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Varied prevalence of Borna disease virus infection in Arabic, thoroughbred and their cross-bred horses in Iran

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Abstract

Borna disease virus (BDV) naturally infects horses and sheep and induces progressive poliomeningoencephalomyelitis. Here, BDV recombinant proteins of the first open reading frame (ORF-I; coding for p40 nucleoprotein) and the second ORF-II (coding for p24 polymerase cofactor) were immunoblotted with plasma derived from 72 healthy (28 Arabic, 17 thoroughbred and 27 cross-bred) race horses at Tehran in Iran to detect anti-BDV antibodies. In addition, their peripheral blood mononuclear cells (PBMCs) were also examined for BDV RNA by a nested reverse transcriptase-polymerase chain reaction (RT-PCR) at ORF-II. The prevalence of BDV antibodies and/or RNA was 41.2% in Arabic, 23.5% in thoroughbred, and 33.3% in cross-bred horses, but only 17.9, 5.9, and 11.1% of them, respectively, showed positive signals for both BDV antibodies and RNA. Especially, cross-bred horses showed a higher prevalence for BDV RNA, which was detected only in females. In addition, significantly higher prevalence for BDV RNA was observed in Arabic males and thoroughbred females. The BDV prevalence did not increase with aging of the horse. Sequencing at the region of BDV derived from Iranian horses revealed a slight difference from those of Japanese horse- and European horse-derived BDVs even in the amino acid residues, although those in the three groups of Iranian horses were quite similar. Thus, the varied prevalence of BDV was observed with the horse strain or sex in Iranian horses, although BDV sequences were very similar among all three groups in Iran compared with those derived from other countries.

Keywords: Borna disease virus; Prevalence; Sequence variation

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1. Introduction

Borna disease virus (BDV) is a neurotropic, yet unclassified, nonsegmented, negative-sense, single-stranded RNA virus (de la Torre, 1994; Schneemann et al., 1995). BDV naturally infects horses and sheep and induces a disease characterized by a progressive meningoencephalopathy (Ludwig et al., 1973). In addition, BDV naturally infects cats, cattle and ostriches (de la Torre, 1994). BDV, or a related agent, has also been suggested to be closely associated with specific psychiatric disorders in humans, compared with healthy people (Rott et al., 1985; Bode et al., 1988, 1992, 1993; Richt et al., 1993b).

Neurological symptoms characteristic of the disease in horses have been recognized since the end of the 18th century, mainly in the endemic areas in Germany (Ludwig et al., 1993). More recently, the infection was demonstrated in Switzerland (Richt et al., 1993b) and the USA (Kao et al., 1993). These epidemiological studies have been carried out by conventional serological assays such as immunofluorescence. However, the antibody titers were usually very low (1:20–1:80). In fact, horses with clinical signs reportedly developed very low antibody titers (< 1:10) even when infectious BDV could be demonstrated in the brain, while other infected horses without apparent clinical signs sometimes displayed higher titers (more than 1:320) (Lange et al., 1987). Thus, more evidence for the BDV infection of horses as well as other BDV-sensitive animals or human patients with psychiatric disorders should be obtained by a more sensitive method such as polymerase chain reaction (PCR) to detect viral RNA to confirm the relevance of the results obtained by serological assays. In fact, PCR revealed the presence of BDV RNA in several tissues including brain from naturally infected horses, donkeys, and sheep (Richt et al., 1993a; Binz et al., 1994; Zimmermann et al., 1994) or in peripheral blood mononuclear cells (PBMCs) or bone marrow cells from experimentally infected rats (Sierra-Honigmann et al., 1993).

We have recently developed a molecular-epidemiological protocol (Kishi et al., 1995a,b; Nakamura et al., 1995, 1996; Nakaya et al., 1996) for BDV RNA in PBMCs using a nested reverse transcriptase-polymerase chain reaction (RT-PCR) at the second open reading frame (ORF-II) which codes a phosphorylated protein (polymerase cofactor), p24 (de la Torre, 1994; Schneemann et al., 1995). By using this technique, we have demonstrated the presence of BDV RNA in several areas in Japan, i.e. 29.8% of the healthy thoroughbred race horses at Hokkaido (Nakamura et al., 1995), 13.3% of hospitalized cats without neurological disorders at Hokkaido (Nakamura et al., 1996), 36.7% of the psychiatric patients at Hokkaido (Kishi et al., 1995b), 12.0% of patients with chronic fatigue syndrome at Osaka (Nakaya et al., 1996), and 4.7% of healthy blood donors at Hokkaido and Tokyo (Kishi et al., 1995a). Bode et al. (1995) also similarly showed higher prevalence of BDV RNA in PBMCs from psychiatric patients, compared with healthy donors. The sequences of amplified products of the samples derived from cats (Nakamura et al., 1996) and human patients with psychiatric disorders or chronic fatigue syndrome (Kishi et al., 1995b, 1996; Nakaya et al., 1996) were closely related to, but significantly different from the reported sequences of the horse-derived BDVs in Europe (Cubitt et al., 1994; Schneider et al., 1994). By contrast, the sequences of BDV RT-PCR products from psychiatric patients in Germany were shown to be highly conserved (Bode et al., 1995). Viral sequences from horses in several regions of Germany were shown to be highly conserved (Binz et al., 1994). However, it is unknown whether the prevalence of BDV infection is similar in different horse strains in the same country or whether the sequences of their BDVs were similar in horses in different countries. These points seem to be quite important for clarifying how BDV is transmitted between horses or among infected animals and humans. Herein, we examined BDV prevalence by immunoblotting for anti-BDV antibodies in plasma and by nested RT-PCR for BDV RNA in PBMCs from Iranian horses with different strains. The sequences of amplified products in the samples derived from horses at Tehran, Iran were comparatively analyzed with those from horses at Hokkaido, Japan.

