

ORIGINAL ARTICLE

Infections of horses and shrews with Bornaviruses in Upper Austria: a novel endemic area of Borna disease

Herbert Weissenböck^{1,*}, Zoltán Bagó^{2,*}, Jolanta Kolodziejek^{3,*}, Barbara Hager⁴, Günter Palmetzhofer⁵, Ralf Dürrwald³ and Norbert Nowotny^{3,6}

Borna disease, a lethal infection with Borna disease virus-1 (BoDV-1), was diagnosed in four horses from Upper Austria in 2015 and 2016. All cases occurred in winter (two cases in February 2015 and two cases in December 2016), and the maximal distance of the affected stables was 17 km. To demonstrate whether the causative agent was also harbored by its reservoir host, the bicolored white-toothed shrew (*Crocidura leucodon*), 28 shrews from this geographic area were collected in 2015 and investigated for the presence of BoDV-1. The shrew species were identified according to taxonomic clues and molecular barcodes. Affected horses and all shrews were investigated using histology, immunohistochemistry (IHC) and reverse transcription PCR. The horses exhibited severe nonpurulent encephalitis. Large amounts of BoDV-1 antigen were identified in their CNS. Among the 28 shrews, nine were identified as *C. leucodon* and 13 as *Sorex araneus* (Common shrew; Eurasian shrew). Six *C. leucodon* (66.7%) and one *S. araneus* (7.7%) had BoDV-1 infections. In accordance with previous findings, the IHC of *C. leucodon* exhibited a high amount of viral antigen in many neural and extraneural tissues. By contrast, the single positive *S. araneus* had an exclusively neural staining pattern. Of all positive samples, whole-genome BoDV-1 sequences were generated. The acquired sequences of the affected shrews were not identical to each other and clustered around the sequences of the diseased horses belonging, surprisingly, to the German 'strain V' cluster.

Emerging Microbes & Infections (2017) 6, e52; doi:10.1038/emi.2017.36; published online 21 June 2017

Keywords: Austria; bicolored white-toothed shrew; Borna disease virus-1; common shrew; *Crocidura leucodon*; endemic area; horse; reservoir

INTRODUCTION

Borna disease virus-1 (BoDV-1) is the causative agent of Borna disease (BD), a neurological condition of horses and other mammals, which has been credibly identified only in certain endemic areas in central Europe to date.^{1–3} The affected regions include eastern and southern Germany, the eastern part of Switzerland and the area bordering Liechtenstein, and the most western federal state of Austria, Vorarlberg.² In an area not associated with these endemic areas, in the Austrian federal state of Styria, a single case of a lethal infection with a divergent bornavirus termed Borna disease virus-2 (BoDV-2) was detected ~20 years ago.⁴ Increasing evidence suggests that the natural reservoir host of BoDV-1 is the bicolored white-toothed shrew, *Crocidura leucodon*, and that disease in large mammals is only an accidental spillover event.^{5–7} In *C. leucodon*, the virus replicates in a large number of tissues and is excreted by different routes, but pathological lesions and clinical signs are not reported.⁸ In large mammals, such as horses, the infection is selectively neurotropic and triggers a massive infiltration of T cells into the central nervous system (CNS), ultimately

leading to nonpurulent encephalitis, characteristic clinical signs and an almost inevitable lethal outcome.⁹

Antibodies to Bornaviruses were also identified in different species outside of the endemic areas in Germany and other countries.^{10–12} Owing to the broad cross-reactivity between mammalian and avian Bornaviruses, antibody findings cannot be considered proof of infection with mammalian Bornaviruses.¹³

Despite the claims of several publications of the presence of BoDV-1 in regions of the globe remote from the classical endemic areas, these findings have not been confirmed epidemiologically, and the recovered virus sequences are compatible with sample contaminations with common laboratory strains.^{14–16}

The recent identification of a divergent bornavirus in squirrels (Variegated squirrel; *Sciurus variegatoides* and Prevost's squirrel; *Callosciurus prevosti*) and the association of this virus with deaths in humans has increased the attention of the scientific community on this viral family.^{17,18}

Thus, in the context of this changing landscape of bornavirus research, the purpose of this study is to present evidence for a

¹Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine Vienna, Vienna A-1210, Austria; ²Institute for Veterinary Disease Control Mödling, Austrian Agency for Health and Food Safety (AGES), Mödling A-2340, Austria; ³Institute of Virology, University of Veterinary Medicine Vienna, Vienna A-1210, Austria; ⁴Veterinary Practice St. Agatha, St. Agatha A-4084, Austria; ⁵Veterinary Practice Hartkirchen, Hartkirchen 4081, Austria and ⁶Department of Basic Medical Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai Healthcare City, Dubai 505055, United Arab Emirates

*These authors contributed equally to this work.

Correspondence: N Nowotny

E-mail: Norbert.Nowotny@mbru.ac.ae

Received 1 February 2017; revised 29 March 2017; accepted 9 April 2017

new endemic area of BoDV-1 documented by both clinically affected horses and the presence of the virus in two different shrew species.

MATERIALS AND METHODS

In February 2015 and December 2016, a total of four horses, all originating from different small family-owned premises in Upper Austria, were humanely killed due to progressive neurological signs (Figure 1). The most common clinical signs included depression, ‘pipe smoking’ behavior, and ataxia (Table 1).

The head and selected organ samples of the affected animals were sent to the national reference laboratory for equine encephalomyelitis to exclude notifiable infectious diseases. After removing the brain under strict biosafety measures, standard histopathological and immunohistochemical investigations and molecular analyses for the exclusion of rabies, flaviviruses and American encephalomyelitides were performed. On the basis of histopathological findings, subsequent analyses for BoDV were carried out.

