Detection and analysis of Borna disease virus in Chinese patients with neurological disorders


Department of Neurology, The First Affiliated Hospital, Chongqing Medical University, Chongqing, China; Institute of Neuroscience, Chongqing Medical University, Chongqing, China; Department of Neurology, The Affiliated Hospital of Ning Xia Medical College, Ning Xia, China; and Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Shizuoka, Japan

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Background and purpose: Borna disease virus (BDV) is a neurotropic RNA virus that is known to cause neurological disturbances in various animal species, potentially even humans. However, the association between BDV infection and human neurological disorders remains unclear. Methods: Between August 2005 and March 2006, 65 patients with neurological disorders were enrolled into our study. The presence of BDV p24 RNA from peripheral blood mononuclear cells (PBMCs) was investigated by using nested reverse transcriptase PCR (RT-PCR) assay. Results: Borna disease virus p24 RNA was detected from PBMCs in six patients with viral encephalitis by using nested RT-PCR assay. However, BDV p24 RNA was not detected in patients with multiple sclerosis or peripheral nerve diseases. Conclusion: There might be possible associations between BDV infection and human viral encephalitis.

Introduction

Borna disease virus (BDV) is a neurotropic, enveloped virus that causes Borna disease in a wide variety of animal species ranging from avian to primates [1–3]. The BDV genome size is approximately 8.9 kb, which consists of six major open reading frames. BDV has been classified as the prototype virus of a newly established family, Bornaviridae, within the order Mononegavirales [4]. Over the years, information has accumulated about unusual features of BDV-induced disease in experimental animals such as rats, mice and tree shrews. In these animals, BDV can induce behavioural disorders with obvious signs of viral encephalitis. Several epidemiological and molecular studies performed in Europe, Asia and USA has once demonstrated that BDV could also infect human beings [5–7].

Detection of BDV in diseased animals, mainly sheep and horses, is achieved by histological, immunohistochemical and serological approaches and/or PCR-based technologies [8,9]. Although a wide variety of tools have been developed for detection of BDV strains, there are still huge controversies between the published results. Isolation of human BDV is extremely difficult and not applicable in clinical practice [10]. In situ hybridization is a promising nucleic acid detection method for the detection of BDV. However, it may yield false-negative results if low concentrations of BDV RNA are present or the BDV RNA is degraded. In previous reports, serological methods have been developed and used for screening of animals with suspected BDV infections. However, the seroprevalence of BDV infections in different studies have shown controversial results. Recently, molecular assays for the detection of BDV are commonly used because they provide highly specific and sensitive results in a timely manner [11,12]. The use of reverse transcriptase PCR (RT-PCR) to detect BDV RNA in peripheral blood mononuclear cells (PBMCs) of infected subjects is a powerful tool to evaluate the role of carrier hosts as potential sources of human BDV infection [13,14]. The purpose of our study was to investigate the possible association between BDV infection and human neurological disorders, by using nested RT-PCR assay.

Materials and methods

Subjects

This study was approved by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from all the participants. Between August 2005 and March 2006, 65 patients (37 male, 28 female; average age: 42 ± 9 year-old) were enrolled into the study. All the patients were from the department of neurology at the Ning Xia Medical College hospital and the first affiliated hospital of Chongqing Medical University. Of these 65 subjects, 40 patients were diagnosed with viral encephalitis, 9 with multiple sclerosis, seven with Guillain–Barre Syndrome, 9 with peripheral nervous disease. All patients diagnosed with viral encephalitis had acute
or subacute onset of disease. The clinical signs and symptoms were suggestive of viral encephalitis. All patients had lumbar punctures immediately after admission and the CSF white blood cell count were found to be 50–500 WBCs, mainly lymphocytes. Patients were excluded from our study if they had clear aetiological diagnosis of viral encephalitis. Blood samples were collected from patients with viral encephalitis within 10 days of disease onset. Forty-six healthy subjects (28 male, 18 female, average age: 37 ± 11.53 year-old) were also recruited as control group. If the nested RT-PCR was positive for BDV p24 RNA, convalescent blood samples were also collected from patients with viral encephalitis once they recovered from symptoms of encephalitis. To confirm the presence of BDV in patients with neurological disorders, western blot analysis was performed to detect anti-BDV antibodies in all BDV positive samples. If the p24 RNA was detected, PBMC RNA from the same patient was screened for the presence of nucleoprotein by nested RT-PCR as previously described [13].

