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ORIGINAL RESEARCH ARTICLE

Detection of Borna disease virus RNA from peripheral blood cells in schizophrenic patients and mental health workers

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Accumulating evidence suggests that Borna disease virus (BDV), a neurotropic, negative-stranded RNA virus, might be associated with certain human mental disorders. Several research groups reported that psychiatric patients had a significantly higher prevalence of BDV serum antibodies than normal controls. In addition, a significantly higher presence of BDV RNA from peripheral blood cells was identified in mental patients than in controls. In our previous study, we first identified the presence of BDV serum antibodies in a cohort of Chinese schizophrenic patients from Taiwan, and we also demonstrated a significantly higher seroprevalence of BDV antibodies among schizophrenic patients than in non-psychiatric controls. Prompted by the positive seroepidemiological result, we set out to investigate the detection of BDV RNA from the peripheral blood cells of our schizophrenic patients. By using the reverse transcription-polymerase chain reaction (RT-PCR) method, 10 out of 74 Chinese schizophrenic patients from Taiwan were found to have BDV RNA in their blood cells, whereas only one out of 69 controls was positive. The BDV RNA detection rate among schizophrenic patients was significantly higher than that in controls (14% *vs* 1.4%, $P < 0.01$). Furthermore, we studied the BDV RNA detection rate among mental health workers, and seven out of 45 mental health workers were found to have positive results. The prevalence rate was significantly higher than that in normal controls (15% *vs* 1.4%, $P < 0.001$), which lends further support to our previous finding that mental health workers have a significantly higher presence of BDV serum antibodies. In summary, our data support the finding that BDV infection might be a contributory factor to the pathogenesis of schizophrenia in the Chinese population.

Keywords: Borna disease virus; schizophrenia; RNA; RT-PCR

Introduction

Borna disease virus (BDV) is a neurotropic, enveloped, negative-strand RNA virus, which was first identified as an agent causing an infectious horse encephalitis endemic in Germany. The horses and sheep are natural hosts of BDV, but the BDV can be experimentally transmitted to birds, rodents and primates.[1] The infected animals manifested neurological deficits combined with various behavioral changes such as aggression, hyperactivity, frenzy, social withdrawal, apathy, and cognitive deficits, which are reminiscent of some human neuropsychiatric symptoms. These characteristic behavioral abnormalities of BDV-infected animals have prompted the study of the possible involvement of BDV infection in the pathogenesis of human mental disorders.[2–5]

Evidence for the possible link between BDV infection and human mental disorders first came from seroepidemiological studies.[6,7] Several research groups have demonstrated a significantly higher seroprevalence rate of anti-BDV antibodies in psychiatric patients than in normal controls. The possible association of BDV infection and human mental disorders was further consolidated by the demonstration of a higher detection rate of BDV RNA from peripheral blood cells in schizophrenic patients than in controls.[8–10] Furthermore, BDV antigens and RNA were detected in human autopsy brain tissues from neuropsychiatric patients.[11–13] More recently, Bode *et al* reported isolation of BDV from the peripheral blood cells from psychiatric patients.[14] A link between BDV infection and neuropsychiatric disorders was further supported by the detection of BDV antigen in cerebrospinal fluid from patients with recurrent depression and multiple sclerosis.[15] These data suggest that BDV

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may contribute to the pathogenesis of human neuropsychiatric disorders. Nevertheless, the role of BDV in human mental disorder is not without controversy, several groups presented contradictory results and indicated the possible methodological defects in previous serological and molecular biological studies.[16–18] Hence, the association of BDV and human mental disorders needs further clarification.

