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Prevalence of Anti-Borna Disease Virus Antibody in Horses and Their Caretakers in Bangladesh

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To elucidate the spread of Borna disease virus (BDV) in Asian countries, we surveyed 48 normal horses in Bangladesh and their 26 caretakers for the BDV antibody by electrochemiluminescence immunoassay. Eleven horses (23%) were found positive. None of the 5 horses at the age of < 1 year was positive. Seven of 23 horses (30%) at the age of 1 year were positive, as well as 4 of 16 horses (25%) at the age of ≥ 3 years. The geometric average of the ECLIA titer of the antibody positive horses at the age of 1 year, 3041, was significantly lower than that found at the age of ≥ 3 years, 6887, by the Mann-Whitney test (P = 0.012). Sexual preference in the prevalence of anti-BDV was not evident. None of the 26 male horse caretakers between the ages of 12 to 54 years was positive, including those who were taking care of the antibody positive horses. Total RNA extracted from the peripheral blood nucleated cells was tested by polymerase chain reaction coupled with reverse transcription capable of detecting 200 molecules of BDV p40 RNA per reaction. None of the 11 seropositive horses and the 5 randomly selected seronegative horses was positive. The results showed that BDV is penetrating the Bangladeshi labor horse population with similar levels reported in Germany, Iran and Japan, although the viral genome in the blood was not detected.

Key words: Borna disease virus; horse; caretaker; reverse transcription-polymerase chain reaction

Recently much attention has been paid to the Borna disease virus (BDV) because of the detection of BDV-positive patients with neuropsychiatric disorders suggesting a possible link between BDV and human disease (Lipkin et al., 1995; Bechter et al., 1996). The wide host range of BDV is consistent with the possibility of zoonotic spread from animals (Waelchli et al., 1985; Ludwig et al., 1988; Bode et al., 1994; Lundgren et al., 1995; Bode and Ludwig, 1997). The current list of natural hosts for BDV includes horses, sheep, cattle, cats and ostriches (Waelchli et al., 1985; Ludwig et al., 1988; Bode et al, 1994; Weisman et al., 1994; Lundgren et al., 1995). Seroepidemiological studies suggested that animal care workers had a higher prevalence of anti-BDV antibody (Weisman et al., 1994; Takahashi et al., 1997). Bechter et al. (1992, 1996) reported neuropsychiatric patients with anti-BDV antibody who had histories of working in BDV-positive animal farms. Some researchers even tried to correlate human mental disorder cases with cats in the household (Torrey et al., 1995).

Still, little is known about the natural reservoir of BDV for human infection. The neurologic manifestations of BDV infection in horses is similar to the neuropsychiatric disorders in human (Bode and Ludwig, 1997). Prehistoric "sad horse disease" or "head disease" has now been proven to be an immune mediated encephalitis caused by BDV. The horse was the first

Abbreviations: BDV, Borna disease virus; ECLIA, electrochemiluminescence immunoassay; RT-PCR, reverse transcription-polymerase chain reaction

animal described as a natural host of BDV (Ludwig et al., 1988). In the early days, it was generally believed that the Borna disease in the horse was endemic only in southern Germany and Switzerland (Rott and Becht, 1995). However, the presence of horses seropositive for anti-BDV antibody in recent studies from the United States, Japan, Iran, Austria and Sweden changed the view that BDV is more widespread than previously thought (Kao et al., 1993; Nakamura et al., 1995; Bahmani et al., 1996; Weissenbock et al., 1998; Berg et al., 1999). As far as we know, there has been no report on BDV in the horses of southeast Asia. In the typical southeast Asian situation, horses are usually reared in the same house together with humans who then have a greater chance of threat from any virus with zoonotic properties. Here we report BDV prevalence by testing the antibody and the genome in normal Bangladeshi horses and their caretakers living in the same house.