To investigate the existence of a local virus reservoir, 28 shrews were collected between April and October 2015 in two districts of Upper

Austria in close proximity to the properties with 3 of the affected horses (Figure 1). These shrews were either caught by cats or trapped inadvertently in commercially available rodent traps. The animals were kept frozen at -20 °C until transportation to the laboratory, where necropsy was performed immediately after complete thawing. The shrew species were identified according to taxonomic clues¹⁹ and molecular barcodes. Molecular confirmation of all shrew species was performed by amplification and sequencing of a fragment within the mitochondrial DNA cytochrome b (mtDNA cytb) gene. For this purpose, published PCR assays were performed using two universal primer pairs: L14724f with H15915r²⁰ and/or L14724f (as before) with H15149r.²¹ For the genomic DNA-PCRs, a Fast Cycling PCR Kit (Qiagen, Redwood, CA, USA) was applied. At necropsy, tissue samples from the brain and skin of all shrews and from the lung, heart, liver, spleen, kidney and intestinal tract of 24 animals were fixed in 10% neutral buffered formalin. Brain samples from all shrews were immediately frozen at -80 °C.

The formalin-fixed horse and shrew samples were processed to paraffin blocks, sectioned at 2 µm and placed on Superfrost plus slides (Thermo Fisher Scientific, Fremont, CA, USA). The slides were stained

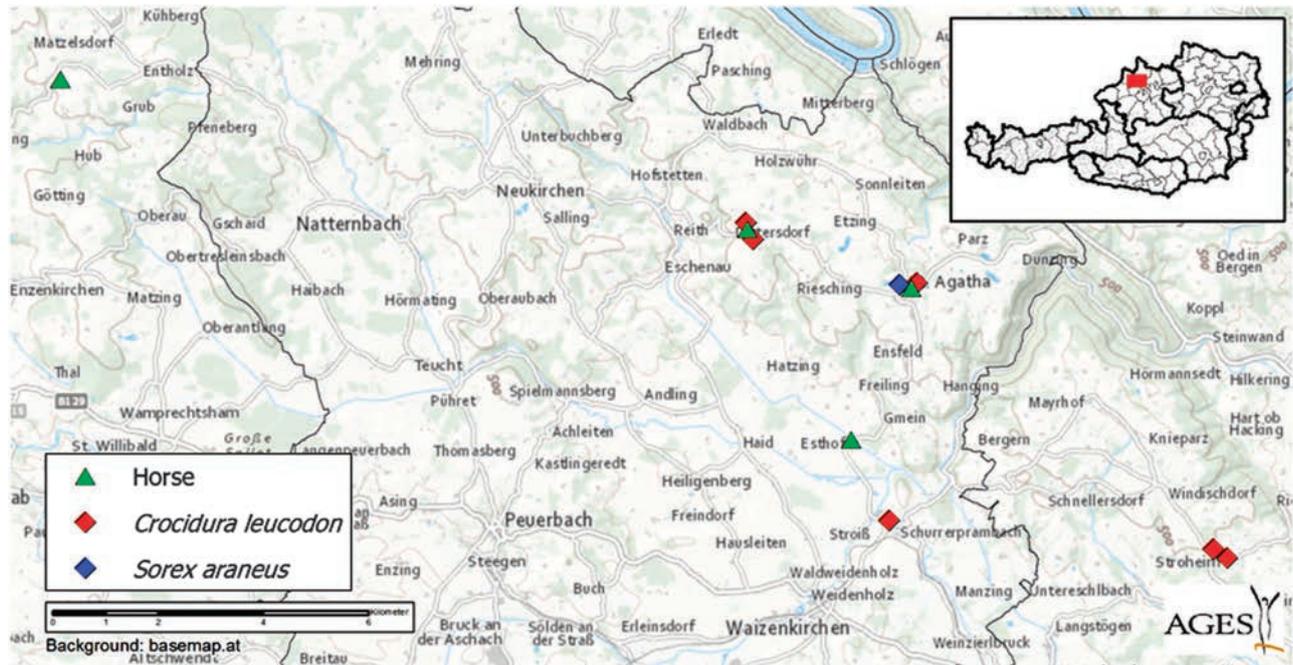


Figure 1 Map depicting the geographical distribution of the BoDV-1-positive horses and shrews. The inset shows the area in Upper Austria, which has been enlarged.

Table 1 Overview of clinical signs and histopathological findings in the affected horses

Number	Breed	Age	Sex	Clinical signs	npE	npG	JD IB	IHC (BoDV)
1 011407/15	Austrian warm blood	6 y	Mare	Constipation, colic, proprioception disturbances, circular movement, ataxia, disorientation.	+++	+	(+)	+++
2 018072/15	Shetland pony	3 y	Mare	Depression, empty chewing/pipe smoking, proprioception disturbances, circular movement, ataxia, stringhalt.	++	++	++	+++
3 134407/16	Noriker	6 y	Mare	Apathy, depression, pipe smoking, swallowing disorders.	++	+	+++	+++
4 135391/16	Trotter	10 y	Gelding	Initially fever (39.7 °C), loss of appetite, somnolence, ataxia, dyspnea, compulsive walking.	+++	+++	++	+++

Abbreviations: BoDV, Borna disease virus; IHC, immunohistochemistry; JD IB, Joest Degen inclusion bodies; npE, nonpurulent encephalitis; npG, nonpurulent ganglionitis; y, year; (+), single, +, mild, ++, moderate, +++, severe.

with hematoxylin and eosin and subjected to immunohistochemistry with the anti BoDV-antibody Bo18. This antibody recognizes a peptide (LYEPPASLP) in the nucleoprotein of the vast majority of known strains of BoDV-1 and BoDV-2.²² The staining procedure was performed with an automated immunostainer (Thermo Autostainer 360-2D System, Thermo Fisher) using the Ultravision LP Detection System (Thermo Fisher). The dilution of the primary antibody was 1:30 000.

