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Citation	Japanese Journal of Veterinary Research, 46(2-3), 73-81
Issue Date	1998-11-30
DOI	10.14943/jjvr.46.2-3.73
Doc URL	<a href="http://hdl.handle.net/2115/2644">http://hdl.handle.net/2115/2644</a>
Type	bulletin (article)
File Information	KJ00003407976.pdf



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## A single-tube RT-PCR method for the detection of Borna disease viral genomic RNA

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(Accepted for publication: Jan. 14, 1998)

### Abstract

For detecting Borna disease virus (BDV) genomic stranded RNA, single-tube reverse transcription-polymerase chain reaction (St RT-PCR) was developed to equal the sensitivity of RT-nested PCR but with reduced risk of contamination. BDV-genomic stranded RNA was synthesized *in vitro* using plasmid cDNA of BDV p24 region as a template and RNA was also extracted from BDV-persistently infected MDCK (MDCK/BDV) cells. Both RNAs were amplified by St RT-PCR in which a single round of RT and a single round of PCR were performed in the same tube. Ten copies of synthesized RNA could be amplified by St RT-PCR, indicating that St RT-PCR method is as sensitive as the ordinary RT-nested PCR method. Furthermore, this method was applied to quantify the exact copy number of genomic RNA in MDCK/BDV cells. Signals were obtained from the samples containing more than 1 pg total cellular RNA. From the results, approximately 100 copies of BDV genomic RNA exist in one MDCK/BDV cell. BDV genomic RNA from the *in vivo* RNA samples using St RT-PCR, indicating this method is applicable for the epidemiological study of BDV without contamination.

Key words: Borna disease virus, Single-tube RT-PCR method

### Introduction

Borna disease virus (BDV) naturally infects horses and sheep, and induces a disease characterized by progressive meningoencephalopathy<sup>14</sup>. Natural infection of BDV is also found in cattle, ostriches and cats<sup>4,15,16</sup> and BDV derived from horses can be experimentally transmitted to both avian and

other mammalian species<sup>21</sup>. Furthermore, in humans, seroepidemiological studies found a significantly higher seroprevalence of BDV in patients with neuropsychiatric disorders than in healthy individuals<sup>1,2,9,12,22,27</sup>. BDV sequences derived from human peripheral blood mononuclear cells exhibited a close genetic relationship to animal-derived BDV sequences<sup>23</sup>. These findings strongly suggest that the central nervous

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system disease caused by BDV infection is an emerging zoonosis.

BDV has been characterized as a negative-stranded RNA virus which replicates and transcribes at a nuclear site and it is unique among nonsegmented negative-stranded RNA viruses, indicating that BDV is a prototype of a new group of animal RNA viruses<sup>7,8,24</sup>. BDV mRNAs are transcribed from a negative-stranded (genomic) RNA, containing at least six open reading frames (ORFs) encoding proteins of 40 kDa (p40), 23 kDa (p24), 10kDa (p10), 16 kDa (gp18), 57 kDa (G) and 190 kDa (pol)<sup>3,7,26,28</sup>. In BDV-infected cells, newly synthesized viral mRNAs are efficiently transported to the cytoplasm, whereas genomic RNA remains associated with the nuclear fraction and is detected in the cell-free fraction at very low levels<sup>6</sup>, indicating that BDV is a cell associated virus. BDV grows only to a low titer and infects persistently *in vivo*, suggesting that the amount of genomic RNA is regulated at a set amount in the nucleus in the persistently infected cells. Detection of BDV genomic RNA *in vivo* is an important key to examine the possibility of animal to animal viral transmission. Therefore, it is important to detect and determine the amount of BDV genomic RNA to understand its mechanism of viral infection *in vitro* and *in vivo*.