Materials and Methods

Blood

One milliliter of blood with EDTA as an anticoagulant was collected from 48 normal horses and 26 horse-caretakers in Bangladesh. The whole blood was preserved at –20˚C until use. After thawing the frozen blood, the fluid portion separated by centrifugation at 3000 rpm for 5 min at room temperature was used for detection of anti-BDV antibody by electrochemiluminescence immunoassay (ECLIA) (Yamaguchi et al., 1999). The nucleated cells in the precipitate were used for the detection of BDV RNA by reverse transcription-polymerase chain reaction (RT-PCR). This method is not ideal but useful in testing for antibodies and viral genomes in field conditions where routine experimental equipment, such as a centrifuge, are unavailable (Kawase et al., 1992). During the period of thawing the frozen whole blood, red cells will burst but nucleated cells keep their cytoplasmic membrane.

Antibody Titration

The antibody was titrated by ECLIA. Briefly, 20 µL of serum at 1/10 dilution was preincubated overnight with 200 µL of normal chicken serum with or without 1 mg each of recombinant BDV peptides, rp24 and/or rp40. Microbeads (magnetic polystyrene beads coated with a polyurethane layer, No. 140.03, Dynal As, Oslo, Norway) coated with the peptides were incubated at 30˚C for 9 min with the preincubated serum. After washing, the beads were incubated with 200 µL of 200 ng/mL anti-horse or anti-human immunoglobulin G (Sigma, St. Louis, MO) coupled with $Ru(bpy)32^{+}$ [ruthenium (II) tris (bipyridyl)-*N*-hydroxysuccinimide ester (IGEN Int. Inc., Gaithersburg, MD)]. At every washing step, the well was washed with 0.05-mol/L Tris-HCl, pH 8.0, 0.01% Tween-20 and 10% normal chicken serum 3 times. The ECLIA count was computed automatically by an automatic ECLIA analyzer (Picolumi 8220, Sanko Jyunyaku, Tokyo, Japan) based on optical density at 620 nm. A sample was considered positive when the ECLIA count was higher than the mean of control blood samples plus 3 SDs, and > 50% of the count was competed out by the recombinant peptide antigen as previously described (Yamaguchi et al., 1999). This criterion was selected since some sera showed non-specific reactivity which could not compete with the purified antigen (Yamaguchi et al., 1999).

Extraction of RNA

RNA was extracted by a guanidium thiocyanate extraction method (Chomczynski and Sacchi, 1987) with minor modifications. Briefly, the cellular fraction obtained from 1-mL whole blood was lysed in 1.8 mL of the denaturing solution using a glass homogenizer. The lysate was collected in a new 15-mL tube, and added with 1/10 volume of 2-mol/L sodium acetate, pH 4.0, an equal volume of water-saturated phenol and 1/2 volume of chloroform and isoamylalcohol mixture (24:1). After incubation for 15 min on ice, the mixture was centrifuged at 3000 rpm for 40 min at 4˚C. The su-

Table 1. Nucleotide sequence of primers

Pair	Polarity	Name	Sequence (5'–3')	Base position
Outer	Antisense	BD980	CATTGTGGGGTTTTCCTTCTTACTC	1004 – 980
	Sense	BD579	CCATCCCATGGTGAGACTGCTACAC	579 – 603
Inner	Antisense	BD908	CTTGATAGCGTCGGGGTGTCTAATG	932 – 908
	Sense	BD653	ACCCTGGGTAGGCTCCTTTGTGTTG	653 – 677

pernatant RNA was precipitated with an equal volume of 2-propanol at –20˚C. After centrifugation in the same conditions, the RNA was dissolved in 300 µL of the denaturing solution, and precipitated by the 2-propanol. Following centrifugation at 15,000 rpm for 15 min at 4˚C, the RNA pellet was washed by 70% ethanol and vacuum dried. Finally, the pellet was dissolved in 50 µL of water and preserved at –80˚C for further use. All the equipment and water used in the study were treated with 0.01% diethylpyrocarbonate and autoclaved.

Primers

Primers were designed to amplify the conserved region coded by the BDV p40 gene based on published sequences (GeneBank: BDU04608 and BDU94867). Customized oligonucleotide primers (Table 1) were purchased from Pharmacia Biotech (Tokyo, Japan).