Four different brain regions (brain stem, cerebellum, hippocampus and cerebral cortex) and the trigeminal ganglia of all horses were investigated by BoDV reverse transcription PCR (RT-PCR). In addition, the spleen of the first horse, cerebrospinal fluid and lymph nodes of the second horse, and basal ganglia and mesencephalon of the third horse were included. All frozen samples of the horses and the frozen brain samples of 28 shrews were thawed quickly and homogenized by addition of nuclease-free water (ROTH, Karlsruhe, Germany) and ceramic beads (VWR International, Vienna, Austria) using an automatic TissueLyser II (Qiagen). The obtained tissue suspensions were centrifuged at 10 000g for 5 min, and the supernatants were processed by automated nucleic acid extraction (QIAcube, Qiagen) using a QIAamp Viral RNA Mini QIAcube Kit (Qiagen) according to the manufacturer's instructions. This method allows the use of the same extracts to detect both viral RNA and genomic DNA.

All BoDV RT-PCR assays were performed with the OneStep RT-PCR Kit (Qiagen). The nucleic acid extracts were first investigated with the published screening BoDV RT-PCR.²³ All BoDV-positive samples were subsequently identified by 6 further RT-PCRs targeting the complete N, X and P genes and the N/X intergenic region. To each reaction mix containing PCR buffer, dNTPs and enzymes (amounts

according to the manufacturer's instructions), 0.4 µM of the corresponding primer pair, and 10% template were added. Reverse transcription and denaturation steps were determined according to the manufacturer's instructions. The DNA amplification was performed in 45 cycles of heat denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and DNA extension at 72 °C for 30 s followed by a final extension at 72 °C for 7 min. Given that all of the horse samples were positive by the screening PCR, only the cerebrum samples were chosen for the subsequent detailed investigations. The specific amplification products were purified using PCR Kleen Spin Columns (Bio-Rad, Hercules, USA) following the manufacturer's protocol and subjected to sequencing by Microsynth (www.microsynth.ch) or by Eurofins (www.eurofinsgenomics.com). Each PCR product was sequenced in both directions.

As the first sequence analysis exhibited the highest identity to BoDV-1 strain V (GenBank acc. no U04608), the subsequent primer pairs for the completion of the whole BoDV-1 genomes were designed based on this nucleotide sequence. A total of 13 new primer pairs (targeting the complete M, G, and L genes) were created using the Primer Designer program (Scientific and Educational Software, Durham, NC, USA) and synthesized by Microsynth. The primer sequences are listed in Table 2. The newly established RT-PCR assays were conducted for each positive sample as described above, and the PCR products were sequenced.

All obtained BoDV-1 sequences were manually verified and compiled to continuous full genome sequences. The sequences were then compared to each other and to the bornavirus sequences from GenBank. Prior to the phylogenetic analysis, ClustalW multiple sequence alignments were conducted using BioEdit Sequence

Table 2 Sequences of 13 primer pairs used in addition to previously described primers² for the generation of complete BoDV-1 genomes

Primer name/position/direction	Primer sequence 5'-3'	Length of PCR product (nt)
BoDV 1566 f	AGA CAT CTC GGC TCG TAT CG	807
BoDV 2372 r	GTC GCC TTA TCT CCA GGT CA	
BoDV 1953 f	CAC ACT GAT GCT TGA GAT AG	860
BoDV 2812 r	GTT CAC GAC TTC TGA CTG TA	
BoDV 2717 f	GTG AGC CAA CAG GAG CTA GA	860
BoDV 3576 r	CCT GAG CCT GTA TCC GTA GA	
BoDV 3332 f	ATG TGG TTC GGC AGG TAC TT	877
BoDV 4208 r	ATT AGG CAG CTT GTC GTG TC	
BoDV 4073 f	TTG CCT ACC AGC GCA TAG TG	883
BoDV 4955 r	AAG GCC GCT GCA TTG TAC TC	
BoDV 4237 f	GAC ACA GCC AAG AGC AGA TG	888
BoDV 5124 r	CTT AGG CAC GAG CAC AGT CA	
BoDV 5003 f	GAC CGT CAC GAC TTG TGA AT	838
BoDV 5840 r	ACA GGT ACA CCA CGG AAG AA	
BoDV 5647 f	GGC CAA GGT GAT AAT CAG AC	829
BoDV 6475 r	CGA ATA AGG CCG ACA TAT CC	
BoDV 6039 f	CCA GGA TGA GTC GCT ATT GA	857
BoDV 6895 r	CCT TGA CAG CCG TAT TGG AT	
BoDV 6327 f	GTG GAT TGA GGA AGC GAT AG	843
BoDV 7169 r	GAG AAT CGA AGC CAC GTA CT	
BoDV 6822 f	GAC ATT GCG GTC ACA CCA TC	884
BoDV 7705 r	TTG GAC CTG TCG CAG CAT AC	
BoDV 7350 f	GTT CGT CCT GGC ATG TGA AC	872
BoDV 8221 r	CGC ACA GGT CCA TCT CAA GT	
BoDV 8034 f	GTC ACG CAA TCA ATC ACA GG	847
BoDV 8880 r	AAG CAC TGC ACC ACT GAC AT	

Abbreviations: BoDV, Borna disease virus; f, forward; r, reverse.
Primer positions refer to the sequence of strain V (GenBank acc. number U04608).

Alignment Editor Version 7.0.9.0. Several phylogenetic trees were created with the MEGA7 program²⁴ using both the neighbor-joining (NJ) and the maximum-likelihood (ML) methods with different algorithms by employing 1000 replicates of bootstrap resampling analysis for each tree. Two trees with the highest supporting bootstrap values were chosen.

The first phylogenetic tree was inferred on the basis of complete coding sequences of 15 representative members of the genus *Borna-virus* and eleven sequences determined in this study. The second phylogenetic tree including only selected BoDVs-1 was constructed from 54 1824-bp long sequences coding for the N gene, the N/X intergenic region, and X and P genes.

For investigation of potential genome integration of BoDV, one primer pair within the N gene²³ and another within the P gene² were used, and PCRs without the RT-step were performed using a Fast Cycling PCR Kit.

