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Survey of Borna disease virus infection in red foxes (*Vulpes vulpes*) in Austria

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Summary

In certain regions of central Europe, the bicolored white-toothed shrew (*Crocidura leucodon*) is the reservoir host of Borna disease virus 1 (BoDV-1), an agent with zoonotic potential that causes a non-purulent meningoencephalitis in some domestic mammals. *Crocidura leucodon* is endemic in Austria and forms part of the diet of the red fox (*Vulpes vulpes*), so it is likely that red foxes are exposed to BoDV-1. Borna disease has been diagnosed in domestic mammals in the federal states of Vorarlberg, Upper Austria and Styria and BoDV-1 infections were found in shrews in Upper Austria. We now report an investigation of the red fox for BoDV infection in Austrian regions where BoDV has occurred in the past.

We tested brain samples (n=365) for the presence of bornavirus RNA by two RT-qPCR assays. We also tested serum samples (n=271) for BoDV-1-reactive antibodies using an indirect immunofluorescence assay (IFAT). Samples with positive reactions were re-analysed with a modified version of the IFAT.

Zusammenfassung

Erhebung zum Vorkommen des Borna-Virus in Rotfüchsen (*Vulpes vulpes*) in Österreich

Einleitung

Die Feldspitzmaus (*Crocidura leucodon*) ist der Reservoirwirt des Borna disease virus 1 (BoDV-1), dem Erreger der Bornaschen Krankheit, die sich als nichteitrige Meningoenzephalitis bei Pferden, Schafen und anderen Säugetieren in bestimmten endemischen Regionen in Mitteleuropa darstellt und zoonotischen Charakter aufweist. *Crocidura leuacodon* ist in Österreich heimisch und gleichzeitig Nahrungsbestandteil von Füchsen (*Vulpes vulpes*), somit ist ein direkter Kontakt von Füchsen zu BoDV während der Beutejagd anzunehmen. In Österreich sind bis dato einzelne Fälle von Bornascher Krankheit bei Pferden und Hunden aus Vorarlberg, Oberösterreich und der Steiermark bekannt. Ziel dieser Studie war es, Füchse aus diesen drei Bundesländern auf eine mögliche BoDV-Infektion zu untersuchen.

Material und Methoden

Insgesamt 365 Gehirnproben von Füchsen aus Oberösterreich, der Steiermark und Vorarlberg wurden mittels zwei verschiedener RT-qPCR Tests auf das Vorhandensein von BoDV spezifischer RNA untersucht, einem für BoDV-1 spezifischen Ansatz und einem Ansatz, welcher ein weites Spektrum von Orthobornaviren detektiert. Zudem wurden 271 Serumproben von Füchsen aus Vorarlberg mittels Indirektem Immunfluoreszenztest (IFAT) auf das Vorhandensein von BoDV-1-reaktiven Antikörpern untersucht. Proben mit positiven Testreaktionen wurden in einem modifiziertem IFAT ein weiteres Mal getestet.

Ergebnisse

Alle Gehirnproben wurden in der RT-qPCR mit negativem Ergebnis getestet. Drei Serumproben (1,1 %) waren im Screeningtest auf BoDV-1-infizierten Verozellen positiv, bei diesen Proben wurde der Test mittels modifiziertem IFAT auf SK6 Zellen wiederholt und es konnte nur bei einer Probe das serologische Ergebnis bestätigt werden. Da im

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All brain samples were negative for BoDV-1 and pan-bornavirus RT-qPCR. Three serum samples (1.1 %) gave positive results in the screening test on BoDV-1-infected Vero cells but only one of them showed comparable IFAT titres in the confirmatory test on SK6 cells. As bornavirus RNA was not detectable in the brain of the animal in question, we assume that the seroreactivity resulted from an unspecific cross-reaction.

We conclude that the prevalence of bornavirus infections in red foxes in Austria is at most very low, even in regions where BoDV-1 or BoDV-2 previously occurred. It remains unclear whether foxes are a dead-end host for BoDV-1 and there is a need for further investigations into the aetiology of encephalitis in the red fox.

Gehirn des betreffenden Tieres keine virusspezifische RNA nachgewiesen werden konnte, muss die ermittelte Seroreaktivität als wahrscheinlich unspezifisch gewertet werden.

Diskussion

Die Studienergebnisse lassen vermuten, dass die Prävalenz von Bornavirusinfektionen bei Füchsen allenfalls sehr gering ist, auch in österreichischen Regionen, aus denen Fälle von BoDV-1 oder BoDV-2 bekannt sind. Ob Füchse an einer BoDV-Infektion erkranken können, muss in weiterführenden Studien untersucht werden.

Abbreviations: BoDV = Borna disease virus; Cq = cycle of quantification; IFAT = indirect immunofluorescence assay; NTC = negative control template; PBS = phosphate-buffered saline; RT-qPCR = Quantitative reverse transcription polymerase chain reaction

Introduction

Borna disease virus 1 (BoDV-1, family *Bornaviridae*, order *Mononegavirales*) is the causative agent of Borna disease, a progressive meningoencephalitis of horses, sheep and other domestic mammals, in certain regions of central Europe (Staeheli et al. 2000; Kuhn et al. 2015). BoDV-1 RNA and antigen have been detected in the brains of human patients with severe encephalitis and bornavirus-reactive antibodies were found in their sera and cerebrospinal fluid (Schlottau et al. 2018; Niller et al. 2020), showing that the virus has zoonotic potential.