Although BDV infection was first identified in Germany, the geographic distribution of BDV is larger than previously appreciated. BDV-specific antibodies were reported in other parts of Europe, North America and parts of Asia, suggesting that BDV appears to have worldwide distribution (for review see Bode).[19] In our recent study, we first identified the presence of anti-BDV nucleoprotein (40 kD, p40) and phosphoprotein (24 kD, p24) serum antibodies in a cohort of Chinese schizophrenic patients from Taiwan using Western blot analysis.[20] We also demonstrated a significantly higher seroprevalence rate of anti-BDV serum antibodies in schizophrenic patients than in controls, which suggests that BDV infection may also be associated with the pathology of psychiatric disorders in the Chinese population. Prompted by the positive seroepidemiological results, we carried out experiments to detect the presence of BDV RNA from peripheral blood cells in our schizophrenic patients, and to determine the prevalence rate of BDV RNA among schizophrenic patients and non-psychiatric controls from Taiwan.

The reservoir and route of transmission of BDV are still unclear.[21] In our previous study, we demonstrated a significantly higher seroprevalence rate of anti-BDV antibodies among patients' unaffected family members and mental health workers than in controls, suggesting a possible human-to-human transmission of BDV.[20] Although the transmitting route of BDV is not very clear, the olfactory neuroepithelium might occasionally play a role as portal of entry. After entering the olfactory nerve, BDV is spread intra-axonally as suggested from animal studies.[21,22] To further elucidate the possible human-to-human transmission of BDV, we also investigated the detection rate of BDV RNA in peripheral blood cells from mental health workers.

Materials and methods

Subjects

Chinese schizophrenic patients from Taiwan fulfilling the diagnostic criteria of schizophrenia according to the DSM-IV (American Psychiatric Association) were recruited from two mental hospitals in Taipei City. The patient group comprised 40 males and 34 females, with a mean age of 40 years. The clinical diagnosis of these patients included schizophrenia, residual type ($n = 35$), paranoid type ($n = 11$), disorganized type ($n = 20$) and undifferentiated type ($n = 8$). Non-psychiatric controls were recruited from an out-patient unit of a local community hospital in Taipei City. The control group consisted of 34 males and 35 females, with a mean age of 50 years. Mental health workers with a working experience of longer than 2 years, were recruited from two mental hospitals, including 11 males and 34 females. The mean age was 35 years. They were nurses, social workers, occupation therapists and psychiatrists. Informed consent was obtained from the subjects or patients' guardians.

Preparation of human peripheral blood mononuclear cells (PBMCs)

Ten milliliters of venous blood samples were taken from each subject with EDTA as anticoagulant. The PBMCs were prepared from the EDTA-treated blood by gradient centrifugation using Ficoll–Paque plus (density $1.077\ g\ ml^{-1}$, Pharmacia Biotech, Uppsala, Sweden) as described. Total RNA was extracted from pelleted PBMCs using an RNA extraction kit (RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The total RNA was eluted in 50 μl DEPC-treated water.

Detection BDV RNA by RT-PCR

BDV p40 and p24 RNA in the total RNA isolated from PBMCs were assayed using the Titan One Tube RT-PCR System (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instruction with modification. In brief, an aliquot (10 μl, 1–2 μg) of total RNA was used for RT-PCR in a total volume of 25 μl. The reaction mixtures contained 1× reaction buffer (pH 7.5), 0.2 mM each of dNTP, 5 mM DTT, 1.5 mM $MgCl_2$, 0.4 mM of sense and antisense primers, and 0.5 μl enzyme mix (AMV and Expand High Fidelity PCR-System). For the detection of p40 BDV RNA, sense primer 5′-gtcacggcgcgatatgtttc-3′ (nucleotide position 242–261, Genebank accession No. U04608) and antisense primer 5′-ccatgcattctgcgaggta-3′ (nucleotide position 801–783) were used. For detecting p24 BDV RNA, sense primer 5′-agacactacgacgggaacga-3′ (nucleotide position 1327–1346) and antisense primer 5′-tgggagctggggataaatgc-3′ (nucleotide position 1838–1819) were used. Reverse transcription was first performed at 50°C for 30 min, followed by denaturation at 95°C for 5 min to terminate the reverse transcription. Subsequently, 30 cycles of PCR amplification were performed in the same tube at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The RT-PCR was carried out in the PCR machine PTC-200 (MJ Research, Watertown, MA, USA). After amplification, an aliquot (20 μl) of the PCR was resolved in 2% agarose gel with electrophoresis, and visualized with ethidium bromide staining. Total RNA isolated from Madin-Darby canine kidney (MDCK) cells persistently infected with BDV was used as positive control, while total RNA isolated from uninfected MDCK cells were used as negative control. The predicted size of the BDV p40 RT-PCR product was 559 bp, while the predicted size of the BDV p24 RT-PCR product was 512 bp. After electrophoresis, the PCR products were transferred onto nylon membrane for Southern blot analysis.

