FULL PAPER *Virology*

Borna Disease Virus in Raccoons (*Procyon lotor*) in Japan

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(Received 19 January 2009/Accepted 27 February 2009)

ABSTRACT. We have examined the seroprevalence of BDV in wild Raccoons (*Procyon lotor*) in Hokkaido, Japan. Serum samples from raccoons were examined using ELISA and Western blot assays to detect the presence of serum antibodies that react specifically to BDV antigens. Among 549 investigated individuals, eleven (2.0%) showed a positive reaction to BDV antigens. Brain tissue samples from five individuals were subjected to RT-PCR, which detected BDV sequences in three of them. Sequence analysis revealed a high degree of genetic conservation between BDV sequences derived from raccoons and previously published sequences derived from other animal species.

KEY WORDS: Borna disease virus, epidemiology, raccoon.

J. Vet. Med. Sci. 71(8): 1009–1015, 2009

Borna disease virus (BDV) is the causative agent of Borna disease (BD), an immune-mediated neurological disease first described in horses more than 200 years ago in southern Germany [15, 28, 29]. Initially, BD was thought to be restricted to horses and sheep in central Europe; subsequent epidemiological evidence has indicated that the prevalence and geographic distribution, as well as the host range of BDV, are broader than previously thought [27–29], though the data supporting this view of the epidemiology of BDV have not been solidly established [3, 4]. Some cases of BDV infection in humans have been reported, and it has been suggested that the virus may be related to certain neuropsychiatric disorders [2, 24].

Experimentally, BDV can infect a wide spectrum of species ranging from birds to rodents to non-human primates [28, 30]. Both host and viral factors contribute to a variable period of incubation as well as significant heterogeneity in the symptoms and pathology associated with infection [15, 29]. BDV-infected animals can develop neurobehavioral abnormalities in the absence of either encephalitis or other clinical manifestations by mechanisms that remain largely unknown [5, 6,17, 25, 26].

Epidemiological studies have documented the presence of serum antibodies to BDV and virus RNA in the brains of domestic animals and companion animals in several countries including Japan [1, 9]. Recently, BDV-infected dogs, cats and macaques have been reported in Japan [12, 21, 23]. In wild animals, however, the host range and prevalence of BDV remain poorly understood.

Raccoons (*Procyon lotor*) are native to North America, but as a result of escapes, raccoons are now distributed across the European mainland and in Japan. In Japan, raccoons are widely distributed in mountainous regions and urban areas, including those of Hokkaido. Hokkaido's population of raccoons is descended from individuals imported from North America as companion animals; unfortunately, many of these raccoons escaped from their owners and began to reproduce in the wild.

Recently, the wild raccoons of Japan have come into close contact with humans. In farming regions, they have caused damage to crops; they have also damaged the natural habitats of other small animals. In response to these problems, the govarnment of Hokkaido developed a control program that involved capture and removal of raccoons. This program provided us with access to valuable biological samples, which we used to examine the prevalence of BDV in Hokkaido's wild raccoons.

MATERIALS AND METHODS

Animals: The raccoons examined in this study were captured as part of an agriculture damage control program carried out by the govarnment of Hokkaido from 2000 to 2003. They were collected in the center area of the island of Hokkaido, Japan, specifically, in an area including parts of Ishikari and Sorachi subprefectures; we divided the capture area into 4 regions labeled A through D, which are shown on a map in Fig. 1 and described here. Region A was hilly terrain including many ranches and farms. Region B was a forested area adjacent to an urban area. Region C was scattered small towns and farms around a mountainous district. Region D was an urban area.