Table 1
Profiles of 72 horses from Iran and summarized results for BDV

Horse no.	Sex	Age (year)	Breed	BDV genome in PBMC	Serum	Antibody
					p24	p40
1	F	3	Arabic	+	+	+
2	F	4	Arabic	−	−	−
3	F	5	Arabic	−	−	−
4	F	5	Arabic	−	−	−
5	F	5	Arabic	−	−	−
6	F	7	Arabic	−	−	−
7	F	7	Arabic	−	−	−
8	F	8	Arabic	−	−	−
9	F	8	Arabic	−	−	−
10	F	8	Arabic	−	−	−
11	F	11	Arabic	−	−	−
12	F	12	Arabic	−	−	−
13	F	14	Arabic	+	−	−
14	F	18	Arabic	+	+	+
15	F	19	Arabic	−	−	−
16	F	19	Arabic	−	−	−
17	F	19	Arabic	−	−	−
18	M	3	Arabic	+	−	+
19	M	3	Arabic	−	−	−
20	M	4	Arabic	+	+	+
21	M	4	Arabic	−	−	−
22	M	4	Arabic	−	−	−
23	M	4	Arabic	−	−	−
24	M	6	Arabic	+	+	+
25	M	13	Arabic	−	−	−
26	M	15	Arabic	−	−	−
27	M	16	Arabic	−	+	+
28	M	17	Arabic	−	−	−
29	F	4	Thoroughbred	−	−	−
30	F	6	Thoroughbred	+	+	+
31	F	6	Thoroughbred	−	+	+
32	F	7	Thoroughbred	−	−	−
33	F	7	Thoroughbred	−	−	−
34	F	8	Thoroughbred	−	−	−
35	F	12	Thoroughbred	−	−	−
36	F	15	Thoroughbred	+	−	−
37	F	18	Thoroughbred	−	−	−
38	M	4	Thoroughbred	−	−	−
39	M	6	Thoroughbred	−	−	−
40	M	12	Thoroughbred	−	−	−
41	M	12	Thoroughbred	−	−	−
42	M	13	Thoroughbred	−	−	−
43	M	14	Thoroughbred	−	+	+
44	M	24	Thoroughbred	−	−	−
45	M	24	Thoroughbred	−	−	−
46	F	3	Cross-bred	+	+	+
47	F	3	Cross-bred	+	+	+
48	F	3	Cross-bred	+	−	+
49	F	3	Cross-bred	−	−	−

Table 1 (*continued*)

Horse no.	Sex	Age (year)	Breed	BDV genome in PBMC	Serum	Antibody
					p24	p40
50	F	3	Cross-bred	−	−	−
51	F	4	Cross-bred	+	−	−
52	F	4	Cross-bred	+	−	−
53	F	7	Cross-bred	+	−	−
54	F	7	Cross-bred	−	−	−
55	F	8	Cross-bred	+	−	−
56	F	8	Cross-bred	−	−	−
57	F	8	Cross-bred	−	−	−
58	F	9	Cross-bred	+	−	−
59	F	10	Cross-bred	−	−	−
60	F	10	Cross-bred	−	−	−
61	F	12	Cross-bred	−	−	−
62	F	14	Cross-bred	−	−	−
63	F	14	Cross-bred	−	−	−
64	F	18	Cross-bred	+	+	+
65	F	19	Cross-bred	−	−	−
66	F	19	Cross-bred	−	−	−
67	M	8	Cross-bred	−	−	−
68	M	9	Cross-bred	−	−	−
69	M	13	Cross-bred	−	−	−
70	M	13	Cross-bred	−	−	−
71	M	15	Cross-bred	−	−	−
72	M	22	Cross-bred	−	−	−

2. Materials and methods

2.1. Plasma and PBMC preparations from animals in Iran

A total of 72 apparently healthy race horses at Tehran in Iran were randomly selected, i.e. 28 (11 male and 17 female) Arabic, 17 (8 male and 9 female) thoroughbred, and 27 (6 male and 21 female) cross-bred horses (Table 1). None of these horses had had neurological disorders. After removal of plasma from EDTA-treated blood, the PBMCs were isolated by centrifugation on Ficoll-paque (density, 1.077 g/ml).

2.2. Virus and cells

The cells used for controls were MDCK cells uninfected and persistently infected with a horse-derived BDV (MDCK/BDV) (Herzog and Rott, 1980).

2.3. Detection of anti-BDV antibodies

Anti-BDV antibodies in the plasma obtained from the same EDTA-treated blood were examined by immunoblotting, as described (Kishi et al., 1995b; Nakamura et al., 1995, 1996; Nakaya et al., 1996). Two BDV antigens, the first open reading frame (ORF-I; p40 nucleoprotein (de la Torre, 1994; Schneemann et al., 1995)) and the second ORF (ORF-II; p24 polymerase cofactor (de la Torre, 1994; Schneemann et al., 1995)), were used for these assays. The MDCK/BDV-derived recombinant full-length p24 fusion protein with glutathione *S*-transferase (GST) was expressed in *Escherichia coli* (Weiss et al., 1992), as described (Kishi et al., 1995b). The recombinant full-length p40 fusion protein with GST was also similarly expressed. The p40 region of BDV in MDCK/BDV was amplified at nucleotides 48–1194 by primers 5′-ATGGATCC ACGCAATGCCACCCAAG-3′ and 5′-GCC-GAATTCGGTTTTTTATTGG-3′, then cloned into pGEX-5X (Pharmacia Biotech AB, Uppsala, Sweden). The underlined portions of the primers indicate the restriction sites of *Bam*HI and *Eco*RI, respectively. A negative control antigen was GST alone. These GST-p24, GST-p40 and GST proteins were used after purification by glutathione Sepharose 4B (Pharmacia Biotech AB) column chro-

matography. The molecular weight values of the proteins after immunoblotting were calculated by comparing their mobilities with those of marker proteins in a calibration kit (Bio-Rad).

