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Detection of Borna disease virus in a pregnant mare and her fetus

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Abstract

A pregnant mare showing pyrexia, reduced appetite, ataxia and paresis was euthanized and examined for the presence of Borna disease virus (BDV). Her brain, showing multiple neuronal degeneration and necrosis with hemorrhage, and the histologically normal brain of the fetus were both positive for BDV RNA. The BDV nucleotide sequences were identical in the mare and fetus in the second open reading frame (ORF). This is the first report of the possible vertical transmission of BDV in a horse. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Borna disease virus; Horse; Vertical transmission

1. Introduction

Borna disease (BD) is a transmissible, progressive meningoencephalopathy of horses and other animals (Dürrwald and Ludwig, 1997). Borna disease virus (BDV), the etiological agent responsible for BD, is a neurotropic non-segmented negative-stranded RNA virus that persistently infects a broad range of vertebrate species (Gosztonyi and

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Ludwig, 1995). Previous epidemiological studies indicated the presence of serum antibodies to BDV and virus RNA in peripheral blood mononuclear cells (PBMCs) in asymptomatic domestic animals in several countries, including Japan (Kao et al., 1993; Nakamura et al., 1995; Bahmani et al., 1996; Hagiwara et al., 1996, 1997a). Thus, the prevalence and geographic distribution of BDV is widespread. Furthermore, experimental infections by intranasal or intracerebral injection have shown a markedly wide host range for BDV, from birds to non-human primates (Anzil, 1972; Kao et al., 1984; Ludwig et al., 1985; Bautista et al., 1994). In general, the natural transmission of BDV is believed to be due to direct contact with secretions or by exposure to contaminated food or water. However, the precise route of transmission of BDV has not been elucidated. In addition, there is no information available regarding the possible vertical transmission of BDV in natural or experimental infection. In this study, we found evidence for the vertical transmission of BDV in a horse.

2. Materials and methods

2.1. Animals and detection of anti-BDV antibodies

An 8-month pregnant 11-year old thoroughbred horse was submitted to a veterinarian in Hokkaido, the northernmost island of Japan, with a clinical history of spontaneous anorexia, pyrexia and paresis with paralysis of the hind limbs. After 12 h (of the symptoms), the horse developed severe central nervous signs with complete inability to move and was euthanized 1 day later.

Samples of sera and cerebrospinal fluid (CSF) from the mare and fetus were examined for the presence of anti-BDV antibodies by immunoblotting as described previously (Kishi et al., 1995a; Hagiwara et al., 1997a). BDV-infected and -uninfected serum from previously tested horses (Hagiwara et al., 1997b) were used as positive and negative controls. The BDV antigens used for this assay were fusion proteins of the BDV first open reading frame (ORF), p40 and second ORF, p24 with glutathione S-transferase (GST) expressed in *Escherichia coli*. The GST-p40 and -p24 proteins were purified by glutathione column chromatography followed by cleavage with PreScission™ Protease (Pharmacia Biotech AB). These BDV proteins obtained after glutathione Sepharose 4B chromatography to remove the free GST protein were used for immunoblotting.

2.2. Detection of BDV-RNA by RT-nested PCR

To detect BDV-specific p24 RNA, we used a reverse transcriptase (RT)-nested PCR method on the brain tissue and PBMCs from both the mare and the fetus. Total cellular RNA was extracted from fresh brain tissue (parietal lobe of cerebrum) and PBMCs using an RNA isolation kit (ISOGEN; Nippon Gene, Tokyo, Japan). The RNA (1 μg) was reverse-transcribed using 200 Units of SuperScript II Rnase H-negative RT (GIBCO BRL) and random hexamers (10 pmol/μl) for 60 min at 42°C. The reaction was terminated by incubation at 70°C for 15 min. BDV-specific cDNA corresponding to the

BDV-p24 sequence was amplified by nested PCR, as described previously (Kishi et al., 1995b). The primers for PCR and nested PCR corresponded to sequences within the BDV-p24 coding region at nucleotides 1387–1405 (5′-TGACCCAACCAGTAGACCA-3′) and 1865–1847 (5′-GTCCCATTCATCCGTTGTC-3′). Samples were amplified for 35 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C, followed by a final polymerization extension step for 7 min at 72°C. Aliquots of 1/10 of the first round PCR product were subjected to a second round of PCR with the nested sets of BDV p24 primers corresponding to nucleotides 1443–1461 (5′-TCAGACCCAGACCAGCCGAA-3′) and 1834–1816 (5′-AGCTGGGGATAAATGCGCG-3′). Amplification was performed using the same cycle conditions as for the first round of PCR.