A total of 549 raccoons (225 male, 324 female) were trapped with box traps (Havahart Model 1089, Woodstream, Lititz, PA, U.S.A.). Prior to euthanasia, they were sedated by intramuscular administration of a mixture of ketamine HCl (5 mg/kg body weight; Sankyo, Tokyo, Japan) and xylazine HCl (1 mg/kg body weight; Bayer, Tokyo, Japan). Euthanasia was performed using a lethal dose of pentobarbital Na (Sankyo, Tokyo, Japan) administered intravenously according to the methods described by the guidelines for capture, handling and care of mammals by the Mammalogical Society of Japan. Each raccoon's age was estimated based on eruption of permanent teeth, cranial suture obliter-

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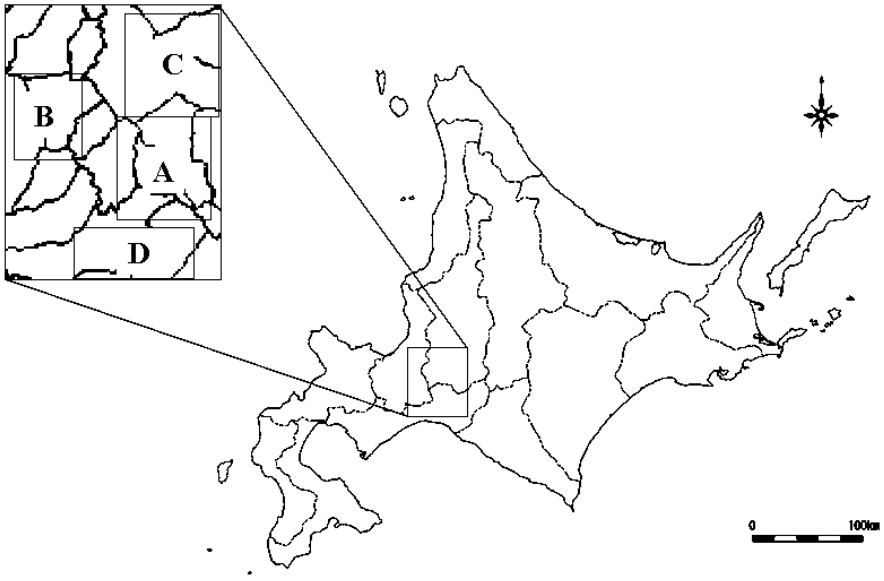


Fig. 1. Map showing the area in Hokkaido where the raccoons were captured. The four capture regions (A to D) are shown in the detail map.

ation and annual incremental lines in the tooth cementum of the canines [7]. Blood samples were collected from all raccoons (N=549), though fresh brain tissue samples could be obtained from only five raccoons; these were stored at –80°C until dissection. Serum and brain tissue samples were likewise stored at –80°C until testing. These studies were performed according to the guidelines for capture, handling, and care of mammals of the Mammalogical Society of Japan.

Detection of anti-BDV serum antibodies: Serum samples were diluted at 1:100 with PBS containing 10% Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) and 0.05% Tween 20 and screened for antibodies to BDV by ELISA using the recombinant BDV nucleoprotein (BDV-N) antigen as described in our previous reports [8, 11]. To detect antigen-bound raccoon immunoglobulin, a peroxidase-conjugated goat affinity purified anti-dog IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA, U.S.A.) was reacted with the samples; positive reactions were identified using a Microplate Imaging System (Ultramark, Bio-Rad, Hercules, CA, U.S.A.) at 405 nm. ELISA-positive samples were further examined by Western blot using recombinant BDV-N as the target antigen. Antibodies and antigen complexes were detected using the same peroxidase-conjugated goat affinity purified anti-dog IgG menthioned above, as described elsewhere [9, 16].