Southern blot analysis

The specificity of products of RT-PCR detection of BDV RNA was assayed by Southern blot hybridization. After

electrophoresis of the RT-PCR products in 2% agarose gel, the PCR products were blotted onto a nylon membrane, then hybridized with biotinylated oligonucleotides internal to the predicted PCR product as probes. Biotinylated oligonucleotide primer 5′-attctttacctgggg actca-3′ (nucleotide position 717–698) was used for detecting BDV p40 sequences, while biotinylated oligonucleotide primer 5′-gcaacatgggtgcagaggtc-3′ (nucleotide position 1797–1778) was used for BDV p24 sequences detection. After a stringent wash, the hybridized biotinylated DNA was detected using a chemiluminescent detection kit (Phototope-Star Detection Kit, New England Biolabs, Beverly, MA, USA) according to the manufacturer's protocol.

RNA quality control
Partial cDNA sequences of human α-galactosidase A gene (Genebank accession No. M13571) were amplified using RT-PCR for quality assurance of total RNA isolated from human PBMCs. In brief, first-strand cDNA were synthesized from an aliquot of total RNA (1–2 μg) using oligo-dT primer at 42°C according to the manufacturer's protocol (SuperScript II, Gibco BRL, Gaithersburg, MD, USA). The reverse transcription was terminated by incubation at 95°C for 10 min. An aliquot (5 μl) of the first cDNA was used for PCR amplification of partial cDNA sequences of human α-galactosidase A gene. A total volume of 25-μl reaction mixtures contained 10 μM α-galactosidase A gene. A total volume of 25-μl reaction mixtures contained 10 μM each of sense primer 5′-tcacagcaaaggactgaagc-3′, antisense primer 5′-aaagaggccactcacaggag-3′, 0.2 mM dNTP, 1× reaction buffer (pH 8.8), 1.5 mM $MgCl_2$, and 0.5 unit of recombinant thermostable DNA polymerase (ProZyme II, Protech Technology, Taipei, Taiwan). The amplification was performed with initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 1 min. The PCR products were analyzed in 2% agarose gel by electrophoresis. The predicted size of the PCR product was 248 bp.

Sensitivity of RT-PCR
Serial dilutions of plasmids containing the BDV p40 cDNA (pCYB2-p40) and p24 cDNA (pCYB2-p24) were used to assess the relative sensitivity of the Titan One Tube RT-PCR System in this study. After amplification, an aliquot (20 μl) of PCR products was analyzed in 2% agarose gel by electrophoresis, visualized by ethidium bromide staining. Subsequently, the PCR products were transferred to nylon membrane and subjected to Southern hybridization as described. The details of the construction of expression plasmids pCYB2-p40 and pCYB2-p24 were described in detail in our previous study.[20]

Sequencing of BDV RT-PCR product
To further verify the authenticity of the positive RT-PCR products identified from PBMCs, the PCR product was size-fractionated by electrophoresis in 1% agarose gel and recovered using PCR purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Purified PCR products were subjected to direct thermocyclic sequencing using AmpliTaq sequencing kit (Perkin Elmer, Norwalk, CT, USA) according to the standard protocol provided by manufacturer, and visualized by autoradiography on sequencing gel. α-[^{33}P]-dATP was used for internal incorporation during sequencing reactions.

