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High Prevalence of Borna Disease Virus Infection in Healthy Sheep in Japan

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Previous seroepidemiological and molecular epidemiological studies of Borna disease virus (BDV) showed considerably high rates of infection in horses, cattle, cats, and humans in Hokkaido, Japan. Here, we further demonstrate high rates of specific antibodies to BDV and BDV RNA in peripheral blood mononuclear cells (PBMCs) from healthy sheep bred on the same island. The BDV prevalences by immunoblotting and/or reverse transcriptase PCR were 0% (0 of 19) in newborns (<1 month old), 51.7% (15 of 29) in lambs (1 to 6 months old), and 36.7% (11 of 30) in adults (>2 years old). Among animals positive for BDV, 60% of lambs and 45.5% of adults contained BDV RNA in PBMCs while 46.7% of lambs and 90.9% of adults contained specific antibodies to BDV. Thus, it is suggested that virus replication in the blood, as observed in lambs, is usually reduced in adulthood by raising immune responses to BDV.

Borna disease virus (BDV) is a neurotropic, as-yet-unclassified, nonsegmented, negative-sense, single-stranded RNA virus (12, 35). BDV naturally infects horses and sheep and induces a disease characterized by progressive meningoencephalopathy (17, 24, 25, 30, 38). In addition, BDV naturally infects cats, cattle, and ostriches. BDV-specific antibodies are readily detected in these animals even when there are no clinical signs, suggesting that BDV is more widespread than previously thought (16, 19, 23, 27, 28). BDV may be closely associated with specific psychiatric disorders in humans, since serum antibodies to BDV have been detected at high rates (1, 4, 6–9, 15, 31–33, 37, 39). Similarly, even healthy individuals or patients without psychiatric disorders may contain antibodies to BDV, although the prevalence is very low (6–8, 31).

We developed a molecular epidemiological protocol (21, 22, 27–29) to detect BDV RNA in peripheral blood mononuclear cells (PBMCs) by a nested reverse transcriptase PCR (RT-PCR) at the second open reading frame (ORF II), which codes a phosphorylated protein (polymerase cofactor), p24 (12, 35). By this technique, we showed that the prevalence of BDV RNA in blood donors was significantly lower (21) than that in patients with psychiatric disorders (22) or chronic fatigue syndrome (29). On the other hand, there have been no reports of Borna disease in horses and sheep in Japan. However, considerably high prevalences have been demonstrated in healthy and neurological-disease-unrelated animals, such as thoroughbred horses (28), cattle (16), and cats (27) in Hokkaido (the northernmost island of Japan), as well as horses in Tehran, Iran (3). In this study, we examined for the first time the BDV prevalence in healthy sheep bred on Hokkaido. Immunoblotting and RT-PCR showed no BDV-positive animals among 19 newborns examined. In sharp contrast, the BDV prevalences in lambs and adults were considerably higher than those found in other animals on this island. The prevalences for samples from lambs and adults were significantly different. The detection rate of BDV RNA in PBMCs, without specific serum antibodies, was higher for lambs, while the detection rate of serum antibodies was significantly higher for adults.

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MATERIALS AND METHODS

Plasma and PBMC preparations from animals. We examined BDV seroprevalences in sera stored at −70°C and prepared from 317 healthy adult sheep at a Hokkaido farm in 1989. In addition, EDTA-treated blood samples were also obtained from 19 (1 through 19) newborns (<1 month old; mean age, 20.3 days), 29 (20 through 48) lambs (1 to 6 months old; mean age, 3.2 months), and 30 (49 through 78) adults (2 to 7 years old; mean age, 4.8 years), all of which were reared on the same farm during 1995 and 1996. No original sheep from 1989 was included in the adult group of 30 sheep tested in 1995 and 1996. None of these animals showed neurological symptoms. After plasma was removed from EDTA-treated blood samples, PBMCs were isolated by Ficoll-Conray centrifugation (density, 1.087 g/ml).

Virus and cells. The controls were uninfected MDCK cells or MDCK cells persistently infected with a horse-derived BDV (MDCK/BDV) (18).