For detection of the new agent Variegated squirrel bornavirus-1 all samples were tested using the recently described BoDV-like RT-qPCR (assay 6).¹⁷

RESULTS

All horses were negative for notifiable infectious viral encephalites, such as rabies, flavivirus and alphavirus encephalites. Histologically, all affected horses exhibited moderate to severe nonpurulent encephalitis with perivascular lymphohistiocytic cuffing, microgliosis, neuronal necrosis and eosinophilic intranuclear inclusion bodies (Joest Degen) in neurons. In addition, nonpurulent inflammation of trigeminal ganglia (in horse no. 2 with demonstration of Joest Degen inclusion bodies in ganglion cells) was consistently identified. Based on immunohistochemistry, high amounts of BoDV antigen were present in neurons, glial and ganglion cells and their processes (Figure 2; Table 1).

Among the 28 shrews, 9 were identified as *C. leucodon*, 13 as *Sorex araneus* (Common shrew, Eurasian shrew), 3 as *Neomys anomalus*

(Miller's water shrew), 2 as *C. suaveolens* (Lesser white-toothed shrew) and one as *S. minutus* (Pygmy shrew).

Six *C. leucodon* (66.7%) and one *S. araneus* (7.7%) were clearly positive for BoDV infections by both IHC and RT-PCR. All *C. leucodon* exhibited a high amount of viral antigen in neural tissues, such as brain, peripheral and vegetative nerve fibers, and in a number of extraneural tissues, such as epidermis, salivary glands, bronchiolar epithelium, smooth muscle cells, myocardium and adipose tissue. The parenchyma of large organs, such as liver, kidney, lung and spleen, exhibited no specific staining signals. Although staining of neural tissue was consistent, diffuse and strong in all animals, the staining pattern in other tissues was variable and not present in each individual. The single positive *S. araneus* exhibited a different staining pattern. In the brain, multifocal staining of groups of neurons and glial cells was noted, where the signals could be clearly attributed to nuclei, perikarya or processes of individual cells. The only other investigated tissue in this case was the skin, where the keratinocytes were negative but periadnexal and dermal nerve fibers exhibited positive signals (Figure 3). All shrews including the BoDV-1-positive individual of *S. araneus* displayed no inflammatory infiltrations in the brain and other organs.

From the brain samples of four horses and seven shrews, whole-genome BoDV sequences were generated. The sequences exhibited 99.6% to 99.9% identity to each other. The smallest divergence was observed between viruses identified in horse no. 1 and in two shrews: CL311/15 and CL690/15 (only 3 nts difference in 8 769 coding nts). The highest divergence was observed between shrews CL1285/15, CL1287/15 and horse no. 4 (30 nts). Sequence identities to the German reference BoDV-1 strains V, H1766, and He/80 were 97.8%–97.9%, 97.5%–97.6% and 94.7%–94.8%, respectively. The sequence identity to the Austrian BoDV-2 strain No/98 was only 81.0%–81.1%.

The analysis of 1824-nt long sequences comprising complete N, P, and X genes of 54 BoDVs-1 revealed the highest identity to the

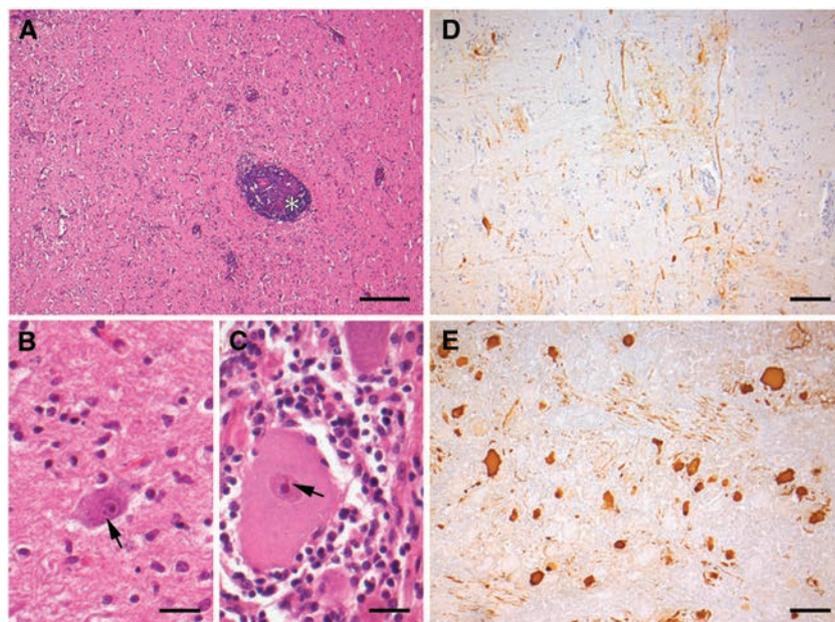


Figure 2 Borna virus encephalitis in horses. (A) Severe nonpurulent encephalitis with perivascular cuffing (asterisk) and microgliosis. Bar=250 μ m. (B and C) Eosinophilic intranuclear inclusion bodies (arrows) in a brainstem neuron (B) and a trigeminal ganglion neuron surrounded by lymphohistiocytic infiltration (C). Bars=25 μ m. (D and E) Immunohistochemical demonstration of BoDV antigen (brown signals) in neurons and glial cells and their processes. Bars=100 μ m. (A–C) Hematoxylin and Eosin staining. (D and E) Bo-18 immunohistochemistry.

