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New World camelids are sentinels for the presence of Borna disease virus

Alexandra J. Malbon[1*] | Ralf Dürrwald[2*] | Jolanta Kolodziejek[3*] | Norbert Nowotny[3,4] | Ralph Kobera[5] | Dietrich Pöhle[6] | Aemero Muluneh[6] | Eva Dervas[1] | Christopher Cebra[7] | Frank Steffen[8] | Giulia Paternoster[9] | Christian Gerspach[10†] | Monika Hilbe[1†]

[1]Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[2]Robert Koch-Institut, Berlin, Germany

[3]Institute of Virology, University of Veterinary Medicine Vienna, Vienna, Austria

[4]Department of Basic Medical Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

[5]Tierarztpraxis Kobera, Dresden, Germany

[6]Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen, Dresden, Germany

[7]Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR, USA

[8]Section of Neurology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[9]Section of Epidemiology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[10]Farm Animal Clinic, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Correspondence
Alexandra J. Malbon, The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush Campus, Midlothian, EH25 9RG UK.
Email amalbon@ed.ac.uk

Abstract

Borna disease (BD), a frequently fatal neurologic disorder caused by Borna disease virus 1 (BoDV-1), has been observed for decades in horses, sheep, and other mammals in certain regions of Europe. The bicoloured white-toothed shrew (*Crocidura leucodon*) was identified as a persistently infected species involved in virus transmission. Recently, BoDV-1 attracted attention as a cause of fatal encephalitis in humans. Here, we report investigations on BoDV-1-infected llamas from a farm in a BD endemic area of Switzerland, and alpacas from holdings in a region of Germany where BD was last seen in the 1960s but not thereafter. All New World camelids showed apathy and abnormal behaviour, necessitating euthanasia. Histologically, severe non-suppurative meningoencephalitis with neuronal Joest-Degen inclusion bodies was observed. BoDV-1 was confirmed by immunohistology, RT-qPCR, and sequencing in selected animals. Analysis of the llama herd over 20 years showed that losses due to clinically suspected BD increased within the last decade. BoDV-1 whole-genome sequences from one Swiss llama and one German alpaca and—for comparison—from one Swiss horse and one German shrew were established. They represent the first published whole-genome sequences of BoDV-1 clusters 1B and 3, respectively. Our analysis suggests that New World camelids may have a role as a sentinel species for BoDV-1 infection, even when symptomatic cases are lacking in other animal species.

KEYWORDS

alpaca, Borna disease virus, Bornavirus, encephalitis, llama, New World camelids

*AJM, RD and JK should be considered joint first author.

†CG and MH should be considered joint senior author.

1 | INTRODUCTION

Borna disease (BD), caused by Borna disease virus 1 (BoDV-1), is a potentially fatal neurological disorder first described in Germany in the 19th century as 'head disease' of horses (reviewed by Dürrwald & Ludwig, 1997). The name is derived from the district around the city of Borna near Leipzig in Germany where from the 1880s until the 1960s, frequent sporadic cases of meningoencephalitis in horses were seen despite no epidemic occurring in the city itself (Dürrwald & Ludwig, 1997).

BoDV-1 is classified within the order *Mononegavirales*, family *Bornaviridae*, genus *Orthobornavirus*, species *Mammalian 1 orthobornavirus*, together with BoDV-2 (Kuhn et al., 2015; Nowotny et al., 2002). Another species, *Mammalian 2 bornavirus*, is represented by variegated squirrel bornavirus 1 (VSBV-1) which was first detected in asymptomatic squirrels and some of their breeders who developed fatal encephalitis (Hoffmann et al., 2015). Other bornaviruses were found in birds, mainly parrots (here causing proventricular dilatation disease) (Honkavuori et al., 2008; Kistler et al., 2008; Weissenböck et al., 2009), reptiles (Hyndman et al., 2018; Stenglein et al., 2014), and fish (Shi et al., 2018).

Bornaviruses are enveloped, non-cytolytic, non-segmented, negative-stranded RNA viruses with a genome of approximately 9 kb which replicate in the nucleus (Briese et al., 1992). They encode six viral proteins, N, X, P, M, G, and L (Briese et al., 1994). Endogenous bornaviral elements in the genome of several species of mammals and other vertebrates indicate an evolutionarily old origin of bornaviruses and several infection events during evolution (Horie et al., 2010).

BoDV-1 is endemically present in certain areas of Germany, Austria, Switzerland, and The Principality of Liechtenstein. In these areas, infected mammals are indicators of its locally restricted distribution. Persistently infected bicoloured white-toothed shrews (*Crocidura leucodon*) tolerate infection while sporadic cases of fatal neurological disease in higher mammals reveal the local presence of the virus (Dürrwald et al., 2006; Hilbe et al., 2006). Locally, the genome of BoDV-1 is strongly conserved (Kolodziejek et al., 2005). Neither the reasons for this strong local genetic conservation of BoDV-1 nor the reasons for the endemic restriction are known (Kolodziejek et al., 2005).

BD results from immunopathology driven by virus-specific T lymphocytes which target BoDV-1-infected cells (reviewed by Tizard et al., 2016). Meningoencephalomyelitis in affected animals is characterised histologically by perivascular lymphocytic cuffing predominantly affecting phylogenetically old regions of the central nervous system (Caplazi & Ehrensperger, 1998).

In contrast to dead-end hosts in which BoDV-1 is mainly found in the central nervous system, shrews harbour the virus not only in the brain, but also in a variety of organs (Dürrwald et al., 2014; Hilbe et al., 2006). Shrews appear not to develop pathological lesions, suggesting that this species might be immunotolerant (Nobach et al., 2015).

In animals with BD, common clinical symptoms include somnolence, apathy, dysphagia, and head pressing (Schmidt, 1912; reviewed by Richt et al., 1997). In humans, initial symptoms commonly include fever, headache, cognitive impairment, and progressive brain disease leading to unsteady gait, seizures, memory deficits, coma, and death but also symptoms of Guillain-Barré syndrome occur in some patients (Coras et al., 2019; Niller et al., 2020). Histopathological changes in humans reflect a non-purulent, lymphocytic sclerosing panencephalitis (Liesche et al., 2019). The typical eosinophilic Joest-Degen inclusion bodies can be detected in the nuclei of neurons (Joest & Degen, 1909; Liesche et al., 2019).

Outbreaks of BD in New World camelids have been described in endemic regions of Germany (Altmann et al., 1976; Jacobsen et al., 2010; Schüppel et al., 1994). In North West Brandenburg, eleven fatalities occurred in a herd of 27 alpacas within 10 months (Schulze et al., 2020). Here, we add data to the knowledge on BD in New World camelids by providing results of long-term analysis of clinically suspected BD in a llama herd in an endemic area of Switzerland and from alpacas of different holdings in Saxony in an area dormant for BD over decades.

2 | MATERIAL AND METHODS

2.1 | Clinical investigation and sampling

In the course of submissions to farm animal clinics and pathological institutes (Zurich, Dresden), BoDV-1 infections were identified in one llama herd in Switzerland and in three alpaca holdings as well as in one horse and one sheep holding in Saxony (Germany). BoDV-1 infection of all German animals and two Swiss llamas 1 (S17–1061) and 2 (S17–1073) was confirmed by RT-qPCR. BoDV-1 from the German alpaca 1 (V 636) and the aforementioned Swiss llamas was additionally sequenced. Discussion with the owner of the Swiss llama herd revealed a greater extent of the problem, and for this reason, clinically suspected cases were included as these data were felt to be relevant and important to add to current knowledge of this disease.