A 1:75 dilution of horse plasma was reacted with the BDV antigens on nitrocellulose membrane. The specificity of the serological reactivity was confirmed by competition experiments using representative positive horse plasma which were pre-absorbed with the BDV antigen. The plasma were incubated with GST-p24 or GST-p40 at a final concentration of 0.5 mg/ml for 1 h at 37°C. The absorbed plasma was diluted to 1:75 and then reacted with the BDV antigens on nitrocellulose membrane.

2.4. Extraction of total cellular RNA

Total cellular RNA was prepared using an RNA extraction kit (RNAzol™ B; Cinna/Biotecx Laboratories International, Inc.), as described (Chomczynski and Sacchi, 1987).

2.5. RT-PCR

The extracted RNA was subjected to amplification by nested RT-PCR, as described (Kishi et al., 1995a,b; Nakamura et al., 1995, 1996; Nakaya et al., 1996), to obtain a fragment of the p24 coding region which is relatively conserved within the BDV genome (Schneider et al., 1994). The control experiment included uninfected MDCK or persistently infected MDCK/BDV. Briefly, 1 μg of cellular RNA was amplified by nested RT-PCR using two sets of primers as follows: for the first PCR, 5′-TGACCCAACCAGTAGACCA-3′ at nucleotides 1387–1405 and 5′-GTCCCATTCATCCGTTGTC-3′ at nucleotides 1865–1847 and for the second PCR, 5′-TCAGACCCAGACCAGCGAA-3′ at nucleotides 1443–1461 and 5′-AGCTGGGGATAAATGCGCG-3′ at nucleotides 1834–1816. RT-PCR consisting of reverse transcription and amplification of the viral cDNA, was performed according to the protocol described for the EZ *rTth* RNA PCR kit (Perkin-Elmer Corporation). This technique was named EZ RT-PCR. In addition, representative RNA (1 μg) samples were reverse-transcribed before amplification, by STRATASCRIPT II (Stratagene) using oligo $d(T)_{16}$ as a primer at 37°C for 1 h, then 42°C for 30 min. The cDNA products were similarly amplified by nested PCR (named Oligo RT-PCR) using the same two sets of primers as above. The final products by EZ RT-PCR and Oligo RT-PCR were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, and Southern hybridized using four ^{32}P-labeled synthetic oligonucleotides, sense nucleotides 1462–1485, 1485–1507 and 1637–1658, and the antisense nucleotide 1811–1791.

2.6. Cloning followed by sequencing of PCR products

The PCR products for the samples derived from Iranian horses were cloned into a pUC18 plasmid vector (Pharmacia Biotech AB). In addition, Japanese horses which were identified as positive for BDV RNA in PBMCs under the same PCR conditions (Nakamura et al., 1995), were also subjected to the cloning as a control. Several representative clones were sequenced by use of a Dye Primer Cycle Sequencing kit (Applied Biosystems) using the same primers used for the 2nd PCR. All the numbers for BDV nucleotide sequences described here correspond to the reported numbering scheme in the He/80 strain of BDV (Cubitt et al., 1994).

2.7. Nucleic acid sequence analysis

The nucleotide and amino acid sequences were analyzed by GENETYX-MAC (Software Development, Tokyo, Japan). The phylogenetic tree was constructed with the GeneWorks computer programs (IntelliGenetics, Inc.).

3. Results

3.1. BDV seroprevalence in horses in Iran

The plasma obtained from the same blood samples as for PBMC preparations was exam-

ined for the presence of anti-BDV antibodies by immunoblot analysis. For this, two viral proteins, ORF-I (p40) and ORF-II (p24), were used after purification by glutathione Sepharose column of their fusion proteins, GST-p24 and GST-p40. As a control, GST protein was used after purification under the same conditions. Clear positive staining to GST-p24 and GST-p40 was detected in 15.2% (11/72) and 18.1% (13/72) of the samples, respectively, by immunoblots with 1:75 dilutions of the plasma. Table 1 summarized these results. Fig. 1(A) shows representative results. The stained bands corresponded to expected sizes, 51 kDa for GST-p24 (Fig. 1(Aa)) and 67 kDa for GST-p40 (Fig. 1(Ab)). Several additional bands showing faster mobilities seemed to be cleaved products of their fusion proteins, since these bands were not detected in the lanes of the control GST alone with the same plasma samples. To confirm the specificity of the serological reactions, competition experiments were carried out using representative horse plasma (# 1 and # 20) which were positive for antibodies to both p24 and p40 (Fig. 1(B)). Pre-absorbed plasma with GST-p40 remained their reactivities to GST-p24, but not GST-p40 on nitrocellulose membrane. Similarly, pre-absorbed plasma with GST-p24 remained the reactivities to GST-p40, but not GST-p24.

3.2. Prevalence of BDV RNA in PBMCs derived from horses in Iran

By the nested EZ RT-PCR technique for the detection of BDV-related RNA at ORF-II in PBMCs from 72 healthy race horses in Iran, clear positive signals were detected in 23.6% (17/72) of the samples by both ethidium bromide staining and Southern blot hybridization, as summarized in Table 1. Fig. 2(A) shows the representative results. All the PCR products showed a discrete band corresponding to the size (392 bp) of the fragment from persistent BDV in MDCK/BDV cells. Similar amplification of the samples from # 1 and # 2 horses was also observed by nested Oligo RT-PCR as described in Section 2 (Fig. 2(B)).