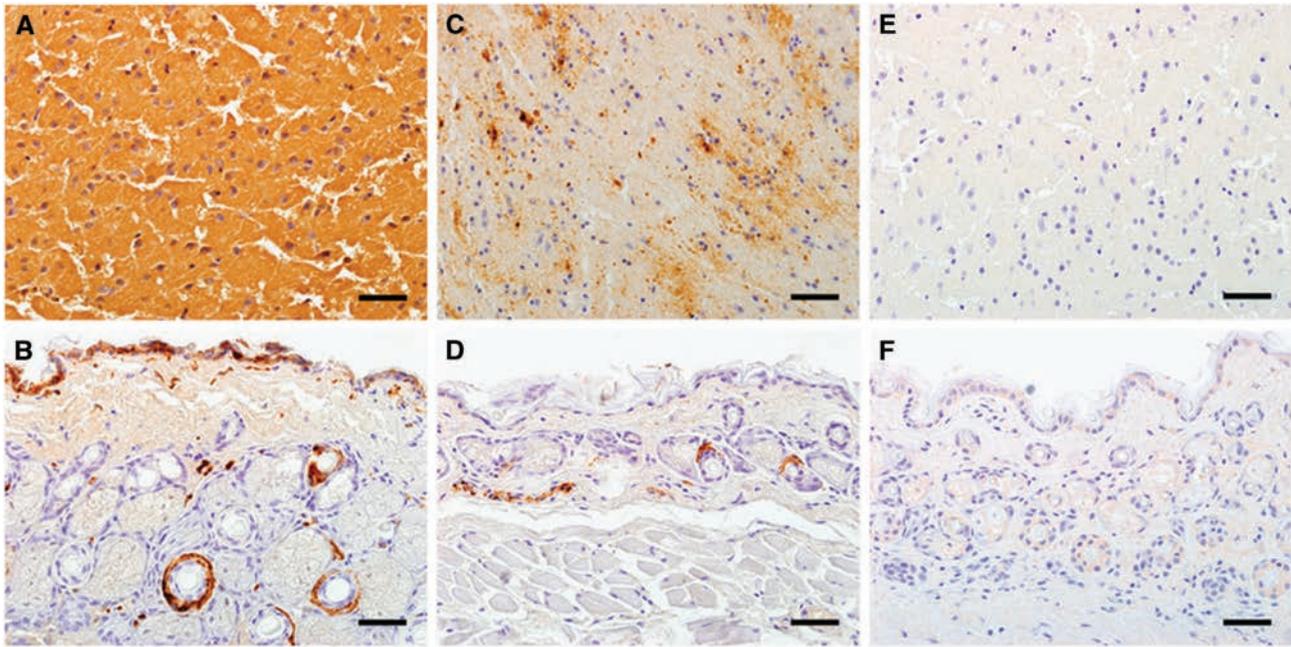


Figure 3 Bornavirus infection of shrews. (A and B) Demonstration of BoDV-1 antigen in an infected *Crocidura leucodon* shrew. In the brain (A), the antigen is diffusely distributed; in skin (B), keratinocytes and periadnexal nerve fibers are positive. (C and D) Demonstration of BoDV-1 antigen in an infected *Sorex araneus* shrew. In the brain (C), the viral antigen has a multifocal, patchy staining pattern, representing individually stained neurons and glial cells and their processes. In skin (D), the viral antigen is confined to periadnexal nerve fibers; keratinocytes are negative. (E and F) Lack of immunoreactivity in brain (E) and skin (F) in a non-infected *C. leucodon* shrew. Bars = 40 μm; Bo-18 immunohistochemistry.

German horse isolate H215/FR (between 98.5% and 98.7%) and to further strains and viruses around strain V (between 97.6% and 98.5%).

Furthermore, in one horse sample (no. 2), a P gene fragment was identified by the BoDV DNA-PCR. Its sequence was 100% identical to that of the viral sequence achieved by the BoDV RT-PCR. In addition, P or N gene fragments were integrated into the genomes of two shrews (CL428/15 and CL690/15). The N gene sequence of the genomic BoDV of shrew CL690/15 was 100% identical to its viral sequence, the genomic- and viral-nucleotide P gene sequences of shrew CL428/15 exhibited one nucleotide substitution associated with an amino acid exchange.

Multiple alignments of the corresponding complete proteins between the new strains demonstrated only one (of 370) amino acid substitution within the N gene (in CL428/15), 1 (of 201) within the P gene (in CL1287/15), 6 (of 503) substitutions within the G gene (in 9 different sequences), and 12 (of 1711) within the L gene (in all sequences). Amino acid sequences of the X gene (87 aa) and M gene (142 aa) exhibited 100% identity to each other.

All samples were negative by the BoDV-like RT-qPCR specific for variegated squirrel bornavirus-1, which belongs to the species *Mammalian 2 bornavirus*.

With the current data sets, the NJ method with the p-distance algorithm of MEGA7 was the best method for phylogenetic analysis. Analysis of the full-length recovered genome sequences demonstrated that the new Austrian viruses represent further members of BoDV-1 belonging to the species *Mammalian 1 bornavirus* of the genus *Bornavirus* (Figure 4). These sequences are clearly segregated from the Austrian reference strain No/98, representing the only member of BoDV-2 (Figure 4).

More detailed phylogenetic investigation of the fifty-four 1824-bp long sequences of only BoDVs-1 exhibits the presence of five different,

geographically confined clusters well-known from previous studies (Figure 5).^{2,7} The first cluster (1A) contains mostly viruses from Southwest Germany closely related to the strains from Switzerland and Liechtenstein (cluster 1B). Cluster 2 represents the South German group and cluster 3 includes Bornaviruses mostly from Southern Saxony-Anhalt and Saxony in Germany. The newly discovered Upper Austrian strains share cluster 4 together with other Central German sequences (Figure 5). These strains cluster close to each other independent of their hosts and collection time. Further, they exhibit close relationships to other German strains and viruses around the oldest BoDV strain V isolated in 1929 in Lower Saxony, especially to strain H215FR, discovered in a horse with clinical BD reported to originate from BoDV-1-non-endemic Rhineland-Palatinate in 1989 and analyzed 25 years later (Figure 5).²⁵

The eleven newly described Upper Austrian complete genome BoDV-1 sequences are available from GenBank under the following accession numbers: KY002071 (Horse 011407/15), KY002072 (Horse 018072/15), KY002073 (CL311/15), KY002074 (CL428/15), KY002075 (CL690/15), KY002076 (CL1285/15), KY002077 (CL1286/15), KY002078 (CL1287/15), KY002079 (SAR313/15), KY490040 (Horse 134407/16) and KY490041 (Horse 135391/16).

