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Genetic stability of the open reading frame 2 (ORF2) of borna disease virus 1 (BoDV-1) distributed in cattle in Hokkaido

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ABSTRACT. Borna disease virus (BoDV) is a neurotropic virus that causes several infections in humans and neurological diseases in a wide range of animals worldwide. BoDV-1 has been molecularly and serologically detected in many domestic and wild animals in Japan; however, the genetic diversity of this virus and the origin of its infection are not fully understood. In this study, we investigated BoDV-1 infection and genetic diversity in samples collected from animals in Hokkaido between 2006 and 2020. The analysis was performed by focusing on the P region of BoDV-1 for virus detection. The presence of BoDV-1 RNA was observed in samples of brain tissue and various organs derived from persistently infected cattle. Moreover, after inoculation, BoDV-positive brains were isolated from neonatal rats. The gene sequences of the P region of BoDV obtained from the rat brain were in the same cluster as the P region of the virus isolated from the original bovine. Thus, genetic variation in BoDV-1 was extremely low. The phylogenetic analysis revealed that BoDV-1 isolates obtained in this study were part of the same cluster, which suggested that BoDV-1 of the same cluster was widespread among animals in Hokkaido.

KEY WORDS: borna disease, genetic variation, Japan, phylogenetic analysis

Borna disease virus (BoDV), a member of the genus Orthobornavirus, family Bornaviridae, and order Mononegavirales, is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome of approximately 8.9 kb. The ssRNA of BoDV comprised six open reading frames (ORFs), encoding six structural proteins including the N protein (p40, nucleoprotein, ORF1), P protein (p24, phosphoprotein, ORF2), M protein (gp18, matrix protein, ORF3), G protein (gp84/94, p57, glycoprotein/envelope, ORF4), L protein (p190, L-polymerase, ORF5), and X protein (p10, ORF6). This virus transcribes and replicates in the nucleus, and the cellular RNA-splicing machinery of the host is used to regulate viral gene expression. The virus can be further classified into eight species according to the International Committee on Taxonomy of Viruses (ICTV), including avian orthobornaviruses (Passeriform 1 orthobornavirus, Passeriform 2 orthobornavirus, Psittaciform 1 orthobornavirus, Psittaciform 2 orthobornavirus, and Waterbird 1 orthobornavirus), reptile orthobornavirus (Elapid 1 orthobornavirus), and two mammalian orthobornaviruses (Mammalian 1 orthobornavirus and Mammalian 2 orthobornavirus). Mammalian 1 orthobornavirus is further classified into borna disease virus 1 (BoDV-1) and borna disease virus 2 (BoDV-2). However, Mammalian 2 orthobornavirus is only represented by variegated squirrel bornavirus 1 (VSBV-1) [2, 27].

BoDV-1 infection can cause severe neurological disease in several animals, and BoDV-1 is a causative agent of human psychiatric disorders. Horses, sheep, and other livestock are natural hosts of this virus, in whom BoDV-1 infection causes severe neurological abnormalities and often culminates in death [4, 6, 26, 38]. In humans, a correlation between BoDV-1 infection and mood disorders has been reported in many countries [5]. Recently, BoDV-1 was detected in patients with encephalitis who also had the clinical presentation of Guillain-Barre syndrome [8, 40]. The detection of BoDV-1 has been reported in several countries, including eastern and southern Germany, Switzerland, Liechtenstein, Austria, Australia, China, and Japan [10, 11, 18, 32, 39]. Although BoDV-1 infection has mainly been reported in horses, infection with this virus has also been reported in many other types of mammals, including sheep, cattle, goats, dogs, cats, alpaca, foxes, voles, raccoons, and monkeys [9, 10, 14, 19, 20, 23, 24,

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30, 31, 45]. Several studies have reported that the bicolored white-toothed shrew (*Crocidura leucodon*) is a natural reservoir host of BoDV-1 [22, 37]. Infected shrews remained healthy without any clinical symptoms, and as dead-end hosts, they may shed the virus through their urine, feces, and saliva to farm animals and humans.