2.3. Analysis of the nucleotide sequence

The PCR products from brain samples were cloned into the pGEM-T Easy vector (Promega, WI, USA). Three clones, randomly selected from individual brain samples, were sequenced according to the protocol supplied with the Thermo Sequence fluorescent-labeled primer cycle sequencing kit (Amersham Life Science) using the fluorescent-labeled M13 universal primer and the fluorescent-labeled M13 reverse primer, in an ALF DNA Sequencer II (Pharmacia Biotech). The nucleotide sequences were analyzed by GENETYX-MAC (Software Development, Tokyo, Japan) and compared with previously published sequences for BDVs isolated from horses (Cubitt et al., 1994; Schneider et al., 1994).

2.4. Histological analysis

Brain tissue samples from both the mare and fetus were fixed in 4% paraformaldehyde and embedded in paraffin; sections approximately 4 μm thick were stained with hematoxylin and eosin (HE), and examined by light microscopy. Immunostaining of tissue sections for BDV antigen was performed with rabbit antiserum specific to BDV p40. After extensive washing, bound antibodies were identified using labeled streptavidin–biotin immunohistochemistry (IHC). We also examined for the presence of equine herpes virus 1 (EHV-1) antigen in the CNS of the mare and fetus by IHC using a polyclonal antibody against EHV-1 (HH1 strain). In situ hybridization (ISH) was carried out on paraffin-embedded brain samples from both mare and fetus using digoxigenin (DIG)-labeled sense and antisense RNA probes specific for BDV p40 generated by in vitro transcription using T7 polymerase (Boehringer Mannheim). The probes were partially hydrolyzed by incubation in buffer containing 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 for 30 min at 60°C, then neutralized by incubation in 300 mM CH_3COONa containing 1% CH_3COOH for 30 min at 80°C. The hybridization mixture consisted of 50% formamide, 3× SSC, 50 mM HEPES (pH 7.0), 2× Denhardt's, and 2.5 mg/ml salmon sperm DNA. Hybridization was performed at 50°C for 16 h, followed by washing with 2× SSC and finally with 0.2× SSC at 57°C for 15 min. Specific hybridization was detected using alkaline phosphatase-labeled anti-DIG polyclonal antibody (Boehringer Mannheim) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the chromogen. Brain samples from an experimentally BDV-infected,

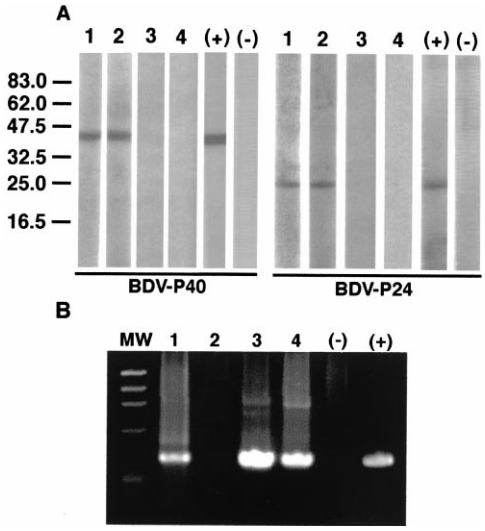

Fig. 1. Detection of anti-BDV antibodies and BDV-RNA. (A) Immunoblots of anti-BDV antibodies in serum and CSF samples obtained from the mare and fetus. A 100-fold dilution of sera and CSF samples from mare and fetus were reacted with nitrocellulose membranes (p40 and p24). Lane 1, serum from mare; lane 2, CSF from mare; lane 3, serum from fetus; lane 4, CSF from fetus. The positive and negative controls were sera from a horse infected with BDV (+) and an uninfected horse (−). Molecular weights were determined by comparison with the marker proteins supplied with the calibration kit (New England Biolabs, Inc.). (B) RT-nested PCR for detection of BDV RNA in PBMCs and the brain of the mare and fetus. Representative results of RT-nested PCR are shown: brain samples from the mare (lane 1) and fetus (lane 3), and PBMC samples from the mare (lane 2) and fetus (lane 4). The positive and negative controls were RNA fraction from MDCK/BDV (+) and MDCK (−), respectively. PCR products of the BDV p24 region were subjected to agarose gel electrophoresis, then stained with ethidium bromide. MW, size markers (φx 174 DNA/Hae III fragments).

20-day old Lewis rat and age-matched uninfected Lewis rat were used as positive and negative control as described by Nakamura et al. (1999).

