/GER-BY/1997
DQ680833.1, horse/GER-BY/1992
AY374525.1, horse/GER-BY/1997
LR722642, human/GER-BY/2017
1A
EU095834.1, Crocidura leucodon/SUI-GR/2006
AY374550.1, horse/SUI-GR/1989
AY374544.1, horse/SUI-GR/1991
AY374548.1, horse/SUI-SG/1995
AY374549.1, sheep/SUI-GR/1998
EU095836.1, sheep/SUI-GR/2006
EU095835.1, Crocidura leucodon/SUI-GR/2006
AY374552.1, horse/SUI-GR/1985
AY374540.1, sheep/SUI-SG/1993
AY374543.1, sheep/SUI-SG/1991
AY374542.1, sheep/LIE/1991
AY374546.1, sheep/LIE/1991
DQ251041.1, Crocidura leucodon/SUI-GR/2005
AY374547.1, horse/SUI-GR/1991
DQ251042.1, horse/SUI-GR/2005
AY374538.1, horse/SUI-GR/1996
AY374545.1, donkey/SUI-GR/1995
AY374539.1, donkey/SUI-GR/1995
AY374551.1, horse/SUI-GR/1988
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KY490041.1, horse/AUT-UA/2016
KY002078.1, Crocidura leucodon/AUT-UA/2015
KY490040.1, horse/AUT-UA/2016
KY002079.1, Sorex araneus/AUT-UA/2015
KY002076.1, Crocidura leucodon/AUT-UA/2015
KY002077.1, Crocidura leucodon/AUT-UA/2015
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MH190827.1, human/GER-BY/2016
MN937375, Crocidura leucodon/GER-BB/2019
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KF724705.1, Crocidura leucodon/GER-ST/2008
KF724703.1, Crocidura leucodon/GER-ST/2008
KF724701.1, Crocidura leucodon/GER-ST/2008
KF724707.1, Crocidura leucodon/GER-ST/2009
KF724708.1, Crocidura leucodon/GER-ST/2009
EU622879.1, Crocidura leucodon/GER-ST/2006
KJ127543.1, Crocidura leucodon/GER-ST/2006
KF724709.1, Crocidura leucodon/GER-ST/2009
EU622878.1, Crocidura leucodon/GER-ST/2006
AY374519.1, horse/GER-ST/1949
DQ116030.1, alpaca/GER-SN/2003
AY374534.1, horse/GER-HE/1992
3
AJ311524.1, horse/AUT-ST/1998
0.02
100
83
98
97
100
100
99
100
75
97
100
100
97
100
100

FIGURE 5 Phylogenetic analysis of BoDV-1 sequences from the endemic areas. Phylogenetic analysis of partial BoDV-1 sequences (1,824 nucleotides, representing genome positions 54–1877 of BoDV-1 reference genome U04608) from the endemic regions in Germany, Austria, Switzerland and Liechtenstein was performed using neighbor-joining algorithm and Jukes–Cantor distance model in Geneious 11.1.5 and the tree was rooted with sequence BoDV-2 No/98 (AJ311524). Novel sequences originating from the alpaca farm (black) and from horses in Brandenburg (grey) are depicted in bold. Values at branches represent support in 1,000 bootstrap replicates. Only bootstrap values ≥ 70 at major branches are shown. Germany (GER): BB = Brandenburg, BY = Bavaria, BW = Baden-Wuerttemberg, HE = Hesse, NI = Lower Saxony, RP = Rhineland-Palatinate, SN = Saxony, ST = Saxony-Anhalt; Switzerland (SUI): GR = Grisons, SG = St. Gall; Liechtenstein (LIE); Austria (AUT): UA = Upper Austria, ST = Styria. Cluster designations, host and geographic origin are indicated according to previously published work (Dürrwald, Kolodziejek, Herzog, & Nowotny, 2007; Dürrwald et al., 2014; Kolodziejek et al., 2005; Niller et al., 2020; Nobach et al., 2015; Schlottau et al., 2018; Weissenböck et al., 2017)

4 | DISCUSSION

Here, we describe detailed investigations on a severe Borna disease outbreak on an alpaca farm in Brandenburg, Germany. During the outbreak, eleven out of totally 27 alpacas (40.7%) of the herd had died within a period of ten months. Although diagnostic material allowing BoDV-1 detection was available only from six of these animals, it is conceivable that also the remaining deaths in this outbreak were associated with BoDV-1 infection, since only few fatalities due to other causes had been reported on the farm prior to December 2018. A similarly high mortality of approximately 30% within 14 months had been reported for the only previously described Borna disease outbreak in a New World camelid herd (Schüppel et al., 1994), possibly suggesting a high susceptibility of these species. In contrast, the mortality in horses and sheep is usually low and often restricted to one or few individuals of an otherwise unaffected herd (Caplazi et al., 1999; Metzler, Frei, & Danner, 1976; Richt & Rott, 2001; Vahlenkamp, Konrath, Weber, & Muller, 2002).

