Antibodies against viral nucleo-, phospho-, and X protein contribute to serological diagnosis of fatal Borna disease virus 1 infections

Highlights
- Borna disease virus 1 causes fatal encephalitis upon zoonotic spillover infections
- An ELISA system using recombinant BoDV-1 N, X, and P proteins has been established
- Antibodies against at least two different BoDV-1 antigens corroborate seroconversion
- Early detection of viral RNA and antibodies could contribute to BoDV-1 diagnosis

Authors
Bernhard Neumann, Klemens Angstwurm, Ralf A. Linker, ..., Hans Helmut Niller, Barbara Schmidt, Markus Bauswein

Correspondence
markus.bauswein@ukr.de

In brief
Neumann et al. provide data on antibody responses to Borna disease virus 1 in eight cases of fatal encephalitis based on a newly developed ELISA using recombinant BoDV-1 nucleoprotein, phosphoprotein, and accessory protein X. The presence of antibodies against at least two different viral antigens contributes to BoDV-1 diagnosis.
Antibodies against viral nucleo-, phospho-, and X protein contribute to serological diagnosis of fatal Borna disease virus 1 infections

Bernhard Neumann,1,2 Klemens Angstwurm,1,13 Ralf A. Linker,1 Gertrud Knoll,3 Lisa Eidenischink,3 Dennis Rubbenstroth,4 Kore Schlottau,5 Martin Beer,4 Patrick Schreiner,2 Erwin Soutschek,5 Merle M. Böhmer,6,7,13 Benedikt M.J. Lampfl,6,9,13 Matthias Pregler,6,10 Alexander Scheiter,11 Katja Evert,11 Saida Zoubaa,12 Markus J. Riemenschneider,12 Benedikt Asbach,10 André Gessner,3,10 Hans Helmut Niller,3,10,13 Barbara Schmidt,3,10,13 and Markus Bauswein3,13,14,*

1Department of Neurology, University of Regensburg, Bezirksklinikum, Regensburg, Germany
2Department of Neurology, Donau-Isar-Klinikum Deggendorf, Deggendorf, Germany
3Institute of Clinical Microbiology and Hygiene, Regensburg University Hospital, Regensburg, Germany
4Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald – Insel Riems, Germany
5Mikrogen GmbH, Neuried, Germany
6Bavarian Health and Food Safety Authority, München, Germany
7Institute of Social Medicine and Health Systems Research, Otto-von-Guericke-University, Magdeburg, Germany
8Public Health Department Regensburg, Regensburg, Germany
9Department of Epidemiology and Preventive Medicine, Faculty of Medicine, University of Regensburg, University Hospital Regensburg, Regensburg, Germany
10Institute of Medical Microbiology and Hygiene, University of Regensburg, Regensburg, Germany
11Institute of Pathology, University of Regensburg, Regensburg, Germany
12Department of Neuropathology, Regensburg University Hospital, Regensburg, Germany
13Bornavirus Focal Point Bavaria
14Lead contact
*Correspondence: markus.bauswein@ukr.de
https://doi.org/10.1016/j.xcrm.2021.100499

SUMMARY

Borna disease virus 1 (BoDV-1) causes rare but often fatal encephalitis in humans. Late diagnosis prohibits an experimental therapeutic approach. Here, we report a recent case of fatal BoDV-1 infection diagnosed on day 12 after hospitalization by detection of BoDV-1 RNA in the cerebrospinal fluid. In a retrospective analysis, we detect BoDV-1 RNA 1 day after hospital admission when the cell count in the cerebrospinal fluid is still normal. We develop a new ELISA using recombinant BoDV-1 nucleoprotein, phosphoprotein, and accessory protein X to detect seroconversion on day 12. Antibody responses are also shown in seven previously confirmed cases. The individual BoDV-1 antibody profiles show variability, but the usage of three different BoDV-1 antigens results in a more sensitive diagnostic tool. Our findings demonstrate that early detection of BoDV-1 RNA in cerebrospinal fluid and the presence of antibodies against at least two different viral antigens contribute to BoDV-1 diagnosis. Physicians in endemic regions should consider BoDV-1 infection in cases of unclear encephalopathy and initiate appropriate diagnostics at an early stage.