Results

Sensitivity of RT-PCR
Serial dilutions of plasmid pCYB2-p40 were used to assess the sensitivity of the Titan One Tube RT-PCR system. As illustrated in Figure 1a, 250 molecules of the pCYB2-p40 can be detected with 30 cycles of amplification. Southern blot analysis can increase the

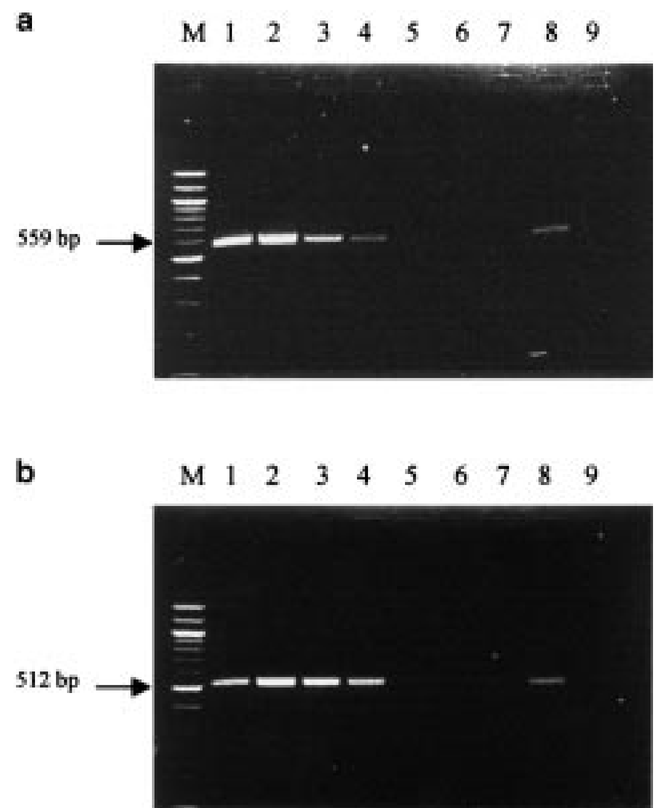

Figure 1 Sensitivity of agarose gel electrophoresis of the Titan One Tube RT-PCR system in detecting pCYB2 plasmids containing BDV p40 and p24 cDNA inserts, respectively. (a) PCR analysis of pCYB-40 with serial dilutions; lanes 1, 2, 3, 4, 5, 6 indicate 10^4, 10^3, 50, 250, 100 and 50 molecules, respectively. Lane 7 is water blank, lane 8 represents RNA isolated from MDCK cells persistently infected with BDV, which was used as positive control. Lane 9 is RNA isolated from MDCK cells without BDV infection used as negative control. M indicates DNA marker. (b) PCR analysis of pCYB-24 with serial dilutions; lanes 1, 2, 3, 4, 5, 6 indicate 10^4, 10^3, 50, 250, 100 and 50 molecules, respectively. Lane 7 is water blank, lane 8 represents RNA isolated from MDCK cells persistently infected with BDV, which was used as positive control, while MDCK cells without BDV infection are used as negative control (lane 9). M indicates DNA marker.

sensitivity 10-fold up to 100 molecules (data not shown). Similarly, sensitivity of up to 250 molecules of the pCYB2-p24 was achieved with 30 cycles of amplification (Figure 1b), while 10-fold sensitivity of up to 100 molecules can be detected with Southern blot analysis (data not shown).

Prevalence of BDV RNA in schizophrenic patients
Seventy-four Chinese schizophrenic patients from Taiwan were studied; 11 patients were detected as having BDV RNA in their PBMCs. Nine patients were positive for p40 RNA, while two patients were positive for BDV p24 RNA. One male patient had both BDV p40 and p24 RNA in his PBMCs. The prevalence rate was 13.5%. In contrast, only one out 69 non-psychiatric controls was positive, having both BDV p40 and p24 RNA in his blood cells. The prevalence of BDV RNA in schizophrenic patients was significantly higher than in controls (odds ratio: 11.9; 95% confidence interval: 2.2–63.9). The results of RT-PCR and Southern blot analysis of detecting BDV p24 and p40 RNA were illustrated in Figures 2 and 3, respectively. The authenticity of the positive PCR products was confirmed by sequencing analysis (data not shown). There are some nucleotide variations in the amplicon sequences between our patients and sequences published in literature, however, the sequences among our patients are consistent. The results of the amplicon sequences from our patients and comparison with other BDV sequences will be published elsewhere.