The alpacas that developed disease and eventually died during this outbreak showed a highly variable disease progression, ranging from sudden death to chronic disease. The animals exhibited typical neurologic signs and behavioural changes, as well as unspecific clinical courses characterized mainly by loss of appetite and progressive weight loss. Similarly diverse forms of Borna disease have been reported not only for New World camelids, but also for other dead-end hosts, such as equids and sheep, and the time until death has been described to vary from few days to several months after disease onset (Altmann et al., 1976; Caplazi et al., 1999; Heinig, 1964; Jacobsen et al., 2010; Katz et al., 1998; Kobera, 2016; Matthias, 1958; Priestnall et al., 2011; Rott & Becht, 1995; Vahlenkamp et al., 2002; Weissenböck et al., 2017). These findings further emphasize that BoDV-1 has to be considered as a possible cause not only for neurologic disorders but also for untypical clinical presentations in potential dead-end hosts in known endemic areas.

In addition to the clinically affected animals, we identified six alpacas that developed bornavirus-reactive antibodies but remained free of apparent clinical disease throughout the study period. Seropositive animals without clinical signs of Borna disease have been previously reported from BoDV-1-affected sheep herds (Metzler, Ehrensperger, & Danner, 1979; Vahlenkamp et al., 2002). Whether such seroconversion results from subclinical virus persistence or from an abortive infection remains elusive.

Four alpacas had seroconverted in June 2019 or later, including a stallion that had been newly introduced into the herd in May 2019 and had been kept only on premise 1. This observation and the occurrence of a BoDV-1-infected bicoloured white-toothed shrew trapped on premise 1 in May 2019 indicate that the infection source had been present on premise 1 and that the exposure lasted until at least early summer. It remains unknown if exposure to BoDV-1 had also occurred on premise 2 or if the mares and foals that died or seroconverted on this premise had encountered the infection already during their stay on premise 1 at the beginning of the outbreak. Information on the incubation period of Borna disease in naturally infected non-reservoir hosts is scarce. In experimentally BoDV-1-infected horses and sheep, the time point of first clinical signs is highly variable, ranging from less than two weeks to several months after intracranial or intranasal inoculation (Heinig, 1964; Katz et al., 1998; Matthias, 1958; Mayr & Danner, 1974; Nitzschke, 1963; Schmidt, 1951). Similar incubation periods may be assumed for natural infections, since a BoDV-1-infected alpaca and horse were reported to develop Borna disease two and five months, respectively, after translocation from endemic regions in Germany to locations outside the known endemic areas (Jacobsen et al., 2010; Priestnall et al., 2011).

The factors determining the incubation period of Borna disease and the fate of persistently infected animals are poorly understood. In this study, the disease occurred simultaneously in five adult alpaca stallions 2 days after immunization with an adjuvanted clostridia vaccine. However, vaccination of all mares and foals, including three seropositive individuals, with the same vaccine in September 2019 did not induce apparent disease. Borna disease is known to result from immunopathogenesis driven by virus-specific T lymphocytes (Bilzer & Stitz, 1994; Richt et al., 1997; Richt, Stitz, Wekerle, & Rott, 1989; Stitz, Bilzer, & Planz, 2002). Triggering clinical signs by vaccine-induced non-specific activation of immune responses appears therefore possible. In the past, provocation of Borna disease by vaccinating BoDV-1-infected horses and sheep with inactivated bacterial vaccines has been discussed, but experimental evidence is lacking (Matthias, 1958).

BoDV-1 is known to possess a broad cell tropism in bicoloured white-toothed shrews, its known reservoir host, leading to shedding of infectious virus (Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). In contrast,