Detection of anti-BDV antibodies. Anti-BDV antibodies in serum and plasma samples were detected by enzyme-linked immunosorbent assay (ELISA) or immunoblotting with peroxidase-conjugated rabbit immunoglobulin G (IgG) fraction to sheep immunoglobulin G (Organon Teknika Corporation, Durham, N.C.), as previously described (2, 22). The BDV antigens for these assays are ORF I and ORF II, coding for p40 nucleoprotein and p24 polymerase cofactor, respectively (12, 35). Horse BDV-derived recombinant full-length p40 and p24 fusion proteins with glutathione *S*-transferase (GST) were expressed in *Escherichia coli*, as previously described (3, 22). The negative control antigen was GST alone. These GST-p40, GST-p24, and GST proteins were purified by glutathione-Sepharose 4B (Pharmacia Biotech AB) column chromatography before being used.

Extraction of total cellular RNA. Total cellular RNA was prepared by using an RNA extraction kit (RNAzol B; Cinna/Biotecx Laboratories International, Inc.), as previously described (10).

RT-PCR. Extracted RNA was amplified by nested RT-PCR, as previously described (22), to obtain a fragment of the p24 coding region which is relatively conserved within the BDV genome (36). The control experiment included uninfected MDCK cells or persistently infected MDCK/BDV cells. Briefly, 1 μg of cellular RNA was amplified by nested RT-PCR with two sets of primers as follows: for the first PCR, nucleotides 1387 through 1405 and 1865 through 1847, and for the second PCR, nucleotides 1443 through 1461 and 1834 through 1816. RT-PCR, consisting of reverse transcription and amplification of viral cDNA, was performed according to the protocol supplied with the EZ *rTth* RNA PCR kit (Perkin-Elmer Corporation). PCR products were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, and Southern hybridized with four ^{32}P-labeled synthetic oligonucleotides, sense nucleotides 1462 through 1485, 1485 through 1507, and 1637 through 1658 and antisense nucleotides 1811 through 1791.

Cloning with subsequent sequencing of PCR products. PCR products were cloned into a pUC18 plasmid vector (Pharmacia Biotech AB). Several representative clones from individual sheep were sequenced according to the protocol of