Detection of BDV-RNA by RT-nested PCR: Total RNA was isolated from the hippocampus regions of brain tissue samples from five seropositive raccoons (RC #3, #5, #10, #38 and #224) using a TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA samples (1 μg) were reverse-transcribed using 200 units of SuperScript II reverse transcriptase (RT) (GIBCO BRL, Carlsbad, CA, U.S.A.) and random hexamers (100 *n*g) for 60 min at 42°C. The reactions were terminated by incubation at 70°C for 15 min. In our epidemiology survey, the detection efficiency for the RT-PCR amplification of the P gene region was better than for the N region. Therefore, we used the P region for detection of BDV-RNA. BDV-specific cDNAs corresponding to the BDV phosphoprotein (BDV-P) ORF were amplified by nested PCR as described elsewhere [16]. The first pair of primers corresponded to nucleotides 1387–1405 (5'-TGACCCAACCAGTAGACCA-3') and 1865–1847 (5'-GTCCCATTCAT CCGTTGTC-3') within the phosphoprotein ORF. The first-round PCR conditions were 35 cycles each consisting of 30 seconds at 94°C, 30 seconds at 58°C and 1 min at 72°C, followed by a final polymerization extension step for 7 min at 72°C. Aliquots (1/10) of the first-round PCR product were subjected to a second round of PCR with a nested set of BDV-P primers corresponding to nucleotides 1443–1461 (5'-TCAGACCCAGACCAGCCGAA-3') and 1834–1816 (5'-AGCTGGGGATAAATGCGCG-3'). The conditions for the nested PCR were the same as those for the first round of PCR.

Analysis of the nucleotide sequence: PCR products were cloned into pGEM-T Easy Vector (Promega Co., Madison, WI, U.S.A.). Three randomly-selected clones derived from brain samples RC #3, #5 and #10 were sequenced using the protocols recommended for the DNA Sequencer II (Pharmacia Biotech, Uppsala, Sweden). Sequences were analyzed using GENETYX-MAC (Genetyx Corporation, Tokyo, Japan) and compared with previously published BDV sequences derived from the horse (AB247158), cow (AB246670), sheep (AY066023), macaque (AB281092), dog (DQ116031) and shrew (DQ251041).

Table 1. Summary of BDV seroprevalence in raccoons

Regions	District	Number of Animals Captured Per Year					Positive (#)	Positive (%)
		2000	2001	2002	2003	Total		
A	1	36	0	0	13	49	1	2.0
	2	46	0	0	17	63	1	1.6
	3	72	0	0	38	110	2	1.8
	4	34	0	0	22	56	1	1.8
	5	0	0	0	19	19	1	5.3
	6	9	0	0	0	9	0	0.0
	7	0	0	0	4	4	0	0.0
	8	0	0	0	1	1	0	0.0
B	1	12	3	0	26	41	2	4.9
	2	28	3	0	19	50	0	0.0
	3	10	4	6	16	36	0	0.0
C	1	6	0	0	0	6	0	0.0
	2	5	0	0	0	5	0	0.0
	3	0	0	0	28	28	1	3.6
	4	0	0	0	38	38	0	0.0
	5	0	0	0	30	30	2	6.7
D	1	0	3	0	1	4	0	0.0
Total		258	13	6	272	549	11	2.0

Region A : Hilly terrain containing many ranches and farms.
Region B : Forest adjacent to an urban area.
Region C: Scattered small towns and farms in a mountainous district.
Region D: Urban area.

RESULTS

Detection of serum antibodies to BDV: A total of 549 raccoons, divided somewhat evenly between the sexes (225 males, 324 females), were captured in this study. Samples that were determined to be BDV seropositive by ELISA were also positive for BDV antibodies when examined by Western blot. Based on serological data, 2.0% (11/549) of the raccoons were identified as BDV seropositives; 72.7% (8/11) of these seropositive raccoons were male (Table 1). All of the seropositive raccoons except one were adult animals (Table 2). Seropositive raccoons were collected in three of the four regions (A, B and C). Six of the 11 seropositive raccoons were captured in region A; one was an infant male raccoon, two were female raccoons and two were captured in the same district (#1) in region B. Three of the seropositive raccoons were captured in region C; one was a female raccoon, but we could not determine whether there was any relationship among these animals. We did not observe any significant correlation between the rate of seropositive status and the region where the animals were captured.

Detection of BDV RNA and sequence analysis: We obtained brain tissue samples from five BDV-seropositive raccoons and examined them for the presence of BDV RNA using RT-nested PCR. We succeeded in amplifying BDV-P sequences from only three of these five raccoons (RC #3, #5 and #10); these samples corresponded exactly to the three seropositive raccoons.