Borna disease is sporadically diagnosed in horses, sheep und South American camelids and there are rare reports of the disease in other mammalian species, such as cats, dogs, cattle and rabbits (Kinnunen et al. 2013). In the dead-end host of the virus, infection causes an immune-mediated pathogenesis resulting in a non-purulent encephalitis that leads to a broad spectrum of neurological symptoms (Caplazi & Ehrensperger 1998; Stitz et al. 2002; Dürrwald et al. 2016). The bicolored white-toothed shrew (*Crocidura leucodon*) is the reservoir host of BoDV-1 (Dürrwald et al. 2014). Infected shrews have a high viral load in many organs. They do not develop pathological alterations or clinical signs but shed the virus via various routes including saliva, urine, skin and faeces (Nobach et al. 2015). The incidence of Borna disease shows a seasonal variation with higher numbers of cases in spring and early summer and yearly fluctuating disease peaks (Dürrwald et al. 2006). BoDV-1 is endemic in parts of eastern and southern Germany, the eastern part of Switzerland, Liechtenstein and parts of the Austrian federal states of Upper Austria and Vorarlberg (Niller et al. 2020; Ebinger et al. 2024). There have been cases of BoDV-1 infection in Upper Austria in four horses (2015–2016) and in several shrews (Weissenböck et al. 2017) and in Vorarlberg in two horses (1993 and 1997) and one dog (1994) (Weissenböck et al. 1998a, b). BoDV-2, a close relative of BoDV-1, was discovered in a single horse that died of neurological disease in Styria in 1998 (Nowotny et al. 2000). Figure 1 shows the verified cases of BoDV in Austria.

The European population of the red fox (*Vulpes vulpes*, hereafter also 'fox') has increased over the last decades and the species has invaded urban areas (Deplazes et al. 2004). In the hunting period 2021–2022, about 69,875 foxes were shot and 3,681 were found dead (game casualties) in Austria (Statistik Austria 2022). As foxes can be carriers of zoonotically important pathogens and sources of infection for humans, e.g. *Echinococcus multilocularis* (Gottstein et al. 2015) and *Rabies lyssavirus* (Matouch 2008), it is important to study the fox population and monitor it for the incidence of potentially zoonotic or emerging infectious diseases. The aetiology of non-suppurative encephalitis, usually caused by viral infection (Grant & Sameh 2007), in foxes is often unclear. In central Europe, canine distemper virus is the most frequently detected pathogen in red foxes with non-suppurative brain lesions (Höche et al. 2022). Other potential viral causes of encephalitis are *Rabies lyssavirus* (Matouch 2008), Aujeszky's disease virus (SHV-1) (Moreno et al. 2020), canine adenovirus type 1 (Walker et al. 2016) and circoviruses (Bexton et al. 2015) but bacteria such as *Streptococcus canis* (Höche et al. 2022) and parasites, e.g. *Toxoplasma gondii* (Verin et al. 2013), may also cause brain lesions with a purulent or granulomatous character.

As the bicolored white-toothed shrew forms part of the diet of red foxes in Austria (Spitzenberger & Bauer 2001), foxes sharing the habitat with BoDV-1-infected shrews may be exposed to the viral agent (Kauhala et al. 1998; Kidawa & Kowalczyk 2011). It is thus possible that foxes may serve as a sentinel species for the presence of BoDV-1 in wildlife. We now report an investi-