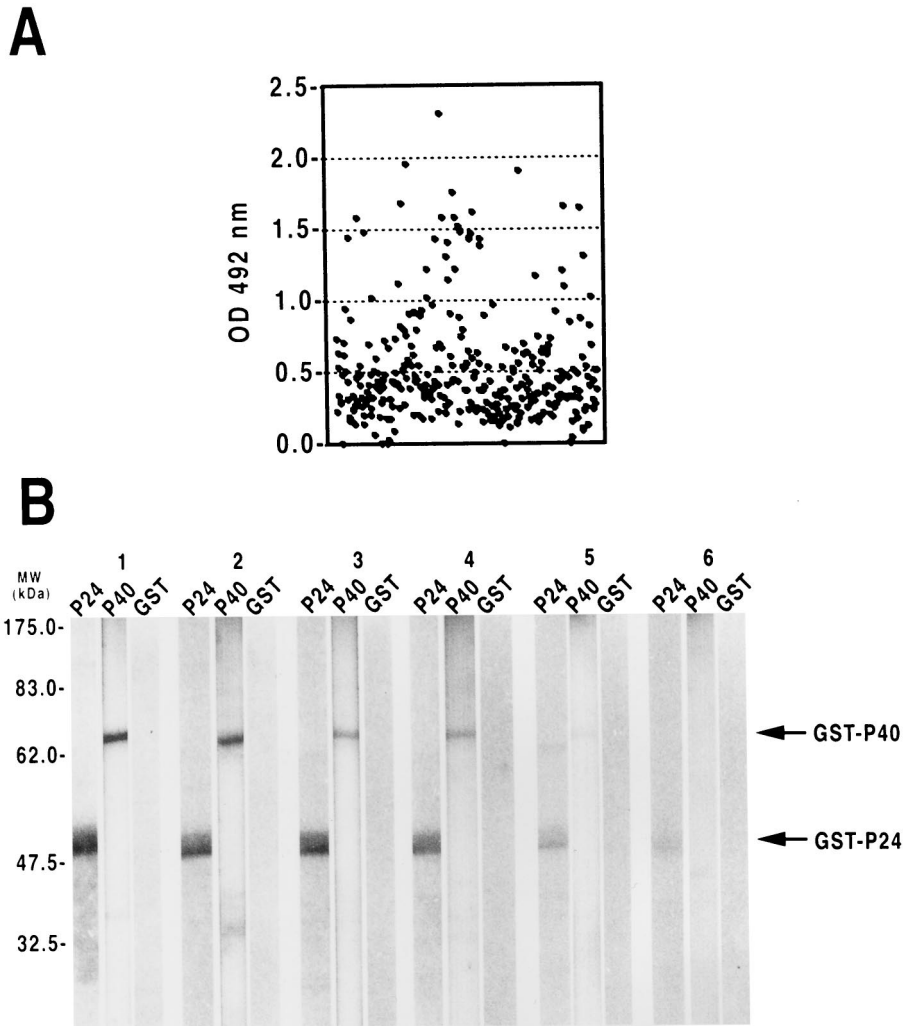

FIG. 1. Detection of BDV-specific antibodies in plasma samples obtained from healthy adult sheep in 1989 by ELISA and immunoblotting. (A) A total of 317 sera from healthy adults obtained in 1989 was examined by ELISA with GST-p24 and with GST alone as the control. Data are absorption (optical density [OD]) values at 492 nm for GST-p24 after the values for GST alone in individual samples have been subtracted. (B) The specificity of the antibody reaction by ELISA was confirmed by immunoblotting six samples (samples with ELISA values of 1.8, 1.2, 0.8, 0.6, 0.5, and 0.4 [lanes 1 through 6, respectively]) with GST-p24 (approximately 51 kDa) and GST-p40 (67 kDa) and with GST alone (27 kDa) as the control. A 100-fold dilution of plasma was reacted with the nitrocellulose membrane. Molecular masses (MW) were determined by comparisons with marker proteins in a calibration kit (New England Biolabs, Inc.).

the dye primer cycle sequencing kit (Applied Biosystems) by using the −21M13 dye primer and M13 reverse dye primer in a 373-A DNA sequencer. The nucleotide sequences were analyzed with GENETYX-MAC (Software Development Co., Ltd., Tokyo, Japan). All the BDV nucleotide sequence numbers described here correspond to the previously reported numbering scheme for strain V of BDV (11, 36).

Nucleotide sequence accession numbers. The BDV p24 sequences are available in the DDBJ, EMBL, and GenBank DNA databases under accession no. AB001470 for clone 49-1, AB001471 for clone 49-5, AB001472 for clone 72-2, AB001473 for clone 72-4, AB001474 for clone 59-1, and AB001475 for clone 59-2.

RESULTS

Varied BDV prevalences in newborns, lambs, and adults. Initially, the sera of 317 adult sheep obtained from one farm on the island of Hokkaido in 1989 were examined by ELISA to determine the presence of anti-BDV antibodies. GST-p24 and GST alone (as a control) were purified by affinity column chromatography. The results were evaluated after the absorbance (at 492 nm) values for GST alone had been subtracted from those of GST-p24 in individual samples (Fig. 1A). The mean absorbance ± standard deviation was 0.5 ± 0.3. Sera from 85 of 317 (26.8%) sheep had an absorbance of >0.6. Immunoblotting of randomly selected sera with GST-p24 and GST proteins confirmed the specific reactions of four sera with an absorption (at 492 nm) of at least 0.6 by ELISA (Fig. 1B). Similar positive reactions to GST-p40 were also detected in these sera. Thus, the seroprevalence in sheep was quite high. Based on these results, we collected EDTA-treated blood samples from sheep at the same farm during 1995 and 1996. These sheep included 19 newborns, 29 lambs, and 30 adults. The plasma samples were immunoblotted to identify anti-BDV antibodies. PBMC fractions from Ficoll-Conray centrifugation were analyzed by nested RT-PCR.