The collection of shrews in the Anhalt region of Saxony-Anhalt was continued (Dürrwald et al., 2014). Among them, one BoDV-1-positive bicoloured white-toothed shrew (*Crocidura leucodon*), CL112, was found dead by chance on 29 December 2011 in Tornau near Roßlau, a new location for BoDV-1 positives within this region.

From the Swiss llama 1 (S17–1061), blood, cerebrospinal fluid, cardiovascular organs, haematopoietic organs, endocrine organs, brain, eyes, trigeminal ganglia, tongue, masseter muscle, diaphragm, appendicular skeletal muscles, gastrointestinal tract, faeces and urogenital tract samples were taken. For the Swiss llama 2 (S17–1073), only brain, trigeminal ganglia, tongue and masseter muscles were available. From the Swiss horse S95–1272, RNA from the brain had been stored from a previous study (Hilbe et al., 2006). From the remaining Swiss animals (llamas S1–S10), no materials were available. From the German animals (alpacas 1–3, horse, sheep, and shrew), a smaller set of samples was taken, mainly from brain and spinal cord. For details, see Table 1.

TABLE 1 Overview of all animals investigated

Species	Animal ID	Holding /newly introduced animal	Disease onset	Clinically suspected case	Date of admission	Sample	Serology (Ab titre)	Gross and histological examination	BoDV–1 RT-qPCR p24 (Cq value)	1,824 bp long sequence (GenBank acc. no.)	Whole-genome sequence (GenBank acc. no.)
Swiss samples, confirmed cases											
Llama 1[a]	S17–1061	C/Nov. 2016	June 2017	yes[d]¶	11.07.2017	Brain	nr	X	X (14.5)	nd	X (MK644607)
						Plasma	X (1:2,560)				
						CSF	X (1:512)				
Llama 2[b]	S17–1073	C/Nov. 2016	June 2017	yes[d]¶	14.07.2017	Brain	nr	X	X (12.5)	X (MK644608)	nd
Horse[c]	S95–1272	A/-	June 1995	yes[d]¶	03.07.1995	Brain	nr	§	X (21.1)	X (DQ251042)§	X (MK644606)
Swiss samples, clinically suspected cases											
Llama S1	S1	C/Nov. 2008	June 2009/ June 2011	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S2	S2	C/Nov. 2009	June 2010	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S3	S3	C/Oct. 2011	June 2012	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S4	S4	C/Oct. 2011	June 2012	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S5	S5	C/Nov. 2013	June 2014	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S6	S6	C/Oct. 2016	June 2017	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S7	S7	C/-	June 2018	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S8	S8	C/-	Oct. 2019	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S9	S9	C/-	June 2020	yes[e]	none	na	nd	nd	nd	nd	nd
Llama S10	S10	C/-	Sept 2020	yes[e]	none	na	nd	nd	nd	nd	nd
German samples, confirmed cases											
Alpaca 1[a]	V 636	Co/-	Oct. 2002	yes[d]¶	08.10.2002	Brain	nd	X	X (22,75)	X (DQ116030)f	X (MT366065)
Alpaca 2[a]	R 131	G/-	Apr. 2009	yes[d]¶	07.04.2009	Brain	nd	X	X (30,20)	nd	nd
Alpaca 3[a,g]	R 369	N/-	Oct. 2014	yes[d]¶	23.10.2014	Brain	nd	X	X (27,80)	nd	nd[g]
Horse[a]	V 218	A/-	Aug. 2009	yes[d]¶	29.08.2008	Brain	nd	X	X (27,42)	nd	nd
Sheep[a]	R 217	H-S/-	Oct. 2014	no[d]¶	17.06.2008	Brain	nd	X	X (24,76)	nd	nd
Crocidura leucodon[a]	CL 112	T/-	nr	nr	29.12.2011	Brain	nd	X	X (12,20)	nd	X (MT366064)

Abbreviations: na, not available; X, done; nd, not done; nr, not relevant.

animal was not newly introduced into the herd.

[a]From these animals, the entire carcass was pathologically investigated

[b]From this llama, the brain sample underwent further investigation.

[c]Hilbe et al., 2006.

[d]These cases were confirmed by BoDV-1 RT-qPCR.

[e]These cases were diagnosed by the referring veterinarian, and from these animals, no materials were available for further investigations because the animals were slaughtered or euthanized without submitting samples for diagnostic investigation.

[f]Dürrwald et al. 2006.

[g]From this animal, residual brain material was provided to the Friedrich-Loeffler Institute for sequencing and sequence data will be published elsewhere.

2.2 | Gross pathological, histological and other investigations

Pathological investigations were performed on the complete carcass of Swiss llama 1 and on the head of Swiss llama 2 as well as on complete carcasses of all German animals. Faeces from llama 1 also underwent parasitological examination.

Histological examination was performed on paraffin-embedded tissue prepared from all above-mentioned animals.

After 24 hr of fixation with 10% neutral-buffered formalin (or up to 7 days for the brains), tissues were routinely processed through a graded series of alcohol, embedded in paraffin and sectioned at 2 μm for haematoxylin and eosin staining.

Consecutive sections of neural tissue were mounted on positively charged slides and used for rabies virus and BoDV-1 immunohistology. Two BoDV-1 monoclonal antibodies were employed, directed against the proteins p24 (MAb 21E7; P protein) and p38/40 (MAb Bo18; N protein). Paraffin-embedded brain and trigeminal ganglion sections were deparaffinized and dehydrated in xylene and alcohol, digested with Dako REAL™ Proteinase K (Dako, Glostrup, Denmark) for 10 min at room temperature and incubated overnight at room temperature with the monoclonal mouse anti-BoDV-1 antibodies p38/40 and p24, both at a dilution of 1:500. Dako REAL™ anti-mouse/rabbit biotinylated secondary antibodies were then used and Dako 3-amino-9-ethylcarbazole (AEC) served as a substrate–chromogen. Sections were counterstained for 30s in haematoxylin. The brain slides for use with the rabies antibody were prepared in the same manner with the same pre-treatment and the same secondary antibody. The polyclonal rabbit antibody against rabies virus (Swiss Rabies Centre) was diluted 1:200 and incubated for 30 min at room temperature. Dako 3,3'-diaminobenzidine (DAB) was used as the substrate–chromogen.

2.3 | PCR, sequencing and phylogenetic analysis

For genetic and phylogenetic investigations, brain samples of both Swiss llamas 1 (S17–1061) and 2 (S17–1073), one German alpaca (V 636), and one German shrew (CL 112) were available. From the Swiss horse (S95–1272), an RNA sample extracted from brain from a previous study (Hilbe et al., 2006) was available. An 1824-bp long BoDV-1 sequence of this horse had been previously deposited in GenBank under accession number DQ251042.