Viral isolation of BoDV-1 in all detected cases has not been successful because of the small amount of virus available in the tissue. Most BoDV-1 sequences available in GenBank are based on a small fragment of the p24 gene. Sequence comparison has revealed high genetic conservation between isolates and no evidence of species–specific infection. Interestingly, the sequences of BoDV-1 obtained from wild shrews have showed high percent identity with BoDV-1 obtained from a horse that died 15 years ago [22]. This finding suggests the unusual genetic stability of BoDV-1. Moreover, phylogenetic analysis has revealed that the clustering of viruses is related to their regional origins [26].

In Japan, seropositive or PCR-positive BoDV-1 infections have been widely reported in domestic animals such as horses, sheep, cats, dogs, and cattle [15–17, 33, 43]. In addition, BoDV-1 has been detected in Japanese macaques (*Macaca fuscata*) and raccoons (*Procyon lotor*) [19, 20]. Although sequence analysis did show a high degree of genetic conservation within the same host species, there were some differences between sequences obtained from wildlife samples and sequences previously reported from farm animals. However, the genetic diversity and phylogenetic relationships of BoDV-1 from Japanese cattle have not been fully studied. In this study, we investigated the BoDV-1 infections in livestock and evaluated the genetic diversity of the virus detected in Japan in 2006–2020.

MATERIALS AND METHODS

Animals

This study was performed using both clinical and non-clinical animals, and different types of samples were collected in 2006–2020. A total of 10 cattle, originating from 8 farms, suspected to be infected with BoDV-1 were admitted to the Animal Hospital of Rakuno Gakuen University for diagnosis. These animals tested positive for the BoDV-1 (N antigen) antibody and had symptoms including difficulty in standing, behavioral abnormalities, and wobbling of the hind legs. The details of each animal are presented in Supplementary Table 1. Necropsy was conducted in cases of animals that showed clinical symptoms such as depression, unusual posture and gait, and imbalanced movement, as well as in cases with seropositive results for the BoDV-N antigen. In addition, a total of 34 aborted fetuses from farms where BoDV-1 antibody-positive cattle were identified were also studied. The care of the animals included in this study was in accordance with the Laboratory Animal Control Guidelines at Rakuno Gakuen University (approval number: VH16C10, VH18B5).

Necropsy and sample collection methods

Brain dissection was performed on 7 cattle to collect different brain regions (JB1106, J524, NF042706, Pr7994, 1663, NF6883, and Pr7288), which were preserved at −80°C prior to RNA extraction. Whole-body necropsy was performed for three dairy cattle (Pr13188, Pr13529, and Pr13530) with ataxia, and internal organs such as the liver, lungs, spleen, and ovaries were collected. For brain dissection, the brain was cut cross-sectionally into small tissue sheets of approximately 100 mg from at least four different regions. In this study, we consistently selected the same cross-sectional plane of the brain for each animal to sample the same brain regions. For the aborted fetus samples, the whole brain of the fetus was used because of the low amount of brain tissue and difficulty in performing dissection. The details of the brain region and type of organ/sample for each animal are summarized in Table 1. The collected tissue was homogenized using QIAGEN TissueLyser (QIAGEN, Hilden, Germany) at a frequency of 23 Hz for 30 sec and were stored at −80°C prior to RNA extraction and animal model experiments. For pathological analysis, the necropsy tissues were subjected to hematoxylin–eosin staining according to a standard method.

Inoculation method

Neonatal rats have been used as a model for BoDV isolation and the study of immunological mechanisms. In this study, the cerebral and hippocampal regions of cattle (Pr13530) were homogenized in 10% DMEM and intracranially inoculated into two newborn rats. The rats were euthanized at 5–7 weeks post-inoculation. The brains of both rats were collected and examined for BoDV-1 detection (the isolates were named Bo#4521-R1/CE and Bo#4521-R1/HIP, respectively).