Clinical characteristics of BDV-positive patients
The clinical diagnoses of these BDV RNA-positive patients are schizophrenia, disorganized type ($n=4$), residual type ($n=3$), paranoid type ($n=2$) and undifferentiated type ($n=2$), including six male patients and five female patients. All the patients are chronic cases with a disease history of more than 10 years. Most of the patients (80%) are refractory to neuroleptics treatment, and manifested prominent psychotic features. The clinical features of these patients contrast with the observations by Waltrip *et al*, who reported that BDV seropositivity is associated with deficit symptoms in schizophrenic patients.[23]

Prevalence of BDV RNA in mental health workers
Seven out of 45 mental health workers were identified as having BDV RNA in their PBMCs. Six subjects were positive for BDV p24 RNA, while one subject was positive for BDV p40 RNA. The prevalence rate was 15.0%, which was significantly higher than that in controls (odds ratio: 12.5; 95% confidence interval: 2.2–70.4) (Table 1). The positive RT-PCR products were also confirmed by Southern blot analysis, and sequencing analysis.

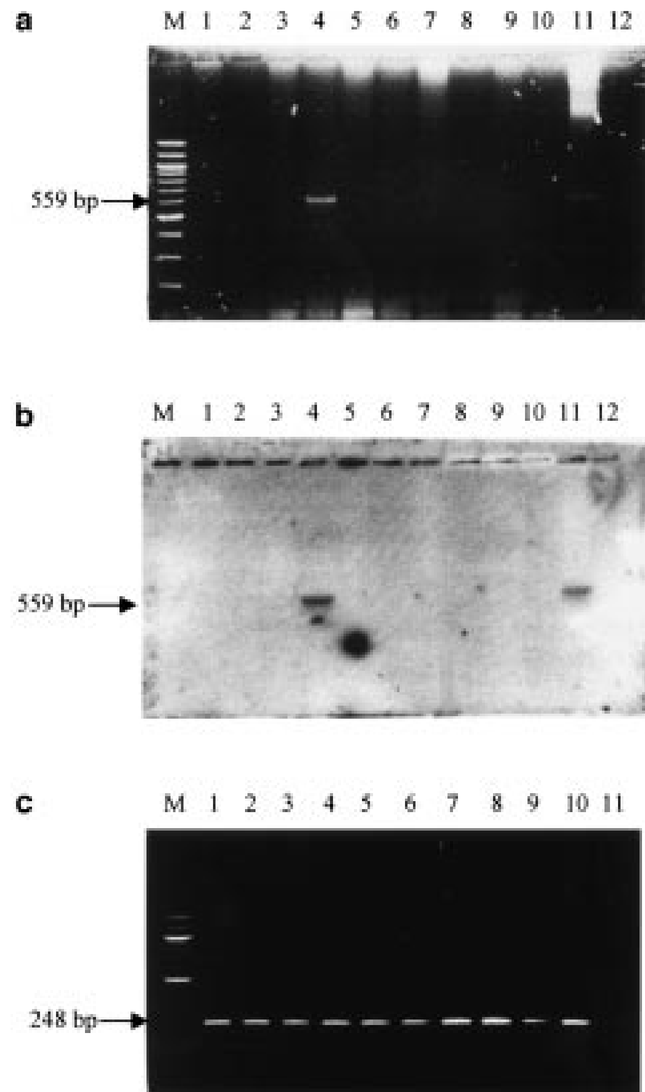

Figure 2 Representative results of RT-PCR for the detection of BDV p40 RNA in peripheral blood mononuclear cells (PBMCs) from schizophrenic patients. (a) Analysis of RT-PCR products of BDV p40 in 2% agarose gel electrophoresis, the predicted size is 559 bp. Lanes 1–10 are schizophrenic patients studied; a positive result was identified in lane 4, while the others were negative. Lane 11 represents RNA isolated from MDCK cells persistently infected with BDV, which was used as positive control, while MDCK cells without BDV infection are used as negative control (lane 12). M indicates DNA marker. (b) The specificity of the RT-PCR analysis in (a) is demonstrated by Southern blot analysis using biotinylated internal oligonucleotides