Although reverse transcription-nested polymerase chain reactions (RT-nested PCR) are performed to demonstrate BDV-infection *in vivo* and *in vitro* in many laboratories, there has been no report concerning quantification of BDV RNA by RT-nested PCR. In addition, RT-nested PCR inevitably has the risk of contamination from cloned DNA and other substances. To resolve these problems, development of an RT-PCR method which is able to distinguish genomic RNA from mRNAs with high sensitivity and to avoid the risk of contamination is necessary. Recently, we developed a novel RT-PCR method for specific detection of negative-stranded hepatitis

C virus (HCV) RNA<sup>17</sup>. This method will be useful for the strand-specific detection of RNA intermediate in virus replication of not only HCV but also other single-stranded RNA viruses. Because more than 100 copies of negative-stranded HCV RNA could be detected by this method<sup>17</sup>, we applied the method for detection of negative-stranded BDV RNA with improved sensitivity. For understanding the regulation of viral replication in persistent infections, it will be helpful to make clear whether the copy number of BDV genomic RNA plateaux in persistently BDV-infected MDCK (MDCK/BDV) cells. Furthermore, detection of a small quantity of genomic RNA, as distinguished from a large quantity of mRNA, is also important for understanding the replication mode of BDV. In this study, the improved method revealed that approximately 100 copies of BDV genomic RNA exist in a MDCK/BDV cell.

## Materials and Methods

### *Cells and virus*

Madin-Darby canine kidney (MDCK) cell line and persistently BDV-infected cell line (MDCK/BDV)<sup>11</sup> was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

### *In vitro synthesized BDV RNA*

DNA fragments from 1461 to 1835 nt of BDV strain V<sup>3</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 (Promega Corp., USA) and the transcription product was digested by DNase QR (Promega Corp., USA) for 15 min at 37°C. The sample was loaded into agarose gel electrophoresis and the appropriate band length was cut and purified by RNaid kit (BIO 101 Inc., USA). The size of RNA was confirmed by agarose gel electrophoresis. The number of BDV RNA copies was determined from OD measurement at 260

nm.

#### *RNA extraction*

RNA derived from confluent MDCK/BDV cells was prepared using RNA extraction kit, ISOGEN (Nippon Gene Corp.) and were serially diluted 10-fold. Lewis rats were inoculated intracerebrally with homogenated MDCK/BDV cells<sup>20</sup>. After three passages among rats intracerebrally, RNAs were extracted from the brains at 11 weeks post-inoculation and were serially diluted 10-fold.

#### *Single-tube RT-PCR (St RT-PCR) method*

In this study, we used two St RT-PCR methods; one was developed for detection of negative-stranded RNA of hepatitis C virus and the other, the improved method for BDV. The former is described in ref. 17. Briefly, RNA solution was added to the reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM dNTP mixture, 1 μM primer nTag-381 [5'-CCTCCGCCGCCGTCATGGTGGCGAATA GGAAACCATCCAGACAGCTC-3', corresponding to positions 1619 to 1638 of BDV strain V<sup>3</sup>) and containing the tag sequence (underlined) in the 5' region]. The reaction mixture was heated at 95°C for 5 min and incubated at 55°C for 60 min after addition of 200 U SuperScript II reverse transcriptase (Gibco BRL Life Technologies, USA) and was heated at 100°C for 60 min to inactivate the reaction. The PCR amplification of the cDNAs was carried out by the addition of 90 μl of reaction mixture containing 1x PCR buffer [10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 70 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 100 μg/ml bovine serum albumin (BSA), 0.1% Triton X-100, pH 8.8], 1 μM sense primer, nTag (5'-CCTCCGCCGCCGTCATGGTGGCGAATA-3'), 1 μM antisense primer, nA10 (5'-GAGGTCCACCTTCTCCATCATGAGCTTC-3', corresponding to positions 1727 to 1754 of BDV strain V<sup>3</sup>), 250 μM dNTP mixture and 5U AmpliTaq Gold DNA polymerase (Perkin Elmer Corp., USA). The reaction mix-

ture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing at 60°C for 30 sec, primer extension at 72°C for 1 min and denaturation at 94°C for 1 min.