Serological diagnosis

Serum samples were diluted to a ratio of 1:200 with phosphate-buffered saline containing 10% Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) and 0.05% Tween 20. The samples were tested for BoDV antibodies using an enzyme-linked immunosorbent assay (ELISA) to screen for the recombinant BoDV nucleoprotein (BDV-N) antigen. To detect antigen-bound bovine immunoglobulin, we used peroxidase-conjugated goat affinity purified anti-bovine IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA). Positive reactions were identified using a microplate imaging system (Ultramark, Bio-Rad, Hercules, CA, USA) at 405 nm. The cutoff value for ELISA was calculated as the mean ± 2 standard deviation at an optical density (OD) of 405 nm from five intact cows (cutoff: OD, 0.4). ELISA-positive samples were further examined by immunoblot analysis to confirm their specificity to the BDV-N antigen. Antibody–antigen complexes were detected using the same peroxidase-conjugated goat affinity purified anti-bovine IgG [18].

Table 1. Details of the sequences of Borna disease virus (BoDV)-1 positive samples reported in this study

No.	Name	Host	PCR positive tissue	Sequences	Accession number	Genotype	Number of positive/total	Positive farm
1	JB1106	Japanese Black	CB	BoDV-JB/CB/HOK/1106	MT199563	HOK-1	1/1	A
2	Pr7994	Holstein friesian	HYP	BoDV-HF/HYP/HOK/7994	MT199564	HOK-1	1/1	D
3	1663	Holstein friesian	PONS	BoDV-HF/PONS/HOK/1663	MT199565	HOK-1	1/1	E
4	NF6883	Holstein friesian	CB	BoDV-HF/CB/HOK/6883	MT199566	HOK-1	1/1	C
			CE	BoDV-HF/CE/HOK/6883	MT199567	HOK-1		C
			PONS	BoDV-HF/PONS/HOK/6883	MT199568	HOK-2		C
5	Pr13188	Holstein friesian	SCN	BoDV-HF/SCN/HOK/13188	MT199570	HOK-1	1/1	H
			ADM	BoDV-HF/ADG/HOK/13188	MT199571	HOK-1		H
			CB (Dentate n.)	BoDV-HF/CB/HOK/13188	MT199572	HOK-1		H
			CE	BoDV-HF/CE/HOK/13188	MT199573	HOK-1		H
			OVC	BoDV-HF/OVC/HOK/13188	MT199574	HOK-1		H
			PIT (ant.)	BoDV-HF/PIT/HOK/13188	MT199575	HOK-1		H
			PONS	BoDV-HF/PONS/HOK/13188	MT199576	HOK-1		H
			SN	BoDV-HF/SN/HOK/13188	MT199577	HOK-1		H
6	Pr13529	Holstein friesian	HYP	BoDV-HF/HYP/HOK/13529	MW715856	HOK-1	1/1	J
			ADC (right)	BoDV-HF/ADC/HOK/13529	MW715857	HOK-1		J
			PSG	BoDV-HF/PSG/HOK/13529	MW715858	HOK-1		J
			AV	BoDV-HF/AV/HOK/13529	MW715859	HOK-1		J
7	Pr13530	Holstein friesian	HYP	BoDV-HF/HYP/HOK/13530	MW715860	HOK-1	1/1	J
			ADC (right)	BoDV-HF/ADC/HOK/13530	MW715861	HOK-1		J
			AV	BoDV-HF/AV/HOK/13530	MW715862	HOK-1		J
8	Aborted fetus	Holstein friesian	Aborted fetus	BoDV-HF/FE/HOK/0996F	MT199581	HOK-3	3/34	E
			Aborted fetus	BoDV-HF/FE/HOK/2173F	MT199582	HOK-1		I
			Aborted fetus	BoDV-HF/FE/HOK/0648F	MT199583	HOK-1		J
9	Inoculated rat (Pr13530)	Lewis rat	Homogenated brain	Bo#4521-R1/CE	MW715863	HOK-1	1/1	-
				Bo#4521-R1/HIP	MW715864	HOK-4	1/1	-