Plasma immunoblots showed positive signals reactive only with GST-p24, not GST, for 0% (0 of 19) of newborns, 24.1% (7 of 29) of lambs, and 33.3% (10 of 30) of adults. Profiles of representative (four positive and two negative) samples are shown in Fig. 2. The four samples positive for anti-p24 were also positive for anti-p40 antibodies. On the other hand, the results of the nested RT-PCR, which detected BDV-related RNA at the p24 region in PBMCs from these animals, were

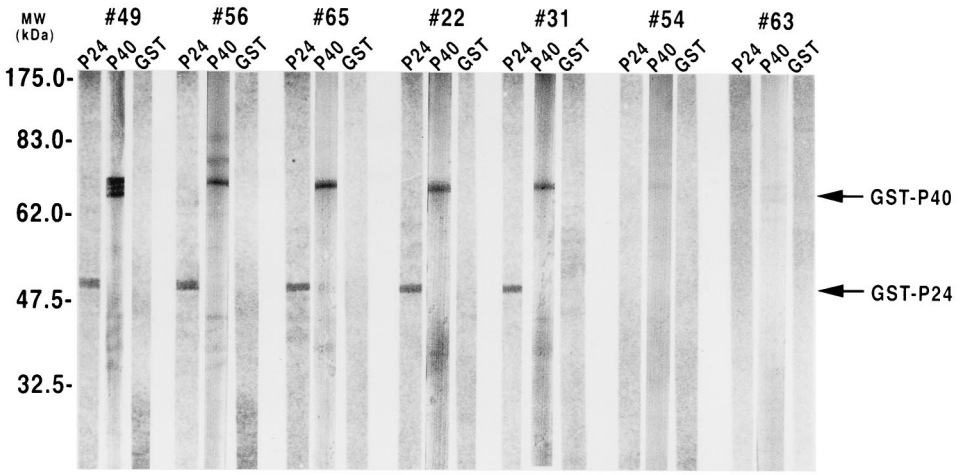

FIG. 2. Immunoblots of anti-BDV antibodies in plasma samples prepared from healthy sheep in 1995 and 1996. A 100-fold dilution of plasma from healthy sheep obtained in 1995 and 1996 was reacted with nitrocellulose membranes prepared as described in the legend to Fig. 1B. Representative sample results (adults 49, 56, and 65 and lambs 22 and 31 [with positive signals to GST-p24 and GST-p40] and adults 54 and 63 [with no signals]) are shown. The positions of molecular mass (MW) markers are indicated on the left.

quite different. Signals were positive in 0% (0 of 19) of newborns, 31.0% (9 of 29) of lambs, and 16.7% (5 of 30) of adults by both ethidium bromide staining and Southern blot hybridization. Figure 3 shows representative results. All PCR products, except that from adult sheep 59, which showed a smaller (~270-bp) band, contained a discrete band corresponding in size (392 bp) to the fragment from persistent BDV in MDCK/BDV cells.

As summarized in Fig. 4, comparisons between the prevalences of lambs and adults for BDV RNA and anti-BDV antibodies revealed a significant difference. Animals positive for BDV RNA in their PBMCs were not always positive for anti-BDV antibodies in their plasma samples. The percentage positive for both anti-BDV and BDV RNA was 3.4% (1 of 29) of lambs and 13.3% (4 of 30) of adults. Consequently, the BDV prevalences by both techniques were 51.7% (15 of 29) in lambs, 36.7% (11 of 30) in adults, and 44.1% (26 of 59) in both adults and lambs, whereas it was 0% (0 of 19) in newborns. Thus, among animals positive for BDV by immunoblotting and/or RT-PCR, 60% (9 of 15) of lambs and 45.5% (5 of 11) of adults contained BDV RNA in their PBMCs while 46.7% (7 of 15) of lambs and 90.9% (10 of 11) of adults contained specific antibodies to BDV.