A method for detection of BDV was improved as described below. For synthesis of cDNA, non-tagged, reverse primer D10 (1 μM, 5'-GGAAACCATCCAGACAGCTC-3', corresponding to positions 1619 to 1638 of BDV strain V<sup>3</sup>) was used. PCR was performed with primer A10 (1 μM, 5'-CCTTCTCCATCATGAGCTTC-3', corresponding to positions 1727 to 1746 of BDV strain V<sup>3</sup>) and primer D10 (1 μM) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin and 250 μM dNTP mixture. PCR cycles consisted of preheating at 95°C for 12 min, denaturing at 94°C for 1 min, annealing at 55°C for 30 sec and primer extension at 72°C for 1 min for 70 cycles. PCR product was detected by ethidium bromide staining after separation by 3% agarose gel electrophoresis.

#### *RT-nested PCR*

We performed RT-nested PCR (Fig. 1B-3), which amplified p24 region, according to a method as described in ref.18 with a slight modification. Briefly, RNA was reversely transcribed with SuperScript II (200U) and primer D3 (1 μM, 5'-TCAGACCCAGACCAGCGAA-3', corresponding to positions 1443 to 1461 of BDV strain V<sup>3</sup>) at 37°C for 60 min after RNA denaturation at 70°C for 3 min. Enzyme was inactivated by boiling for 60 min. Amplification by PCR with 5U of TaKaRa Taq DNA polymerase (TaKaRa Shuzo Corp.) was performed with primer A2 (1 μM, 5'-AGCTGGGGATAAATGCGCG-3', corresponding to positions 1816 to 1834 of BDV strain V<sup>3</sup>) and primer D3 (1 μM) for the first round, and an internal primer pair, A10 and D10 (each 1 μM), was used for the second round. Each PCR cycle consisted of denaturing at 94°C for 1 min, annealing at 55°C for 45 sec and primer

extension at 72°C for 1 min. This reaction was performed for 35 cycles.

#### Southern blot analysis of PCR products

Oligonucleotide probe (5'-ATTGTCTCCAT CATTGTCTTCATGGAGCGATCTAGTATCT-3', corresponding to positions 1687 to 1726 of BDV strain V<sup>3</sup>), which is an internal region of the A10 and D10 primers (Fig. 1), was labeled with digoxigenin (DIG)-11-dUTP using terminal deoxynucleotidyl transferase at the 3'-end. Each PCR product was separated by 3% agarose gel electrophoresis and blotted onto a nylon membrane. The membrane was incubated at 55°C for 16 hrs with a hybridization buffer which consisted of 500 mM sodium-phosphate buffer, pH 7.2, 7% SDS, 1 mM EDTA and 2 pmole per ml of the DIG-labeled oligonucleotide probe. After hybridization, the membrane was washed at

55°C for 20 min with 40 mM sodium-phosphate buffer (pH 7.2) and 1% SDS. Hybridized probe was detected by DIG luminescent detection kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions.

#### Results

To detect BDV negative-stranded RNA, we applied a method, which was developed for detection of HCV RNA with strand specificity using tagged primer<sup>17</sup>. The p24 region of negative-stranded BDV RNA, which is known a remarkably stable sequence<sup>25</sup>, was detected by this method for more than 10 pg (Fig. 2). Our aim is to detect 10 copies of BDV genomic RNA. The sensitivity of the tagged primer method for HCV is, however, 100 copies as found in the examination of *in vitro* synthesized RNA<sup>17</sup>.