Sequences derived from horse BDV and sequences derived from field isolates from different species and geographic locations usually exhibit a high degree of genetic conservation. This frequently raises the concern that inadvertent contaminations with laboratory-adapted BDV could be responsible for BDV cases that are presented as naturally occurring infections. To rule out this possibility, aliquots of the same tissue sample were collected using disposable blades. In addition, control reactions in the absence of RT failed to amplify BDV-P sequences from samples collected from #3, #5 and #10; this result ruled out the possibility that these samples tested positive for BDV RNA due to contamination with plasmid DNA.

Table 2. Sex and maturity of seropositive raccoons

Region	ID	SEX	Maturity	BW*
A	RC-003	Male	Adult	5.8
A	RC-008	Female	Adult	7.7
A	RC-010	Male	Adult	6.1
A	RC-023	Male	Infant	2.0
A	RC-135	Female	Adult	6.7
A	RC-169	Male	Adult	8.0
B	RC-002	Male	Adult	8.1
B	RC-038	Male	Adult	5.7
C	RC-005	Female	Adult	5.4
C	RC-224	Male	Adult	9.1
C	RC-369	Male	Adult	7.5

* BW: Body weight (kg).

Sequence analysis of the amplified PCR product revealed a high degree of homology compared to previously-reported BDV sequences derived from the horse (AY247158), cow (AB246670), sheep (AY066023), dog (DQ116031),

Table 3. Matrix comparison of the nucleotide and amino acid differences between raccoon (RC) and previously reported BDV-P sequences

AA \ **NA**	AY247158 horse	AB246670 cow	AY066023 sheep	DQ116031 dog	AB281092 macaque	DQ251041 shrew	AB469325 RC 3	AB469326 RC 5	AB469327 RC 10
AY247158 horse		**81.5**	**95.8**	**95.8**	**99.4**	**96.1**	**99.1**	**99.7**	**99.4**
AB246670 cow	85.7		**84.1**	**82.0**	**81.5**	**82.7**	**81.3**	**81.7**	**82.0**
AY066023 sheep	99.1	85.0		**96.4**	**95.8**	**97.2**	**95.5**	**96.1**	**96.4**
DQ116031 dog	99.1	85.0	98.3		**95.8**	**98.6**	**95.5**	**96.1**	**96.4**
AB281092 macaque	99.1	85.0	98.3	98.3		**96.1**	**99.1**	**99.7**	**99.4**
DQ251041 shrew	99.1	85.0	98.3	98.3	98.3		**95.8**	**96.4**	**96.6**
AB469325 RC 3	98.3	84.2	97.5	97.5	97.5	97.5		**99.4**	**99.1**
AB469326 RC 5	100	85.7	99.1	99.1	99.1	99.1	98.3		**99.7**
AB469327 RC 10	100	85.7	99.1	99.1	99.1	99.1	98.3	100	

NA: nucleotide acid differences (bold). AA: amino acid differences.

macaque (AB281092) and shrew (DQ251041). Comparison with the previously-reported BDV sequences showed that the homology of the nucleotide sequence was more than 95.5%. On the other hand, the homology with the cow was low (81.3–82%). Comparison of amino acid sequence showed that the homology with the cow was 84.2–85.7%; however, the homology with the other animals was high, more than 97.7% (Tables 2 and 3). Nevertheless, the BDV-P sequence derived from the brain tissue sample collected from #3 differed from the sequences derived from the other animals at nucleotide positions 269 and 337 (Fig. 2a). The BDV-P sequence derived from #10 differed from the sequences derived from the horse, macaque and the other two seropositive raccoons (#3 and #5) at nucleotide position 84. It is worth noting that this difference at position 84 was conserved in the sequences derived from the cow, sheep, dog and shrew, whereas the two differences in the sequence derived from #3 were not conserved in any of the other sequences. A similar degree of conservation was observed among the corresponding deduced amino acid sequences; among the amino acid substitutions detected, two amino acid residues (positions 90 and 113) corresponded to non-synonymous changes (E to G and A to T) in the sample collected from #3 (Fig. 2b).