Analysis of BDV RNA by nested RT-PCR

The reaction mixture of reverse transcription in a volume of 10 µL contained 50-mmol/L Tris-HCl, pH 8.3, 75-mmol/L KCl, 3-mmol/L $MgCl_2$, 1 µL of the RNA stock (equivalent to the nucleated cells in 20 µL of blood), 2-mmol/L dithiothreitol, 0.5 mmol/L each dNTP (Takara Shuzo, Kyoto, Japan), 5-µmol/L of the sense primer BD579, 200 U of Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) and 1 U of RNasinA (Promega, Madison, WI). After incubation at 45˚C for 60 min, the whole reaction mixture was served to the following PCR.

The cDNA was amplified in a programmable incubator (API300, ASTEC, Fukuoka, Japan) for 35 cycles. The 50-µL reaction mixture contained the cDNA, 20-mmol/L Tris-HCl, pH 8.5, 50-mmol/L KCl, 1.5-mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 0.5-µmol/L the outer primer pair (BD980 and BD579) and 1.25 U of Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). Each cycle consisted of 1 min at 94˚C (2 min on the first cycle), 1 min at 52˚C and 1 min at 72˚C (10 min on the final cycle). A 5-µL aliquot of the first PCR product was served to the second PCR with the inner primer pair (BD908 and BD653) for another 35 cycles. A 7-µL portion of the nested PCR product was electrophoresed on a composite gel containing 2% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) and 1% LO3 agarose (Takara Shuzo), and stained with ethidium bromide. To avoid possible contamination, separate rooms served as sites for RNA extraction, PCR reaction and gel electrophoresis.

Results

BDV seroprevalence in normal Bangladeshi horses

Out of 48 normal horses tested for the BDV antibody by the ECLIA method, 11 (23%) were positive (Table 2). Although 5 additional horses (identification number 5, 11, 21, 29 and 35) showed an ECLIA titer of > 1000 in the anti-(rp24 + rp40) assay, they did not show a significant reduction in ECLIA count compared to the competing antigen. None of these showed a significant reaction in either the anti-rp24 or anti-rp40 assay. Therefore, we judged the reactions in the anti-(rp24 + rp40) assay as false. All of the positive sera were positive only for anti-p24, but not significantly for anti-p40 of BDV.

Table 2. Seroprevalence of BDV in normal horses in Bangladesh

ID No.*	Sex	Age (year)	Anti-(rp24 + rp40) ECLIA	Anti-(rp24 + rp40) % Inhibition†	Anti-rp24 ECLIA	Anti-rp40 ECLIA	Judgment
3	m	0.1	68	0	0	0	–
39	m	0.1	372	0	0	0	–
41	m	0.1	652	0	0	0	–
47	m	0.1	754	0	0	0	–
37	f	0.8	73	0	0	0	–
2	m	1.0	159	0	0	0	–
27	m	1.0	59	0	0	0	–
21	f	1.1	1144	33	0	0	–
28	m	1.1	2583	64	3456	395	Anti-p24
14	m	1.2	309	0	0	0	–
18	m	1.2	1922	78	2674	244	Anti-p24
22	f	1.2	111	0	0	0	–
31	m	1.2	160	0	0	0	–
33	m	1.2	1820	84	2587	283	Anti-p24
42	m	1.2	266	0	0	0	–
48	m	1.2	967	0	0	0	–
5	m	1.3	1749	0	0	0	–
35	m	1.3	1818	10	0	0	–
43	m	1.3	1776	74	2996	257	Anti-p24
8	f	1.4	167	0	0	0	–
15	m	1.4	695	0	0	0	–
32	m	1.4	224	0	0	0	–
4	m	1.5	1790	87	2445	299	Anti-p24
13	f	1.6	261	0	0	0	–
45	f	1.6	1742	74	2554	243	Anti-p24
6	m	1.8	206	0	0	0	–
19	m	1.8	211	0	0	0	–
38	m	1.8	3597	97	5388	68	Anti-p24
20	m	2.0	773	0	0	0	–
26	m	2.0	245	0	0	0	–
46	f	2.0	133	0	0	0	–
24	f	2.2	937	0	0	0	–
25	m	3.0	739	0	0	0	–
30	m	3.0	273	0	0	0	–
1	m	3.2	3572	96	5483	72	Anti-p24
9	m	5.0	127	0	0	0	–
36	m	6.0	54	0	0	0	–
17	m	6.2	325	0	0	0	–
10	m	8.0	2752	87	3813	395	Anti-p24
16	m	8.0	217	0	0	0	–
29	m	8.0	1617	11	0	0	–
40	m	8.0	201	0	0	0	–
44	f	8.0	196	0	0	0	–
7	f	10.0	6757	97	10420	129	Anti-p24
11	f	10.0	1488	0	0	0	–
12	f	10.0	258	0	0	0	–
23	m	11.0	460	0	0	0	–
34	f	11.0	6839	97	10352	156	Anti-p24