Among the subjects examined here, there were three cases positive for BDV between mothers and offspring i.e., lambs 21 and 22 (negative for RNA but positive for antibodies), 31 (positive for both RNA and antibodies), and 48 (negative for RNA but positive for antibodies) were born to mothers 53 (positive for RNA but negative for antibodies) and 62 and 78 (negative for RNA but positive for antibodies), respectively. In contrast, newborns 10 and 11, 12 and 13, 15 and 16, and 19 were born to mothers 72 (positive for both RNA and antibodies), 73, 75, and 77 (negative for RNA but positive for antibodies), respectively.

Comparison of p24 sequences in PCR products. Three PCR-positive animals, adults 49, 59, and 72, were randomly selected, and p24 sequences were compared. PCR fragments were cloned into the pUC18 plasmid vector. Figure 5 shows the sequences of two representative clones from each of these three individuals. The cDNA clones from adult 59 were derived from the smaller PCR product (Fig. 3). The reported p24 sequences from horse-derived BDVs in Europe, strains V,

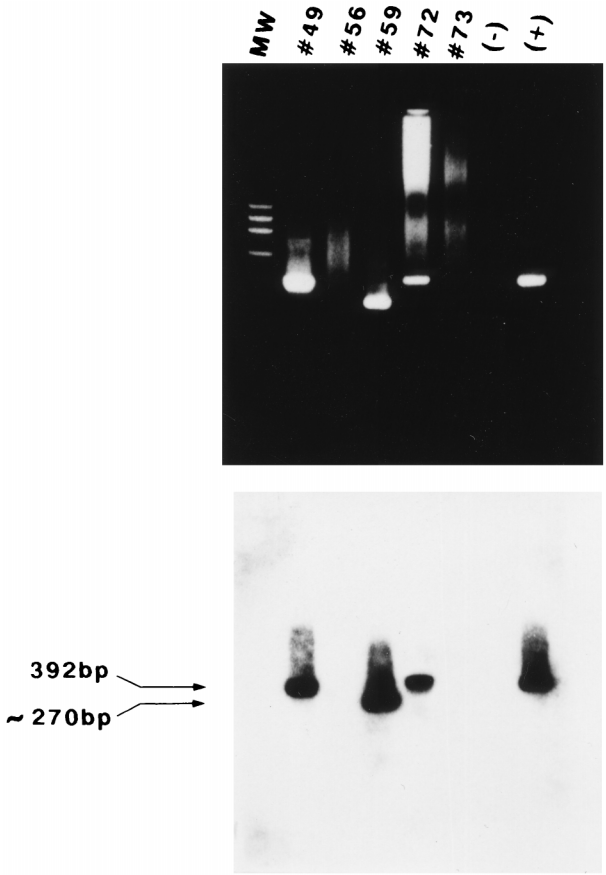

FIG. 3. Nested RT-PCR for the detection of BDV RNA in PBMCs prepared from healthy sheep in 1995 and 1996. Representative results of nested RT-PCR samples derived from three positive samples (adults 49, 59, and 72) and two negative samples (adults 56 and 73) are shown. (Top) The products of PCR amplification of the BDV p24 region were resolved by agarose gel electrophoresis and then stained with ethidium bromide. (Bottom) The results of Southern blot hybridization with four oligomers as probes are shown. The positive and negative controls were RNA fractions from MDCK/BDV (+) and MDCK (−) cells, respectively. Lane MW, size markers (ϕx DNA/*Hae*III fragments).