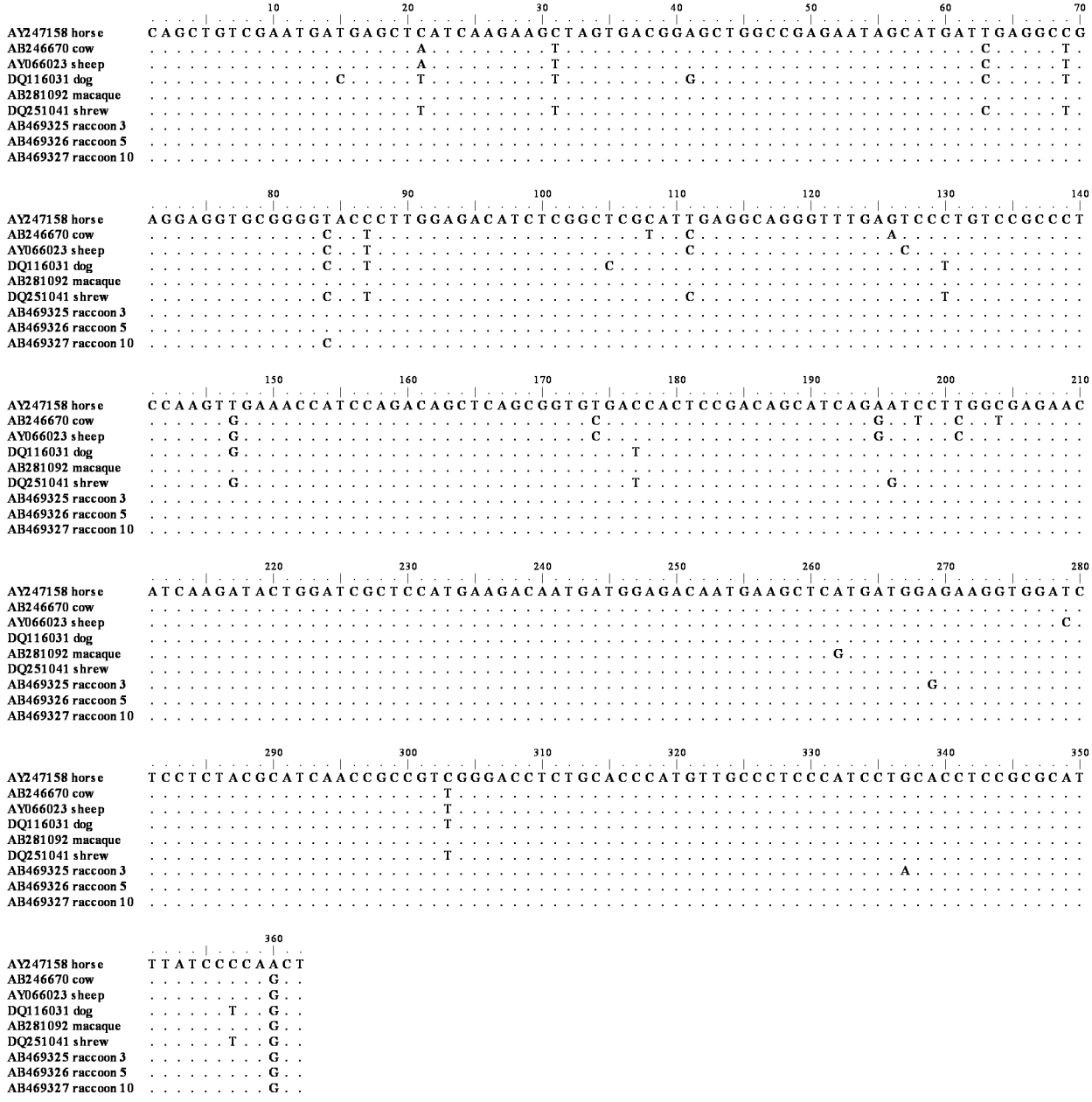
DISCUSSION

The raccoon is originally from the North American continent and is not indigenous to Japan. Over the past 20 years, however, raccoons have been imported from North America as companion animals, and subsequently released into the wild. They now inhabit farmlands and fields in many parts of Japan, including Hokkaido, and their numbers increase every year. In this study, we determined the prevalence of seropositive raccoons in Hokkaido and observed BDV RNA in raccoon tissue. BDV phosphoprotein sequences showed high rates of conservation compared to previously-reported sequences from several other animals.

Epidemiological data from other studies support the view of a worldwide distribution of BDV and a natural host range of BDV that is broader than previously thought [15, 27, 28, 29]; though these findings remain controversial [3, 4]. Notably, with the exception of a unique case [22], sequence analysis of field isolates has revealed a high degree of genetic conservation both among isolates from different species and among isolates of the same species from different geographic locations and among isolates of the same species isolated at different times [15]. This feature of the epidemiology of BDV is suggestive of the existence of reservoir species in which the virus has reached a certain degree of evolutionary equilibrium. These reservoirs would be the source of infections in species known to develop disease upon BDV infection. This explanation could account at least in part for the genetic stability of BDV isolates from BD-infected animals. Recent evidence from Switzerland indicates that the bicolored white-toothed shrew, *Crocidura leucodon*, is probably one of these BDV reservoir species [13], but this does not exclude the existence of additional BDV reservoir species that could account for the presence of BDV in geographic regions where *C. leucodon* is not present.

Several previous studies have documented the detection of BDV serum antibodies and virus RNA in a variety of domestic animals (sheeps, cows and horses) in Japan [9–11, 20]. These findings led us to investigate the possible presence of BDV in companion animals (dogs and cats) and wild animals (such as a non-human primates) in Japan [19, 23]. As part of this series of experiments, we took advantage of an opportunity to access samples from Japanese raccoons captured as part of a control program undertaken by the government of Hokkaido Prefecture.