3. Results

3.1. Detection of antibodies to BDV and BDV RNA

Positive signals were observed for both p40 and p24 in serum and CSF from the mare, while fetal serum and CSF were negative for both antigens (Fig. 1A).

The brain of the mare, and the brain and PBMCs of the fetus, were positive for BDV RNA by RT-nested PCR. These PCR products showed the expected size of 392 bp. In contrast, the PBMCs of the mare were negative for BDV RNA (Fig. 1B).

3.2. Sequence analysis

Automated DNA sequencing of the cloned p24 PCR products from both mare and fetal brain samples revealed the same sequence. Previously reported p24 sequences from

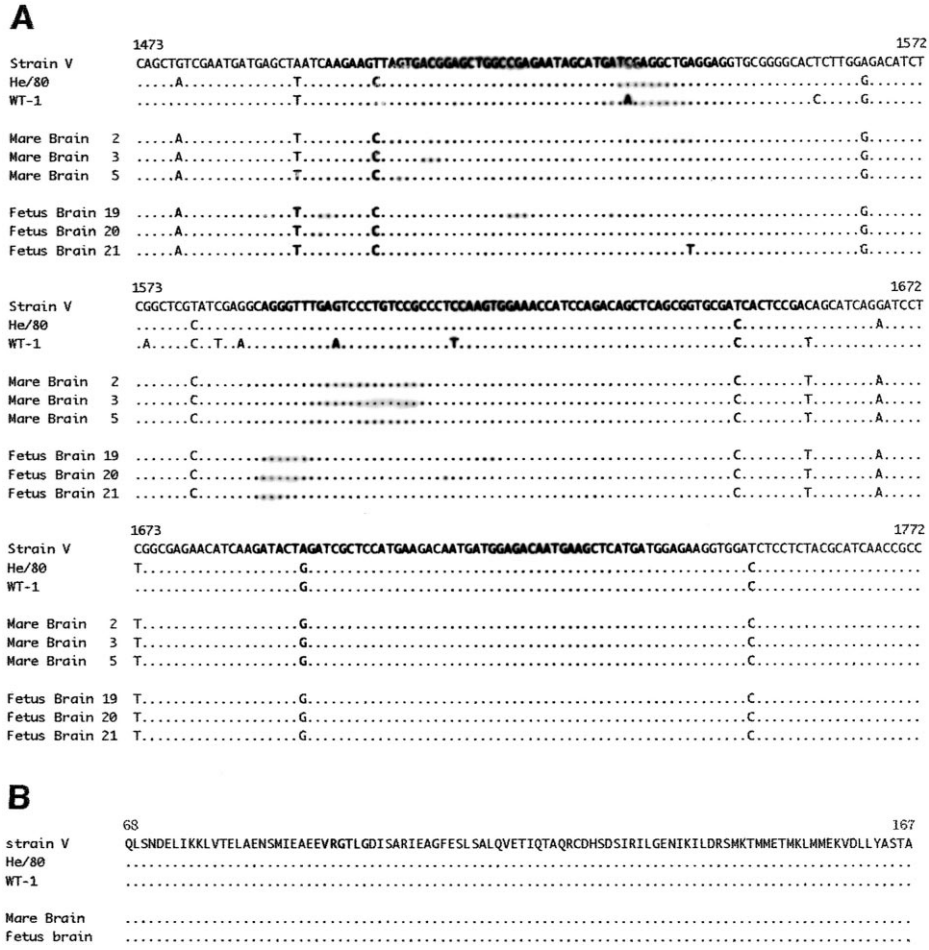

Fig. 2. Comparison of the BDV p24 nucleotide sequence. Comparison of the BDV p24 nucleotide sequence at nucleotides 1473–772 between the BDVs in the brain of the mare and the fetus. cDNA clones obtained from the mare and the fetus are shown in (A). The published sequences of horse-derived BDVs (Strain V, He/80 and WT-1) are also shown as controls. Nucleotides identical to those of Strain V are indicated by dots. The deduced amino acids according to these sequences are shown in (B).

horse-derived BDVs from Europe (Cubitt et al., 1994; Schneider et al., 1994) were used as standards (Fig. 2). The sequences of randomly selected clones were identical in both mare and fetal brain, and were also highly homologous to standard horse-derived BDVs (96.3–99.7% homology at the nucleotide level and 100% at the amino acid level). Nucleotide 1658 was the same as WT-1, but differed from those of Strain V and He/80, although other nucleotide substitutions were the same as those of He/80. Nucleotide 1658 was also the same in BDVs from the brain of the mare and fetus. The nucleotide sequence data for BDV from the mare and the fetus have been lodged in the GenBank nucleotide sequence database with accession number AB022025. These sequences were obtained