INTRODUCTION

Borna disease virus 1 (BoDV-1), the causative agent of Borna disease in horses, sheep, and other domestic mammals, induces severe to fatal encephalitis in humans in endemic regions of southern and eastern Germany.1–4 The bicolor white-toothed shrew (Crocidura leucodon) is the only known natural reservoir host so far.4,7 The pathogenesis of BoDV-1 infections is mainly attributed to an immunopathology mediated by virus-specific CD8+ and CD4+ T cells.3 Residence in mainly rural environments has been discussed as a risk factor for BoDV-1 infections.3,4 To date, there is no established therapy. In cell cultures, however, ribavirin and favipiravir (T-705) showed antiviral activity against bornavirus.10,11 As diagnostic tools, BoDV-1-specific qRT-PCR, indirect immunofluorescence assay (iIFA), and a line blot assay have been established.5,6 Sensitivity of qRT-PCR is limited for cerebrospinal fluid (CSF) but reliable for biopsies or post mortem tissue samples from the brain.4,5 As iIFA is laborious and its assessment is, to some extent, subjective, while the sensitivity of the line blot assay is limited, there is a need for developing additional serological tests, such as ELISA.5 The earliest possible time for a reliable diagnosis in the course of disease and the existence of a possible window for curative treatment are poorly understood. Here, we report a case of BoDV-1 infection that was diagnosed by qRT-PCR from CSF and via seroconversion using a newly established ELISA system with recombinant BoDV-1 proteins.
RESULTS

The affected individual, who was over 70 years of age, was admitted to the department of neurology of a tertiary care hospital in a BoDV-1-endemic region of southern Germany with a 2-week history of confusion, dizziness, memory impairment, and recurrent vomiting. Their medical history was insignificant, especially without immunosuppression or organ transplantation. An electroencephalogram (EEG) displayed mild diffuse slowing, and CSF parameters were nonspecifically altered, indicating a blood-brain barrier disruption with a normal cell count (2 leucocytes/μL, lactate 1.98 mmol/L, protein 925 mg/L) on the day after admission (Figure 1). The next day, the individual was found unconscious and was intubated based on the assumption of severe aspiration pneumonia. The individual remained in a deep coma after stopping light sedation on day 5. CSF analysis on day 8 showed a CSF leucocyte count of 41 cells/μL (Figure 1) and negative PCR results for herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, and enterovirus. Serological indicators of Lyme disease, tick-borne encephalitis, or autoimmune encephalitis were not detectable. On day 12, BoDV-1 infection was diagnosed by a positive qRT-PCR result from CSF that had been collected on day 8 after admission. At the time of the diagnosis, the individual was in a deep coma, showing no motor responses. Multiple brainstem reflexes were absent. Two brain magnetic resonance images (MRIs) on days 4 and 11 remained unremarkable. Following the individual’s reported will, we decided against experimental therapies and for palliative care. The individual died on day 15 after admission. Other available CSF samples of the individual were retrospectively examined by qRT-PCR. The first CSF obtained on day 1 after admission yielded positive results for BoDV-1 RNA ranging between 515 and 1,850 copies/mL, whereas a CSF obtained on day 4 tested negative (detection limit: 300 copies/mL; Figure 1). Post mortem, all samples from nonneuronal tissue tested negative, while samples from the frontal cortex as well as from the optical nerve and peripheral nerve tissue contained a high RNA load (Table 1). A histological work-up showed marked necrotizing lymphocytic neuritis of the peripheral femoral and small visceral nerves (data not shown).

Serological tests were performed with all available serum and CSF samples of the current individual. Using iIFA, seroconversion
was detected on day 13 (titer 320), whereas BoDV-1-specific antibodies were not detected in serum or CSF samples obtained earlier. To further characterize the immune response against different BoDV-1 antigens, recombinant BoDV-1 nucleoprotein (N), accessory protein X (X), and phosphoprotein (P) proteins were expressed in *E. coli*. Purity of these proteins was confirmed by silver staining (Figure 2). Further, the plasma sample of the current affected individual, which tested positive by iIFA, as well as selected iIFA-positive samples of 7 additional previously confirmed BoDV-1-infected individuals were evaluated by western blot using the recombinant BoDV-1 proteins (Figure 2). Samples of all affected individuals showed at least two reactive bands.