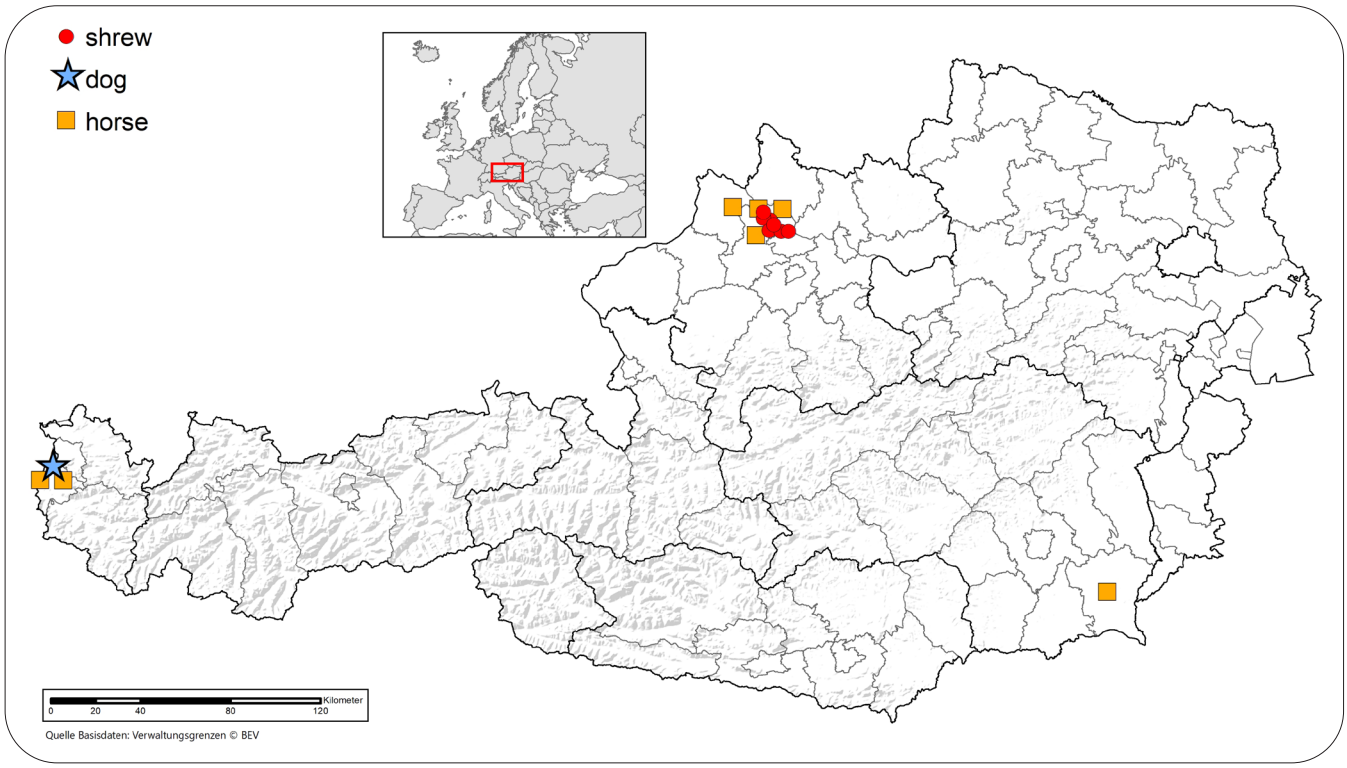

Fig. 1: Geographical distribution of previous cases of Borna disease virus 1 (BoDV-1) and BoDV-2 in Austria. / Geografische Darstellung bestätigter Fälle von Borna Disease Virus in Österreich.

Tab. 1: Number, origin and date of collection of the samples from red foxes (*Vulpes vulpes*) tested for Borna disease virus by RT-qPCR and indirect immunofluorescence assay. / Übersicht über die Anzahl, Herkunft und Probenahmedatum der auf Borna disease virus untersuchten Proben von Füchsen (*Vulpes vulpes*) mittels RT-qPCR und indirektem Immunfluoreszenztest.

Region	Brain samples (n) / Gehirnproben (n)	Serum samples (n) / Serumproben (n)	Sample collection date (month/year) / Probennahmedatum (Monat/Jahr)
Styria / Steiermark	70	-	01/2015–12/2016
Upper Austria / Oberösterreich	29	-	01/2015–08/2016
Vorarlberg	266	271 (204 paired with the brain sample / von 204 Füchsen wurden sowohl Gehirn- als auch Serumproben untersucht)	11/2013 – 12/2016 most from November to February / die meisten Proben zwischen November und Februar
Total / Gesamt	365	271	11/2013 – 12/2016

gation of BoDV-1 and -2 infection in free-ranging red foxes in regions of Austria where BoDV-1/-2 infections previously occurred.

Materials and methods

Sample origin

We investigated 365 brain tissue samples and 271 blood serum samples from red foxes. The samples originated from three Austrian federal states in which BoDV-1 or BoDV-2 occurred in the past: Upper Austria, Vorarlberg and Styria, as described in Table 1. The majority of the brain tissue samples and all of the blood samples were obtained from a prevalence study on *Echinococcus multilocularis* (2013–2016) in Vorarlberg. Additional brain tissue samples came from the national rabies monitoring programme of 2015 and 2016 (Styria: n=70, Upper Austria: n=29, Vorarlberg: n=9). These samples had previously tested negative for rabies. The origins of the samples are shown in Figures 2 (brain samples) and 3 (blood samples). Most animals were hunted during the winter hunting season (November–February); only eight animals were found dead and three killed because they showed behaviour

indicative of rabies. Table 1 presents the collection dates of the samples. The tissue samples were taken from 160 juvenile animals, defined as younger than one year, and 96 adults, with the age not specified for 109 samples; 132 of the animals were female and 125 male, with the sex not specified for 108 samples. The blood samples came from 180 juveniles and 90 adults, with the age not specified for one sample; 128 of the animals sampled were female and 142 male, with the sex not specified for one sample. Carcasses in a good state of preservation were sent to the laboratory, where sampling was conducted. Brain tissue (approx. 2 g), mainly brain stem, was taken with a spoon accessing from the *Foramen magnum* and blood samples (approx. 2 ml)