CB (dentate n.): Dentate nucleus of cerebellum, CB (hemi): Cerebellum hemisphere, CE: Cerebrum, HIP: Hippocampus, HYP: Hypothalamus, PONS: Pons, PIT (ant.): anterior pituitary, PIT (mid.): middle pituitary, PIT (post.): posterior pituitary. VN: Vagus nerve, SPL: Spleen, KID: Kidney, SN: Sinus node, PF: Purkinje fiber, LUNG: Lung, LIV: Liver, OV: Ovary. TRG: Trigeminal ganglion, ADC: Adrenal cortex, ADM: Adrenal medulla, PSG: Parasympathetic ganglion, SCG: Sciatic. UP: unusual posture, UP*: unusual posture found in some individual, SP: Seropositive, SP*: Seropositive in farm level, NA: Data is not available ganglion, AV: Atrioventricular node. *Fetus were come from 3 different farm: #2173F male I farm, #0648F female J farm, #0996F male E farm.

RNA extraction, cDNA construction, and nested PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA of each sample was constructed using a cDNA synthesis kit (ReverTra Ace -α-®TOYOBO, Co., Ltd., Osaka, Japan) following the manufacturer's instructions and was preserved at −30°C until further use. Since endogenous Borna-like N elements (EBLN) have been identified in most animal species, the P region (p24), which confirms virus distribution without overlapping with EBLN, is commonly used as the target region for BoDV-1 detection. Thus, cDNA is the standard preliminary investigation to detect the presence of BoDV-1 specific to the P region by using a nested PCR method. The p24-specific PCR was performed using the following primers: 1st BDV-D1.1 (5′-CTGAAGGACCTCAGGAAGA-3′), BDV-A1.1 (5′-TGGTATGATGTCCCAYTCATC-3′), nested PCR BDV-D3.1 (5′-TCAGACCCAGACCAGCGAA-3′), and BDV-A2.1 (5′-GAGCTGGGGATAAATGCG-3′) with the proofreading polymerase (Takara Ex-Taq® polymerase, Takara Bio Inc., Kusatsu, Japan) following the manufacturer's instructions. Contamination countermeasures were also taken into consideration; for example, DNAse treatment and sample handling were conducted in a separate room using a special glove box under UV treatment. Moreover, the extracted RNA was concurrently tested for contamination with BoDV-DNA in this experiment by performing a BoDV-specific PCR directly with RNA template without the RT step. The BoDV gene was not found to be amplified in any of the samples, and contamination was ruled out. PCR amplicons were observed using 1.5% agarose gel electrophoresis. Positive PCR amplicons were purified using the FastGene kit (NIPPON Genetics, Co., Ltd., Japan) and sequenced by a commercial laboratory (Hokkaido System Science Co., Ltd., Sapporo, Japan).

Sequence alignment and phylogenetic analysis

The BLAST® tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify sequences. Nucleotide alignment was performed using Unipro UGENE V33.0. [35]. Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA X) [28] using a maximum-likelihood method based on the Kimura 2-parameter model with 1,000 bootstrap replicates. The Templeton, Crandall, and Sing (TCS) network infers the relationships among the observed and unobserved genetic variants (or genotype) by relaxing the assumption that all genotypes have been sampled and thus allowing the unobserved genotype to be incorporated into network relationships. In addition, the TCS methods have been used to estimate relationships among organisms to infer population-level genealogies when divergences are low. Thus, the genealogy of BoDV was estimated to reveal the origin

(ancestral) sequences among virus populations by using the TCS network [7] and PopART software [29]. Nucleotide alignment and phylogenetic analysis were established through comparison with previously reported BoDV sequences in Japan (AB281092, AB469325, AB469326, AB469327, AB022025, AB00147, and AB246670) and GenBank. The accession numbers of the sequences used in this study are listed in Supplementary Table 2.