–, negative; BDV, Borna disease virus; ECLIA, electrochemiluminescence immunoassay; f, female; m, male.

* Identification number of the horses.

† % Inhibition of ECLIA counts by purified competing antigen. Samples with ≥ 50% inhibition were considered as specific for BDV.

Table 3. Age-based distribution of total and anti-BDV antibody-positive horses

Age group (year)	Total of tested horses		Anti-BDV antibody-positive horses		
	Number	Male/female ratio	Number	Male/female ratio	Percentage
< 1	5	4:1	0	0:0	0
1	23	18:5	7	6:1	30
2	4	2:2	0	0:0	0
≥ 3	16	11:5	4	2:2	25

BDV, Borna disease virus.

None of the 5 horses at the age of < 1 year was positive (Table 3). However, 7 of 23 horses (30%) at the age of 1 year were positive, as well as 4 of 16 horses (25%) at the ages of ≥ 3 years. Although the positive rate was not significantly higher in the older age group, the geometric average of the ECLIA titer of the antibody-positive horses at the age of 1 year was significantly lower than those at the ages of ≥ 3 years by the Mann-Whitney test ($P = 0.012$) (Table 4). With this small sized sample, sexual preference in the prevalence of anti-BDV was not evident.

BDV antibody in the horses caretakers

The 26 horse caretakers were tested for anti-BDV antibody. All the individuals were male with an age range of 12 to 54 years who have been taking care of the horses under the same roof. None of them was positive including those who were taking care of the antibody-positive horses (Table 5). Although 2 caretakers (identification number 6 and 25) had a ECLIA titer of > 1000 in the anti-(rp24 + rp40) assay, they were considered negative because their reactivity showed no significant competition from preincubation with the purified rp24 and rp40.

BDV-RNA in the horse blood

To check the presence of BDV in the blood, total RNA extracted from the nucleated cell fraction in the blood was tested in the RT-PCR directed toward the p40 region of BDV. The sensitivity of the RT-PCR was 200 molecules of in vitro synthesized BDV p40 RNA per reaction (Fig. 1A). None of the 11 seropositive horses (Fig. 1B) and none of 5 randomly selected seronegative horses (data not shown) gave any positive signal for BDV RNA by RT-PCR. Both of the 2 positive control tubes containing a double amount of threshold level BDV RNA (Fig. 1B, Lanes P) showed a clear positive signal at the expected 280 nt length.

Discussion

Eleven (23%) of 48 healthy domestic Bangladeshi horses were found positive for anti-BDV by ECLIA. Since there is no report on the prevalence of BDV in horses in southeast Asia, this report has expanded the area of BDV infiltration in the world. Overall prevalence was similar to that reported in other countries, such as Japan, Iran, Germany and Sweden, although the test

Table 4. ECLIA titer of anti-BDV antibody-positive horses

Age group (year)	ECLIA titer		P^*
	Geometric average	95% Confidence interval	
1	3041	2192 – 4121	0.012
≥ 3	6887	3126 –15171	

BDV, Borna disease virus; ECLIA, electrochemiluminescence immunoassay.
* Mann-Whitney test.