Here, for the first time, we present serological and biochemical evidence of naturally-occurring BDV infection in the raccoon, a recently introduced terrestrial species that became wild. We detected antibodies to BDV antigens in sera from 2.0% (11/549) of the raccoons examined. It is difficult to compare this antibody prevalence with those in other wild species due to the limited amount of available data, but we can state that this seroprevalence was lower than those in previously studied domestic species. Of the eleven seropositive raccoons trapped in three different regions, ten were adult animals, and one was a young male. There was a higher incidence of BDV-seropositive cases among male raccoons, though the control program, which



was primarily intended to decrease the raccoon population, captured raccoons of each sex at a somewhat equal rate (225 males, 324 females). It was not possible, however, to examine correlations between BDV seroprevalence and sex.

We isolated RNA from brain tissue samples from five BDV-seropositive cases and examined the RNA for the presence of BDV sequences. Three of the five cases (#3, #5 and #10) were positive for BDV RNA. The reasons why not all of the samples from seropositive animals contained BDV RNA are unknown. Differences in sample handling, especially in the amount of time that passed between collection and storage at low temperature, might have contributed to our failure to detect BDV RNA in some samples. Likewise, differences among individual raccoons with respect to the course of infection could account for this outcome. Although BDV is characterized by its ability to persist, we cannot rule out the possibility that, in some cases, infected raccoons can clear the virus and retain only serum antibodies as a signature of infection. Likewise, significant heterogeneity in BDV distribution within the brains of infected animals has frequently been reported, which suggests that our technique of sampling a single brain region (the hippocampus) in raccoons might have caused us to overlook the presence of BDV RNA in other brain regions. Interestingly,