RESULTS

Prevalence of BoDV-1 in animal and tissue samples

Seven out of 10 animals (70%) and 3 out of 34 (8.8%) aborted fetus samples tested positive in the nested p24-specific PCR. Viral nucleic acids were detected using nested PCR in different brain regions. Several tissues from three clinical cases where whole-body necropsy was conducted (Pr13188, Pr13529, and Pr13530) were investigated for the presence of BoDV-1. Surprisingly, PCR showed positive results for samples from the pituitary gland, trigeminal ganglion, sciatic nerve, sinus node, adrenal gland, liver, spleen, and ovary in addition to samples from the major brain regions (e.g., cerebrum, cerebellum, hypothalamus, hippocampus). Histopathological analysis showed perivascular lymphocyte infiltration in the temporal lobe, pons, and pituitary gland. Pyogenic vasculitis was observed in the sciatic nerve ganglion (Supplementary Tables 1 and 2). Interestingly, BoDV-1 RNA was detected only in the anterior region, and not in the posterior region, of the pituitary gland. Likewise, BoDV-1 RNA was detected in the dentate nucleus of the cerebellum, but not in the cerebellar hemisphere. Moreover, BoDV-1 was detected in the samples from the aborted fetuses, suggesting the possibility of vertical transmission in the population. The details of the positive samples and tissues are summarized in Table 1. Interestingly, the homogenate of the brain of inoculated newborn rats (Bo#4521-R1/HIP and Bo#4521-R1/CE) tested positive in the PCR for BoDV-1 with a strong signal.

Genetic variation in BoDV-1 isolates from in Hokkaido

BLAST results confirmed that the obtained sequences were BoDV-1. The BoDV-1 sequence showed a high percent identity to strains of BoDV-1 previously reported in Japan and other countries. In this study, nucleotide alignment revealed four different genotypes that were detected in virus samples isolated from cattle and rat brain tissues (Table 1). The major genotype found in this study was named HOK-1, which was found mainly in cattle. Multiple infections with different BoDV-1 genotypes have been detected in cattle and inoculated rats. Interestingly, heterogenous infection with two BoDV-1 genotypes (HOK-1 and HOK-2) was detected in brain tissue in one cow (NF6883); furthermore, sequences obtained from the two inoculated rats (Bo#4521-R1/HIP and Bo#4521-R1/CE that inoculated with brain of Pr13530) were also found to include two genotypes, HOK-1 and HOK-4. The nucleotide and amino acid substitutions are summarized in Table 2.

Phylogenetic tree and network analysis

Here, we constructed the BoDV-1 phylogenetic tree by using our obtained sequences and sequences that were previously reported in Japan and other countries. The phylogenetic tree showed that all BoDV-1 sequences obtained from samples of livestock and inoculated rats were clustered together with a high similarity to BoDV-1 that was previously reported in Europe (livestock group) (Fig. 1). In addition, the BoDV-1 genotype HOK-1 showed 100% identity to BoDV-1 isolated from a horse in Hokkaido in 1998 (AB022025) and clustered closely with BoDV-1 isolated from a sheep in 1995 (AB001473). Interestingly, the BoDV-1 strain Bo/04w (AB246670), previously reported in cattle in Hokkaido since 2006, clustered with the central Germany and V groups, which is dissimilar to the sequences obtained in this study (Fig. 1). Moreover, the BoDV-1 sequences obtained from samples of wild animals (AB281092, AB469325, AB469326, AB469327) were clustered with the BoDV-1 strain “RW98”. This indicates that BoDV-1 infection in livestock and wildlife in Japan does not share the same origin (Fig. 1).

Here, the TCS network was constructed using BoDV-1 sequences derived from animals in Japan to verify the genetic diversity of BoDV-1 that caused outbreaks in different farms. In the study of cattle in Hokkaido, the HOK-1 genotype was mainly detected, and other genotypes noted in the livestock in Hokkaido may have originated from this cluster (the largest circle shown in the haplotype network) (Fig. 2). The Bo/04 strain was completely separate from the major genotype group. Likewise, the TCS network analysis showed different origins of BoDV-1 infection in livestock and wildlife (Fig. 2).

Table 2. Details of the nucleotide and amino acid substitutions found in this study

Genotype	Nucleotide substitutions/Amino acid substitutions				
	Positions				
	37/13	140/47	142/48	174/58	214/72
HOK-1	G/E	A/Q	G/R	T/L	A/M
HOK-2	A/K	A/Q	G/R	T/L	A/M
HOK-3	G/E	A/Q	A/Q	T/L	A/M
HOK-4	G/E	G/R	G/R	C/L	G/V

The position of nucleotide and amino acid in this table refer to MT199563.