used as probes. The arrow indicates the position of the positive band. (c) The quality assurance of RNA isolated from PBMCs in patients was demonstrated by RT-PCR amplification of a partial cDNA of a housekeeping gene α-galactosidase A gene. The predicted size is 248 bp. Lanes 1–10 represent patients 1–10 respectively; lane 11 is negative control.

Discussion

In this study, we demonstrate a significantly higher detection rate of BDV p40 and p24 RNA from peripheral blood monocytes in a cohort of Chinese schizophrenic patients from Taiwan than that in non-psychiatric controls. The result is comparable to studies from other research groups that also report a higher BDV RNA detection rate in schizophrenic patients than in

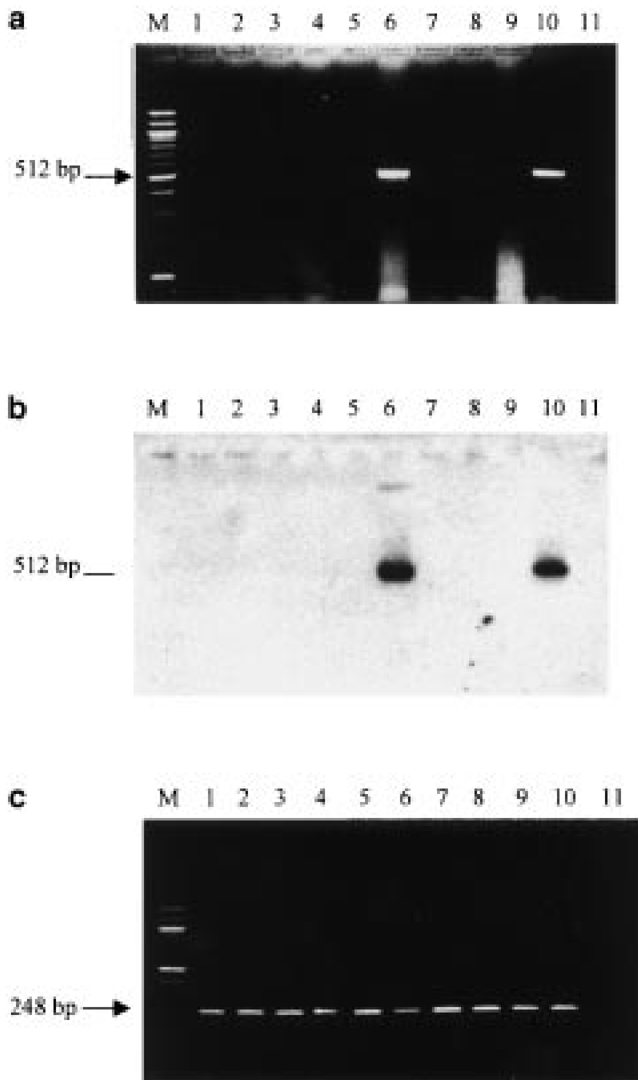

Figure 3 Representative results of RT-PCR for the detection of BDV p24 RNA in PMBCs from schizophrenic patients. (a) Analysis of RT-PCR products of BDV p24 in 2% agarose gel electrophoresis, the predicted size is 512 bp. Lanes 1–9 are schizophrenic patients studied; a positive result was identified in lane 6, while the others were negative. Lane 10 represents RNA isolated from MDCK cells persistently infected with BDV, which was used as positive control, while MDCK cells without BDV infection are used as negative control (lane 11). M indicates DNA marker. (b) The specificity of the RT-PCR analysis in (a) is demonstrated by Southern blot analysis using biotinylated internal oligonucleotides used as probes. The arrow indicates the position of the positive band. (c) The quality assurance of RNA isolated from PBMCs in patients was demonstrated by RT-PCR amplification of a partial cDNA of a housekeeping gene α-galactosidase A gene. The predicted size is 248 bp (arrow). Lanes 1–9 are patients studied; lane 10 is RNA isolated from a human hepatoma cell line (T2), which was used as positive control. Lane 11 is water blank as negative control.