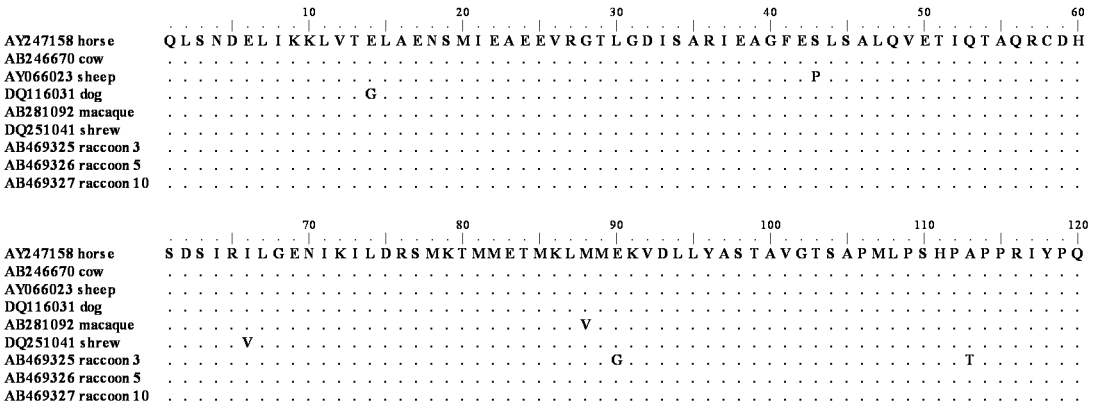


Fig. 2. Comparison of BDV-P nucleotide sequences. A: Comparisons of nucleotides 1473 to 1834 of the BDV-P sequences derived from raccoon brain tissue samples #3, #5 and #10 with those of previously reported BDV-P sequences from other species. The raccoon (*Procyon lotor*) nucleotide sequence data shown here appears in the GenBank nucleotide sequence database under the following accession numbers: AB469325, AB469326 and AB469327. Previously published BDV-P sequences derived from the horse (AY247158: France 2003), cow (AB246670: Japan 2006), sheep (AY066023: Germany 2001), dog (DQ116031: Austria 2005), macaque (AB281092: Japan 2006) and shrew (DQ251041: Austria 2005) are shown as controls. Nucleotide identity is indicated by dots. Nucleotide changes are indicated by the single-letter abbreviation for the nucleotide. B: Comparison of BDV-P amino acid sequences. The amino acids deduced from the corresponding nucleotide sequences are shown. Previously published BDV-P sequences derived from the horse, cow, sheep, dog, macaque and shrew are also shown as controls. Amino acid identity is indicated by dots, whereas amino acid changes are indicated by their single-letter database codes (SLC).

the BDV-P sequence derived from case #3 exhibited some differences from previously-reported sequences derived from domestic animals.

The close proximity of regions A and C allows for the possibility of contact between the raccoon groups inhabiting these two regions and therefore prevented us from establishing any direct correlation between seroprevalence and the geographic distribution of BDV in the raccoon population. Two of the seropositive raccoons (#3 and #10) were captured in region A, while the third (#5) was captured in region C (Fig. 1). The highest seroprevalence was observed in region A. Region A contains many ranches and farms, and several farmers in this region reported that they had witnessed raccoons going in and out of sheds located on farms. Thus, proximity to livestock may be one route of infection for BDV in raccoons, but there is no evidence to confirm this hypothesis.

The home ranges of wild raccoons in North America are larger for males than for females [18]. Moreover, while many females stay close to the home range of their mothers, males will sometimes move more than 20 km away [14]. The tendency of male raccoons to travel farther than females is one explanation for their higher incidence of seropositive cases. Although we could not confirm any relationship between region of capture and seropositive status, our results suggest that there is contact between raccoons and domestic animals in region A and that the virus may be spreading from there. To understand the origin of BDV in raccoons, we need to investigate large numbers of samples from wild raccoons in different regions of Japan as well as in North America and compare their BDV sequences.

ACKNOWLEDGMENTS. This study was supported in part by a grant-in-aid to the High Technological Research Center (Rakuno Gakuen University) and a grant-in-aid for Scientific Research (project number: S0891002) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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