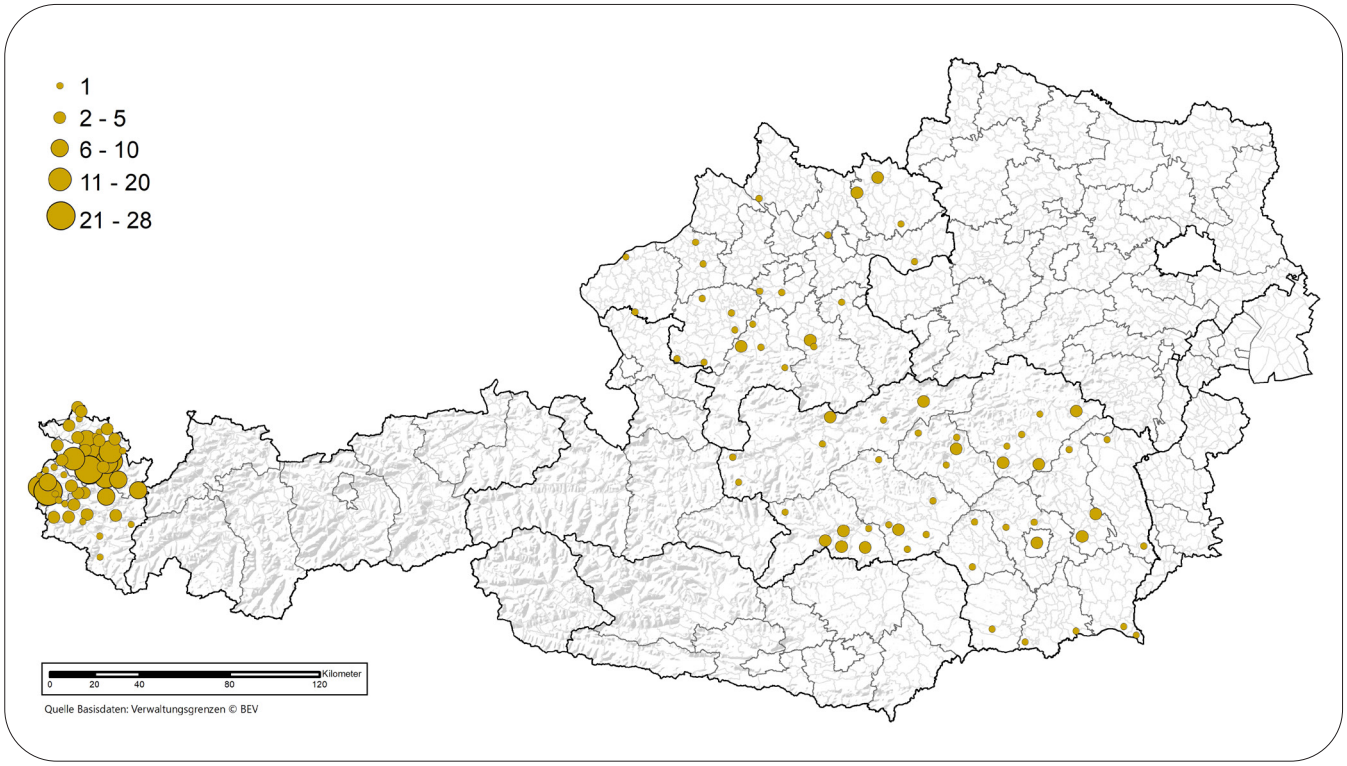

Fig. 2: Origin of 365 brain samples from red foxes *(Vulpes vulpes)* that tested negative by RT-qPCR for Borna disease virus 1 (BoDV-1) and other Orthobornaviruses, originating from Styria, Upper Austria and Vorarlberg. The diameters of the dots represent the number of samples per municipality. / Geographische Darstellung der 365 Gehirnproben von Füchsen *(Vulpes vulpes)*, welche mittels RT-qPCR negative auf Borna Disease Virus 1 (BoDV-1) und andere Orthobornaviren getestet wurden. Die Proben stammen aus den österreichischen Bundesländern Steiermark, Oberösterreich und Vorarlberg. Der Durchmesser der Kreisfläche gibt die Probenanzahl pro Gemeinde an.

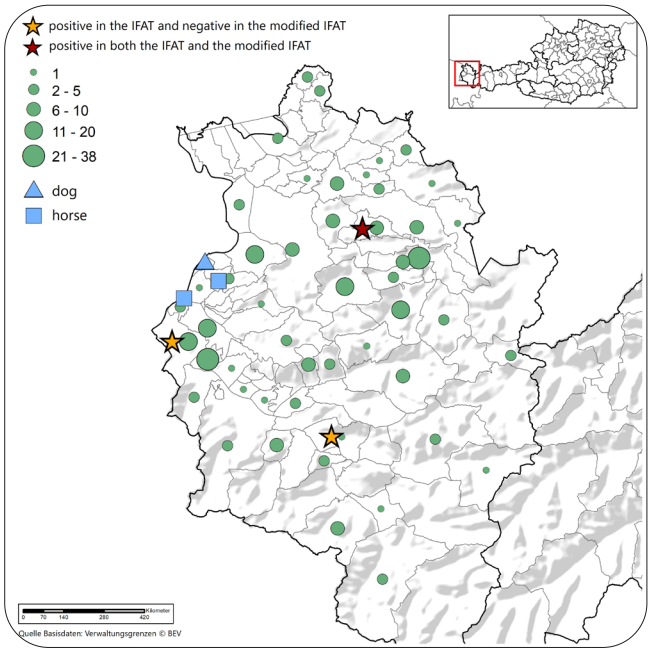

Fig. 3: Origin of serum samples (n=271) from red foxes *(Vulpes vulpes)*, originating from Vorarlberg, tested for Borna disease virus 1 (BoDV-1)-reactive antibodies using an indirect immunofluorescence assay (IFAT) and previous cases of BoDV-1 in Vorarlberg. The sample shown in red gave positive results in both the IFAT and the modified IFAT; the two samples shown in yellow gave positive results in the IFAT and negative in the modified IFAT. The samples shown in green gave negative results in the IFAT. The diameters of the dots represent the numbers of samples per municipality. / Geographische Darstellung der untersuchten Serumproben (n=271) von aus Vorarlberg stammenden Füchsen *(Vulpes vulpes)*, welche mittels Indirektem Immunfluoreszenztest (IFAT) auf das Vorhandensein von Borna Disease Virus 1 (BoDV-1)-reaktiven Antikörpern untersucht wurden sowie bestätigter Fälle von BoDV-1 in Vorarlberg. Die rot eingezeichnete Probe wurde sowohl im IFAT als auch im modifizierten IFAT positiv getestet, die zwei gelb dargestellten Proben waren im IFAT positiv und im modifizierten IFAT negativ und die Proben in Grün hatten im IFAT negative Ergebnisse. Der Durchmesser der Kreisfläche gibt die Probenanzahl pro Gemeinde an.

either from the heart or from blood accumulation within the body cavities. Samples were stored in tubes at -15 °C until investigation. The corresponding brain sample was available for 204 blood samples and not available for 67 blood samples.