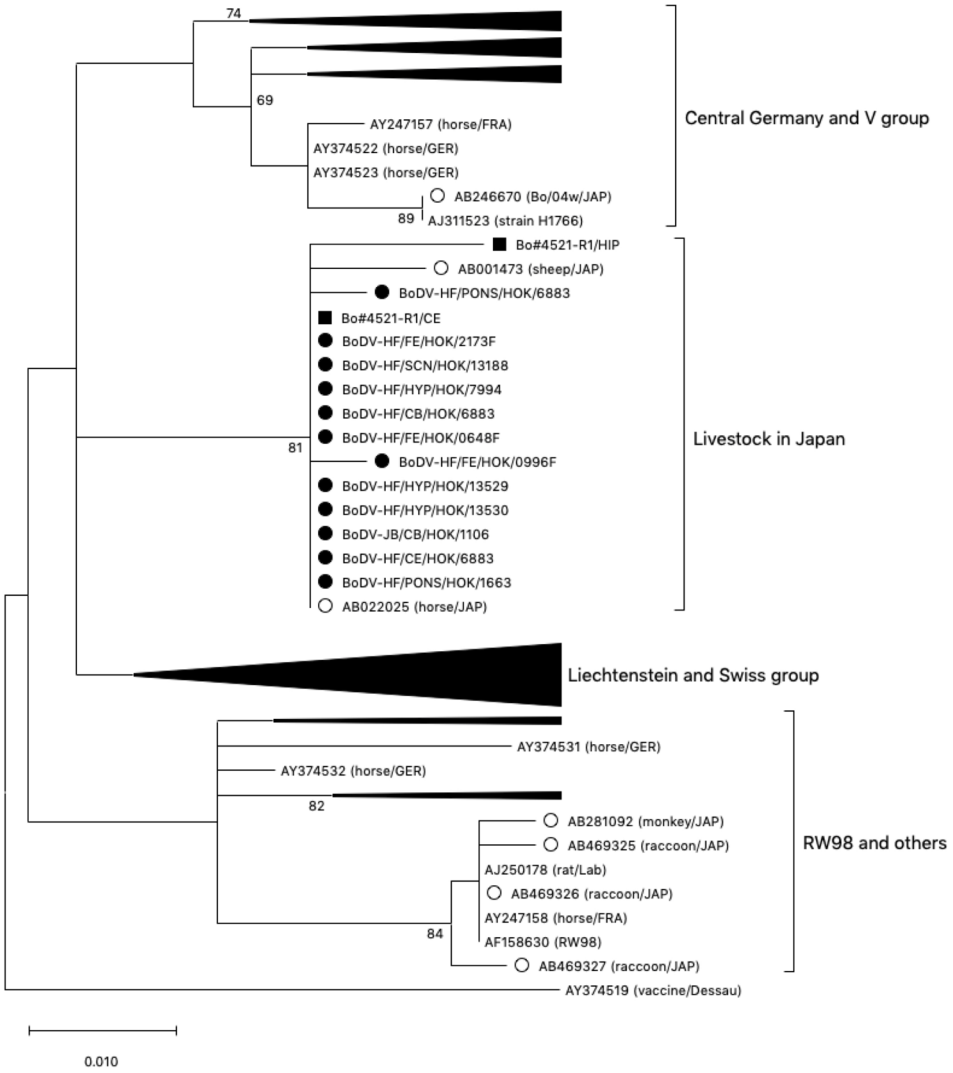

Fig. 1. Phylogenetic analysis using 268 bp of the P region. The clustering of monophyletic clade is displayed by a compress tree. Sequences obtained in this study are indicated with a black circle and square for cattle and inoculated rat, respectively. Previously reported sequences from Japan are indicated with open circles. Each bracket indicates the group of Borna disease virus (BoDV)-1 according to geographic origins [26]. The number at the node denotes the bootstrap value ≥60%.

