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Single-step reverse transcriptase-polymerase  
chain reaction for detection of Borna disease  
virus RNA *in vitro* and *in vivo*

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Abstract

There are few copies of Borna disease virus (BDV) genome in peripheral blood mononuclear cells and no reliable standard reverse transcriptase-polymerase chain reaction (RT-PCR) method for the detection of BDV RNA, which is both highly sensitive and free of contamination. Single-step RT-PCR, in which both reverse transcription and amplification by *Taq* DNA polymerase work efficiently in a single buffer, was applied to detect the p24 region of BDV RNA *in vitro* and *in vivo*. Using *in vitro* synthesized RNA, it was demonstrated that at least 100 copies of BDV RNA could be detected and the sensitivity and specificity were nearly equal to those obtained by RT-nested PCR. We could detect BDV RNA from more than 1 pg of cellular RNA obtained from BDV-persistently infected MDCK cells. Furthermore, this method was successfully performed on brain specimens obtained from a BDV-infected rat at 11 weeks post-inoculation. This single-step RT-PCR method will be convenient for detecting limited amounts of BDV RNA in various cells and tissue samples.

Key words : Borna disease virus ; single-step reverse transcriptase-polymerase chain reaction

Introduction

Borna disease virus (BDV) has been characterized as a nonsegmented negative-stranded RNA virus<sup>2,4</sup> which is replicated and transcribed at a nuclear site<sup>3</sup>. BDV mRNAs are transcribed

from a negative-stranded (genomic) RNA, containing at least six open reading frames encoding proteins of 40 kDa (p40), 23 kDa (p24), 10 kDa (p10), 16 kDa (gp18), 57 kDa (G) and 190 kDa (Pol.)<sup>2,4,13,15,17</sup>. Borna disease in horses has been known as a chronic encephalomyelitis ende-

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mic in Germany and several other European countries<sup>6)</sup>. Natural infection of BDV has been reported in sheep, cattle, cats, rabbits and ostriches<sup>12)</sup>. The possible relationship between BDV infection and human psychiatric disorders has been reported<sup>1,5)</sup>. However, there is no standardized diagnostic method for the epidemiological study of BDV infection in animals and humans.

The reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) is important for the assessment of BDV infection in animals and humans since BDV grows only to a low titer. Because RT-nested PCR is highly sensitive, the risk of contamination is increased and false-positive results may happen. It is important to avoid contamination of DNA templates to make correct diagnosis. Therefore, a simple method in which RT and PCR could be combined in a single-step is desired. Recently, we developed a practical and highly sensitive single-step RT-PCR method for the detection of hepatitis C virus<sup>9)</sup>. In this study, we demonstrated that the single-step RT-PCR is a useful method for detection of BDV from tissue culture *in vitro* and from experimental animal models *in vivo*.

## Materials and Methods

### *Cells and virus*

MDCK cell line and persistently BDV-infected cell line (MDCK/BDV)<sup>7)</sup> were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

### *RNA extraction*

RNA was extracted from MDCK/BDV cells using an RNA extraction kit (ISOGEN, Nippon Gene Co., Ltd).

### *In vitro synthesized BDV RNA*

DNA fragment from 1461 to 1835 nt of BDV strain V<sup>2)</sup> were inserted into pBluescript II KS(+) with *EcoRI* and *HindIII* sites. Genomic strands of BDV RNA were transcribed from the linearized plasmid using T3 RNA polymerase.

The transcription product was digested with DNase I for 15 min at 37°C. The sample was loaded on agarose gel electrophoresis and the band with appropriate length was cut and purified by RNA extraction kit. The size of RNA was confirmed by agarose gel electrophoresis. The number of BDV RNA copies was determined by OD measurement at 260 nm.

### *Single-step RT-PCR*

RNA solution (1  $\mu$ l) was added to the reaction mixture (9  $\mu$ l) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.5 mM dNTP mixture, 1  $\mu$ M sense primer against genomic strand RNA (5'-CCTTCTCCATCATGAGCTTC-3', corresponding to positions 1727 to 1746 of BDV strain V [2]), 1  $\mu$ M antisense primer (5'-GGAAACCATCCAGACAGCTC-3', corresponding to positions 1619 to 1638 of BDV strain V [2]), 5 U AMV reverse transcriptase XL (Life Science, USA) and 10 U AmpliTaq Gold (Perkin Elmer, USA). The reaction mixture, with 2 drops of mineral oil in the tube, was heated at 65°C for 1 min (to denature RNA) and 42°C for 60 min (RT reaction), and then at 95°C for 20 min (activation of AmpliTaq Gold) followed by 70 cycles of amplification using DNA thermal cycler 480 (Perkin Elmer, USA). Each PCR cycle consisted of annealing at 55°C for 30 sec, primer extension at 72°C for 1 min and denaturation at 94°C for 1 min. The PCR product was detected by ethidium bromide staining after separation by 3% agarose gel electrophoresis.

## Results

Because BDV genome has a remarkably conserved sequence in the p24 region<sup>14)</sup>, we selected p24-encoding mRNA as our target. To confirm the limit of detection by single-step RT-PCR method, the p24 RNA was synthesized *in vitro* as described in Materials and Methods. Synthesized RNA was purified using DNase I and RNA purification kit to avoid contamination of the

template by Plasmid DNA. This RNA was 10-fold serially diluted and RT-nested PCR was performed. Signals were obtained from the samples containing more than 10 copies of RNA in a reaction tube which is the limit of detection by RT-nested PCR method (data not shown).