RNA extraction and detection of bornavirus RNA by RT-qPCR

We pre-treated tissue samples as follows: 25–30 mg of each brain sample was transferred to a 2 ml tube with a screw cap containing 300 mg glass beads, size 0.1 mm (Retsch GmbH, Haan, Germany). One ml of PowerBead Solution (Qiagen GmbH, Hilden, Germany) was added and the sample homogenized for 4 min at 25 Hz using a TissueLyzer II (Qiagen GmbH). The tube was centrifuged for 1 min at 14,000 rpm and either 200 µl of the supernatant was extracted immediately, or 300 µl of the supernatant was transferred to a fresh tube and stored at -15 °C until extraction. After thawing, these samples were centrifuged for 1 min at 14,000 rpm and 200 µl of the supernatant was used for extraction. Viral RNA was extracted with the IndiMag® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) and the KingFisher™ Flex Purification System (Thermo Fisher Scientific, Vienna, Austria), following the manufacturers' instructions; the elution volume was 60 µl. BoDV-1-positive tissue samples from a horse were used as a positive control for preparation and extraction (dilution 1:4 with PowerBead Solution). Extracts were stored at 4 °C (short-term) or at -15 °C (long-term) and subjected to two RT-qPCR assays.

For the specific detection of BoDV-1, we used *p40* primers and probes targeting the BoDV-1 nucleoprotein N (Schindler et al. 2007). Each reaction contained 6.25 µl 2x qScript XLT one-step RT-qPCR ToughMix (Quanta BioSciences, Gaithersburg, USA), 1.0 µl of the primer-probe-mix (10 pmol/µl primer and 2.5 pmol/µl probe), 1.0 µl of beta-actin mix (2.5 pmol/µl primer and probe) (Toussaint et al. 2007), 1.75 µl RNase-free water and 2.5 µl RNA template or RNase-free water for the no template control (NTC) in a total volume of 12.5 µl. The thermal programme consisted of 1 cycle of 50 °C for 10 min and 95 °C for 1 min, followed by 45 cycles of 95 °C for 10 s, 57 °C for 30 s and 68 °C for 30 s. All RT-qPCRs were performed using a CFX96 Touch Real-Time PCR Detection System with the CFX Maestro V1-1 Software (Bio-Rad Laboratories, Vienna, Austria) or an Mx3005P qPCR System with the MxPro qPCR Software (Agilent, Santa Clara, USA).

To detect a broad spectrum of orthobornaviruses, including BoDV-1 and BoDV-2, we used panBorna mix 7.2, as described (Schlottau et al. 2018). The RT-qPCR was performed using either the AgPath-ID™ One-Step RT-PCR Reagents or the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (both Thermo Fisher Scientific, Vienna, Austria). Each reaction contained 0.5 µl of enzyme mix and 6.25 µl of reaction mix, 0.94 µl of each primer (10 pmol/µl) and 0.32 µl of probe (10 pmol/µl), 1.25 µl of beta-actin mix (2.5 pmol/µl primer and probe), 0.3 µl RNase-free water and 2.0 µl RNA template or NTC in a total volume of 12.5 µl. With AgPath-ID™ One-Step RT-PCR Reagents, the thermal programme consisted of 1 cycle of 45 °C for 10 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 45 s. The thermal programme for the SuperScript™ III One-Step RT-PCR System assay consisted of 1 cycle of 50 °C for 15 min and 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. All RT-qPCRs were performed with the CFX96 Touch Real-Time PCR Detection System and the CFX Maestro V1-1 Software.

We used primers and probes targeting the beta-actin gene (Toussaint et al. 2007) to assess the RNA quality of the samples and the efficacy of the RT-qPCR. We used RNA dilutions from BoDV-1-positive tissue as positive controls. Results were determined as cycle of quantification (Cq) values.

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

Serum samples were tested for the presence of bornavirus-reactive antibodies by an immunofluorescence antibody test (IFAT) according to described protocols (Zimmermann et al. 2014; Schlottau et al. 2018). Confluent overnight cultures of either non-infected Vero cells or non-infected Vero cells mixed with 30 % Vero cells persistently infected with BoDV-1 isolate Z65-1 (Niller et al. 2020) were fixed with 4 % paraformaldehyde and permeabilized with 0.5 % Triton X-100. Two-fold dilution series of samples were prepared in Tris-HCl buffer with Tween 20 (T9039; Sigma-Aldrich, Schnelldorf, Germany) and 50 µl of each dilution were added in parallel to the BoDV-1-positive and -negative wells. After incubation for one hour, the plates were washed three times with phosphate-buffered saline (PBS), followed by incubation with goat anti-dog-IgG Cy3 conjugate (Jackson Immunoresearch, Ely, UK) for another hour. After a final washing cycle, endpoint titres were determined by fluorescence microscopy. We considered a dilution as positive if we observed a staining pattern typical for bornaviruses (particularly intranuclear inclusions) in the expected 30 % BoDV-1-infected cells of the test well but not in the corresponding control well. Samples with positive reactions were re-analysed using a modified version of the IFAT. Confluent uninfected and BoDV-1 Z65-1-infected porcine SK6 cells were air-dried for 2.5 hours and incubated with the diluted sera and conjugate as described above. SK6 cells were selected due to their stability upon air drying. A potentially BoDV-1-specific staining was assumed if both assays yielded reactivities with comparable titres. A mixture of monospecific polycloncal rabbit sera directed against BoDV-1 N and