DISCUSSION

This study clearly demonstrates that the map of endemic areas for BoDV-1 is far from complete and that new endemic pockets remote from the well-known affected regions may emerge from time to time. In the present scenario, confirmed BD cases occurred within a short time span in four horses housed in stables not much more than 17 km apart. Another horse with matching clinical signs and seropositive for BoDV-1 was already registered in the same area in late 2014. This case was not subjected to laboratory investigation post mortem; thus, a definitive diagnosis has not been made (personal observation,

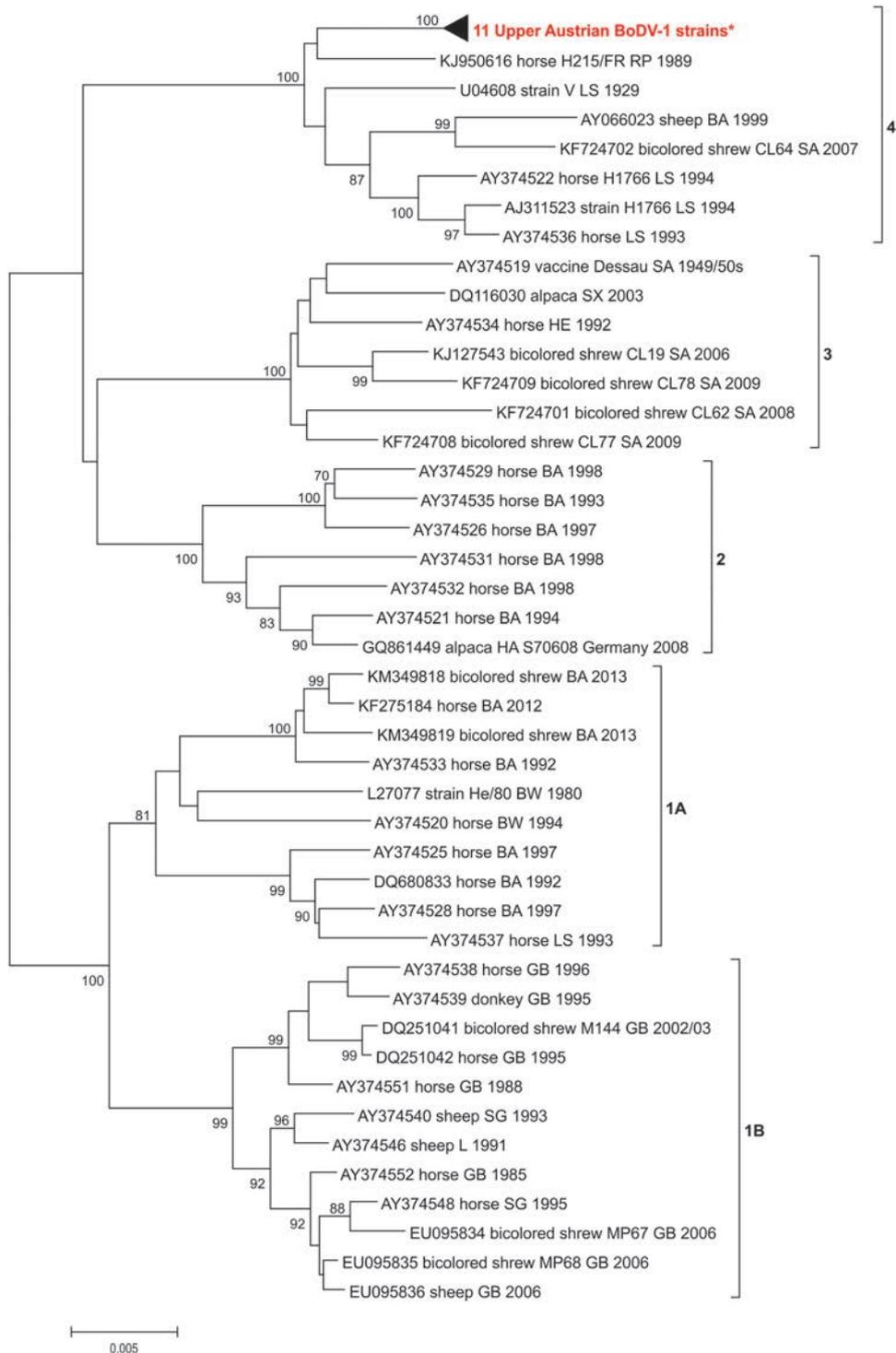


Figure 5 Phylogenetic tree of an 1824-bp long sequence stretch (coding for N protein, intergenic N/X, as well as for X and P proteins) of 43 selected BoDVs-1 from the GenBank and eleven sequences determined in this study (which are collapsed). The five major clusters are indicated. The GenBank accession numbers, host or strain names, geographic location and years of isolations are indicated at the branches. Supporting bootstrap values $\geq 70\%$ are displayed next to the nodes. The horizontal scale bar indicates genetic distances (here 0.5% nucleotide sequence divergence). BA, Bavaria; BW, Baden-Württemberg; GB, Graubünden; HE, Hesse; L, Liechtenstein; RP, Rhineland-Palatinate; SA, Saxony-Anhalt; SG, Sankt Gallen; SX, Saxony; TH, Thuringia. *GenBank acc. nos: KY002971-79 and KY490040-41.

overt BD, no inflammation was noted in the brain of this common shrew, indicating a natural resistance against BoDV-1-induced immunopathology.