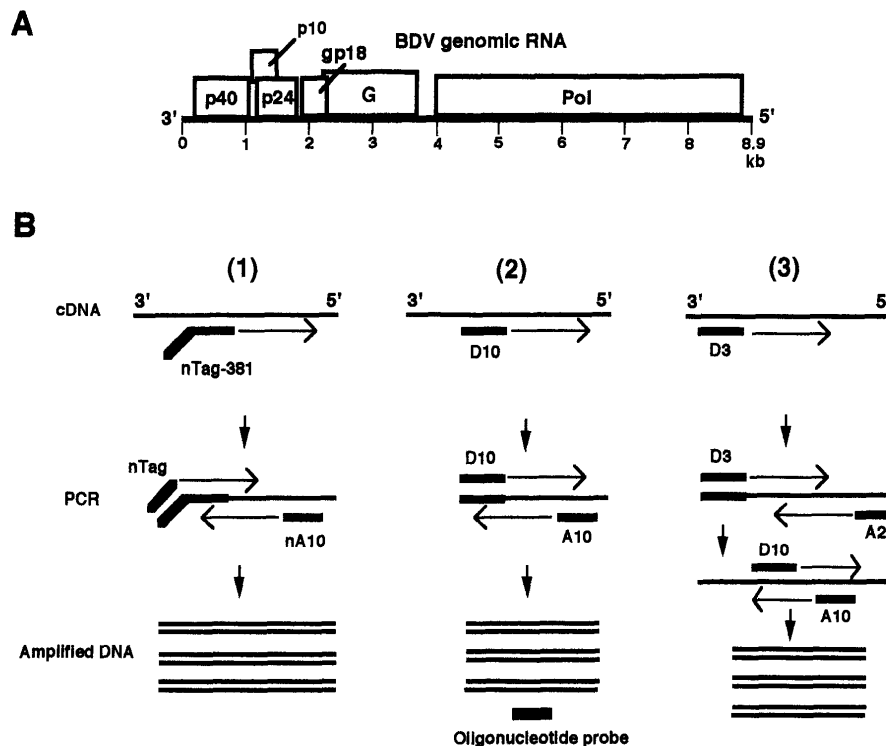


Fig. 1. Genomic organization of BDV (A) and schematic drawing depicting the design of the strand-specific RT-PCR assays and RT-nested PCR assays (B). (1) RT-PCR method developed for HCV detection using a tagged primer; (2) Single-tube RT-PCR method developed for BDV detection, not using tagged primer; (3) RT-nested PCR method. These methods are described in Materials and Methods in detail.

RT-nested PCR is thought to be the most sensitive method which can detect 10 copies of target RNAs. We performed RT-nested PCR to detect *in vitro* synthesized genomic stranded BDV RNA encoding the p24 region. Serially 10-fold dilutions of RNA were amplified. Signals were detected from the samples containing more than 10 copies of RNA in a reaction tube (Fig. 3).

To detect BDV genomic RNA with strand specificity, we developed an RT-PCR method (St RT-PCR) which was an improvement on the method for HCV without using the tagged primer as described in Materials and Methods and Fig. 1. We determined the end point of detection by St RT-PCR method using serially diluted *in vitro* synthesized RNA. The results showed that

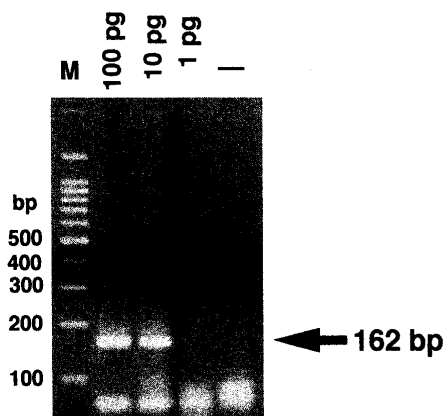


Fig. 2. Detection of BDV genomic RNA by a method which was developed for HCV in samples from MDCK/BDV cells. BDV genomic RNA was detected from the RNA samples equivalent to 10 pg. Lane M. 100 bp ladder DNA marker.

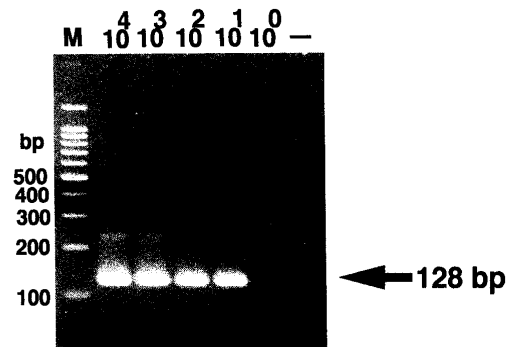


Fig. 3. Detection of *in vitro* synthesized BDV genomic stranded RNA by RT-nested PCR methods. BDV RNA which had been serially diluted 10 times was amplified. PCR products (128 bp) were detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis.