Brain samples were homogenized with 2.8 mm ceramic beads (Bertin Technologies, Montigny-le-Bretonneux, France) and nuclease-free water (Carl Roth, Karlsruhe, Germany) in a TissueLyser (Qiagen, Hilden, Germany). After centrifugation, all supernatants were extracted automatically in a QIAcube (Qiagen) using QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

BoDV-1 RT-qPCR within the p24 gene region was applied for screening (Schindler et al., 2007). However, while the forward primer (5′-TCCCTGGAGGACGAAGAAGAT-3′) and the probe (5′-FAM-CCA GACACTACGACGGGAACGA-TAMRA-3′) were used exactly as described, the reverse primer was slightly modified by the addition of the degenerate base Y (5′-CTTCCGTGGY**C**TTGGTGACC-3′) to allow successful detection not only of Swiss but also of known German BoDV-1 strains. Additionally, PCR amplification was optimized for Quanta qScript XLT One-Step RT-qPCR ToughMix Kit (Quantabio, MA, USA) with primers and probe concentrations of 0.5 μM each. The PCR conditions were as follows: 15 min at 50°C for reverse transcription, 2 min at 95°C for denaturation followed by 45 cycles of denaturation at 95°C for 15 s then annealing and elongation at 60°C for 32 s.

Thereafter, conventional RT-PCRs targeting the complete N, X, and P genes as well as the intergenic region N/X and subsequent sequencing were performed for all samples as described (Kolodziejek et al., 2005). As the approximately 2,000 nt long BoDV-1 nucleotide sequences of both Swiss llamas 1 and 2 exhibited 100% identity to each other, further RT-PCRs for the completion of the whole BoDV-1 genome and sequencing were conducted on only one of these samples (llama 1, S17–1061) as well as on the Swiss horse (S95–1272), the German alpaca 1 (V 636), and the German shrew (CL 112). For this purpose, primer pairs designed for a previous study (Weissenböck et al., 2017) were applied using the One-Step RT-PCR Kit (Qiagen). For the Swiss samples however, two additional specific primer pairs had to be designed as follows: BoDV-1 4531f (5′-GTTCTCCTCCTCATGGTCTC-3′) with BoDV-1 5358r (5′-CCGTTGCACCAGGAACTAT-3′) and BoDV-1 6742f (5′-GCCGAGCAATTCCATCCTCA-3′) with BoDV-1 7070r (5′-ATGCTAGGTCGAGCGTCAGA-3′). Primer positions refer to the sequence of strain V (GenBank acc. number U04608). All oligonucleotides were synthetized by Microsynth (https://www.microsynth.ch/). Sequencing of all specific PCR products was performed in both directions by Eurofins Genomics (https://www.eurofinsgenomics.eu/).

After manual verification and compilation of the individual sequences, ClustalW multiple sequence alignments were conducted using BioEdit Sequence Alignment Editor version 7.2. Phylogenetic trees were created with the MEGA X program (Kumar et al., 2018) by employing neighbour joining method and p-distance algorithm with 1,000 replicates of bootstrap resampling analysis.

One phylogenetic tree was constructed with 111 BoDV-1 sequences, 1,824-bp in length, covering the N, X, and P protein gene regions as well as the N/X intergenic region, including five sequences generated in this study. Another tree was inferred on the basis of the complete coding sequences of all 30 current members of BoDV-1 from GenBank and four sequences generated in this study with BoDV-2 as out-group. For the whole-genome sequences, determination of mean distances between and within the five major whole-genome sequence clusters (MEGA X) was performed.

The whole-genome BoDV-1 sequences generated in this study have been submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: MK644606 Swiss horse (S95–1272), MK644607 Swiss llama 1 (S17–1061), MT366064 German shrew (*Crocidura leucodon*) CL112, and MT366065 German alpaca 1 (V 636). The 1,824-bp long sequence of the Swiss llama 2 (S17–1073) is available under GenBank accession number MK644608. Details of the corresponding sequences are listed in Table 1.

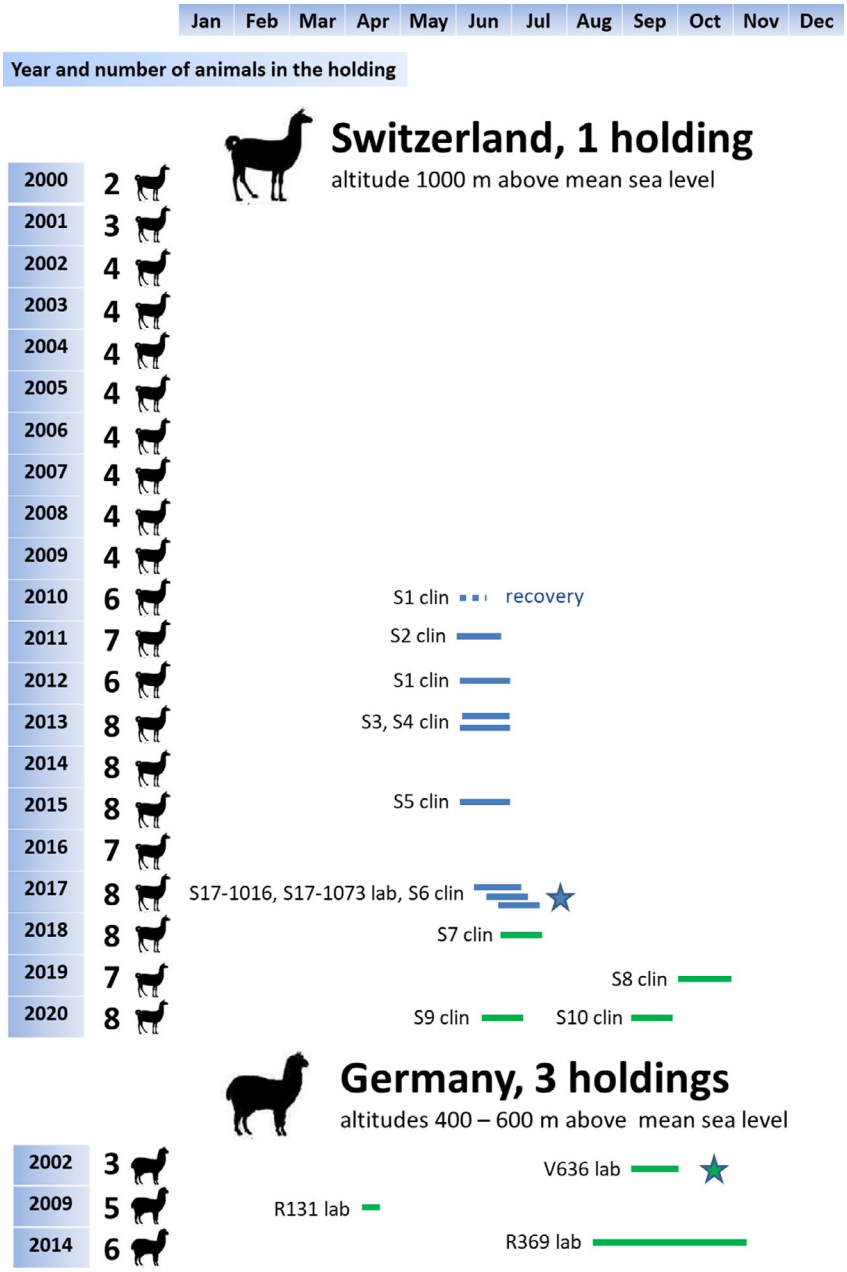

FIGURE 1 Overview of Borna disease cases in four holdings of New World camelids; the numbers on the left represent the number of adult animals kept in the farm in the corresponding year; bars adjacent to animal case numbers represent the duration of symptoms from onset until death in replacement animals (blue) and in animals born or having lived > 1 year on the farm (green); stars show cases from which BoDV-1 sequences were established, clin—clinically suspected cases, lab—laboratory-confirmed cases of BD; the numbers represent the identification of the animal (see Table 1)

2.4 | Serology

BoDV-1-specific antibodies were determined on serum and CSF samples of Swiss llama 1 (S17–1061) by indirect immunofluorescence assay at the Institute of Virology, University of Giessen, Germany, as described previously (Herzog et al., 2010).