3.3. Varied BDV prevalence with the horse strain in Iran

Consequently, we demonstrated 27.8% (20/72) prevalence of BDV in 72 horses in Iran (Table 1). Comparison between the prevalences for BDV RNA and anti-BDV antibodies revealed a signifi-

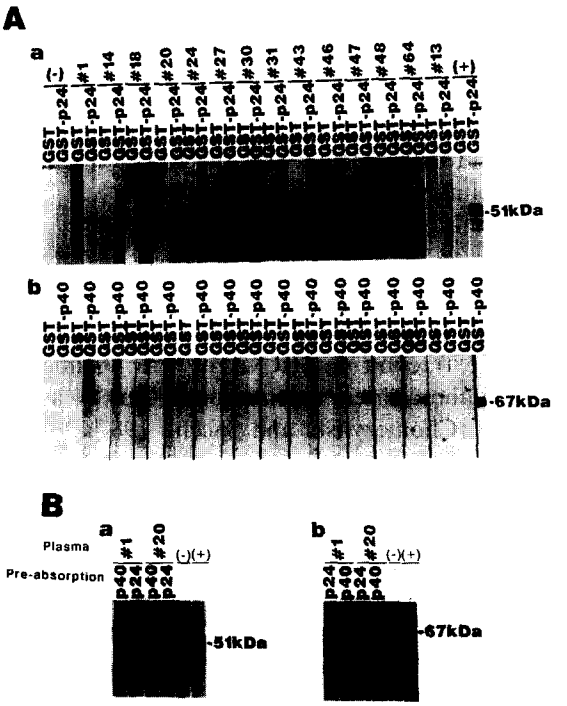


Fig. 1. Anti-BDV antibodies in the plasma samples from healthy race horses in Iran. The purified GST-BDV p24 (51 kDa), GST-BDV p40 (67 kDa) and GST alone as a control were similarly resolved by SDS–PAGE followed by its blotting onto a nitrocellulose membrane for immunoblotting (A). A seventy-five-fold dilution of the plasma samples derived from horses was used for antibodies to p24 (a) and p40 (b). The results of all 11 samples positive for both antibodies to p24 and p40 (# 1, # 14, # 20, # 24, # 27, # 30, # 31, # 43, # 46, # 47, and # 64) and all two samples positive only for antibodies to p40 (# 18 and # 48), in addition to one negative sample (# 13) are shown. The results of competition experiments using representative plasma from # 1 and # 20 horses are shown in B. The plasma were pre-incubated with GST-p40 or GST-p24 as described in Materials and Methods. The absorbed plasma were reacted with GST-p24 (a) and GST-p40 (b) on nitrocellulose membrane. Positive (+) and negative (−) controls were the plasma samples which we previously showed as horse sera positive and negative for antibodies to BDV (Nakamura et al., 1995).

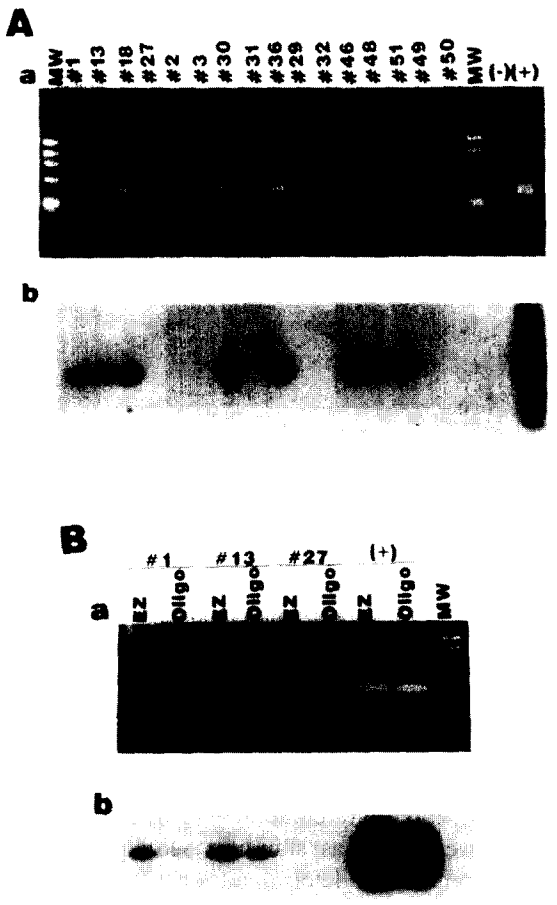


Fig. 2. Nested RT-PCR for the detection of BDV RNA in PBMCs from healthy race horses. The results of 16 samples from Arabic (# 1, # 13 and # 18 as positive; and # 27, # 2 and # 3 as negative samples), thoroughbred (# 30 and # 36 as positive; and # 31, # 29 and # 32 as negative samples), and cross-bred (# 46, # 48 and # 51 as positive; and # 49 and # 50 as negative samples) horses by nested EZ RT-PCR are shown here as representatives (A). # 1, # 27, # 30, # 31, and # 46 are positive for both antibodies to p24 and p40. # 18 and # 48 are positive only for antibodies to p40, but not to p24. The other horses are negative for BDV antibodies. The products of PCR amplification within the BDV p24 region were subjected to agarose gel electrophoresis, then stained with ethidium bromide (a). The results of the Southern blot hybridization using four oligomers as a probe are also shown (b). As positive and negative controls, the RNA fractions from MDCK/BDV (+) and uninfected MDCK (−) were similarly subjected to nested RT-PCR. MW, size markers (Ø × 174 DNA/*Hae*III fragments). In the RNA samples from Arabic (# 1 and # 13 positive and # 27 negative) horses, PCR amplification was carried out by two techniques, EZ RT-PCR and Oligo RT-PCR as described in Section 2 (B). Similarly, the products were subjected to agarose gel electrophoresis, stained (a), then Southern-hybridized (b).