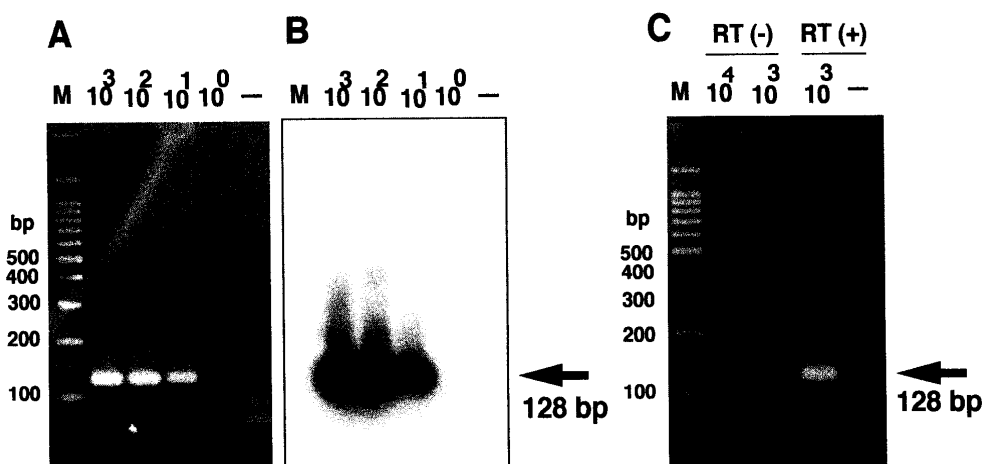


Fig. 4. Detection of *in vitro* synthesized BDV genomic stranded RNA by St RT-PCR method. After amplification of serially diluted RNA (A), PCR product was transferred onto a nylon membrane and hybridized with the DIG-labeled probe specific to BDV RNA (B). These RNA samples did not contain the plasmid DNA in that no signal was obtained from the samples without reverse transcriptase (C). RT; reverse transcriptase.

more than 10 copies of BDV RNA in a reaction tube could be detected (Fig. 4A). To confirm that the amplified 128 bp DNA fragment was specific to *in vitro* synthesized BDV RNA, Southern blot hybridization was performed with a DIG-labeled oligonucleotide specific to the internal region of primer A10 and D10 (Fig. 1). Fig. 4B showed that the 128 bp DNA fragment amplified by St RT-PCR was of BDV RNA. No signal was obtained from the samples containing 0.1 copy or less RNA (data not shown). Furthermore, BDV RNA was not detected in reactions without reverse transcriptase (Fig. 4C). These results suggested that St RT-PCR could detect BDV genomic RNA of more than 10 copies in a reaction tube. This sensitivity was equivalent to that of RT-nested PCR method (Fig. 3).

To determine the BDV genomic RNA copies per cell, we attempted to detect and to quantify BDV RNA in MDCK/BDV cells. RNA extracted from MDCK/BDV cells was subjected to St RT-PCR after being serially diluted. As shown in Fig. 5A, the p24 region of genomic BDV RNA was detected from the samples containing more than 1 pg RNA of the cells. This result indicated that the St RT-PCR method is more sensitive than the tag-used RT-PCR method (Fig. 2).

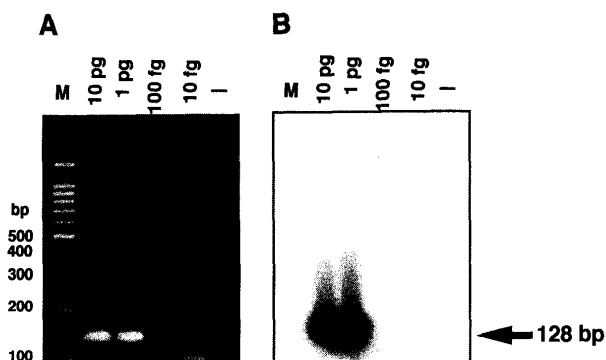


Fig. 5. Detection of BDV genomic RNA by St RT-PCR method in samples from MDCK/BDV cells. BDV genomic RNA was detected from the RNA samples equivalent to 1 pg (A) and hybridized with BDV RNA specific probe by Southern hybridization (B).