This is the first report of BoDV-1 infections in Upper Austria. It is unknown when and how the virus became established in this particular region. Given that even in classical endemic areas clinical

equine infections occur rarely and unpredictably, overt BD in spillover hosts, such as horses and sheep, is an unreliable indicator of the presence of BoDV-1. In such areas, equine cases may occur at intervals of several decades,³³ and there are areas with a high viral burden in shrews in which BD has never been recognized.⁷ Thus, the virus could have been present and unnoticed in the region for a long period of time. Recently, favorable events may have caused an increase in the reproductive rate of shrews and driven a larger number of them into horse stables compared with previous years. Thus, the likelihood of equine infections by excretions and sheddings of the shrews into the horse environment increased the risk of infection and ultimately culminated in the lethal spillover infection of the reported four horses. The point in time of overt disease in the four reported horses is more uncommon compared with the seasonal pattern of BD described in Germany, which has a nadir in late autumn and early winter.¹⁴ Considering the long time period from infection to expression of disease (the incubation period is considered to be up to five months),⁷ the infection may have been the result of previously unknown conditions.

The clinical signs and pathological changes in the four affected horses match the descriptions of classical BD in this species.^{34,35} Immunohistochemistry revealed a strictly neurotropic infection with clear restriction to the CNS. In this host, the virus did not spread via the peripheral or autonomic nerves, and there were no viral signals in any tissue except the CNS. This tight confinement of the virus to the CNS compartment, which is most likely the result of a strong antiviral cellular immune response, argues against the possibility of viral shedding by infected horses. By contrast, infection in *C. leucodon* is clinically inapparent and seems to persist for the entire life span of the animal. In addition, infectious virus is continuously shed by various routes, such as the saliva, lacrimal fluid, urine, and exfoliated skin.⁸ Contrary to numerous other proven or suspected reservoir hosts for viruses of the order *Mononegavirales*, especially bats and rodents,^{36,37} the reservoir status of *C. leucodon* has not only been defined virologically but also by demonstration of large amounts of viral proteins and nucleic acids in many organs and tissues. Viral replication in these sites does not seem to negatively influence cell functions and does not lead to any degenerative or inflammatory changes. There is obviously a perfect equilibrium between host and virus that underlines the presumptive natural host status of this shrew species. As previously mentioned,⁸ whether lack of any tissue reaction in BoDV-1-infected *C. leucodon* is due to attenuated pathogenicity, differences in viral entry and circumvention of the antiviral host immune system remains unexplained. The lack of any immunopathological response despite the replication of the virus in a broad range of tissues is compatible with an immunotolerant state, which is reminiscent of experimental rat models of BoDV-1 infection. In certain rat strains, infection in newborns does not trigger an immune response that protects the animals from encephalitis on one hand and that enables the replication of the virus outside the CNS compartment on the other hand.³⁸ The same effect is produced by immunosuppressive drugs in adult rats.³¹ Although not fully comparable, intriguing parallels are noted between these experimental models and the naturally infected *C. leucodon* shrews. At present, it can only be hypothesized that the animals are exposed to the virus as newborns by their persistently infected mothers when their immune system is still immature, and that they are immunotolerant to the virus for the remainder of their lives. Given that a few bicolored shrews in Germany also reflected the restriction of BoDV-1 to the brain,⁷ both infection cycles—infections of newborns and spillover to adults—may exist in shrews. Despite the restriction of bornavirus to the brain in these

shrews, BoDV-1-positive PCR signals could be detected in the lungs and stomach, reflecting a viral burden in their surroundings and possible mechanisms of virus uptake.⁷ In addition to the finding of BoDV-1-positive shrews in Switzerland and Germany and related spillovers of the infection to horses, this report confirms the role of shrews in the transmission of BoDV-1 in a third country.

ACKNOWLEDGEMENTS

We thank Sybille Herzog (Institute of Virology, Justus-Liebig-University Giessen, Giessen, Germany) for the generous gift of the Bo18 antibody. We also thank Katharina Dimmel and Michael Kolodziejek (both: Institute of Virology, University of Veterinary Medicine, Vienna, Austria), Petra Kodajova (Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine, Vienna, Austria), and the laboratory staff of the national reference laboratory of equine encephalomyelitis (Institute for Veterinary Disease Control Mödling, Austrian Agency for Health and Food Safety (AGES), Mödling, Austria) for the excellent technical assistance and Christian Franz (Equine Clinic Tyllisburg, Sankt Florian, Austria) for submitting one of the horses. We are grateful to Klaus Bittermann (Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine, Vienna, Austria) and Michael Schwarz (Data, Statistics and Risk Assessment, AGES, Vienna, Austria) for their help with the digital figure files.