cant difference. The prevalences by assays for p24 RNA, anti-p24, and anti-p40, in the three groups of Iranian horses were comparatively shown in Fig. 3. Total BDV prevalence by all these measures in three groups was also included. The prevalence of BDV RNA in PBMCs was the highest in cross-bred horses, i.e. 33.3% (9/27) in cross-bred, 21.4% (6/28) in Arabic, and 11.8% (2/17) in thoroughbred horses. On the other hand, the seroprevalence was the highest in Arabic horses, i.e. 21.4% (6/28) in Arabic, 17.6% (3/17) in thoroughbred, and 14.8% (4/27) in cross-bred horses. Thus, the horses positive for BDV RNA in PBMCs were not always positive for anti-BDV antibodies in plasma, although the seroprevalence to p24 and p40 viral proteins was consistent, except for # 18 Arabic and # 48 cross-bred horses which showed a positive reaction only to p40, but not to p24 (Table 1). Thus, the phenotypes of BDV-positive horses were separated into four categories: p24 RNA^+/p24 $antibody^+$/p40 $antibody^+$; p24 RNA^+/p24 $antibody^-$/p40 $antibody^+$; p24 RNA^-/p24 $antibody^+$/p40 $antibody^+$; and p24 RNA^+/p24 $antibody^-$/p40 $antibody^-$ (Fig. 3). The principal characteristics of BDV-positive Arabic, thoroughbred, and cross-bred horses were to be the highest in the category of p24 RNA^+/p24 $antibody^+$/p40 $antibody^+$, the category of p24 RNA^-/p24 $antibody^+$/p40 $antibody^+$, and the category of p24 RNA^+/p24 $antibody^-$/p40 $antibody^-$, respectively. Consequently, the total prevalences of BDV infection including all these categories was 25% (7/28) in Arabic horses, 23.5% (4/17) in thoroughbred horses, and 33.3% (9/27) in cross-bred horses.

Next, when we focused on the difference with the horse sex, significant differences were also observed with the horse group (Fig. 4). Positive reactions to BDV were observed only in female cross-bred horses (0% in males and 42.9% in females), while a higher prevalence was observed in male Arabic horses (36.4% in males and 17.6% in females) and in female thoroughbred horses (12.5% in males and 33.3% in females).

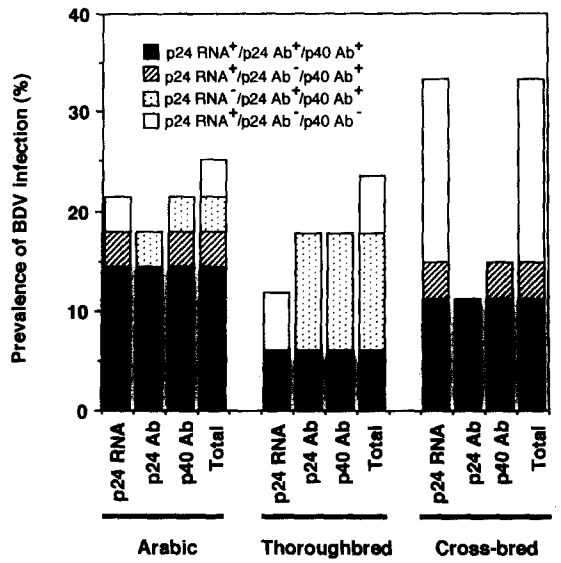


Fig. 3. Varied BDV prevalence with the horse strain. The results of BDV RNA in PBMCs and anti-BDV antibodies in plasma derived from a total of 28 Arabic, 17 thoroughbred, and 27 cross-bred horses were re-arranged to be separated into four categories: p24 RNA$^+$/p24 antibody$^+$/p40 antibody$^+$; p24 RNA$^+$/p24 antibody$^-$/p40 antibody$^+$; p24 RNA$^-$/p24 antibody$^+$/p40 antibody$^+$; and p24 RNA$^+$/p24 antibody$^-$/p40 antibody$^-$.

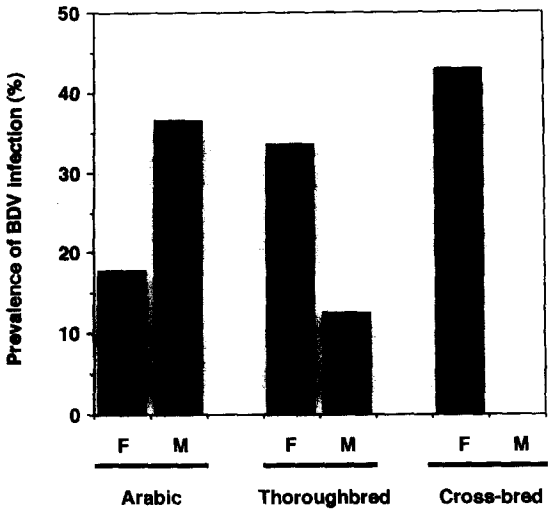
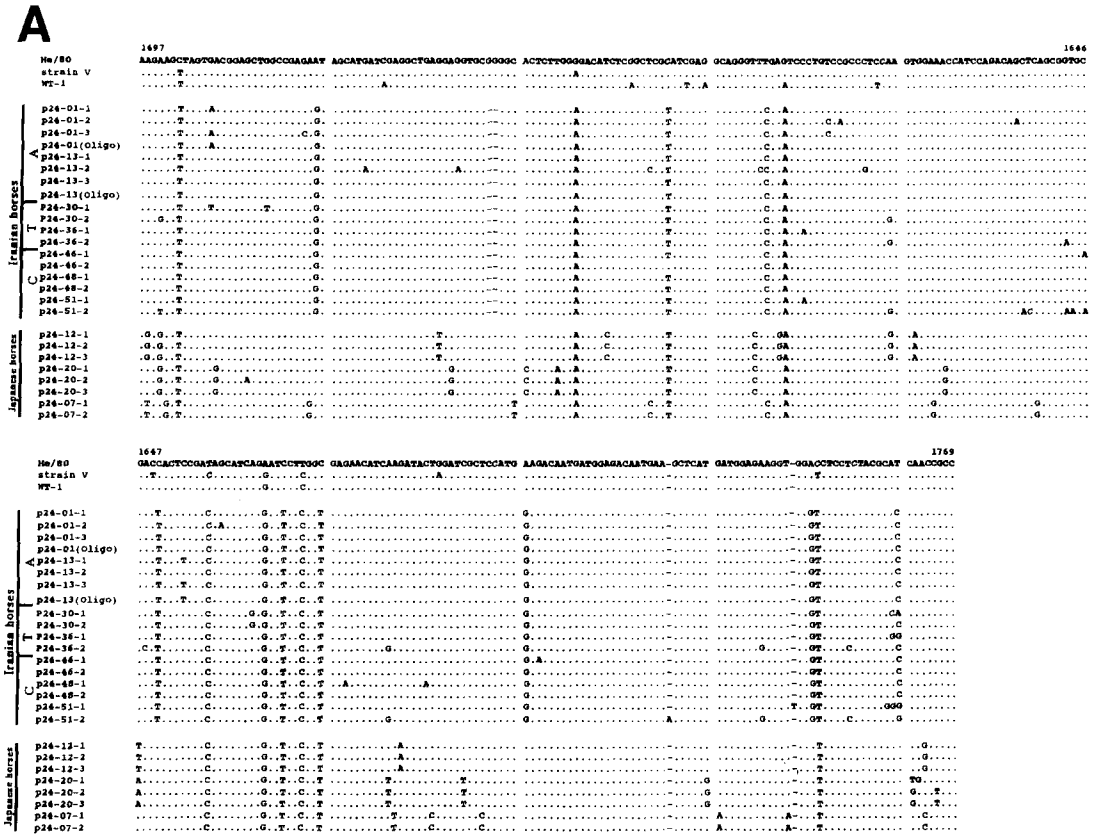