**Primers and probes**

For the amplification of BDV RNA, primers and probes were designed based on the second open reading frames (p24) of BDV genome sequence obtained from GenBank. The primers used for the reverse transcription and the first amplification reaction were BD1 (5’-TCAGACCCAGACCCAGCGAA-3’, nt 1443–1461) and BD2 (5’-ATCTGGGGATAAATGCGCG-3’, nt 1816–1834). The primers used for the second amplification reaction were BD3 (5’-CCCTCAAGTGGAACCAT-3’, nt 1609–1617) and BD4 (5’-CAGTATCTTGTGTCTCGCA-3’, nt 1673–1694). They were provided by Invitrogen corp., Carlsbad, NM, USA. The internal probe used for quantitation of PCR products was 5’-TCAGCGGTGAGCCTAGCGC-3’ (nt 1637–1661, 25 bp) labelled at the 5′ end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher 6-carboxytetramethylrhodamine (TAMRA).

**Preparation of PBMCs**

A total of 10–20 ml blood samples were collected from subjects in the presence of EDTA as an anticoagulant. PBMCs were separated from serum by Ficoll-Conray density gradient centrifugation. Pelleted PBMCs were stored at −70°C for future use.

**RNA Extraction**

Total RNA was extracted from PBMCs with Trizol reagents (Trizol LS; Invitrogen) according to the manufacturer’s protocols. The approximate concentration and the purity of extracted RNA were determined by spectrophotometry.

**Nested RT-PCR**

The nested RT-PCR assay was prepared with special precaution for contaminations. The in vitro transcribed RNA from BDV p24 plasmids was used as the positive control and aliquots which contain all reagents except the target sequence was used as negative controls. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed. For nested PCR, RT-PCR amplicon tubes were spun before the tubes were opened using separate Eppendorf tube openers for transferring products to the nested PCR mix. Negative control was incorporated for every five nested PCRs to monitor cross-contamination. Reverse transcription was performed at 70°C for 15 min followed by 60°C for 15 min in a 20-µl mixture. β-actin was used as internal control to correct variation of different samples. A blank mixture was prepared using all reagents except RNA samples for each reverse transcription. This mixture was used as blank control.

After reverse transcription, the first round PCR was carried out in a total volume of 50 µl, containing 10 µl of reverse transcription products, 1 µM p24-OF and p24-OR nested primers each, deoxynucleoside triphosphates at a concentration of 0.2 mM each, and 1.5 unit of Taq polymerase. The reaction conditions of the first round PCR were as follows: pre-denaturation at 93°C for 3 min, followed by 20 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 1 min in a thermal cycler (OmniGene; Hybaid, Teddington, UK). The final step was extended for an additional 10 min at −72°C. For the second round PCR, 5 µl of the first PCR product was amplified in a 50-µl mixture containing 1 µl of fluorescent DNA probe, 1 µM p24-OF and p24-OR nested primers each, deoxynucleoside triphosphates at a concentration of 0.2 mM each, and 1.5 unit of Taq polymerase. The reaction conditions of the second round PCR were as follows: pre-denaturation at 93°C for 2 min, followed by 40 cycles of 93°C for 30 s, 55°C for 45 s. The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The expected PCR products were used as a query sequence in a BLAST search at GenBank. Considering the access to relative large quantities of samples in our laboratory, all BDV positive samples were sent to an independent laboratory for validation of the results under the same code. All verification experiment was done at the institute of neuroscience, Chongqing Medical University.
Cloning and sequencing of PCR products.

Positive PCR products were cloned and sequenced by using ABI 377 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The reported p24 gene sequences from horse-derived BDV from Europe were used as standards. Nucleotide sequences of those positive products were compared with that of standard strain V and strain He/80.