- 1 Staeheli P, Sauder C, Hausmann J *et al*. Epidemiology of Borna disease virus. *J Gen Virol* 2000; **81**: 2123–2135.
- 2 Kolodziejek J, Dürrwald R, Herzog S *et al*. Genetic clustering of Borna disease virus natural animal isolates, laboratory and vaccine strains strongly reflects their regional geographical origin. *J Gen Virol* 2005; **86**: 385–398.
- 3 Kuhn JH, Dürrwald R, Bao Y *et al*. Taxonomic reorganization of the family Bornaviridae. *Arch Virol* 2014; **160**: 621–632.
- 4 Nowotny N, Kolodziejek J, Jehle CO *et al*. Isolation and characterization of a new subtype of Borna disease virus. *J Virol* 2000; **74**: 5655–5658.
- 5 Hilbe M, Herrsche R, Kolodziejek J *et al*. Shrews as reservoir hosts of Borna disease virus. *Emerg Infect Dis* 2006; **12**: 675–677.
- 6 Bourg M, Herzog S, Encarnação JA *et al*. Bicolored white-toothed shrews as reservoir for Borna disease virus, Bavaria, Germany. *Emerg Infect Dis* 2013; **19**: 2064–2066.
- 7 Dürrwald R, Kolodziejek J, Weissenböck H *et al*. The bicolored white-toothed shrew *Crociodura leucodon* (Hermann 1780) is an indigenous host of mammalian Borna disease virus. *PLoS ONE* 2014; **9**: e93659.
- 8 Nobach D, Bourg M, Herzog S *et al*. Shedding of infectious Borna disease virus-1 in living bicolored white-toothed shrews. *PLoS ONE* 2015; **10**: e0137018.
- 9 Caplazi P, Ehrensperger F. Spontaneous Borna disease in sheep and horses: Immunophenotyping of inflammatory cells and detection of MHC-I and MHC-II antigen expression in Borna encephalitis lesions. *Vet Immunol Immunopathol* 1998; **61**: 203–220.
- 10 Kinnunen PM, Billich C, Ek-Kommonen C *et al*. Serological evidence for Borna disease infection in humans, wild rodents and other vertebrates in Finland. *J Clin Virol* 2007; **38**: 64–69.
- 11 Kinnunen PM, Palva A, Vaheri A *et al*. Epidemiology and host spectrum of Borna disease virus infections. *J Gen Virol* 2013; **94**: 247–262.
- 12 Bourg M, Nobach D, Herzog S *et al*. Screening red foxes (*Vulpes vulpes*) for possible viral causes of encephalitis. *Virology* 2016; **13**: 151.
- 13 Zimmermann V, Rinder M, Kaspers B *et al*. Impact of antigenic diversity on laboratory diagnosis of avian bornavirus infections in birds. *J Vet Diagn Invest* 2014; **26**: 769–777.
- 14 Dürrwald R, Kolodziejek J, Muluneh A *et al*. Epidemiological pattern of classical Borna disease and regional genetic clustering of Borna disease viruses point towards the existence of to-date unknown endemic reservoir host populations. *Microb Infect* 2006; **8**: 917–929.
- 15 Dürrwald R, Kolodziejek J, Herzog S *et al*. Meta-analysis of putative human bornavirus sequences fails to provide evidence implicating Borna disease virus in mental illness. *Rev Med Virol* 2007; **17**: 181–203.
- 16 Lipkin WI, Briese T, Hornig M. Borna disease virus—fact and fantasy. *Virus Res* 2011; **162**: 162–172.
- 17 Hoffmann B, Tappe D, Höper D *et al*. A variegated squirrel bornavirus associated with fatal human encephalitis. *N Engl J Med* 2015; **373**: 154–162.
- 18 Schlottau K, Jenckel M, van den Brand J *et al*. Variegated squirrel bornavirus 1 in squirrels, Germany and The Netherlands. *Emerg Infect Dis* 2017; **23**: 477–481.
- 19 Blatt C, Resch S. *Internethandbuch über Kleinsäugerarten im mitteleuropäischen Raum: Körpermerkmale, Ökologie und Verbreitung*. Available at <http://www.kleinsaeuger.at> (accessed 23 January 2017).
- 20 Irwin DM, Kocher TD, Wilson AC. Evolution of the cytochrome B gene of mammals. *J Mol Evol* 1991; **32**: 128–144.

- 21 Tsai LC, Huang MT, Hsiao CT *et al*. Species identification of animal specimens by cytochrome B gene. *J Forens Sci* 2007; **6**: 63–65.
- 22 Billich C, Sauder C, Frank R *et al*. High-avidity human serum antibodies recognizing linear epitopes of Borna disease virus proteins. *Biol Psychiatry* 2002; **51**: 979–987.
- 23 Sorg I, Metzler A. Detection of Borna disease virus RNA in formalin-fixed, paraffin-embedded brain tissues by nested PCR. *J Clin Microbiol* 1995; **33**: 821–823.
- 24 Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016; **33**: 1870–1874.
- 25 Richt JA, Pfeuffer I, Christ M *et al*. Borna disease virus infection in animals and humans. *Emerg Infect Dis* 1997; **3**: 343–352.
- 26 Encarnação JA, Herzog S, Eickmann M *et al*. Landscape features and reservoir occurrence affecting the risk for equine infection with Borna disease virus. *J Wildl Dis* 2013; **49**: 860–868.
- 27 Vahlenkamp TW, Konrath A, Weber M *et al*. Persistence of Borna disease virus in naturally infected sheep. *J Virol* 2002; **76**: 9735–9743.
- 28 Horie M, Honda T, Suzuki Y *et al*. Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature* 2010; **463**: 84–87.
- 29 Kinnunen PM, Inkeroinen H, Ilander M *et al*. Intracerebral Borna disease virus infection of bank voles leading to peripheral spread and reverse transcription of viral RNA. *PLoS One* 2011; **6**: e23622.
- 30 Spitzenberger F. [The white-toothed shrews (Crocidae) of Austria]. *Mitt Abt Zool Landesmus Joanneum* 1985; **35**: 1–40 German.
- 31 Stitz L, Schilken D, Frese K. Atypical dissemination of the highly neurotropic Borna disease virus during persistent infection in cyclosporine A-treated, immunosuppressed rats. *J Virol* 1991; **65**: 457–460.
- 32 Jacobsen B, Algermissen D, Schaudien D *et al*. Borna disease in an adult alpaca stallion (*Lama pacos*). *J Comp Pathol* 2010; **143**: 203–208.
- 33 Dürwald R, Ludwig H. Borna disease virus (BDV), a (zoonotic?) worldwide pathogen. A review of the history of the disease and the virus infection with comprehensive bibliography. *Zentralbl Veterinärmed B* 1997; **44**: 147–184.
- 34 Gosztonyi G, Ludwig H. Borna disease—Neuropathology and pathogenesis. *Curr Top Microbiol Immunol* 1995; **190**: 39–73.
- 35 Richt JA, Rott R. Borna disease virus: A mystery as an emerging zoonotic pathogen. *Vet J* 2001; **161**: 24–40.
- 36 Meerburg BG, Singleton GR, Kijlstra A. Rodent-borne diseases and their risks for public health rodent-borne diseases and their risks for public health. *Crit Rev Microbiol* 2009; **35**: 221–270.
- 37 Drexler JF, Corman VM, Müller MA *et al*. Bats host major mammalian paramyxoviruses. *Nat Commun* 2012; **3**: 796.
- 38 Herzog S, Kompter C, Frese K *et al*. Replication of Borna disease virus in rats: age-dependent differences in tissue distribution. *Med Microbiol Immunol* 1984; **173**: 171–177.



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017