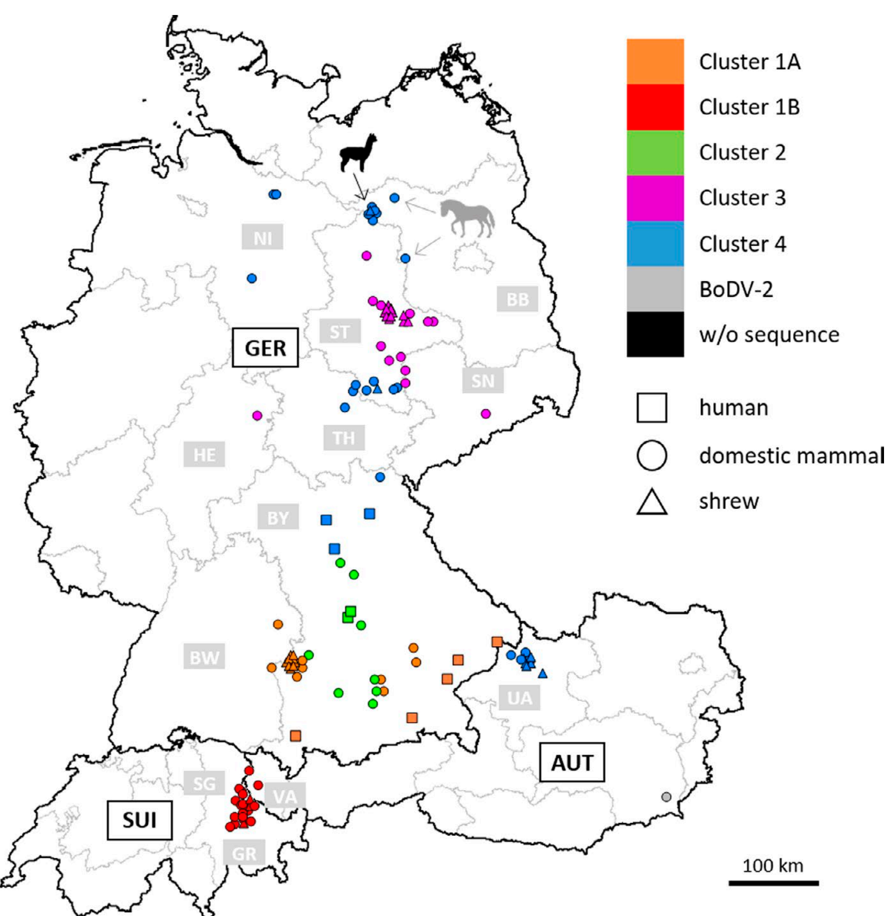

FIGURE 6 Geographic distribution of confirmed BoDV-1 infections of animals and humans. BoDV-1 infections detected in alpacas and horses in Brandenburg reported in this study are presented together with published sequence-confirmed BoDV-1 infections of shrews (triangles), domestic mammals (circles) and humans (squares) with available geographic localization (Dürrwald et al., 2007, 2014; Kolodziejek et al., 2005; Niller et al., 2020; Nobach et al., 2015; Weissenböck et al., 2017). Colours represent regional BoDV-1 sequence clusters (see Figure 5). Germany (GER): BB = Brandenburg, BY = Bavaria, BW = Baden-Wuerttemberg, HE = Hesse, NI = Lower Saxony, SN = Saxony, ST = Saxony-Anhalt, TH = Thuringia; Switzerland (SUI): GR = Grisons, SG = St. Gall; Austria (AUT): UA = Upper Austria, VA = Vorarlberg. w/o = without

it is described as strictly neurotropic and almost exclusively restricted to the CNS in incidentally or experimentally infected immunocompetent non-reservoir hosts (Bilzer, Planz, Lipkin, & Stitz, 1995; Enbergs, Vahlenkamp, Kipar, & Müller, 2001; Herzog, Kompter, Frese, & Rott, 1984; Korn et al., 2018; Liesche et al., 2019; Lipkin, Briese, & Hornig, 2011; Richt & Rott, 2001; Schlottau et al., 2018; Zwick, Seifried, & Witte, 1927). While RT-qPCR confirmed the predominantly CNS-restricted BoDV-1 distribution for the majority of the analysed alpacas, one animal had clearly detectable levels of viral RNA widely distributed in its peripheral nerves and additional tissues, such as nasal mucosa, salivary glands, adrenal gland and gastrointestinal tract, possibly suggesting a retrograde spread of the virus during the particularly long course of disease of this individual. However, IHC analysis confirmed viral antigen in peripheral organs to be clearly restricted to neuronal structures, indicating viral shedding by infected alpacas to be an unlikely event. In congruence, viral RNA was hardly detectable in mucosal swabs collected from several animals with confirmed or suspected BoDV-1 infection during this study. No bornavirus-reactive antibodies were demonstrated in humans who lived on the farm and had close contact to the diseased animals, underscoring that infected dead-end accidental hosts do not transmit the virus to people.

To date, the bicoloured white-toothed shrew is the only known reservoir of BoDV-1 (Bourg et al., 2013; Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). Despite extensive testing of other small mammals within known endemic areas, only a single common shrew tested positive for BoDV-1 (Weissenböck et al., 2017). In congruence with these findings, BoDV-1 infection was detected only in the single bicoloured white-toothed shrew during this study, while a common shrew and all rodents collected during pest control measures on the farm tested negative.