In a next step, these proteins were used to establish an ELISA. Thresholds were determined using 24 serum samples that had tested negative for BoDV-1 using iIFA (Figure 3A). For each antigen, an individual cutoff was defined as the mean plus six standard deviations. In accordance with the iIFA result, seroconversion for the current affected individual, which tested positive by iIFA, as well as selected iIFA-positive samples of 7 additional previously confirmed BoDV-1-infected individuals were evaluated by western blot using the recombinant BoDV-1 proteins (Figure 2). Samples of all affected individuals showed at least two reactive bands.

In a next step, these proteins were used to establish an ELISA. Thresholds were determined using 24 serum samples that had tested negative for BoDV-1 using iIFA (Figure 3A). For each antigen, an individual cutoff was defined as the mean plus six standard deviations. In accordance with the iIFA result, seroconversion for the current affected individual was detected by the ELISA on day 13, mainly based on reactivity against BoDV-1 X protein, whereas anti-BoDV-1-N-IgG was just above the cutoff in the last plasma sample obtained, and anti-BoDV-1-N-IgG was not detectable (Figure 3C).

To further evaluate the sensitivity of the ELISA, longitudinal serum/plasma and CSF samples from the seven previously confirmed BoDV-1-infected individuals were tested. Both anti-BoDV-1-X-IgG and anti-BoDV-1-P-IgG were detected in the serum/plasma of all individuals during the course of the disease, while anti-BoDV-1-N-IgG did not exceed the cutoff value in two affected individuals (Figure 3D). All samples with iIFA titers above 100 were detected by IgG ELISA, showing values above the cutoff against at least one BoDV-1 antigen (Figures 3D and S1A). In the seven selected samples tested by western blot, the ELISA showed a high agreement in terms of positive antigen reactions; only a borderline-positive anti-N-IgG ELISA result for individual 4 did not show a corresponding positive band in western blot (Figures 2 and 3D). The ELISA remained negative for two CSF samples with iIFA titers of 40 and 20 (individual 1) and a serum with an iIFA titer of 40 (individual 4) collected during early stages of disease, which is in congruence with the 100-fold sample dilution used for the ELISA. In addition, iIFT titers significantly correlated with ELISA values in BoDV-1-infected individuals (Figure S2).

To further assess the specificity of the ELISA, we took advantage of an independent cohort of 56 individuals with suspected tick-borne encephalitis whose serum/plasma samples were tested for BoDV-1 IgG antibodies for diagnostic purposes. Corresponding CSF samples of all affected individuals tested negative for BoDV-1 by qRT-PCR. Regarding serum/plasma, six, two, and two samples were reactive with values above N, X, and P cutoffs as tested by ELISA (Figures 3B and S1B) but were negative or not evaluable due to unspecific background in iIFA.
resulting in specificities of the single antigen ELISA tests of 89%, 96%, and 96%, respectively (Figure S2B). Specificity increased to 98% when the diagnosis was based on the presence of antibodies against at least two viral antigens. The serum that tested positive for two ELISA antigens (N and P) also showed an unspecific background in iIFA, while other positive ELISA reactions did not correlate with unspecific background iIFA patterns (Figure S1B).

To address whether the recombinant BoDV-1 proteins used in the newly established BoDV-1 ELISA cross-react with variegated squirrel bornavirus 1 (VSBV-1), the only other virus within the family Bornaviridae known to infect humans, plasma samples of four rabbits experimentally infected with BoDV-1 or VSBV-1 were tested in a modified version of the ELISA using an anti-rabbit-IgG secondary antibody. All infected animals showed reactivity against at least one antigen (Figure S3). Optical density (OD) values of the ELISA corresponded to the titers of iIFA. As one VSBV-1-infected rabbit tested highly positive for all three BoDV-1 proteins, a cross-reactivity of all antigens used in the BoDV-1 ELISA can be assumed.

In addition, all confirmed BoDV-1-affected individuals were screened for BoDV-1-specific IgM antibodies, which were detected in available samples of six affected individuals (Figure 4). Only in one individual (individual 4) did they precede IgG detection by ELISA by six days, while the iIFA IgG titer at the time of positive IgM was 40.