2.5 | Epidemiology

The epidemiology of BD was investigated by studying records provided by the farmers. The location of the cases was analysed, and the regional distribution of the disease was compared to that evaluated in former studies.

3 | RESULTS

3.1 | Case signalment and clinical history

The female llama herd in Eastern Switzerland was built up from 2000 starting with two animals. Cattle, horses, goats, cats, dogs, rabbits, and chickens were also kept on the farm; none of these animals developed BD. Until 2010, four llamas were in the herd, thereafter six to eight adult llamas. Breeding with llamas started in 2009 by purchasing pregnant females. Since 2011 almost each year, a male llama was brought in to breed the females (at different times in the year, later regularly from October to December). Between one to eight crias were born in most of the years.

Offspring were either sold, kept for breeding, or slaughtered for meat consumption. In 2009, a llama had shown symptoms of unusual

prehension of feed but recovered; two years later, it succumbed to BD. Between 2010 and 2020, twelve llamas in total, mostly replacement animals from other areas of Switzerland introduced in autumn, showed similar neurological symptoms (Figure 1). Clinical findings were observed from late June to early July the year following introduction; the exceptions were 2019 and 2020 in which llamas also developed symptoms in October or September. Abnormal prehension of food was the first sign (Figure 2a). The animals separated themselves from the herd and preferred to stay in the shade. They moved constantly but did not exhibit the aimless circling seen in horses with BD. They showed unusually trusting behaviour, rubbed their heads against the ground, bit structures (Figure 2b), and at the late stages showed a tendency to run into fences or into walls (Figure 2c), with clear signs of visual impairment. Two llamas were confirmed to be BoDV-1-positive by RT-qPCR. These two llamas were investigated in detail (see supplemental material). Ten of the llamas died or were slaughtered or euthanized without diagnostic testing or necropsy and are referred here as clinically suspected cases. Shrews were present in an old barn which was taken down in 2017; no shrews have been seen in the new barn. Despite this, from 2018 to 2020 four llamas were euthanized owing to BD symptoms. Two of these four llamas, born on the farm, were daughters of llamas introduced to the herd before. The other two llamas were resident llamas which had been already some years on the farm.

The temperature data for the region show increasing temperatures over the last decade, with the years 2011, 2012, 2014, 2015, 2017, 2018, and 2019 being particularly warm: https://www.meteoschweiz.admin.ch/content/dam/meteoswiss/en/climate/climate-change-in-switzerland/doc/klimawandel_schweiz_fig2_multilanguage.pdf. In six of these seven hot years, BD cases occurred at the holding but not in the cooler years of 2013 and 2016. The years 2009 and 2010 exhibited moderate temperature and clinically suspected cases of BD occurred at the holding.

The German alpacas were each single cases of BD from three different holdings (Weißeritz district, East Ore mountain region in Saxony). They developed neurological symptoms either in spring or in autumn (for details, see supplemental material; Figure 1; Kobera, 2016; Kobera & Poehle, 2004). The owners were contacted again in May 2020. Over the entire period, no other cases of BD were seen despite alpacas still being kept at the corresponding location. Table 1 summarises all reported cases and diagnostic methods used.

FIGURE 2 Llama with Borna disease (llama 1: S17–1061). A, abnormal prehension of feed; B, demonstrating aimless activity; C,biting structures within the stall

3.2 | Gross pathological, histological and immunohistological findings

There were no significant macroscopical abnormalities in any organ system of the animals (for details on pathology, parasitological and other investigations, see supplemental material).

3.2.1 | Swiss llamas

Lymphoplasmacytic perivascular cuffing was seen to varying degrees in the retina, brain, cervical spinal cord, and trigeminal ganglion. In the retina, the infiltrate affected only rare, scattered vessels of the ganglion layer, but was moderate when present. All levels of the brain were affected but predominantly the hippocampus where cuffs exceeded 10 cells in depth (Figure 3a). Inflammation was largely restricted to Virchow-Robin spaces but in places cells extended into the parenchyma. Lower numbers of macrophages were also visible. The meninges and pineal gland were similarly though more mildly affected. Neuronal degeneration accompanied by gliosis was again most predominant in the hippocampus, which is where pathognomonic eosinophilic intranuclear Joest-Degen inclusion bodies were most frequently observed within neurons (Figure 3b).

Immunohistology for BoDV-1 demonstrated strongly positive staining in both llama brains, largely restricted to the nuclei of hippocampal neurons (Figure 3c). Rabies virus stains were negative. Parasite-associated cholangiohepatitis was detected in llama 1 and deemed an incidental finding.

3.2.2 | German alpacas

The alpacas exhibited similar histological alterations to the llamas (for details, see supplemental material).

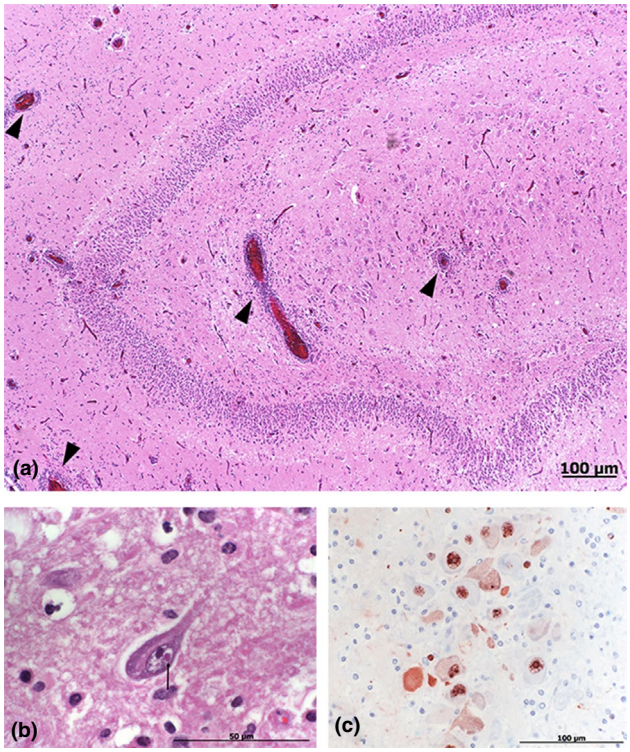

FIGURE 3 Histological investigation of llama 1. A, Low power view of brain at the level of the hippocampus, exhibiting multifocal perivascular cuffing (arrowheads); HE staining, x100; B, close-up view of a single neuron with a characteristic Joest-Degen inclusion (arrow); HE staining, x 1,000; C, immunohistology for BoDV-1 antigen (p38/40; N protein) showing positively stained neurons (brown staining), x400

3.3 | PCR, sequencing and phylogeny

In brain samples from all animals, a large amount of BoDV-1 RNA was detected (Cq values are listed in Table 1).

3.4 | Swiss llamas and horse

The BoDV-1 sequences (1,824 bp) of both Swiss llamas 1 and 2 (S17–1061 and S17–1073) exhibited 100% identity to each other and 98.8%–99.8% identity to previously established Swiss bornaviruses. Phylogenetic analysis confirmed the close relationship within the cluster 1B (Figure 4).

The complete-genome BoDV-1 sequence established for the Swiss llama 1 showed 99.2% nucleotide identity to that originating from the Swiss horse S95–1272 which had died in the same endemic area in 1995. Both sequences (first complete-genome sequences of BoDV-1 cluster 1B) were 95.0%–95.1% identical to the other BoDV-1 sequences and 81.0% to the only BoDV-2 sequence (Figure 5).