The bicoloured white-toothed shrew is an insectivorous small mammal that feeds on beetles, their larvae and molluscs. They prefer dry open habitats in a dispersal area ranging from Western Europe to Ukraine and Southern Russia (Burgin & He, 2018; Krapp, 1990). Territories of bicoloured white-toothed shrews are usually small with diameters of only up to 120 m and their activity range rarely exceeds 1 km (Burgin & He, 2018). This territory-bound behaviour may well explain the restriction of BoDV-1 infection to populations only in particular parts of Central Europe with apparently little tendency of spreading to neighbouring populations to the East and West (Dürrwald et al., 2014; Weissenböck et al., 2017).

While BoDV-1 is well known to be endemic in parts of Eastern and Southern Germany, the federal state of Brandenburg had not been confirmed as a BoDV-1-endemic region in the published literature prior to this work (Dürrwald et al., 2014; Kolodziejek et al., 2005; Weissenböck et al., 2017). Initial epidemiological investigations on the alpaca farm identified several potential links to known endemic regions in Bavaria. These links included a temporary stay of

the complete herd in Bavaria during a flood in their home region in summer 2013 and the import of hay from Bavaria in the winters of 2015/16 and 2017/18. However, the detection of a BoDV-1-positive shrew on the alpaca farm, which possessed a BoDV-1 sequence that was virtually identical to those identified in the alpacas, as well as the detection of closely related BoDV-1 sequences from horses in the same region, unequivocally confirmed a local infection source. Thus, a previously not described endemic area exists in North-Western Brandenburg. The retrospective identification of a BoDV-1-infected alpaca from the herd, which had died in 2016, and a further possibly infected animal in 2015 indicated a previous exposure to the virus already several years before the current outbreak.

The precise route of BoDV-1 transmission from the infected reservoir to spillover hosts, including alpacas, remains elusive. Experimentally, intranasal inoculation of rats, horses and sheep resulted in persistent infection and disease (Carbone, Duchala, Griffin, Kincaid, & Narayan, 1987; Heinig, 1964; Kupke et al., 2019; Matthias, 1958; Morales, Herzog, Kompter, Frese, & Rott, 1988). In rats, viral entrance was shown to occur via olfactory receptor cells and epithelial cells in the nasal and pharyngeal mucosa followed by spread to the CNS via the olfactory nerve (Kupke et al., 2019; Morales et al., 1988). Likewise, the virus may reach the brain by intra-axonal transport in peripheral nerves after inoculation of rats into the footpad (Carbone et al., 1987).

In summary, alpacas appear to be highly susceptible to BoDV-1 infection and they exhibit highly variable progression of infection, including atypical courses of Borna disease, such as sudden fatalities, as well as apparently subclinical infections. This study led to the identification of a previously undescribed endemic area in Northern Germany. The BoDV-1 susceptibility and growing numbers in Europe may render New World camelids ideal sentinels for the identification of BoDV-1 risk areas for domestic animals and humans.

ACKNOWLEDGEMENTS

The authors like to thank the owners and workers of the alpaca farm for supporting these investigations. Anne Günther, Nicole Huth, Cindy Krenz, Alicia Linares, Kathrin Steffen and Patrick Zitzow provided excellent technical assistance. Kathrin Jeske provided advice and support for small mammal trapping. Grit Priemer (Rostock, Germany) and Dagmar Senta Trachsel (Berlin, Germany) submitted samples from BoDV-1-infected horses and Franziska Aßmuth (Bad Langensalza, Germany) submitted archived alpaca tissue. Christian Kiesling provided valuable background information on alpaca husbandry in Germany.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

ETHICAL APPROVAL

Sera, cerebrospinal fluid (CSF) and swab samples were collected from alpacas and goats during this outbreak for veterinary diagnostic purposes. Handling and sampling of live animals were performed by trained personnel, with animal safety and welfare as first priority. Organ samples from alpacas and horses originated from animals that had died or had to be euthanized due to animal welfare reasons. Small mammal carcasses originated from pest control measures. No animals were killed for the purpose of this study. Informed consent was obtained from the human subjects, who were serologically screened for bornavirus-reactive antibodies in this study. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All relevant guidelines for the use of animals in scientific studies were followed.

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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Schulze V, Große R, Fürstenau J, et al. Borna disease outbreak with high mortality in an alpaca herd in a previously unreported endemic area in Germany. *Transbound Emerg Dis*. 2020;67:2093–2107. https://doi.org/10.1111/tbed.13556