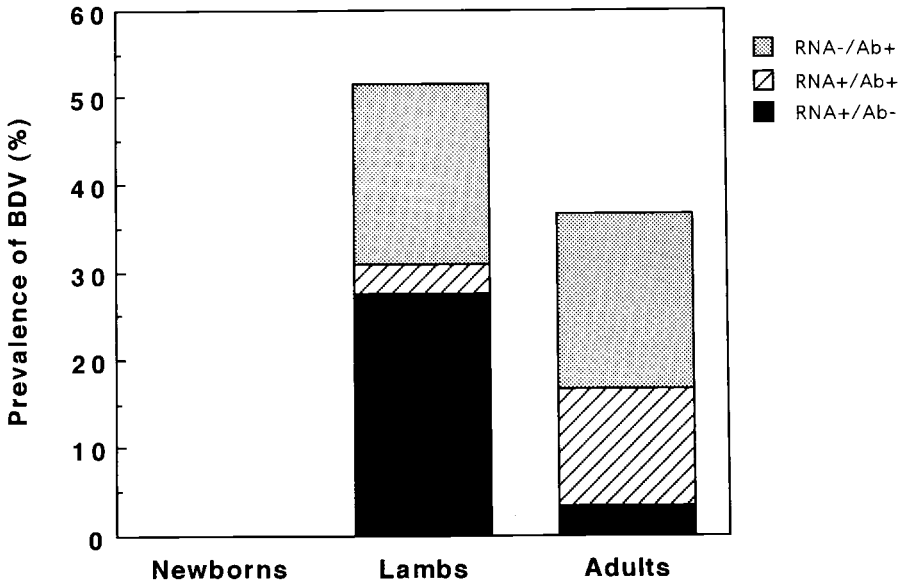

FIG. 4. Summarized results for BDV prevalence in healthy sheep on Hokkaido. The results for anti-BDV antibodies (Ab) in plasma samples and BDV RNA in PBMCs obtained from 19 newborns, 29 lambs, and 30 adults were derived by immunoblotting and nested RT-PCR, respectively. +, positive; −, negative.

He/80, and WT-1 (11, 36), were used as standards. The p24 sequences of two clones derived from one individual were very similar. The nucleotide sequences were also similar between BDVs derived from adults 49 and 59, which were also related to the sequence of horse-derived strain V. The nucleotide sequence from adult 72 was related to that of He/80.

The deletion in the p24 sequence of adult 59 could be due to the repeat sequence (AAG) found in the deletion junction (Fig. 5), which is also found in PCR products from psychiatric patients (22) and dairy cattle (16).

Demonstration of BDV RNA in the brains of sheep positive for serum antibodies to BDV but negative for BDV RNA in PBMCs. Among animals positive for BDV by immunoblotting and/or RT-PCR, 90.9% (10 of 11) of adults, in contrast to 46.7% (7 of 15) of lambs, contained specific antibodies to BDV (Fig. 4). Most of them were negative for BDV RNA in PBMCs. Therefore, we searched for the BDV reservoir in lamb 44, which was positive only for anti-BDV. The negative control was lamb 45, which was negative for both anti-BDV and BDV RNA.

Total RNA samples extracted from the cerebrum cortex, cerebrum (white matter), pons, medulla oblongata, hippocampus, lateral ventricle, olfactory bulb, and cerebellum of the brain were examined by nested RT-PCR under the conditions described in the legend to Fig. 3. RNA samples from the spinal cord, lymph node, liver, kidney, and spleen as well as PBMCs were also similarly characterized for BDV RNA. RT-PCR revealed BDV RNA only in the olfactory bulb of the brain from lamb 44, not in other regions of the brain, internal organs, spinal cord, or PBMCs (data not shown). BDV RNA signals were undetectable in any of these organs from lamb 45 (data not shown).

DISCUSSION

In this study, we focused on the BDV prevalence in healthy sheep bred on the island of Hokkaido in Japan, since we had found that horses, cattle, and cats on this island were infected with BDV at considerably high rates (16, 27, 28). By immunoblotting and nested RT-PCR, we found that the prevalence in sheep was higher than those in the animals listed above (Fig. 4). The susceptibility of sheep to BDV infection was previously reported (30, 38). In fact, it was also reported that one sheep exhibiting signs typical of Borna disease was positive for anti-BDV antibodies and BDV RNA in the cerebrum (5).