Table 5. Seroprevalence of anti-BDV antibody among horse caretakers in Bangladesh

ID No.*	Sex	Age (year)	Anti-(rp24+rp40) ECLIA	Anti-(rp24+rp40) % Inhibition†	Anti-rp24 ECLIA	Anti-rp40 ECLIA	Judgment
4	m	12	199	0	0	0	–
12	m	12	73	0	0	0	–
3	m	16	48	0	0	0	–
10	m	16	462	0	0	0	–
1	m	18	6	0	0	0	–
6	m	18	1493	2	0	0	–
9	m	18	56	0	0	0	–
11	m	18	152	0	0	0	–
19	m	18	97	0	0	0	–
25	m	18	1204	0	0	0	–
18	m	19	116	0	0	0	–
26	m	19	15	0	0	0	–
5	m	20	79	0	0	0	–
13	m	20	114	0	0	0	–
17	m	20	20	0	0	0	–
7	m	21	33	0	0	0	–
16	m	21	225	0	0	0	–
2	m	22	5	0	0	0	–
8	m	22	30	0	0	0	–
14	m	22	36	0	0	0	–
15	m	22	39	0	0	0	–
24	m	22	65	0	0	0	–
20	m	23	468	0	0	0	–
21	m	32	124	0	0	0	–
23	m	32	72	0	0	0	–
22	m	54	69	0	0	0	–

–, negative; BVD, Borna disease virus; ECLIA, electrochemiluminescence immunoassay; m, male.
* Identification number of the horse caretakers.
† % Inhibition of ECLIA counts by purified competing antigen. Samples with ≥ 50% inhibition were considered as specific for BDV.

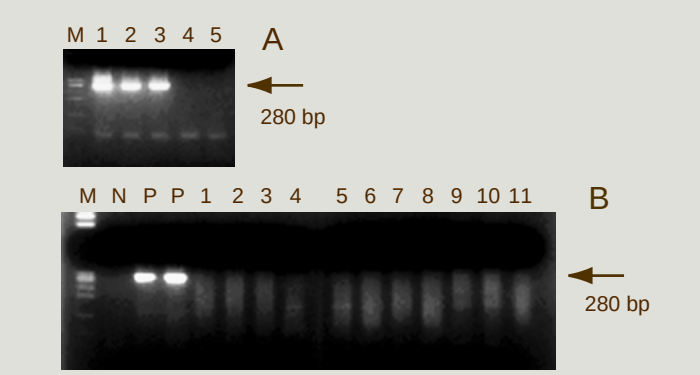

Fig. 1. Nested reverse transcription-polymerase chain reaction (RT-PCR) for Borna disease virus (BDV). Nested PCR products were electrophoresed in a composite gel containing 2% NuSieve GTG agarose and 1% LO3 agarose and stained with ethidium bromide. The expected size of the product was 280 nucleotides. **A:** Sensitivity of the nested RT-PCR. Lane M: ϕX174 *HaeIII* digest. Lanes 1 to 5: 800, 400, 200, 100 and 50 copies of in vitro transcribed BDV RNA/reaction, respectively. **B:** The nested RT-PCR of the horse blood. Lane M: ϕX174 *HaeIII* digest. Lane N: template-free control. Lane P: 400 copies of the in vitro transcribed BDV RNA (the double amount of the threshold shown in **A**-Lane 3). Lanes 1 to 11: total RNA extracted from nucleated cells in 20-µL equivalent of whole blood of the 11 BDV antibody-positive horse blood specimens (horse identification number 1, 4, 7, 10, 18, 28, 33, 34, 38, 43 and 45, respectively). bp, base pair.

Table 6. Geographical prevalence of BDV among the normal horse population

Country	Method	Anti-(p24 + p40)		Anti-p24	Anti-p40	RNA in PBMCs		Reference number
		Ratio†	Positivity (%)	Positivity* (%)	Positivity* (%)	Ratio†	Positivity (%)	
United States	WB	18/295	6.1	NR	100	NR		10
Japan	WB	15/ 57	26.3	100	NR	17/57	30	16
Iran	WB	13/ 72	18.0	85	100	17/72	24	1
Germany	IF	29/100	29.0	NR	NR	NR		18
Austria	IF	2/100	2.0	NR	NR	NR		26
Sweden	ELISA	13/ 53	24.5	NR	NR	1/53	2	4
Japan	ECLIA	16/ 89	18.0	100	13	NR		27
Bangladesh	ECLIA	11/ 48	23.0	100	0	0/11	0	This study

BDV, Borna disease virus; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; IF, indirect immnofluorescence; NR, not reported; PBMC, peripheral blood mononuclear cell; WB, Western blotting.