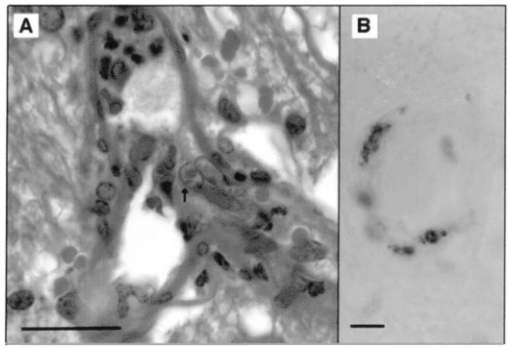

Fig. 3. Histopathology of brain sections from BDV-infected mare. The endothelial cells in the diencephalon contained single eosinophilic intranuclear inclusion bodies (arrow). HE staining is shown in (A); bars: 20 μm. Immunohistochemistry to detect EHV-1 protein expression in the histological lesions is shown in (B); bars: 7 μm.

from the RT-PCR products amplified in two independent laboratories (Rakuno Gakuen University, Hokkaido University). Also, there was no PCR product from either mare and fetus brain samples when we performed PCR without RT process (data not shown).

3.3. Histological analysis

Histological examination of the CNS of the mare revealed non-suppurative meningoencephalitis which was characterized as follows: infiltration of macrophages and lymphocytes in meningitis, perivascular infiltration of macrophages and lymphocytes; neuronal necrosis in the cerebral cortex, and spinal cord; and swelling of endothelial cells consisting of vasculitis with hemorrhage. Some endothelial cells in the brain contained single eosinophilic intranuclear inclusion bodies (Fig. 3A). The histological diagnosis of EHV-1 by IHC disclosed the presence of EHV-1 antigens in the histological lesions of the mare brain sections (Fig. 3B). Immunoreactivity for BDV antigen was not presented in areas with severe encephalitis. ISH revealed positive hybridization signals in several regions of the CNS from both the mare and the fetus (Fig. 4). Although few positive cells were observed in several microscopic fields of each brain section, the positive brain regions of the mare and the fetus were widely distributed as follows: parietal lobe, occipital lobe, mesencephalon, cerebellum and spinal cord of the mare, and frontal lobe, parietal lobe and hippocampus of the fetus (Table 1). The positive cells seemed to be predominantly neurons as evidenced by their morphology. In addition, no positive hybridization signals were detected in the tissue samples from the uninfected control rat using the same probes (Fig. 4).

4. Discussion

Samples of sera and CSF from the mare and fetus were examined for the presence of anti-BDV antibodies. Antibodies to BDV p40 and p24 antigens were detected in serum

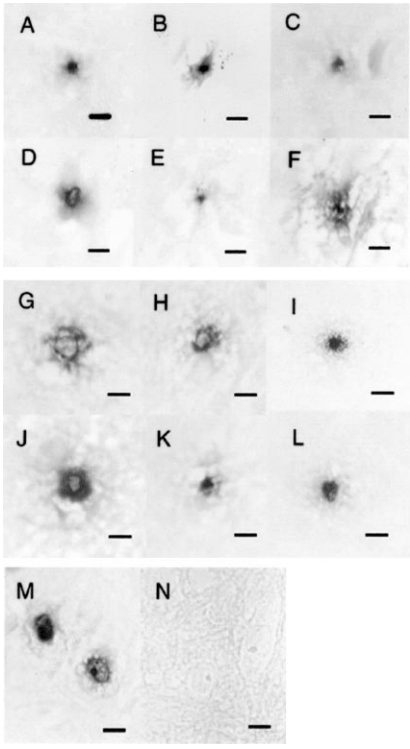

Fig. 4. In situ hybridization of BDV RNA in brain sections from the mare and the fetus. Brain sections were hybridized with a DIG-labeled BDV p40-specific antisense riboprobe, then counterstained with methyl-green. Parietal lobe (A, B), occipital lobe (C, D), mesencephalon (E, F) from the mare brain; frontal lobe (G, H), parietal lobe (I, J), hippocampus (K, L), from the fetal brain. The positive and negative controls were brain samples from the rat infected with BDV (M) and from uninfected rat (N), respectively. Bar: 20 μm.