3.5 | German alpaca and bicoloured white-toothed shrew

The BoDV-1 sequences (1,824 bp) of German alpaca 1 (V 636) and shrew (CL 112) showed 98.4% identity to each other and shared cluster 3 (Figure 4). While the BoDV-1 alpaca sequence was mostly identical (98.9%) to the vaccine strain Dessau, the investigated shrew CL112 showed 98.7%–99.4% identity to previously established bornaviruses identified in several shrews trapped in the same region in the previous years.

The complete-genome BoDV-1 sequences established for the German alpaca and shrew (first complete-genome sequences of BoDV-1 cluster 3) showed 98.2% nucleotide identity to each other. They were 94.6%–95.2% identical to the other BoDV-1 sequences and 81.1% to BoDV-2 (Figure 5).

3.6 | All samples

The lowest evolutionary divergence over individual sequence pairs was calculated between clusters 1A and 1B and the highest between clusters 1A and 3 (Table 2). Evolutionary divergences between remaining cluster combinations were quite uniform (Table 2). Evolutionary divergences of all individual BoDV-1 clusters compared with BoDV-2 were estimated at 0.188–0.190 (which corresponds to 188–190 base differences per 1,000 bp).

3.7 | Serology

The BoDV-1 antibody titres in plasma and CSF of the Swiss llama 1 were 1:2,560 and 1:512, respectively.

3.8 | Epidemiological analysis

BoDV-1 infections in New World tylopods were analysed in the context of data of former epidemiological studies (Caplazi et al., 1999; Dürrwald, 1993). The locations at which BD emerged were visualized geographically (Figures 6 and 7). Whereas in the valleys of Switzerland BD has been observed at a low level over the past decades and extended to higher altitudes in the last years, in Saxony there was a high level in the 1960s which decreased in the decades thereafter. Specifically, in the Weißeritz and East Ore mountain districts of Saxony there has been no BD in recent decades. However, BoDV-1 is still present in this region as indicated by BD in the alpacas.

Prior to 1990, PCR for BoDV-1 was not available. The data collection of the years 1960 – 1990 was based on cases of BD which were all confirmed by histological demonstration of typical mononuclear infiltrates and Joest-Degen inclusion bodies at the veterinary diagnostic institutes of the corresponding region. In order to validate the data, all German samples from the years 1991–1992 were taken from the veterinary diagnostic institutes and investigated by RT-PCR. The initial results of the veterinary diagnostic institutes were confirmed in every case, indicating a high validity of the data (Dürrwald, 1993).

The cases outside the known endemic areas in Switzerland (e.g. Bern, Zurich, Western part of St. Gallen; see Figure 6) represent immunohistologically confirmed BoDV-1 infections, and the displayed locations

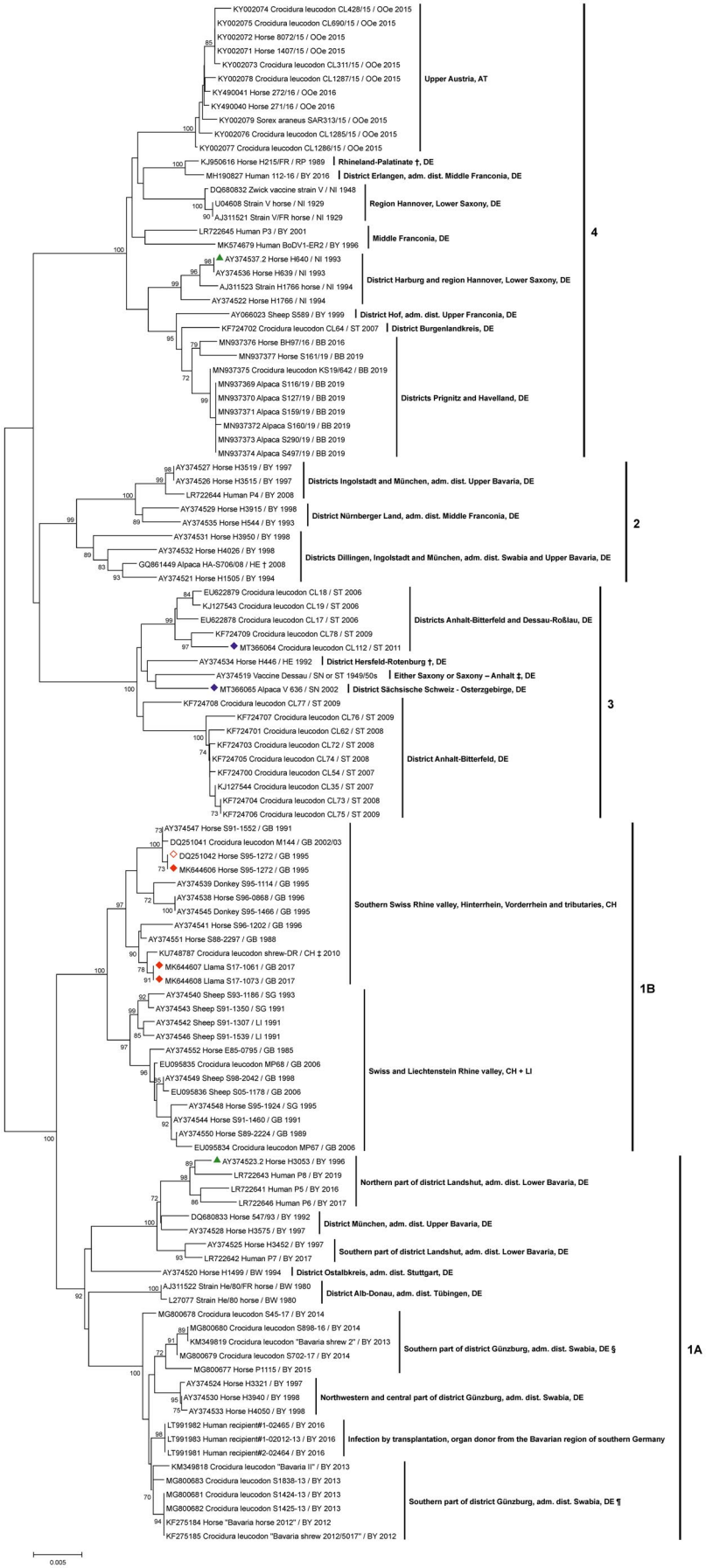

FIGURE 4 Phylogenetic tree of a 1824 nt stretch (genome positions 54–1877 of BoDV-1 reference genome U04608) of 111 BoDV-1 sequences. Red and blue diamonds: sequences determined in this study. Hollow red diamond, previously determined sequence which was completed during the current study (see Figure 5). Green triangles: Of these sequences there are second (corrected) versions in GenBank. GenBank accession numbers, host or strain names, geographic locations and years of isolations are indicated at the branches. The 5 major clusters of BoDV-1 and 28 regional subclades are indicated on the right side. Supporting bootstrap values of ≥70% are displayed next to the nodes. The horizontal scale bar indicates genetic distances. Abbreviations: Austria (AT): OOe = Upper Austria; Germany (DE): BB = Brandenburg, BY = Bavaria, BW = Baden-Wuerttemberg, HE = Hesse, NI = Lower Saxony, RP = Rhineland-Palatinate, SN = Saxony, ST = Saxony-Anhalt; Switzerland (CH): GR = Grisons, SG = St. Gallen; Liechtenstein (LI); Adm. dist. = administrative district.
† Animals developed disease at these locations; true origin unknown (import?);
‡ Exact location unknown;
§ Personal communication Dr. Dennis Rubbenstroth;
¶ Personal communication Dr. Sibylle Herzog.
[Correction added on 08 November 2021, after first online publication: The labelling of the phylogenetic tree in Figure 4 has been corrected to provide accurate location data of the historical samples, and figure caption revised accordingly. Errors do not impact the conclusions of this study.]

represent confirmed locations of the animals' residence. Epidemiological links to confirmed endemic areas are tributaries of the Rhine which allow for the movement of reservoir hosts such as shrews. A further factor supporting the extension of the reservoir host may be climate change which is clearly represented for the region: https://www.meteoschweiz.admin.ch/content/dam/meteoswiss/en/climate/climate-change-in-switzerland/doc/klimawandel_schweiz_fig2_multilanguage.pdf.