Fig. 4. Varied BDV prevalence with sex of horse in Iran. The results of BDV RNA in PBMCs and anti-BDV antibodies in plasma derived from a total of 28 (17 female and 11 male) Arabic, 17 (9 female and 8 male) thoroughbred, and 27 (21 female and 6 male) cross-bred horses are separately shown for females and males.

3.4. Comparison of p24 sequences among different horses

Representative horses were selected from different groups of horses for the comparison of p24 sequences, i.e. Arabic # 1 and # 13; thoroughbred # 30 and # 36; and cross-bred # 46, # 48 and # 51 horses. Fig. 5 shows the sequence results of two or three representative cDNA clones obtained from the EZ RT-PCR products. In addition, the sequence results of one cDNA clone obtained from the Oligo RT-PCR products of the samples from # 1 and # 13 Arabic horses. The reported p24 sequences from horse-derived BDVs in Europe (Cubitt et al., 1994; Schneider et al., 1994) were used as standard sequences. As another control, we determined the p24 sequences of EZ RT-PCR products derived from thoroughbred race horses (three cDNA clones from horse # 12, three clones from # 20, and two clones from # 07) at Hokkaido in Japan which we previously reported to be positive for BDV RNA in PBMCs under the same conditions (Nakamura et al., 1995). The p24 sequences were similar among BDVs derived from Arabic, thoroughbred, and cross-bred horses in Iran, indicating similar BDV

Fig. 5. Nucleotide and deduced amino acid sequences of BDV p24 region in PBMCs. Sequence results of 24 cDNA clones obtained from the products by EZ RT-PCR technique, 16 from Iranian horses and eight from Japanese horses, at nucleotides 1497–1769 are comparatively shown with those of horse-derived BDV, He/80 (Cubitt et al., 1994), strain V (Schneider et al., 1994) and WT-1 (Schneider et al., 1994) (A). Two or three clones derived from two Arabic (A, # 1 and # 13), two thoroughbred (T, # 30 and # 36), and 3 cross-bred (C, # 46, # 48, and # 51) horses in Iran were sequenced. Three clones from # 12 and # 20 and two clones from # 7 horses in Japan which were shown to be positive by EZ RT-PCR previously (Nakamura et al., 1995) were also sequenced. In addition, two cDNA clones obtained from the # 1 and # 13 Arabic horse-derived products by Oligo RT-PCR technique (Oligo) were also sequenced. Nucleotide sequences identical with those of He/80 were indicated by dots. Broken lines indicate deleted nucleotides. The deduced amino acids according to these sequences were also similarly shown (B). X indicates an amino acid residue which is not determined because of the presence of nucleotide deletion within a corresponding codon. * means stop codon, while # means insertion.