Vertical transmission from infected mothers may explain the spread of BDV. However, none of 19 newborns were positive for either BDV RNA or anti-BDV. At least seven of the newborns were born to BDV-positive mothers. Thus, these results do not support the notion of vertical transmission of BDV in sheep. However, we detected BDV-positive signals in four lambs born to BDV-positive mothers. The findings for human infants born to mothers infected with human immunodeficiency virus (HIV) indicate that this virus is transmitted in this manner. Blood HIV titers consistent with primary viremia are increased in most infants born to HIV-infected mothers at several months of age (13, 14, 26). Therefore, the presence of HIV cannot usually be diagnosed at birth, suggesting that viral transmission occurs during late pregnancy and/or delivery. Similarly, we did not detect BDV RNA in the PBMCs of any of the newborns examined. In addition, the prevalences identified by both techniques varied for different generations of sheep, with a much higher prevalence in terms of BDV RNA than of serum antibodies in lambs (60.0 versus 46.7%) but the reverse in adults (45.4 versus 90.9%) (Fig. 4). Similarly, BDV RNA was not always detected in PBMCs even from anti-BDV antibody-positive individuals, such as horses (3, 28), cattle (16), cats (27), and humans (21, 22, 29, 34). Thus, most of the BDV-positive lambs examined carried BDV RNA in PBMCs without BDV-specific antibodies, which might correspond to primary viremia. Thereafter, BDV RNA in the blood would be easily eliminated by the immune response to BDV, since we detected a high rate of specific-antibody-positive, BDV RNA-negative adults. Of particular note is the detection of BDV RNA only in the brain of an adult sheep positive for anti-BDV but negative for BDV RNA in PBMCs (data not shown). Therefore, it is likely that some animals become persistently infected with BDV in the brain even after the virus is cleared from blood cells. Thus, possible vertical transmission of BDV