4 | DISCUSSION

BD is sporadic and mostly affects single domestic animals with no indisputable evidence of in-herd transmission (Dürrwald et al., 2014; Schulze et al., 2020; Weissenböck et al., 2017). From data of the Institute of Veterinary Pathology, University of Zurich, 109 BD cases, mostly consisting of horses and sheep, were registered in Switzerland and Liechtenstein from 1977 until 2017 (Figure 6) (Caplazi et al., 1999). The occurrence of BD in twelve llamas on one farm within ten years is unusually high. The onset of symptoms in the majority of llamas was mainly in June and the disease more frequent in hot years. The only exception was two llamas that developed disease in September or October. Because this was in a period which had been unusually warm, climatic factors may also contribute to activity of virus reservoir hosts and transmission. The clinical and pathological findings matched those previously reported, with neurological disorder and non-suppurative meningoencephalitis and retinitis dominated by lymphocytes, known to be predominantly $CD4^+$ T cells, and showing a predilection for phylogenetically old brain regions such as the hippocampus (Caplazi & Ehrensperger, 1998).

The majority of the clinically suspected cases and the two Borna disease-confirmed llamas in the Swiss farm were purchased rather than bred on the farm. Within the first decade of the holding, the herd size was low and no BD was seen. After enlargement of the herd and introduction of new animals, the first cases of BD occurred. The first eight cases of BD were without exception all new arrivals which were introduced in autumn and succumbed to the disease next summer. Among the last four cases were two llamas born on the farm but derived from mothers who had been newly introduced, and two resident cases. Thus, it could be that the higher number of BD cases among new arrivals occurred by chance due to enlargement of the herd. A higher number of animals on the premises may have increased risk of exposure to BoDV-1. On the other hand, further possible reasons include: (a) different exposures of the new arrivals to an unknown source of infection (these animals may be further down the hierarchy and thus show a tendency to retreat to areas which are not visited by the other llamas of the herd), (b) immunity in the indigenous members of the llama herd (less likely because a few resident llamas also developed disease, but already in the first report of BD in llamas, further cases of an outbreak appear to have been prevented by vaccination (Altmann et al., 1976); thus, although not yet fully understood, immunity may contribute to the complex pattern of BD) and (c) increased susceptibility associated with transportation and introduction into a new environment (this could be indirectly associated with a higher vulnerability to parasites and vectors or other pathogens which stimulate the immune system due to placing of the animals in a new environment containing new microbiota).

During a BD outbreak in an alpaca herd in North-Western Brandenburg, the disease occurred simultaneously in five adult alpaca males after they received the second injection of an adjuvanted clostridial vaccine (Schulze et al., 2020). Thus, in this outbreak the alpacas were most probably already infected and disease was provoked by immunization. This could indicate that a status of tolerance is possible after infection with BoDV-1 in which the immunological response to the bornaviral antigen is not so strong that it provokes disease. Naturally occurring latent infections had been described in horses as early as the 1960s (Ihlenburg & Brehmer, 1964). It is not known whether these animals would have developed disease in time or could also have tolerated BoDV-1 infection long term. The single case of one clinically suspected llama in this study which developed initial symptoms and recovered in 2009 but succumbed to BD two years later could hint at a longer period of individual tolerance to bornaviral antigens despite immunocompetence.

The frequent development of BD in llamas but not in other animals at the same farm suggests that the vulnerability of New World camelids allows them to be sentinels for BoDV-1. The cases of BD in the three alpaca holdings are interesting in this respect. All alpacas had