Results

Efficacy of the nested RT-PCR assay

To determine the sensitivity of the nested RT-PCR assay, in vitro transcribed RNA from plasmids was generated. Ten-fold dilution series of plasmids containing BDV p24 open reading frame were examined to determine the number of DNA molecules representing reverse transcribed template detectable by nested RT-PCR assay. The reaction efficacy was calculated by using the BDV p24 standard curve. For the nested RT-PCR assay, the standard curve remained linear in a range from 100 to $10^7$ copies per reaction and the reaction efficacy was 84%. A nested RT-PCR using 1000 copies of p24 RNA was negative when the reverse transcriptase was omitted, suggesting that the RNA was free of contamination. Specificity test using aliquots which contain all reagents except the target sequence was also performed throughout each experiment. A specificity of 100% was exhibited by the nested RT-PCR assays, as none of the negative controls gave a positive PCR result.

Detection of BDV p24 RNA in PBMCs

Total RNA was successfully isolated from PBMCs of all subjects. In our present study, the concentration of RNA was quantitated using spectrophotometric OD260 measurements and purity was assessed by OD260/OD280 ratio. We randomly selected four RNA samples and measured their values of OD260 and OD280 by ultraviolet spectrophotometer. All RNA isolates had an OD260/OD280 between 1.8 and 2.0. In our present study, the integrity of RNA samples was analyzed by electrophoresis on a denaturing agarose gel containing formaldehyde. After agarose gel electrophoresis of total RNA samples, the 28S and 18S and 5S RNA bands were clearly visible, suggesting that the isolated total RNA was of high integrity.

To evaluate the prevalence of BDV p24 RNA in patients with neurological disorders, RNA samples from PBMCs were investigated by nested RT-PCR assay. All PCR positive products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The RT-PCR assay specifically yielded a predicted 86-bp DNA fragment. From the 46 healthy subjects, peripheral blood cells were screened for the presence of BDV p24 RNA and none of the healthy subjects was positive in our cohort. BDV p24 RNA was detected from PBMCs in six patients with viral encephalitis by using nested RT-PCR assay. (Fig. 1). However, BDV p24 RNA was not detected in patients with multiple sclerosis or peripheral nerve diseases. A total of 10 ml blood samples from six BDV positive viral encephalitis patients were collected and validated in another independent laboratory under the same code. In consistent with our laboratory results, BDV RNA was detected in all six patients with viral encephalitis. To determine whether BDV RNA sequences can still be detected in patients that recovered from disease, convalescent blood samples were collected in all patients with BDV positive results. Interestingly, BDV p24 RNA could only be detected in one of six patients with viral encephalitis at the convalescent phase. To confirm the presence of BDV RNA, blood samples from six BDV p24 positive patients were screened for the presence of BDV nucleoprotein sequences by nested RT-PCR. BDV nucleoprotein was detected in all six patients with viral encephalitis. We also investigated the prevalence of BDV antibodies in plasma specimens from six BDV positive patients. Similarly, antibodies against BDV phosphoprotein and

![Figure 1](https://example.com/figure.png)

Figure 1 Nested RT-PCR assay of BDV p24 RNA from PBMCs in patients with viral encephalitis. The figure shows the results from ABI PRISM 7700 Sequence Detector (Applied Biosystems, Carlsbad, CA, USA). Negative controls are not shown. The plot indicates that the PBMCs from patients with viral encephalitis are positive for BDV p24 RNA.
nucleoprotein can be detected in all six viral encephalitis patients by western blot analysis (Fig. 2). All positive PCR products were cloned and sequenced.

In order to identify potential functions of the gene product of the ORF, the GenBank database was searched for sequences exhibiting homology with the predicted gene product. To register the obtained fragment (86 bp) at GenBank, we compared the basic sequence of the target gene sequence with the query sequence in BLAST. Nucleotide sequence analysis has identified significant homology (97%) between the screening recombinant plasmid DNA and BDV C6BV p24 sequence. Consistency silent mutation occurred at three points (nt 1658 T → C, nt 1667 A → G, nt 1670 C → T).