A
1697
1646
He/80
strain V
WT-1
Iranian horses
A
T
C
p24-01-1
p24-01-2
p24-01-3
p24-01(Oligo)
p24-13-1
p24-13-2
p24-13-3
p24-13(Oligo)
p24-30-1
p24-30-2
p24-36-1
p24-36-2
p24-46-1
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p24-48-2
p24-51-1
p24-51-2
Japanese horses
p24-12-1
p24-12-2
p24-12-3
p24-20-1
p24-20-2
p24-20-3
p24-07-1
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1647
1769
He/80
strain V
WT-1
Iranian horses
p24-01-1
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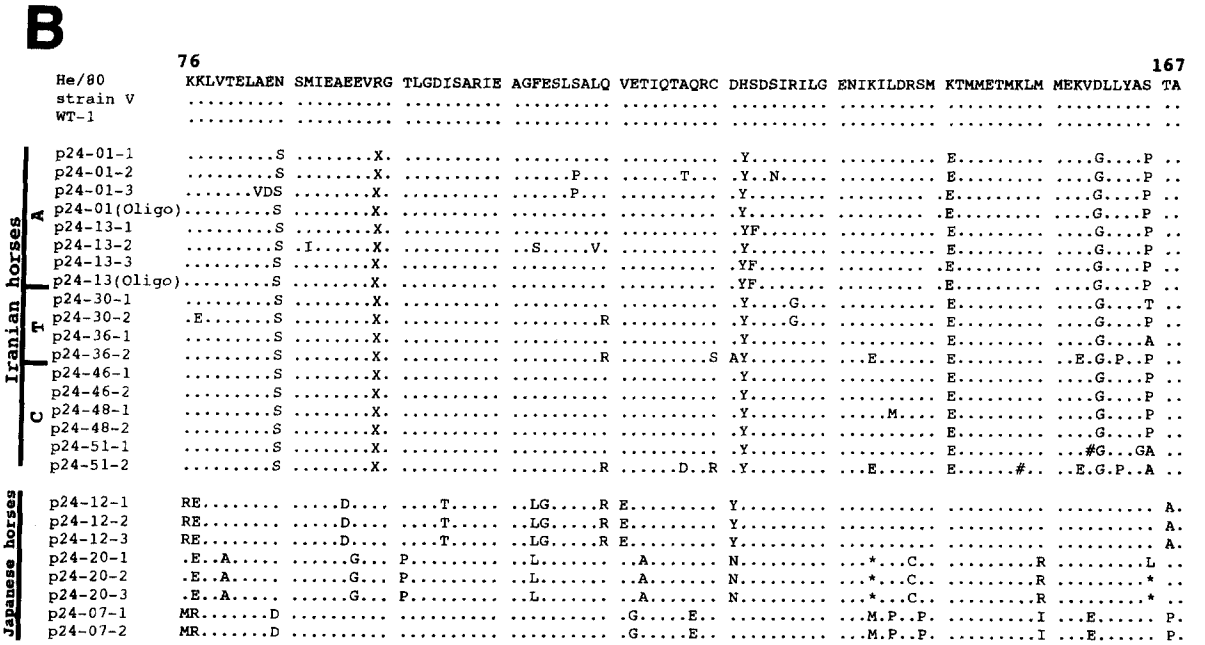
p24-07-1
p24-07-2


B
76
167
He/80 KKLVTELAEN SMIEAEEVRG TLGDISARIE AGFESLSALQ VETIQTAQRC DHSDSIRILG ENIKILDRSM KTMMETMKLM MEKVDLLYAS TA
strain V
WT-1
Iranian horses
A
T
C
p24-01-1
p24-01-2
p24-01-3
p24-01(Oligo)
p24-13-1
p24-13-2
p24-13-3
p24-13(Oligo)
p24-30-1
p24-30-2
p24-36-1
p24-36-2
p24-46-1
p24-46-2
p24-48-1
p24-48-2
p24-51-1
p24-51-2
Japanese horses
p24-12-1
p24-12-2
p24-12-3
p24-20-1
p24-20-2
p24-20-3
p24-07-1
p24-07-2

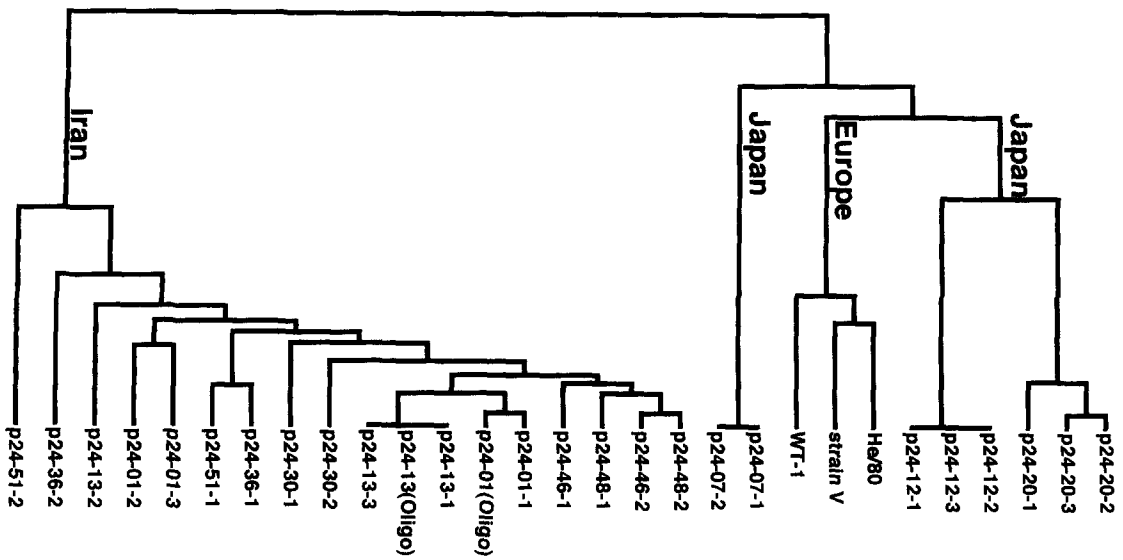


Fig. 6. Phylogeny of the p24 region of BDV derived from horses in Iran and Japan. The virus sequences were the same as for those described in Fig. 5(A). The phylogenetic tree was constructed based on p24 sequences using GeneWorks 2.2 software. As control sequences, the p24 sequences of He/80, strain V, and WT-1 (derived from European horses) were also included.

transmission among the horses in Iran. All cDNA clones from horses in Iran had two-base deletions at the same site. In addition, a one-base insertion and/or substitution at several sites were identified in clones p24-51-1 and p24-51-2. There were no deletions or insertions in any sequences in the clones from Japanese horses. There was no apparent difference between the sequences of p24 products from #1 and #13 horses, which were amplified by EZ RT-PCR and Oligo RT-PCR techniques. The findings showed that several deduced amino acid residues at p24 were identified as common to the BDVs derived from horses in Iran, i.e. Ser^{85}, Tyr^{127}, Glu^{146}, and Gly^{160} which were substituted from Asn, His, Lys, and Asp, respectively, in the sequences of He/80, strain V, and WT-1 reported previously (Cubitt et al., 1994; Schneider et al., 1994). On the other hand, the deduced amino acid residues at BDV p24 derived from three horses in Japan showed higher divergencies, compared with the reported BDV sequences derived from European horses (Cubitt et al., 1994; Schneider et al., 1994). The deduced amino acid residues from all three clones derived-from horse #20 in Japan showed truncation at the same site.