* Of samples with anti-(p24 + p40) antibody.

† Number of positive horses/number of tested horses.

method is not uniform (Table 6). A recent report by ECLIA on Japanese feral horses (Yamaguchi et al., 1999) was also consistent with this research. In contrast, the prevalence reported in the United States and Austria was significantly lower than these reports (Kao et al., 1993; Weissenbock et al., 1998). This may suggest that the penetration of BDV in horses may not be homogeneous in the world. Alternatively, the difference may depend on methodology, since the technology of anti-BDV antibody assay systems is still controversial. Every assay system has a non-specific reactivity. The antibody titer against p40, the major internal protein of BDV, is not always predominant in various host species (Yamaguchi et al., 1999), although the major internal protein is the predominant target of antibodies in most virus-host interactions. Development of a durable antibody assay system for the envelope protein of BDV may solve the problem.

Studies on horses in the United States and Iran done with the Western blotting described the predominant reactivity in the anti-p40 antibody (Table 6) (Kao et al., 1993; Bahmani et al., 1996). Two other independent reports from Japan (Nakamura et al., 1995; Yamaguchi et al., 1999) and this report found that most seropositive horses were only positive for anti-p24 antibody, but not for anti-p40 antibody by Western blotting and/or ECLIA. Since the BDV infection is cell-associated and notoriously dormant (Ludwig et al., 1973), the replication status or genetic background in each horse may result in this phenotypic difference. As far as we know, all the sequence data available on horse BDV are only from Germany (Bode and Ludwig, 1997). The p40 antigenic determinants of BDV in different areas of the world may be heterogeneous. If that is the case, since the ECLIA system used a cloned rp40 antigen derived from the Madian-Darby canine kidney/BDV cell line chronically infected with German horse BDV, anti-(Asian p40) in the Bangladeshi horse may not be as reactive as German anti-p40. Isolation of a BDV of Asian origin may solve the problem.

All of the 5 horses below the age of 1 year were negative, and the difference in antibody titer in the age of 1 year and ≥ 3 years was statistically insignificant. The result suggests that BDV is spreading horizontally in the younger horses. However, we do not have an explanation of how the virus spreads.

Transmission of BDV from horses to humans has been suggested by a higher prevalence of BDV in blood donors near a horse farm (Takahashi et al., 1997). Contradictory to these reports, we could not find any BDV-seropositive caretakers in our study in spite of the fact that all of them were living under the same roof with highly prevalent horses. Therefore, we

could not find any evidence to suggest BDV transmission from horses to humans.

Recently Nakamura et al. (1995) detected the BDV RNA in normal healthy horses in Japan, and Berg et al. (1999) in horses with behavior disorders in Sweden. We failed to prove that BDV RNA in seropositive horses was consistent with the results of Richt et al. (1997). Most of the studies which have tried to detect the BDV genome in human samples have used peripheral blood mononuclear cells. Bode et al. (1995) claimed that they detected the BDV RNA in the blood of psychiatric patients. On the other hand, Richt et al. (1997) failed to detect BDV RNA in psychiatric patients and argued that the most positive signals in the RT-PCR were due to laboratory contamination. They stated that their laboratory had no prior exposure to BDV experimentation. The absence of BDV-specific RNA sequences in the cellular fraction of the blood in our results does not exclude the possibility that BDV stays in the restricted areas of the brain as in the cases of naturally and experimentally infected animals (Rott and Becht, 1995).

This is the first report of the presence of BDV in horses in a southeast Asian country, Bangladesh. We failed to detect BDV RNA in horse blood, nor could we obtain evidence which suggests the zoonotic spread of BDV from infected horses to closely associated caretakers.

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