Using the *in vitro* synthesized RNA, we determined the end point of single-step RT-PCR method. The results showed that more than 100 copies of BDV RNA in a reaction tube could be detected (Fig. 1). The amplified DNA fragments were specifically hybridized with a DIG-labeled oligonucleotide by Southern blot hybridization (data not shown).

RNA extracted from MDCK/BDV cells was subjected to single-step RT-PCR method after being serially diluted. As shown in Fig. 2, the p24 region of BDV RNA was detected in samples containing at least 10 pg of RNA.

To confirm that single-step RT-PCR method was useful for *in vivo* specimens, we applied the method to BDV-infected rats. Lewis rats were inoculated intracerebrally with homogenated MDCK/BDV cells. After three intracerebral

passages among rats, RNA was extracted from the brains at 11 weeks post-inoculation<sup>11</sup>). RNA extracted from the brain was 10-fold serially diluted and single-step RT-PCR was performed. As shown in Fig. 3, at least 100 pg of RNA was

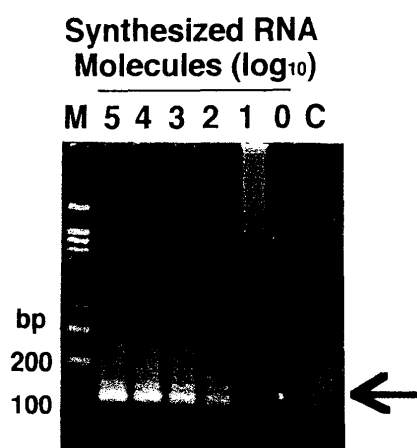


Fig. 1. Detection of *in vitro* synthesized BDV RNA by single-step RT-PCR. A 10-fold serial dilution of *in vitro* synthesized BDV RNA was subjected to single-step RT-PCR. PCR products (128 bp, indicated by arrows) were visualized by ethidium bromide staining after 3% agarose gel electrophoresis. Lane M, a 100 bp DNA ladder as a size marker; lane C, H<sub>2</sub>O was amplified.

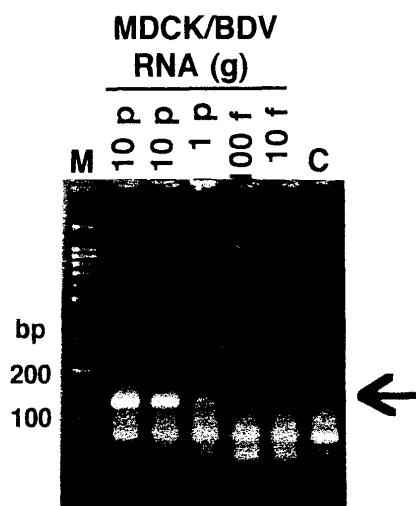


Fig. 2. Detection of BDV RNA in samples from BDV-persistently infected cells. The 10-fold serially diluted RNAs from MDCK/BDV cells were subjected to single-step RT-PCR. PCR products were detected as shown in Fig. 1.

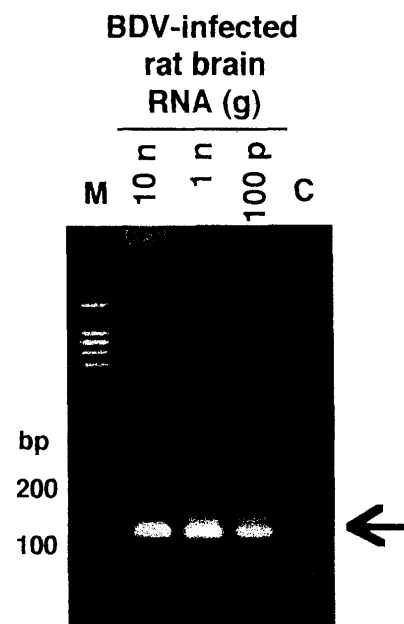


Fig. 3. Detection of BDV RNA in samples from rat brain. The 10-fold diluted RNAs from a BDV-infected rat brain were subjected to single-step RT-PCR. PCR products were detected as shown in Fig. 1.

detected. In another experiment, we could detect 10 pg of p24 RNA (data not shown). This result indicated that the single-step RT-PCR method is useful for *in vivo* specimens.

#### Discussion

The RT-nested PCR method is the most sensitive of all diagnostic methods for detecting RNA, but carries the risk of contamination. It is thought that DNA contamination occurs in many cases during transfer of the first-round PCR product to the new reaction tube for the second-round PCR. Therefore, single-step RT-PCR method is necessary to avoid such contamination.

With RNA from BDV-infected MDCK cells, BDV p24 specific products were detectable from total RNAs of more than 10 pg. Considering the sensitivity level (100 copies) determined with *in vitro* synthesized BDV RNA, 10 pg of RNA solution is suggested to contain at least 100 copies of BDV p24 RNA. When we and other laboratories used RT-nested PCR for BDV RNA from cultured cells, p24 RNA was detected from 100 fg to 10 pg (ref. 10 and data not shown). Thus, single-step RT-PCR could be fairly sensitive even in the presence of co-existing RNA from cultured host cells, being comparable to the most sensitive RT-nested PCR.

Recently, BDV seroprevalence and BDV RNA in peripheral blood mononuclear cells have been reported in blood samples from human patients diagnosed with psychiatric disorders<sup>1,5)</sup>. Horizontal transmission from ostriches<sup>8,18)</sup> and horses<sup>16)</sup> to humans has been suggested by a seroepidemiological study of BDV. This possible transmission of BDV from animal to human is of serious concern. Furthermore, all the reservoir animal species of BDV have not yet been identified. The single-step RT-PCR method established for BDV is anticipated to gain acceptance as a powerful means of detecting a small amount of RNA without risk of contamination in the future epidemiological study of BDV.

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