DISCUSSION

Our case shows that BoVD-1 RNA can be detected in CSF at early stages of the disease when CSF leucocyte counts may still be normal. However, since the viral load in CSF is usually close...
to the lower limit of detection of the applied qRT-PCRs, a negative result does not rule out BoDV-1 infection, as demonstrated in our case by the intermittent detection of BoDV-1 RNA in a series of consecutive CSF samples. The low negative predictive value of PCR from CSF is in accordance with the previously described cases. Post mortem, high viral RNA loads were detected in neuronal tissues of the affected individual, unequivocally confirming BoDV-1 infection. Of note, BoDV-1 was detected in the optic nerve as well as in some peripheral nerves. As the available serological tests for BoDV-1 have limitations—the interpretation of iIFA is subjective, and a line blot assay available for research purposes has limited sensitivity and detects antibodies against N and P proteins only—there is a need for the development of additional serological tests for high-throughput analyses.

Using recombinant BoDV-1 N, X, and P proteins expressed from codon-optimized sequences in E. coli, we established three ELISA systems. In the reported case, anti-BoDV-1-X-IgG showed seroconversion and thus reflected the findings of the iIFA, whereas anti-BoDV-1-N-IgG was not detectable, and anti-BoDV-P-IgG was just above the cutoff. Testing available serum and CSF samples from seven additional affected individuals confirmed anti-BoDV-1-X-IgG as a reliable serological marker but also showed that BoDV-1 antibody profiles were remarkably variable. In this respect, all affected individuals developed IgG responses against P and X. In the current case, anti-X-IgG preceded the detection of anti-P-IgG, whereas in others, anti-P-IgG was detected first. Anti-N-IgG was detectable in only five of eight individuals and usually reached lower levels. In three out of eight affected individuals, IgG antibodies against N, P, or X proteins were already detectable in the first available serum sample after hospital admission. In one immunosuppressed individual, seroconversion occurred before hospitalization (individual 3), while in four affected individuals, BoDV-1-specific IgG was first detected 10 to 68 days after hospitalization. In general, ELISA values reflected iIFA titers in serum and CSF (Figures S1 and S2A). Higher iIFA titers corresponded to higher OD values in ELISA and to the presence of antibodies directed against more than one viral antigen during the course of disease. Overall, the results show that the usage of three different BoDV-1 antigens contributes to a more
sensitive and reliable serological diagnosis of BoDV-1 infection. Inclusion of further BoDV-1 antigens such as the glycoprotein expressed in a eukaryotic system may further improve sensitivity and specificity of the ELISA.

In at least one immunocompromised individual (individual 4), detection of BoDV-1-reactive IgM preceded IgG seroconversion, as measured by ELISA, by some days, but in most affected individuals, IgM became detectable later and at lower levels compared with IgG. Thus, detection of IgM may contribute to early diagnosis in individual cases. The need for early and reliable diagnostic markers remains, in particular because most BoDV-1-infected individuals were already symptomatic at hospital admission and quickly deteriorated thereafter. In addition, one survivor of a BoDV-1 encephalitis described so far, who suffered from BoDV-1 encephalitis as part of a liver transplant, was treated early on with ribavirin. The crucial findings in our case from BoDV-1 encephalitis as part of a liver transplant, was survivor of a BoDV-1 encephalitis described so far, who suffered admission and quickly deteriorated thereafter. In addition, one early diagnosis in individual cases. The need for early and reliable detection of IgM may contribute to early diagnosis in individual cases. The need for early and reliable diagnostic markers remains, in particular because most BoDV-1-infected individuals were already symptomatic at hospital admission and quickly deteriorated thereafter. In addition, one survivor of a BoDV-1 encephalitis described so far, who suffered from BoDV-1 encephalitis as part of a liver transplant, was treated early on with ribavirin. The crucial findings in our case from BoDV-1 encephalitis as part of a liver transplant, was survivor of a BoDV-1 encephalitis described so far, who suffered admission and quickly deteriorated thereafter. In addition, one early diagnosis in individual cases. The need for early and reliable diagnostic markers remains, in particular because most BoDV-1-infected individuals were already symptomatic at hospital admission and quickly deteriorated thereafter. In addition, one survivor of a BoDV-1 encephalitis described so far, who suffered from BoDV-1 encephalitis as part of a liver transplant, was treated early on with ribavirin. The crucial findings in our case from BoDV-1 encephalitis as part of a liver transplant, was survivor of a BoDV-1 encephalitis described so far, who suffered admission and quickly deteriorated thereafter. In addition, one early diagnosis in individual cases. The need for early and reliable diagnostic markers remains, in particular because most BoDV-1-infected individuals were already symptomatic at hospital admission and quickly deteriorated thereafter. In addition, one survivor of a BoDV-1 encephalitis described so far, who suffered from BoDV-1 encephalitis as part of a liver transplant, was treated early on with ribavirin.