Table 1 Prevalence rate of detecting BDV transcripts from peripheral blood mononuclear cells, among schizophrenic patients, mental health workers and non-psychiatric controls

	BDV-positive	*BDV-negative*	*Prevalence*	*OR (95% CI)*
Schizophrenia	11	63	13.5%	11.9 (2.2–63.9)
Mental health workers	7	38	15.0%	12.5 (2.2–70.4)
Controls	1	68	1.4%	1

OR: odds ratio.
CI: confidence interval.

controls.[9–11] Our data strengthen our previous seroepidemiological study showing a significantly higher presence of BDV serum antibodies in schizophrenic patients than in controls. Hence, taken together, our data indicate that BDV infection may contribute partly to the pathogenesis of schizophrenia in Chinese patients. In our previous study, we also demonstrated a significantly higher seroprevalence of BDV serum antibodies in mental health workers, and proposed a possible human-to-human transmission of BDV. In this present study, we further demonstrate that mental health workers have significantly higher detection rate of BDV RNA in their peripheral blood monocytes than normal controls.

In this study, we were able to detect BDV RNA in the peripheral blood monocytes using the one-tube RT-PCR method, which is distinct from other investigators. Most of the other research groups use nested PCR to detect BDV RNA in the peripheral blood cells. However, nested PCR is prone to artifacts and cross contamination. In fact, there have been arguments about the possible false positivity of nested PCR method in detecting BDV transcripts. In our pilot study developing the BDV RNA detection method, we first tested the nested RT-PCR method as performed by several other research groups. However, non-specific bands are present, and it is difficult to control cross contamination. After several experiments, we found that the Titan One Tube RT-PCR System provides enough accuracy and sensitivity for carrying out molecular epidemiological survey. In combination with Southern blot analysis, we were able to detect 100 molecules of BDV p40 and p24 RNA with high specificity. Hence, our BDV RNA detection method should provide a better option to avoid the cumbersome contamination-prone nested RT-PCR method, despite the fact that the sensitivity of our method may not be as great as the nested RT-PCR method. The development of the one-tube RT-PCR assay of detecting BDV RNA in PBMCs should facilitate comparative studies by other research groups.

In our study, only one control subject and one patient were found to have both BDV p40 and p24 RNA, whereas the other positive subjects have only either p40 or p24 RNA. Furthermore, poor correlation

between the presence of BDV serum antibodies and BDV RNA in peripheral blood was observed in our subjects. Only two patients were found to have BDV serum antibodies and BDV RNA in their blood at the same time. This finding is similar to previous studies from other research groups.[8–10]

Although the possible link between BDV infection and pathogenesis of schizophrenia in Chinese population was demonstrated in our serial studies, further isolation of BDV from patients is important in the future. In addition, the pleiotropic effects of BDV infection with various clinical manifestations have been reported in the literature. BDV infection has been observed in various neuropsychiatric diagnoses.[15,19] Similarly, it is likely that the presence of BDV serum antibodies and the BDV RNA may also be identified in other mental disorders in our population. Further systemic study is indicated to address this issue.

Either BDV seroepidemiological or molecular epidemiological studies illustrated that only some of the mental patients were susceptible to BDV infection. In the future, it is essential to identify the modifying factors that may influence the clinical characteristics of BDV infection, and to identify constitutional factors that may underline the susceptibility to BDV infection, which will also help prevent the occurrence of mental illnesses.

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