DISCUSSION

In this study, we report the detection of BoDV-1 RNA in different types of samples obtained from both clinical and non-clinical animals in Japan in 2006–2020. Consistent with previous studies, we demonstrated the global applicability of serological and molecular methods for the detection of BoDV-1, including in Japan. Since 1995, the detection of BoDV-1 has been reported in various types of domestic animals such as horses, sheep, cattle, cats, and dogs, as well as in wild animals such as raccoons and Japanese macaques [15–17, 19, 20, 33, 43].

BoDV-1 is highly neurotropic, and microscopic lesions in the nervous system are difficult to detect. Non-suppurative encephalomyelitis or neuronal degeneration has been detected in many regions of the brain, including the midbrain, hypothalamus, hippocampus, and brainstem. In this study, BoDV-1 was detected in at least one region of the frozen brain, including the cerebrum, cerebellum, and hypothalamus. Because the frozen-brain sampling method includes only part of the brain of each animal, the detection rate of the virus in the sample may have been underestimated.

Because we had the opportunity to perform whole-body necropsy for three clinical cases (Pr13188, Pr13529, and Pr13530), other brain regions and several organs were also used to investigate the presence of BoDV-1. Interestingly, BoDV-1 was detected in the anterior (n=3/3) and middle (n=2/3) regions, but not the posterior region, of the pituitary gland. The anterior pituitary comprises sex hormone-secreting endocrine tissue that determines the reproductive fitness of an animal. Fertilization failure was observed in cattle seropositive for BoDV-1 with no detection of BoDV-1 in the reproductive organ [21], suggesting that the fertilization failure was linked to infection in the central nervous system. Accordingly, our findings provided evidence of BoDV-1 infection

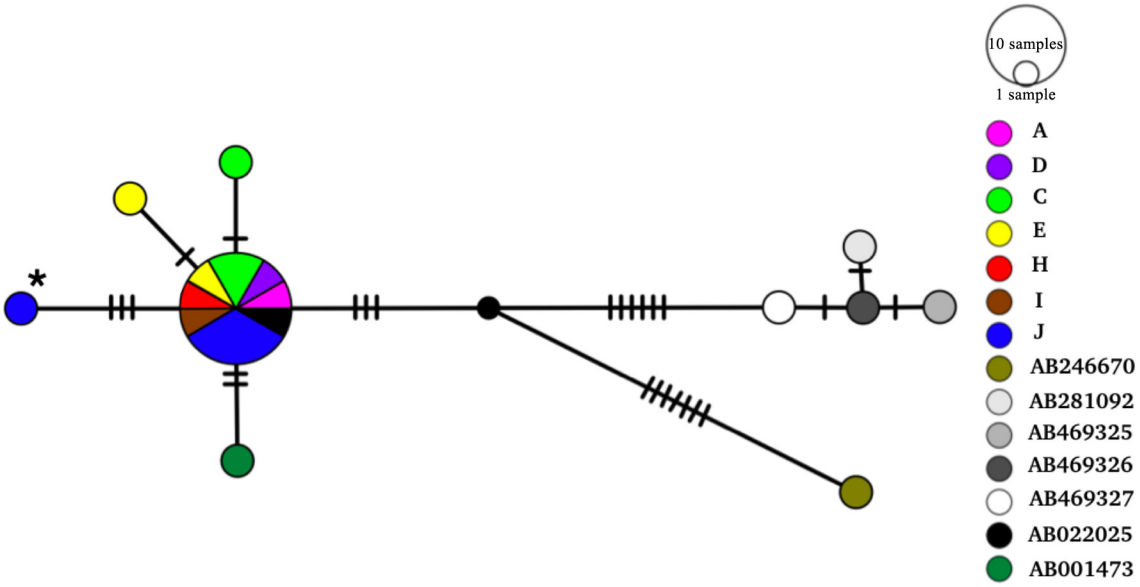

Fig. 2. A Templeton, Crandall, and Sing (TCS) network showing different clusters of Borna disease virus (BoDV)-1 isolated from livestock and wildlife animals in Japan. Each circle represents unique genotype of BoDV-1. The size of the circle represents the number of sequences that are considered as the same genotype. The different colored circles denote the different geographic origins of the sequences from livestock samples. The gray-scale color labelling represents BoDV-1 from wildlife and other related strains. The hatch mark represents the mutation between each genotype. The asterisk refers to the Bo#4521-R1/HIP sequence.