The crucial findings in our case from BoDV-1 encephalitis as part of a liver transplant, was survivor of a BoDV-1 encephalitis described so far, who suffered admission and quickly deteriorated thereafter. In addition, one early diagnosis in individual cases. The need for early and reliable diagnostic markers remains, in particular because most BoDV-1-infected individuals were already symptomatic at hospital admission and quickly deteriorated thereafter. In addition, one survivor of a BoDV-1 encephalitis described so far, who suffered from BoDV-1 encephalitis as part of a liver transplant, was treated early on with ribavirin.

**Limitations of study**

The present study was performed on a rather small number of samples, which is attributed to the low incidence of BoDV-1 infection. Our main conclusion—that reactivity against two viral antigens contributes to BoDV-1 diagnosis—needs to be validated in a larger panel of samples. In addition, the positive predictive value of the ELISA with its current specificity is limited due to the very low prevalence of the disease. A further limitation is the cross-reactivity of our BoDV-1 ELISA with VSBV-1 serology, which was only demonstrated for rabbit plasma samples. The modified rabbit BoDV-1 ELISA has not been formally validated with cutoffs using a panel of negative rabbit sera.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials and availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - RNA extraction and PCR
  - Indirect immunoﬂuorescence assay (IIFA)
  - Protein expression, silver staining and western blots
  - Enzyme-linked immunosorbent assay (ELISA)
  - Generation of BoDV-1/VSBV-1-reactive rabbit sera
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100499.
11. Jordan, I., Briese, T., Averett, D.R., and Lipkin, W.I. (1999). Inhibition of Borna disease virus replication by ribavirin. J. Virol. 73, 7903–7906.
12. Hoffmann, B., Tappe, D., Höper, D., Herden, C., Boldt, A., Mawrin, C., Niederstraßer, O., Müller, T., Jenckel, M., van der Grinten, E., et al. (2015). A Variegated Squirrel Bornavirus Associated with Fatal Human Encephalitis. N. Engl. J. Med. 373, 154–162.
13. Dreier, J., Störmer, M., and Kleesiek, K. (2005). Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J. Clin. Microbiol. 43, 4551–4557.
14. Morrissey, J.H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117, 307–310.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Cy3 AffiniPure Goat Anti-Human IgG (H+L) | Jackson ImmunoResearch | Cat# 109-165-003; RRID: AB_2337718 |
| Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) | Jackson ImmunoResearch | Cat# 111-165-003; RRID: AB_2338000 |
| Polyclonal Rabbit Anti-Human IgG/HRP antibody | Agilent | Cat# P021402-2; RRID: AB_2893418 |
| Polyclonal Rabbit Anti-Human IgM/HRP antibody | Agilent | Cat# P021502-2; RRID: AB_2893505 |
| Goat Anti-Rabbit IgG (H+L)-HRP Conjugate antibody | Bio-Rad | Cat# 172-1019; RRID: AB_11125143 |
| **Bacterial and virus strains** | | |
| BoDV-1, 2 strains isolated from human CNS tissue | Institute of Clinical Microbiology and Hygiene, University Hospital Regensburg, Germany | N/A |
| **Biological samples** | | |
| Plasma samples of 2 BoDV-1 infected rabbits. The animal experiments were evaluated and approved by the ethics committee of the State Office of Agriculture, Food safety, and Fishery in Mecklenburg – Western Pomerania (LALLF M-V: LVL MV/TSD/ 7221.3-2-010/18). | Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald – Insel Riems, Germany | N/A |
| Plasma samples of VSBV-1 infected rabbits. The animal experiments were evaluated and approved by the ethics committee of the State Office of Agriculture, Food safety, and Fishery in Mecklenburg – Western Pomerania (LALLF M-V: LVL MV/TSD/ 7221.3-2-010/18). | Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald – Insel Riems, Germany | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| BoDV-1 nucleocapsid Np40 protein, His-tagged | Mikrogen | N/A |
| BoDV-1 phosphoprotein Pp23, His-tagged | Mikrogen | N/A |
| BoDV-1 X protein Xp10, GST-tagged | Mikrogen | N/A |
| PBS | GibCO | Cat# 14190-094 |
| Tween 20 | Caelo | Cat# 3472 |
| Fat free milk powder | Heirler | N/A |
| RF Absorbent | Virion/Serion | Cat# Z2000 |
| Mikrogen TMB | Mikrogen | Cat# 12003 |
| Mikrogen Stopp (24.9% H3PO4) | Mikrogen | Cat# 12004 |
| TRIS buffer with Tween 20, pH 8.0 | Sigma-Aldrich | Cat# T9039-10PAK |
| TapPath™-1-Step RT-qPCR Master Mix, CG | Applied Biosystems | Cat# A15299 |
| NxtScript Reaction Mix | Roche/Penzberg | Cat# 07368372103 |
| NxtScript RT 85U/μL | Roche/Penzberg | Cat# 07371527103 |
| ROX-Reference Dye | Life technologies | Cat# 12223-012 |
| RNase free DEPC water | Roth | Cat# 143.3 |
| PageRuler Prestained Protein Ladder | Thermo Fisher Scientific | Cat# 26617 |
| Roti-Block | Roth | Cat# A151.2 |