and CSF from the mare, but no antibodies to these antigens were detected in fetal serum or CSF. The brain of the mare, and the brain and PBMCs of the fetus were positive for BDV RNA by RT-nested PCR. In contrast, the PBMCs of the mare were negative for BDV RNA. Similar results, i.e. BDV RNA-negative PBMCs from seropositive individuals, have been reported in humans (Bode et al., 1995; Kishi et al., 1995a, b), horses (Nakamura et al., 1995; Bahmani et al., 1996; Hagiwara et al., 1997b), sheep (Hagiwara et al., 1997a) and cattle (Hagiwara et al., 1996). Also, seropositive sheep and horses showed positive signals for BDV RNA in the brain, while PBMCs were negative (Hagiwara et al., 1997a, b). Thus, the BDV RNA signals in the PBMCs do not correspond to seroconversion, probably because of the extremely low copy number of BDV RNA in PBMCs, even in infected rats (Sauder and de la Torre, 1998). In order to minimize false positives by RT-PCR, we performed the experiments as a multicenter study, distributing aliquots of brain samples derived from the same brain region using disposable knives. These RT-PCR assays have been carried out in the sterile facilities of two independent laboratories (Rakuno Gakuen University, Hokkaido University). BDV RNA specificity

Table 1
Distribution of BDV RNA in brain regions from the mare and the fetus[a]

Region	Mare	Fetus
Olfactory bulb	−	−
Frontal lobe	−	+
Corpus striatum	−	−
Parietal lobe	+	+
Temporal lobe	−	−
Diencephalon	−	−
Hippocampus	−	+
Occipital lobe	+	−
Mesencephalon	+	−
Pons	−	−
Medulla oblongata	−	−
Cerebellum	+	−
Spinal cord	+	NE[b]

[a] +: Positive signals, detected by sense and antisense BDV p40-specific riboprobes; −: negative.
[b] Not examined.

was confirmed by double-checked RT-nested PCR using RNA-loaded samples. Moreover, these samples were examined using PCR without RT, to confirm the absence of contamination by plasmid DNA. In the controls, there was no PCR product from either the mare or the fetus samples.

The sequences of the p24 PCR products from both the mare and the fetal brain samples showed a high degree of homology and were also highly homologous to horse-derived BDVs from Europe (Cubitt et al., 1994; Schneider et al., 1994). Thus, it seems likely that the fetus was infected with the BDV from the mother, although we sequenced only a small fragment at p24.

Histological examination of the CNS of the mare revealed non-suppurative meningoencephalitis and swelling of endothelial cells consisting of vasculitis with hemorrhage. These histological observations were similar to those reported for EHV-1 encephalitis (Jackson et al., 1977; Platt et al., 1980). Therefore, we examined for the presence of EHV-1 viral antigen in the CNS of the mare and the fetus by IHC. The histological diagnosis of EHV-1 by IHC disclosed the presence of EHV-1 antigens in the histological lesions of the mare brain sections. The neuronal necrosis and swelling of endothelial cells as well as vasculitis correspond to the changes associated with EHV-1 infection in horses (Jackson et al., 1977). Thus, from the histopathological features, we assume that the neuronal necrosis and vasculitis seen in the mare brain were due to infection with EHV-1, rather than primary effects of BDV. IHC and HE staining revealed no EHV-1 antigens and no significant abnormalities in the fetal brain, respectively.

In this study, we provide evidence for the vertical transmission of BDV in a pregnant mare which was also infected with EHV-1. It is not clear whether dual infection with BDV and EHV-1 was the reason for the vertical transmission of BDV in this pregnant mare, EHV-1 being a major cause of respiratory disease and abortion (Campbell and Studdert, 1983). Endothelial necrosis is characterized by accumulation of inflammatory cells resulting in vasculitis in infected horses (Charlton et al., 1976; Jackson et al., 1977;

Platt et al., 1980). Consequently, it is possible that the histopathological features of EHV-1 may facilitate the vertical transmission of BDV.

Antibodies to BDV were found in the serum and CSF from the mare but the fetus was seronegative. Neonatal infection results in immunotolerance to virus antigen and such an infected horse might be an asymptomatic carrier of BDV (Carbone et al., 1991). In fact, our epidemiological studies showed that animals with PBMCs positive for BDV RNA did not always show anti-BDV antibodies in their serum (Nakamura et al., 1995, 1996; Bahmani et al., 1996; Hagiwara et al., 1996, 1997b). Taking these results into account, vertical transmission is one of the possible routes for the transmission of BDV in horses; however, vertical transmission of BDV in other vertebrate species including humans remains to be determined.

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