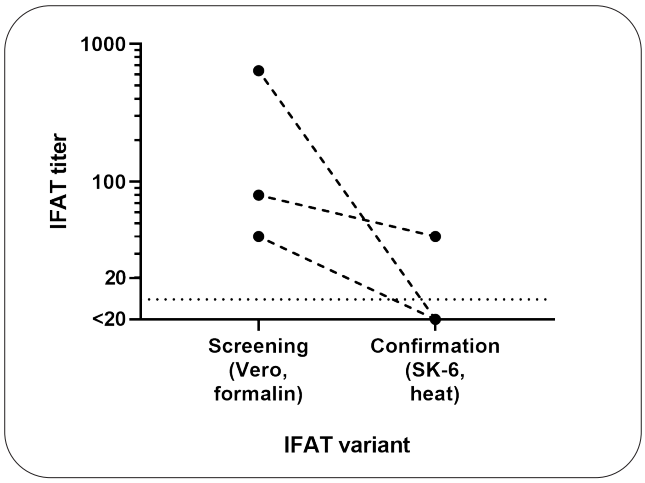

Fig. 4: Detection of bornavirus-reactive antibodies. We used the immunofluorescence antibody test (IFAT) to test serum samples from red foxes *(Vulpes vulpes)* for bornavirus-reactive antibodies. Initial screening of all 271 samples was performed using formalin-fixed Vero cells. Three samples gave positive reactions and were subsequently tested using heat-fixed SK6 cells. Only samples with comparable titres in both assays were considered to show potentially bornavirus-specific seroreactivity. / Untersuchung auf Bornavirus-reaktive Antikörper. Serumproben von Füchsen *(Vulpes vulpes)* wurden mittels indirekter Immunfluoreszenz (IFAT) auf Bornavirus-reaktive Antikörper getestet. 271 Proben wurden mit einem Screeningtest auf formalinfixierten Verozellen untersucht. Drei Proben zeigten eine positive Reaktion und wurden einem Bestätigungstest auf hitzefixierten SK6 Zellen unterzogen. Nur Proben mit vergleichbaren Antikörpertitern in beiden Tests wurden als Proben mit potentieller Bornavirus-spezifischer Seroreaktivität gewertet.

P protein (Zimmermann et al. 2014) was used to confirm the antigen expression of the cells. The crossreactivity of the anti-dog-IgG conjugate with other members of the order Carnivora was confirmed by including a bornavirus-reactive cat serum (Figure 5).

Results

With the initial screening test on BoDV-1-infected Vero cells, three out of 271 (1.1 %) serum samples showed a positive reactivity with low to moderate IFAT titres of 40 (animal 236), 80 (animal 111) and 640 (animal 212) on PFA-fixed Vero cells. However, only animal 111 showed a comparable titre in the confirmatory IFAT on heat-fixed SK6 cells, with animals 236 and 212 (the sample with the highest titre on Vero cells) negative in this assay (Figure 4; Figure 5). No bornavirus RNA was detected in any of the 365 brain samples from red foxes tested using the BoDV-1-specific or the pan-Borna RT-qPCR assay. Brain samples were available for two of the animals with seroreactivity, animals 111 and 212. Both of these brain samples similarly tested negative by RT-qPCR.