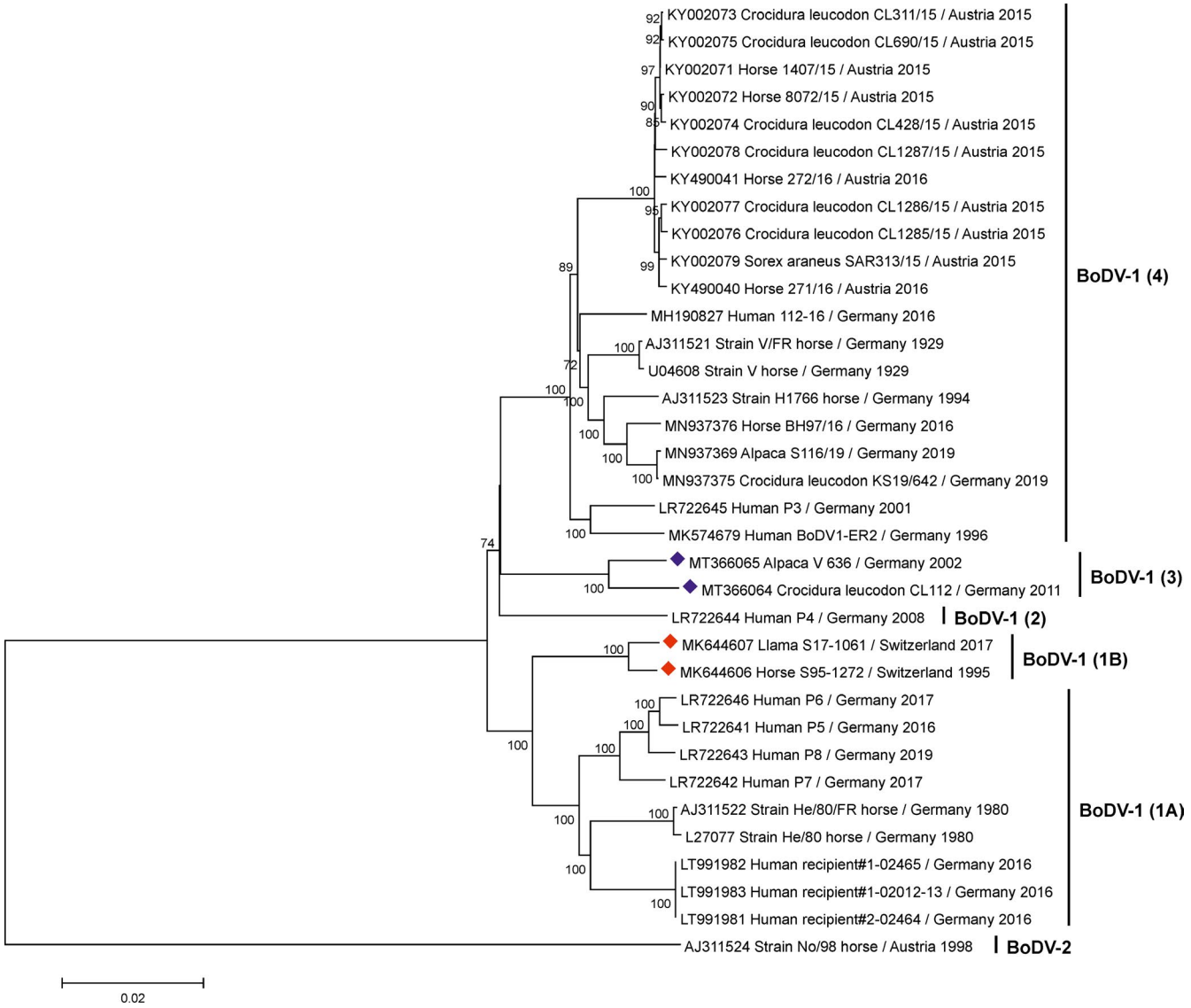

FIGURE 5 Phylogenetic tree of 34 complete-genome sequences of BoDV-1 rooted by strain No/98 (BoDV-2). Red and blue diamonds: sequences determined during this study. GenBank accession numbers, hosts, strain or sample names, countries and years of isolations are indicated at the branches. The major clusters of BoDV-1 and 1 BoDV-2 are indicated. Supporting bootstrap values are displayed next to the nodes. The horizontal scale bar indicates genetic distances

already been on the respective holdings for a long time. Therefore, it is expected that they had been infected at these locations. This region of Saxony was known only for sporadic cases of BD in the 1950s and 1960s, whereas in the Western part of Saxony, a high incidence of BD was noted (Dürrwald, 1993). In the years following the 1960s, BD cases dropped in general (Dürrwald et al., 2006). This drop was associated with a complete freedom of Eastern and Middle Saxony from cases of BD. The data of the 1960s/80s are from identical reporting systems. During this period, BD infection was notifiable in Germany (it was notifiable in this country until 26 February 2011 and has again been notifiable since 31 March 2020). Possible contributing factors to the decrease in cases were discussed by Dürrwald et al. (2006), indicating changes in agriculture as a main factor (industrialization in agriculture caused a reduction in horse livestock and was also associated with increased use of pesticides and herbicides which may have influenced potential reservoir species). BD in three alpacas, one horse, and one sheep in the Weißeritz district and East Ore mountain region of Saxony indicates that BoDV-1 still exists in this area despite an absence of clinical cases for decades.

Investigations of an outbreak within an alpaca herd in the northwestern part of the federal state of Brandenburg in Germany revealed strong differences in duration of disease and time of onset of disease (December to April) (Schulze et al., 2020). This was also confirmed with the alpacas in our study (one case in April and two cases in October). It may be that climatic peculiarities of the higher altitude at the Swiss llama holding restricted transmissions to a closer window. Furthermore, it is noteworthy that the llama stallion, placed into the herd short term in autumn (October to December), never developed BD.

The BoDV-1 complete-genome sequences of clusters 1B und 3 had not yet been analysed (phylo)genetically. The Swiss sequences

TABLE 2 Estimates of evolutionary divergence over sequence pairs between main clusters of BoDV-1 and BoDV-2

Cluster	BoDV−1 (1A)	BoDV−1 (1B)	BoDV−1 (2)	BoDV−1 (3)	BoDV−1 (4)	BoDV−2
BoDV−1 (1A)	X	0.002	0.002	0.002	0.002	0.004
BoDV−1 (1B)	0.038	X	0.002	0.002	0.002	0.004
BoDV−1 (2)	0.052	0.050	X	0.002	0.002	0.004
BoDV−1 (3)	0.054	0.050	0.048	X	0.002	0.004
BoDV−1 (4)	0.051	0.049	0.047	0.047	X	0.004
BoDV−2	0.188	0.190	0.189	0.189	0.188	X

Note: The analysis involved 35 complete-genome nucleotide sequences. The number of base differences per site from averaging over all sequence pairs between groups is shown. Standard error estimates are shown above the diagonal. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

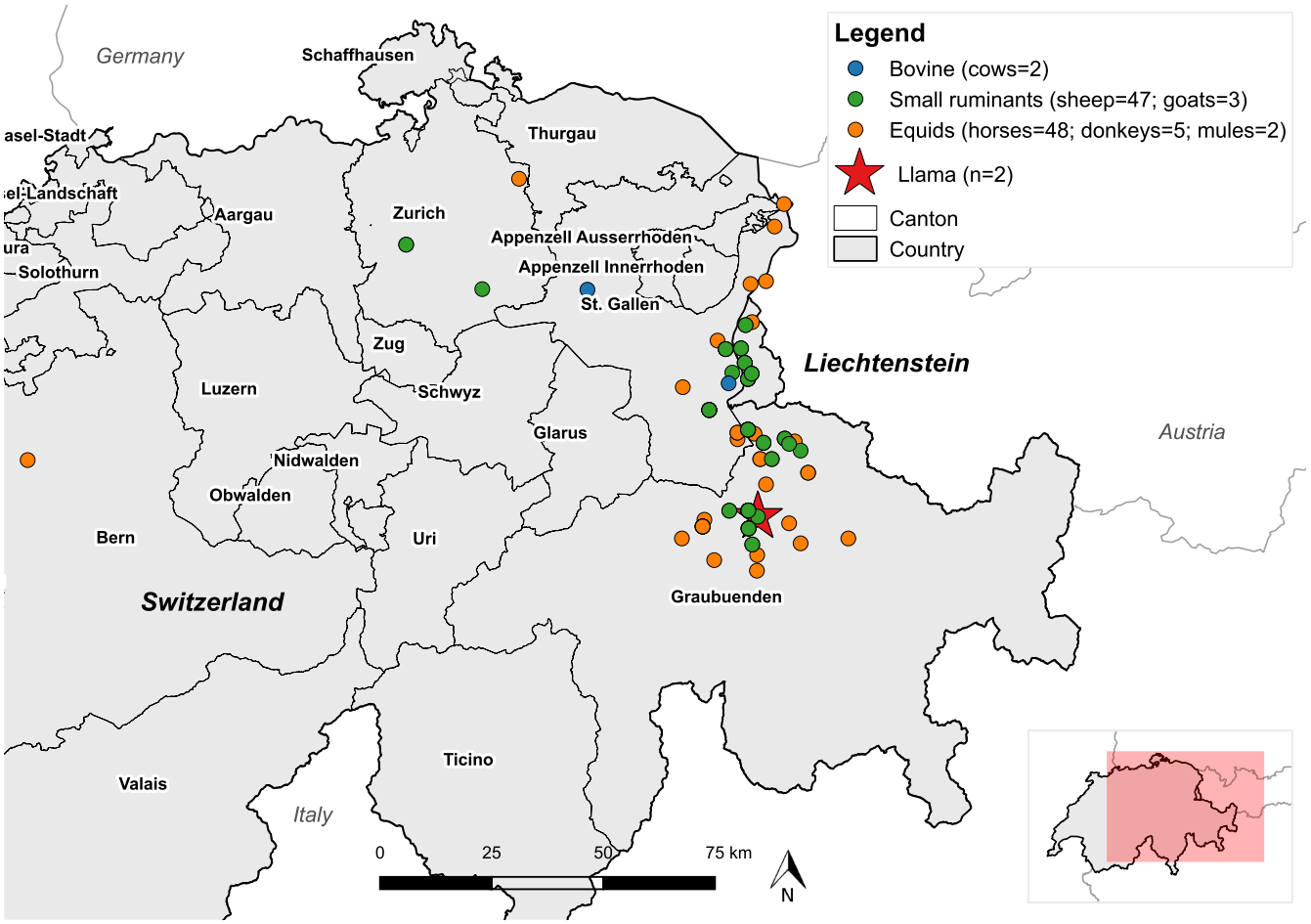

FIGURE 6 Confirmed cases of Borna disease (immunohistologically positive with consistent clinical and histological findings) in different animal species in Switzerland and Liechtenstein between 1977 and 2017