Next, the relatedness of these horse-derived BDVs in Iran and Japan with reported BDVs in p24 sequences was analyzed by a phylogenetic tree (Fig. 6). Although a slight difference was observed among the cDNA clones obtained here by EZ RT-PCR and Oligo RT-PCR techniques, all cDNA clones derived from BDVs in three horse groups in Iran were essentially closely related, compared with those derived from horses in Japan or reported sequences in European horses.

4. Discussion

In this study, we focused on the BDV prevalence in race horses in Tehran, Iran, because this country has never imported or exported any horses for at least the last 15 years. Our study demonstrated the presence of BDV RNA in the PBMC fraction even from apparently healthy race

horses which have been bred in Iran (Fig. 2 and Table 1). The prevalence was similar to that in our previous examination for thoroughbred race horses in Hokkaido, Japan (Nakamura et al., 1995). However, the prevalence of BDV infection in Iran significantly varied with the strain (Fig. 3) and sex (Fig. 4) of the horse. Especially, higher prevalence of BDV RNA was observed in PBMCs from male Arabic and female thoroughbred horses. On the other hand, the BDV RNA in cross-bred horses was identified only in females. In addition, the prevalence did not increase with aging of the horse (Table 1). The viral sequence results (Fig. 5) as well as the phylogenetic tree (Fig. 6) suggested the transmission of BDV specific to the horses in each country (Iran and Japan) as well as in Europe (Cubitt et al., 1994; Schneider et al., 1994). Especially, there was a similarity between BDVs derived from Japan and Europe (Fig. 6). This might be presumably due to the fact that the Japanese horses we examined had originated from Europe. Further studies are necessary to confirm this difference in the prevalence. One Iranian horse # 24 showing positive signals for both BDV RNA and antibodies to BDV was born to mother # 13 showing positive signals only for BDV RNA, although father # 28 was negative for BDV (Table 1), indicating a higher possibility of vertical transmission rather than horizontal transmission in # 24 horse. No information for other horses was available.

The association of BDV with psychiatric disorders in humans is hypothesized according to the evidence of the high prevalence of anti-BDV antibodies in the patients compared with healthy people (Rott et al., 1985; Bode et al., 1988, 1992, 1993; Richt et al., 1993b). The demonstration of BDV RNA in PBMCs of healthy horses (Nakamura et al., 1995) and cats (Nakamura et al., 1996), as well as human patients with psychiatric disorders (Kishi et al., 1995b), patients with chronic fatigue syndrome (Nakaya et al., 1996) and blood donors (Kishi et al., 1995a) suggests a broader route of natural transmission of this virus as a hematopoietic cell-related virus than believed previously as a highly neurotropic virus. Therefore, molecular-biological analyses of BDV sequences derived from PBMCs in infected animals and humans seems to be extremely urgent for understanding the route of natural transmission of BDV in individual host species. At present, the sequencing results in horses as shown here (Fig. 5) may suggest that the BDV in Japanese patients with psychiatric disorders (Kishi et al., 1995b, 1996) is not derived from infected horses, although higher similarity between BDVs derived from Iranian horses and Japanese patients with chronic fatigue syndrome (Nakaya et al., 1996) was observed (Fig. 5). There might be a possibility that the mutations observed here were PCR artefacts by using EZ *rTth* PCR kit. However, the results of EZ and Oligo RT-PCR (Fig. 2) using the same RNA samples derived from # 1 and # 13 Arabic horses, and their sequence results (Fig. 5) showed no apparent difference between the products by both techniques. This indicates that the technique would not be a major reason for the mutations.

Among the 20 Iranian horses showing BDV signals, only eight horses were positive for all RNA and antibodies to both p24 and p40 (Table 1). Two horses were positive for RNA and anti-p40 antibodies, but not anti-p24 antibodies. The other seven and three horses were positive only for RNA and only for antibodies, respectively. In addition, all clones from seven Iranian horses examined contained two-base deletions in p24 sequences (Fig. 5(B)), as found at the same site in the sequences derived from patients with chronic fatigue syndrome (Nakaya et al., 1996). Among them, three (# 1, # 30, and # 46) horses were positive for anti-p24 antibodies (Table 1). In addition, one clone from Iranian horse # 51 and all clones from Japanese horse # 20 had a nonsense mutation in p24 sequences. One explanation for the group that is positive only for RNA signals is that immune complexes might contribute to the lack of detection of circulating antibodies. Alternatively, BDV with an intact p24 sequence could proliferate in the cells in tissues but not blood, although a certain cell population in PBMCs could become reservoirs of BDV with p24 containing two-base deletions.

In conclusion, our findings suggest that BDV infects hematopoietic cells of apparently healthy horses, although the expression seems to be very

low. BDV may be more widespread in horses, since BDV RNA and/or anti-BDV antibodies were detected even in healthy horses at a higher rate (Table 1). However, to our knowledge, no clinical evidence of BDV infection has been found among the horses in either Iran or Japan, although we can not rule out the possibility that the disease may develop only in a part of the carriers after long-term persistence. Therefore, comparative investigations on the BDV whole genomes will be required to determine the BDV pathogenicity in individual species including humans.

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