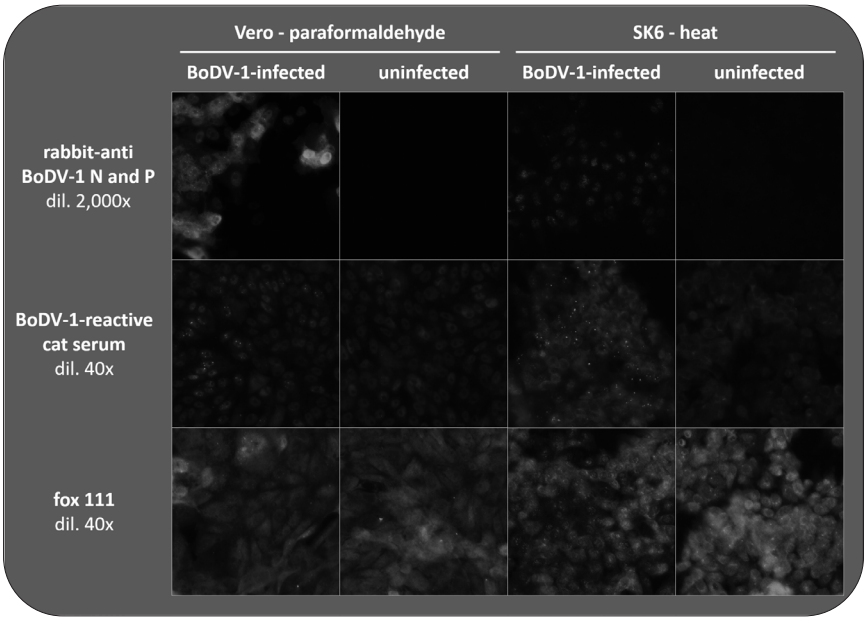

Fig. 5: Detection of bornavirus-reactive antibodies by immunofluorescence antibody test (IFAT). IFAT was performed using paraformaldehyde-fixed Vero cells and heat-fixed SK6 cells. Samples were tested in parallel on BoDV-1-positive cultures containing approximately 30 % infected cells and on uninfected control cells. A mixture of monospecific polyclonal rabbit hyperimmune sera directed against the BoDV-1 N and P protein was used to confirm the presence of antigen in the cells (upper panel; 2,000-fold dilution). The cross-reactivity of the anti-dog-IgG conjugate with other members of the order Carnivora was confirmed by including a bornavirus-reactive cat serum (middle panel; 40-fold dilution). Of the fox samples, only serum 111 showed a positive, albeit weak, reactivity on both cell types (bottom panel; 40-fold dilution). A positive reactivity is characterized by a granular nuclear staining (or nuclear and cytoplasmic staining in case of the hyperimmune sera) that is observed in the BoDV-1-infected but not in the uninfected control wells. / Untersuchung auf Bornavirus-reaktive Antikörper mittels Immunfluoreszenz-Antikörpertest (IFAT). Für den IFAT wurden paraformaldehydfixierte Vero-Zellen und hitzefixierte SK6 Zellen verwendet. Die Proben wurden auf BoDV-1-positiven Kulturen getestet, welche etwa 30 % infizierte Zellen enthielten, und gleichzeitig auf nicht infizierten Kontrollzellen. Zur Bestätigung der Antigenexpression wurde eine Mischung aus monospezifischen polyklonalen Kaninchen-Hyperimmunseren gegen das BoDV-1 N und P Protein verwendet (obere Reihe, 2000-fache Verdünnung). Die Kreuzreaktivität des anti-Hund-IgG Konjugats mit anderen Vertretern der Ordnung Carnivora wurde mittels eines Bornavirus-reaktivem Katzenserums bestätigt (mittlere Reihe; 40-fache Verdünnung). Von den untersuchten Füchsen zeigte nur Serumprobe 111 eine schwach positive Reaktivität auf beiden Zelltypen (untere Reihe; 40-fache Verdünnung). Eine positive Reaktivität ist gekennzeichnet durch eine granuläre Kernfärbung (oder Färbung von Kern und Zytoplasma im Fall der Hyperimmunseren) auf BoDV-1-infizierten Zellen, jedoch nicht auf den uninfizierten Kontrollzellen.

Discussion

We investigated free-ranging red foxes for infection with BoDV-1 or -2 in regions of Austria where the viruses previously occurred. We detected no bornavirus RNA by RT-qPCR in 365 brain samples from foxes; of the 271 serum samples tested by IFAT, only one (0.4 %) yielded a positive result with comparable titres on two cell lines, suggesting a potentially bornavirus-specific seroreactivity. Two additional sera yielded a positive result when the IFAT was performed with PFA-fixed Vero cells but the finding was not confirmed using heat-fixed SK6 cells. As sera originating from patients with BoDV-1 infection confirmed by direct detection of the virus yield nearly identical titres in both variants of the assay (our unpublished data), such discrepant results are likely to represent unspecific reactivities. Binding to cellular epitopes present in the nucleus of one but not the other cell line is a possible explanation, or the result may relate to reactivity of a serum with a single conformative epitope that is not accessible when the cells are fixed differently.

The brain of the potentially seropositive animal showing seroreactivity using both IFAT variants was available for testing. We found no viral DNA, so did not confirm BoDV-1 infection. There are several potential explanations for the discrepancy between supposed seropositivity and the absence of direct virus detection in the brain. Orthobornaviruses establish life-long persistent infections with a highly neurotropic tropism in non-reservoir hosts and the highest viral loads are found in the central nervous system (CNS) (Niller et al. 2020; Schulze et al. 2020; Fürstenau et al. 2024). As the animal originated from Vorarlberg, where BoDV-1 had been detected in the past, it might have been previously exposed to the virus. However, there is no evidence that natural exposure to BoDV-1 can induce seroconversion without establishing persistent infection. In confirmed cases of BoDV-1 infection, seroconversion is often detectable only during the later course of the disease, when the virus has already reached the brain (Niller et al. 2020; Schulze et al. 2020). Seroreactivity may also result from the high antigenic cross-reactivity among the members of the genus *Orthobornavirus* (Zimmermann et al. 2014), although the panBorna RT-qPCR did not give evidence of other orthobornaviruses in this animal. The two variants of the IFAT may have given positive results because the antibodies reacted with cellular antigens that are preferentially expressed in infected cells or because they incidentally detected singular epitopes on a BoDV-1 antigen, as shown for human sera (Billich et al. 2002; Schwemmle & Billich 2004). The seroreactivity of the animal cannot be considered as evidence of BoDV-1 infection from past exposure to the pathogen.

The lack of evidence of BoDV-1 infection in foxes is consistent with previous studies on foxes in endemic and non-endemic regions of Germany. 16.4 % of the red foxes tested (37/225) showed seroreactivity, with titres ranging between 40 and 2,560 (Bourg et al. 2016), although no BoDV-1 RNA was detected in any of the 13 brain samples available for animals with seroreactivity, suggesting false positive serological results. Consistent with this assumption, there was no significant difference in seroprevalence between endemic and non-endemic areas. The study also failed to detect viral RNA in the brain tissue of 16 foxes that showed a non-suppurative encephalitis highly suspicious of a viral aetiology. Subsequent work found no BoDV-1 in brain samples of nine foxes with encephalitis from Schleswig-Holstein tested by RT-qPCR (Lempp et al. 2017) – this region is not known to be endemic for BoDV-1 (Ebinger et al. 2024) – or in 280 foxes with non-suppurative encephalitis from Saxony-Anhalt (Höche et al. 2022), where BoDV-1 is endemic (Ebinger et al. 2024).