(Continued on next page)
Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Markus Bauswein (markus.bauswein@ukr.de).

Materials and availability
BoDV-1 N, X, and P proteins generated in this study will be made available on request, but we may require a completed materials transfer agreement if there is potential for commercial application.

Data and code availability
ELISA datasets generated in this study have been uploaded to Mendeley Data: https://doi.org/10.17632/wpvkdhmgz4.1.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Gender and age of the patients are not revealed for ethical reasons. Patients 1 to 5 of this publication correspond to patients 1 to 5 published by Niller et al. Patient 6 is the kidney transplant recipient 1 published by Schlottau et al. Patient 7 corresponds to patient 8 published by Niller et al. The use of retrospective patient samples has been approved by the ethical commission of the Faculty for Medicine, University of Regensburg (reference number 18-1248-101).

METHOD DETAILS

RNA extraction and PCR

RNA extraction and BoDV-1 specific RT-qPCR were performed following a recently published protocol. Briefly, total RNA from native samples was extracted using EZ1 Virus Mini Kit v2.0 on an EZ1 Advanced XL system (QIAGEN), according to the manufacturer’s instructions. BoDV-1 RNA was detected using two real-time RT-qPCR assays: mix 1 and 6 were used for amplification of RNA from BoDV-1 x/phosphoprotein gene and matrix/glycoprotein gene, respectively. RT-qPCR was performed on a StepOnePlus System (Applied Biosystems). In vitro-transcribed RNA molecules were used as standards for quantification. For extraction and inhibition control, samples were spiked with MS2 phage solution and MS2 phage RT-qPCR was performed. All precautions of accredited laboratories, particularly separate rooms for nucleic acid extraction, amplification, and detection, were taken to avoid any cross-contamination. All samples were tested in two replicates. Positive single values of only one replicate were considered negative. RNA copies were calculated as mean of two positive replicates.

Indirect immunofluorescence assay (iIFA)

Indirect immunofluorescence assay (iIFA) was performed as previously described. Briefly, Vero cells persistently infected with BoDV-1 were mixed 1:2 with uninfected Vero cells and cultured overnight in 96-well microtiter plates (ibidi) to achieve confluent cell layers. Wells with uninfected Vero cells served as negative controls. After removal of the supernatant, plates were dried for 2h and fixed at 80°C for 2h. Thereafter, heat-inactivated patient samples were added in a 2-fold dilution series (starting with 1:20 for sera and 1:2 for CSF samples, dilution in TRIS buffer). After incubation for 1 h, plates were washed three times with PBS and incubated with a 1:200 dilution of Cy-3-conjugated polyclonal rabbit anti-human-IgG antibody for 1 h. For rabbit samples, a 1:500 diluted Cy3-conjugated goat anti-rabbit-IgG antibody was used. After a final washing cycle, the assays were analyzed using fluorescence microscopy. Samples with characteristic fluorescing spots in the nuclei of BoDV-1-infected Vero cells were considered positive. When similar fluorescence signals were detected in non-infected and BoDV-1-infected Vero cells, samples were considered unspecific.