belonging to cluster 1B, achieved from one of the llamas and one horse, exhibited very high identity to each other (99.1%) despite more than two decades between the cases. The Austrian whole-genome sequences of cluster 4 were also pairwise highly identical to each other (99.6%–99.9%) (Weissenböck et al., 2017), while the exclusively German sequences from this cluster and from cluster 1A exhibited lower sequence identities (up to 97.4% and 97.1%, respectively). These differences in sequence identities were not only due to the numbers of analysed sequences but particularly due to geographical distances between individual cases. While the Swiss and Austrian BoDVs-1 originated from surrounding selective areas, the German strains of the clusters 1A and 4 covered wider areas with much larger distances (see detailed description in Figure 4).

The BoDV-1 sequences from the German alpaca and the shrew fit to cluster 3 (Figures 4 and 5). Our data show that the geographic distribution of BoDV-1 cluster 3 viruses extends from the Anhalt region of Saxony-Anhalt to the East Ore mountains in Saxony including the district around the city of Borna. The data extend the collection time of a former

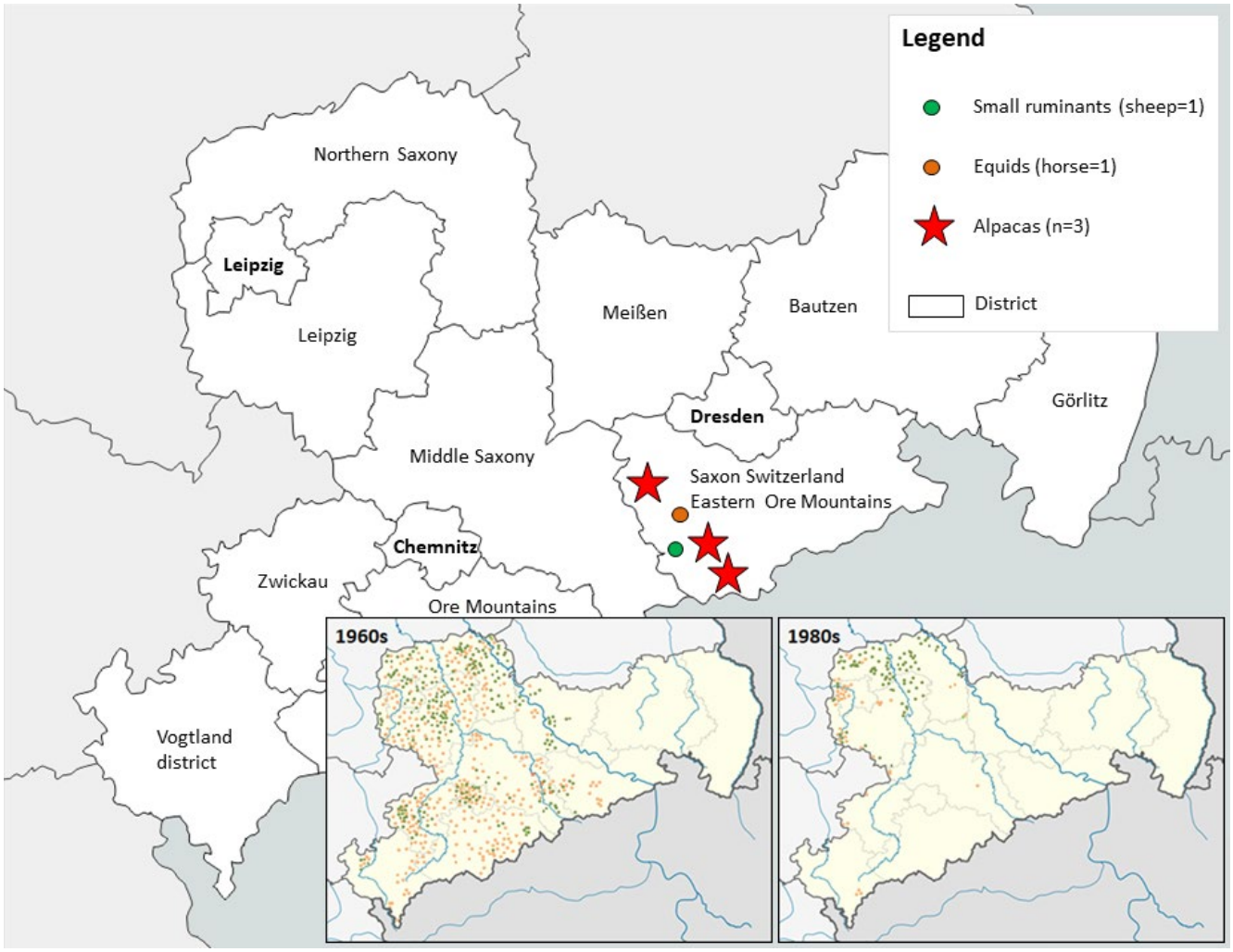

FIGURE 7 The analyses of Borna disease (BD) in the Weißeritz district and East Ore mountain region (Saxon Switzerland-Eastern Ore Mountains, Saxony, Germany) of the last two decades reflect activity at low level with alpacas being sentinels that were more frequently affected than horses or sheep in this region; inserts: BD activity in Saxony in the 1980s and 1960s according to data of Dürrwald (1993); green, sheep; red, horses; question marks, data for Western Saxony were not evaluated in the present study

study (Dürrwald et al., 2014) by some years and indicate a strong local conservation of BoDV-1 sequences within this cluster (98.2% identity).

No zoonotic spread of BoDV-1 to humans has been reported in Switzerland and Saxony to date (Rubbenstroth et al., 2019). Reports from other regions (Bavaria) demonstrate that this very rare but potentially devastating event in humans is possible (Coras et al., 2019; Korn et al., 2018; Niller et al., 2020). BD resulting from organ transplantations from an asymptomatic donor was also reported (Schlottau et al., 2018). The virus sequences from human patients were closely related to those previously obtained from BoDV-1 viruses of animals from the same region. Our study emphasizes the need to include BoDV-1 as a differential diagnosis for moderately progressive diffuse neurological disease in camelids. Other clinical differentials must also be considered (Whitehead & Bedenice, 2009). The growing interest in keeping New World camelids, especially alpacas, in farms and private holdings, requires a greater awareness of BD. In the Swiss farm, twelve of 30 breeding llamas succumbed to BD within 20 years, but none of the other animals which were also kept on the farm. In the Weißeritz/East Ore mountain districts of Saxony, three of five confirmed cases of BD in total (2000–2020) were seen in alpacas, indicating a higher probability of developing BD in New World camelids. Since these can act as sentinels, it is expected that our knowledge of endemic regions may extend in future regarding not only BoDV-1 but also BoDV-2 (so far only known in Styria, Austria) and also for other, as yet unknown bornaviruses.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

ORCID

Alexandra J. Malbon https://orcid.org/0000-0002-5144-3333
Norbert Nowotny https://orcid.org/0000-0002-3548-571X

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