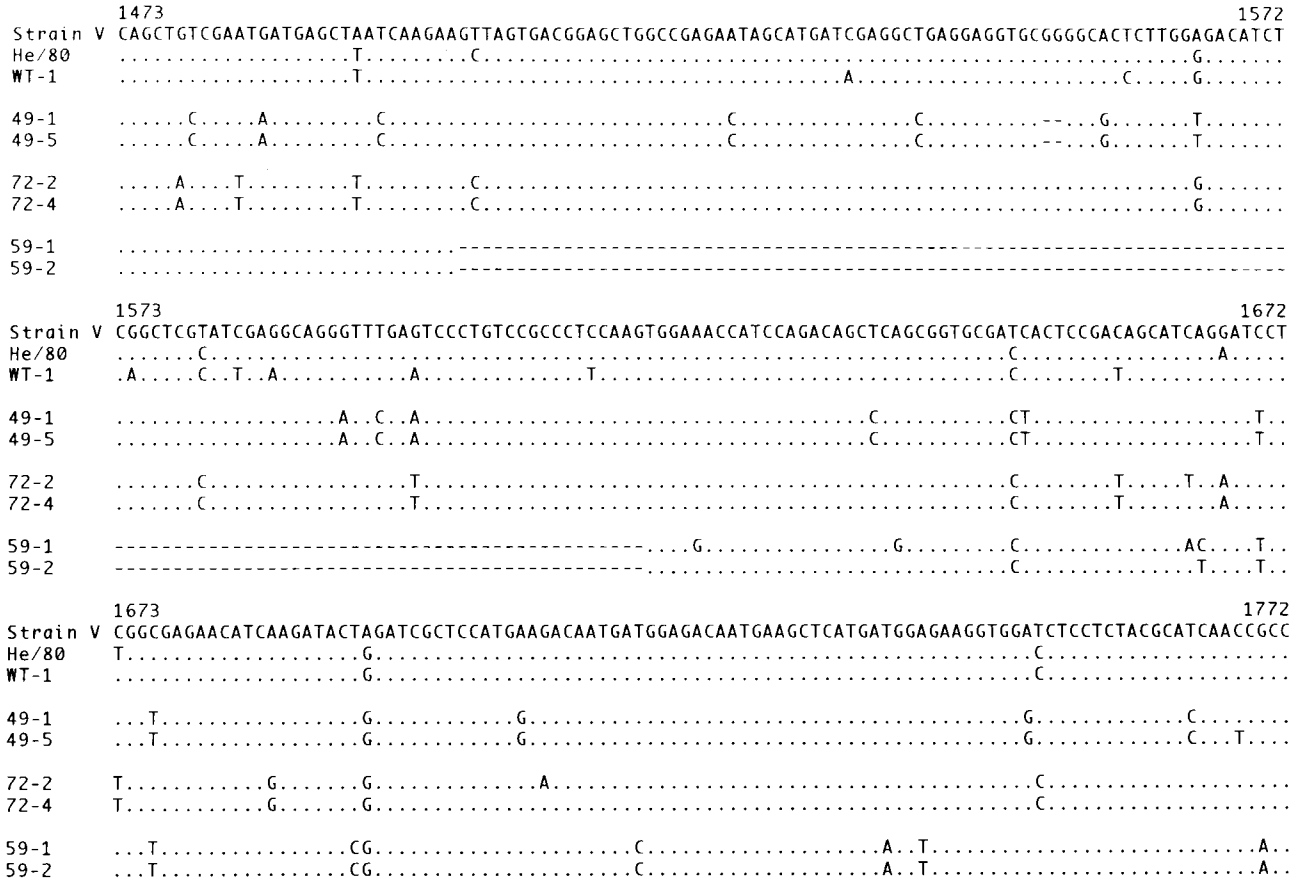

FIG. 5. Comparison of the p24 nucleotide sequences of BDVs derived from sheep PBMCs and horses. The sequences of p24 (nucleotides 1473 to 1772) in two cDNA clones obtained from adults 49, 59, and 72 are shown. The reported sequences of horse-derived BDVs (strains V, He/80, and WT-1) (11, 36) are also shown as controls. Dashes indicate deleted nucleotides. Both cDNA clones from adult 59 contained a large deletion which corresponds to the smaller band in Fig. 3. Dots indicate nucleotides identical to those of strain V.

in sheep remains to be determined by extensive follow-up studies, including cellular immune response to BDV.

The association of BDV with psychiatric disorders in humans has been postulated from the high prevalence of anti-BDV antibodies in these patients compared with that of healthy people (6–8, 31, 33). The demonstration of BDV RNA in PBMCs of healthy horses (28) and cats (27), as well as of patients with psychiatric disorders (22), patients with chronic fatigue syndrome (29), and blood donors (21), suggests a broader route of natural transmission as a hematopoietic-cell-related virus than as a highly neurotropic virus. Therefore, further comparative studies of BDVs derived from animals and humans are quite important for clarifying how BDV is transmitted among infected animals and humans. The sequencing of PCR products derived from PBMCs of infected animals and humans seems to be useful for understanding the route of natural transmission of BDV in individual host species. At present, the sequencing results for sheep (Fig. 5) suggest close relationships between adult 49 and 59-derived PCR products and horse-derived strain V (11, 36) and between the adult 72-derived PCR product and He/80 (11, 36). The close relation of sheep-derived BDV to horse-derived BDV in the p24 region was also reported by Binz et al. (5). The nucleotide sequences of adult 49 and 59-derived products show high levels of similarity with those of BDVs derived from some patients with psychiatric disorders (20, 22) and chronic fatigue syndrome (29) and from horses (3) in Japan. The deletion in the PCR product derived from adult 59 (Fig. 5) occurred at the same site as those found in products derived from psychiatric patients (22) and dairy cattle (16). The mutations observed here may have been PCR artifacts from the EZ *rTth* PCR kit, as recently described (34). However, such mutations would be random events and would not cause mutations at a few nucleotide positions in cDNA clones from each animal. In fact, a comparison between this method and another [reverse transcription with oligo(dT) primers followed by amplification of the resulting cDNA by a similar nested RT-PCR] with the same RNA samples from horses showed no apparent differences between the products (3). Thus, these results indicate that the technique would not be a major reason for the mutations at least at the sites commonly observed in cDNA from each animal.

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