Red foxes are generalist predators of small mammals and mainly feed on rodents, especially *Microtus* voles. Shrews (Soricidae) are eaten less than expected from their availability (Lanszki et al. 2007) but are a component of the fox diet, mainly when food is scarce (Kauhala et al. 1998; Kidawa & Kowalczyk 2011). Bicolored white-toothed shrews are the only known reservoir host of BoDV-1 (Nobach et al. 2015) and it is conceivable that they have contact with foxes. As we do not know what proportion of the fox diet is composed of *C. leucodon* we cannot assume that foxes have periodic contact to shrews. This represents one of the limitations of our study, along with the fact that the precise route of transmission of BoDV-1 infection is unknown.

All samples we tested came from areas in which BoDV-1 or -2 had previously occurred. Epidemiological investigations have confirmed that Upper Austria is an endemic area, with BoDV-1 detected in numerous shrews in regions close to cases of confirmed Borna disease in horses (Weissenböck et al. 2017). Our samples originated from different areas of Upper Austria (Figure 2), not from the precise location of previous BoDV-1 cases in horses, where presumably the infection rate is highest. There have been no tests for virus in the reservoir host in Vorarlberg but the proximity to a BoDV-1-affected area in Switzerland (Niller et al. 2020) combined with several documented cases of Borna disease in domestic mammals (Figure 1 and 3) make an endemic setting plausible. There has only been one documented case of BoDV-2 infection in Austria, which occurred in a horse suffering from Borna disease in Styria (Nowotny et al. 2000). Whether BoDV-2 is endemic in Styria and whether *Crocidura leucodon* also serves as reservoir for this virus remain to be elucidated. The precise origin of a BoDV-1 infection is often unclear, as the period between infection and disease outbreak can last up to several months (Jacobsen et al. 2010; Priestnall et al. 2011).

Because of the seasonal variation in incidence of Borna disease (Dürrwald et al. 2006), the investigation period may affect the detection rate of the virus. We tested samples collected between November 2013 and December 2016. Samples originating from Upper Austria (n=29) were collected between January 2015 and August 2016, a period during which the region saw cases of BoDV-1 infection in horses and shrews (Weissenböck et al. 2017), so it is conceivable that the foxes from Upper Austria had been at risk of exposure to infected shrews. In contrast, no BoDV-1 or BoDV-2 cases were reported during the period of investigation in Styria and Vorarlberg.

We used brain stem to test for bornavirus RNA by RT-qPCR. PCR has been used to investigate the tissue distribution of BoDV-1 RNA in clinically diseased horses and alpacas. High viral RNA loads were found in the olfactory bulb and hippocampus but most brain stem samples were also positive (Lebelt & Hagenau 1996; Schulze et al. 2020). While our choice of sampling within the CNS is unlikely to cause the failure to detect viral RNA, we cannot completely exclude the possibility that it affected the sensitivity of the procedure. Most foxes we tested had been hunted and were thus likely to have been in good health prior to death. Eight animals were found dead and three were killed because of behaviour suspicious of rabies. We cannot rule out encephalitis in these cases, as no pathological examination was carried out. We expect that it is more likely that we would detect an infectious agent in a study population showing characteristic brain lesions.

Alpacas are highly susceptible to BoDV-1 and show fatal neurological disease. The number of South American camelids in Austria is growing and these species may be ideal sentinels for the identification of new areas where BoDV-1 is endemic (Schulze et al. 2020). A recent study found that 11.9 % of clinically healthy South American camelids in all Austrian federal states had seroreactivity in BoDV-1 IFAT, regardless of an endemic occurrence of BoDV-1, with a higher rate in the eastern provinces (Danner et al. 2023). It is conceivable that many of the positive findings are due to unspecific reactivity of the serological tests: the authors are unaware of a single case of clinical Borna disease in llamas or alpacas in Austria over the last decades.

In conclusion, the prevalence of bornavirus infections in red foxes is at most very low, even in regions of Austria where BoDV-1 or BoDV-2 occurred previously. Only a very small proportion of the animals tested demonstrated seroreactivities and we assume that these were unspecific as they could not be confirmed in a second assay and/or the corresponding brain samples were negative for bornavirus RNA. Consistent with previous studies, we did not detect bornavirus RNA in brain samples of foxes. It remains unclear whether foxes serve as a dead-end host for BoDV-1, developing encephalitis and neurological disease. One documented BoDV-1 infection each in a dog and a cat (Bornand et al. 1998; Weissenböck et al. 1998) suggest that carnivores may be susceptible to infection and disease, although cases appear to be rare. There is a need for further investigations into the aetiology of encephalitis in red foxes from BoDV-1 endemic areas.

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Fazit für die Praxis:
Wir konnten molekularbiologisch keine Bornaviren in Gehirnen von Füchsen nachweisen, und auch die niedrige und wahrscheinlich unspezifische Seroreaktivität lässt eine allenfalls sehr geringe Prävalenz von Bornavirusinfektionen in der Fuchspopulation vermuten. Bei Füchsen mit Enzephalomyelitiden ungeklärter Ätiologie sollte eine Bornavirusinfektion als mögliche Differentialdiagnose jedoch abgeklärt werden. Aus unseren Ergebnissen kann geschlussfolgert werden, dass das Risiko der Übertragung von Bornaviren durch den direkten Kontakt zu Füchsen (z.B. bei der Jagd) in Österreich gering ist. Dennoch sollte beachtet werden, dass Bornaviren ein zoonotisches Potential haben und, solange der Übertragungsweg der Infektion auf Menschen ungeklärt ist, Unsicherheiten in der Prävention der Infektion bleiben.

Conflict of interest statement
The authors declare no conflict of interest.

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