in the sex hormone-secreting region of the pituitary, which may have contributed to reproductive failure. However, the impact of BoDV-1 infection on reproductive physiology requires further deliberation on the basis of detailed research. The reproductive index and sex-hormonal profile were not examined in this study; both of these would provide crucial preliminary data for further necessary investigation of the impact of BoDV-1 infection on the function of the pituitary gland. In this study, the detection of the BoDV genome only in a part of the brain was attributed to the low viral load in the brain. BoDV-1 has been reported to spread to non-neural tissue via the peripheral nervous system (PNS) [1, 12, 24, 41, 42]. In this study, BoDV-1 was detected in the sciatic ganglion, trigeminal ganglion, parasympathetic ganglion, sinus node, adrenal gland, spleen, liver, and ovary. Gait imbalance was observed in the cattle, which may have resulted from BoDV-1 infection in the CNS or sciatic neurons. Similarly, clinical signs of viral infection of PNS ganglia should be noted in future clinical observations. Vertical transmission of BoDV-1 has been demonstrated in mouse models as well as in humans, horses, and cattle [3, 5, 17, 34]. Interestingly, BoDV-1 was detected in aborted fetuses obtained from cattle farms in this study. However, the role of BoDV-1 in fetal development during gestation has not been clearly elucidated.

The genomic stability of BoDV in persistently infected cell cultures is extremely high. Few nucleotide substitutions have been observed in long-term passages *in vivo* [11]. In addition, the BoDV sequence recently obtained from wild shrews was highly identical to that obtained from horses that died in the same area 15 years ago and only differed by one or two nucleotides [22]. Thus, the minor changes in the viral genome samples obtained from the clinical cases were not caused by contamination by laboratory strains [13, 44]. Similarly, many studies on the genetic diversity of BoDV-1 have shown that the genome of BoDV-1 is extremely conserved [11] and strongly reflects the geographic origin in central Europe [26]. Therefore, BoDV-1 is phylogenetically clustered into a few specific groups, for example, RW98, strain V, and strain No/98 [25, 26, 36]. In this study, four different genotypes of BoDV were found to have a high sequence similarity of 99% within 268 bp of the p24 region (represented as HOK-1, HOK-2, HOK-3, and HOK-4 in Table 2). The sequence obtained from an inoculated rat (Bo#4521-R1) was matched that obtained from the brain of cattle Pr13530 (HOK-1 in Table 2). Moreover, the p24 sequence obtained from another inoculated rat (Bo#4521-R1 / HIP) brain showed a nucleotide mutation, which was different from Bo#4521-R1 / CE (Table 2).

The major genotype of BoDV-1 found in this study was HOK-1. Heterogenous infection with two BoDV-1 genotypes (HOK-1 and HOK-2) was observed in tissues from different brain regions of one cattle (NF6883). The global transportation of live animals began a long time ago. Since most livestock or grandparent livestock have been imported mainly from European countries, Japan is considered to be a "BoDV-importing country." It is also speculated that BoDV-1, which has high genetic stability, may have been selectively maintained in a Japanese environment with strict livestock hygiene management. Thus, it may be possible that these virus strains persistently infected European-origin livestock in the past and have been stably maintained in livestock ever since.

In conclusion, we demonstrated that BoDV-1 with low genetic variation was detected in cattle in Japan. In this study, the BoDV-1 genome was detected in samples not only from the CNS, but also from other organs. The major genotype detected in Hokkaido (Japan) was HOK-1, which showed high similarity to the ancestral sequence of BoDV-1. The reservoir host and transmission dynamics remain unknown and should be studied further. In-depth and detailed genetic analysis of the isolated virus using multiple samples will clarify some of the uncertainties reported here.

CONFLICT OF INTEREST. The authors declare no competing interests.

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