Discussion

Borna disease is a neuro-infectious disorder that is found to occur in a wide range of animals including birds, cattle, cats and primates. Previous studies suggested that BDV infection may cause immune-mediated meningitis and encephalomyelitis in sheep and horses [15]. BDV is highly neurotropic in natural hosts. The infection of BDV in the CNS of animal species may also cause behavioural abnormalities. There is growing evidence that BDV also infect human beings. Recent epidemiological studies have shown that BDV infection may be related to human psychiatric disorders. It is reported that PBMCs from some neuropsychiatric patients express BDV antigen. Compared with health controls, BDV p24 RNA has been detected at significant higher levels in PBMCs from patients with neuropsychiatric symptoms [16,17]. The potential role of BDV infection in human beings has raised concern in the clinical investigation of BDV in patients with neurological disorders.

Detection of BDV RNA in brain and blood samples from animals or human beings can be achieved by serological assays as well as immunohistological methods [9,18]. In previous studies, various serological methods have been developed to detect BDV infection in diseased hosts. However, none of these methods is sensitive enough to be used alone for a sure diagnosis due to variable results. In BDV-infected animals, BDV antibodies from body fluids including serum or CSF are useful indicators, but immunohistological confirmation of the brain tissues is required for a definite diagnosis. Thus, this is not practical in clinical screening of suspected patients with BDV infections. Recently, PCR assay has been emerged as a promising tool for the specific diagnosis of various neurotropic virus infections. The highly sensitive nature of the molecular assay has ensured its central role in detection of BDV RNA in animals and human beings [19,20]. In our present study, we have successfully established a reliable method to detect BDV p24 RNA from sera specimens by using nested RT-PCR.

In our present study, we investigated the presence of BDV p24 RNA by using nested RT-PCR in patients with neurological disorders. The PCR assay was established for the detection of BDV in PBMCs from human beings, using loci for the p24 phosphoprotein genes. Detection of the p24 transcripts of BDV was carried out with fluorescently labelled probes in a sequence detector. By using our established nested RT-PCR assay, the viral nucleotides could be identified in PBMCs of patients with viral encephalitis. In addition, the RT-PCR parameters, including the cycles, temperature, and some other reaction conditions, were optimized. In consistent with previous reports, we detected BDV p24 RNA in our present study because the p24 target was detected at higher rates than p40 in tissues from BDV-infected hosts [21,22]. Therefore, the p24 RNA is supposed to have higher value in the detection of BDV infections in diseased hosts.

In order to eliminate potential contaminations, positive and negative controls were included in each run, and the nested RT-PCR assay was prepared with special precautions. To prevent false positive results by cross contamination, the preparation area for sample RNA was different to that of where the PCR controls are prepared. Furthermore, all the buffers, primers, nucleotides and the enzyme are prepared as a ready and cooled master mix. The negative control, which contains all reagents except the target sequence, gives information if there is cross contamination. In our present study, the negative control was always negative.
throughout our experiment, suggesting the sample was free of contamination.

The positive PCR products were proved to be BDV p24 RNA by sequencing and homology searching in NCBI. The nucleotide sequence generated from PBMCs of patients by nested RT-PCR showed high homology to that of GenBank. In our case series, BDV p24 RNA was detected in PBMCs from patients with viral encephalitis, suggesting a possible link between BDV infection and human viral encephalitis. However, there is no evidence of BDV infection in patients with multiple sclerosis or peripheral nervous diseases. In retrospective review of the history of BDV positive cases, we noticed that these patients were nomads who engaged in raising horses, cattle, and sheep in Ning Xia province. However, animal contact was not a prerequisite for a human BDV infection. Further investigation of the eating habits of BDV positive patients revealed that they used to kill and eat raw meat of diseased animals. Since BDV was an animal-borne virus, butchering and eating diseased animals might put these nomads at particularly high risk for BDV infection. Thus, further studies are needed to evaluate the role of diseased hosts as potential source for human infections, and to establish a rigorous link between BDV infection and human viral encephalitis.

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References