Protein expression, silver staining and western blots

Sequences for BoDV-1 nucleoprotein (N), X protein and phosphoprotein (P) were codon-optimized for expression in E. coli and cloned into expression vectors pET30a, pGEX6P, and pET82, respectively. His-tagged N and P protein as well as GST-tagged X protein were purified by chromatography.

Purity of proteins was demonstrated by silver staining after SDS-PAGE according to a published protocol. Lanes were loaded with 200 ng of His-tagged Np40, 200 ng of GST-tagged Xp10 and 100 ng of His-tagged Pp23 protein. 5% and 15% polyacrylamide were used for stacking and separating gel, respectively.

For western blots, an SDS-PAGE was performed using a 12% polyacrylamide separating gel. Lanes were loaded with 100 ng Np40, 200 ng Xp10 and 100 ng Pp23. After separation, proteins were blotted onto a 0.2 μm nitrocellulose membrane using a wet tank transfer system. Membranes were blocked with Roti Block (containing Tween 20) and then stained with a 1:100 dilution of selected samples (plasma, serum, CSF). A horseradish-peroxidase-conjugated polyclonal rabbit anti-human-IgG diluted 1:3,000 was used as secondary antibody. Proteins were detected by chemiluminescence.

Enzyme-linked immunosorbent assay (ELISA)

Recombinant BoDV-1 proteins diluted in PBS were used to coat Nunc MaxiSorp plates (Thermo Fisher Scientific). N and X proteins were coated with 800 ng/well (in 100 μL PBS), P protein with 150 ng/well (in 100 μL PBS) at 4°C overnight and then blocked with 200 μL 5% fat-free milk powder in PBS with 0.1% Tween 20 at room temperature (RT) for 1 h. After washing three times with 200 μL PBS containing 0.1% Tween 20 (PBS-T), 100 μL of diluted patient samples were added. For IgG-ELISA, all samples (serum, plasma, CSF) were diluted 1:100 in 1% fat-free milk powder in PBS with 0.1% Tween 20. For IgM-ELISA, IgG was absorbed by pre-incubation of samples with RF Absorbent (Virion/Serion) for 15 min at RT according to the manufacturer’s protocol. Plates were incubated for 1 h at RT and then washed nine times with 200 μL PBS-T. Subsequently, 50 μL horseradish-peroxidase-conjugated polyclonal rabbit anti-human-IgG diluted 1:5,000 in PBS-T were added as secondary antibody. To test for cross-reactivity with VSVB-1, samples of two BoDV-1- and two VSBV-1-infected rabbits were stained with a 1:3,000 diluted goat anti-rabbit-IgG antibody. For IgM-ELISA, 50 μL horseradish-peroxidase-conjugated polyclonal rabbit anti-human-IgM diluted 1:3,000 in PBS-T were used. After 1 h incubation at RT, plates were washed nine times with 200 μL PBS-T. 50 μL substrate solution (TMB) were added to each well, incubated for 4 min at RT, and stopped by adding 50 μL of stop solution (phosphoric acid). After 5 min of incubation, optical density was determined at
450 nm (OD450) and 630 nm (OD630) in three technical replicates using a plate reader (Microplate Reader Model 680, Bio-Rad). For evaluation, OD630 background values were subtracted from OD450 values. OD of blank was subtracted from all OD values. To control for inter-assay variability, all sample values were normalized to an external standard sample. Cut-offs for each ELISA system were determined using serum samples from 24 patients that had been tested negative by iIFA (IgG) and/or whose brain tissue had been tested negative by RT-qPCR. Cut-offs were calculated by the sum of arithmetic mean plus 6 standard deviations.

**Generation of BoDV-1-/VSBV-1-reactive rabbit sera**

Neonatal rabbits were inoculated within 24h after birth with either VSBV-1 or BoDV-1. All animals received $5 \times 10^3$ tissue infectious dose 50 (TCID$_{50}$) intracerebrally. The animals were monitored daily for clinical signs and euthanized at different time points regarding a defined humane endpoint. Tissues as well as sera were collected for serological and virological analysis. The animal experiments were evaluated and approved by the ethics committee of the State Office of Agriculture, Food safety, and Fishery in Mecklenburg – Western Pomerania (LALLF M-V: LVL MV/TSD/ 7221.3-2-010/18). All procedures were carried out in approved biosafety level 3 (BSL3) facilities.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Graphs and descriptive statistical analysis were created using GraphPad Prism V9.2.0.