The amplified 128 bp DNA fragments were hybridized with the internal oligonucleotide probe as shown in Fig. 5B. The majority of MDCK/BDV cells were infected since viral antigens were detected in almost all the cells by immunofluorescence assay (data not shown and ref. 5). The amount of BDV genomic RNA contained in 1 pg of cellular RNA is equivalent to 10 copies. On the other hand, similar result was obtained from Fig. 2. Since the limit of detection of RNA by RT-PCR method using tagged primer is 100 copies<sup>17)</sup>, 10 pg of MDCK/BDV cells RNA contained 100 copies of negative-stranded BDV RNA (Fig. 2). We obtained nearly 10  $\mu$ g total RNA from about  $10^6$  cells using ISOGEN RNA extraction kit, indicating that one cell had approximately 10 pg total RNA containing 100 copies of BDV genomic RNA. From these results, we estimated the number of BDV genomic RNA copies per cell to be about one hundred.

To confirm that St RT-PCR method was useful for *in vivo* specimens, we applied the method to BDV-infected rats<sup>20)</sup>. As shown in Fig. 6, at least 1 ng of RNA was detected. This result indicates that the single-step RT-PCR method is useful for *in vivo* specimens.

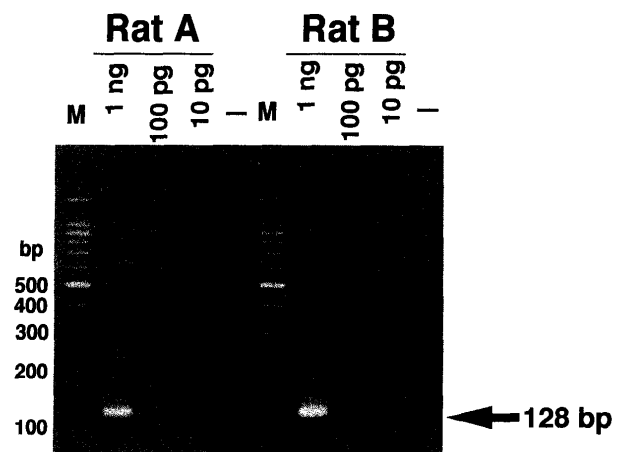


Fig. 6. Detection of BDV genomic RNA by St RT-PCR method in samples from BDV-infected rat's brain. BDV genomic RNA was detected from the RNA samples equivalent to 1 ng derived from two rats' brains.

## Discussion

Methods for detection of single-stranded RNA virus lack strand specificity and have a high level of PCR amplification which produces incorrect strands that are not easily avoided. In the case of poliovirus, detection of negative-stranded RNA is greatly complicated by the presence of positive-stranded RNA. To overcome this problem, a two-cycle RNase protection procedure that removes the excess positive strands at first cycle was developed<sup>19</sup>). In the case of HCV, since false priming of the incorrect strand occurred when there was a great excess of positive-stranded RNA for cDNA synthesis, PCR products were often obtained after reverse transcription in the absence of reverse primer. Although some groups reported strand-specific RT-PCR methods to prevent the false priming which used; chemical modification of RNA<sup>10</sup>), a tagged cDNA primer<sup>13</sup>) and thermostable reverse transcriptase<sup>13</sup>), detection of the incorrect strand was not easy. Using a tagged primer, we recently developed a novel RT-PCR method to detect negative-stranded HCV RNA with strand specificity<sup>17</sup>).

Essential points to develop an RT-PCR method for detection of single-stranded RNA virus (BDV and HCV) are assumed to be as follows. 1) Single-step PCR procedure with equivalent sensitivity to nested PCR is necessary to avoid contamination of DNA which often occurs during transfer of products from the 1st PCR to 2nd PCR solution in nested PCR. 2) Reverse transcription is necessary to have high specificity to target RNA for single-step PCR procedure. 3) RT-PCR should be reacted in a single tube with the hot start method. For the reasons mentioned above, we thought that both conditions of reverse transcription and PCR should be taken into account. We tested several enzymes for reverse transcription including SuperScript II. Because the SuperScript II has a

strong advantage in which cDNA can be synthesized at high temperatures (50–65°C), specific binding of reverse primer to target RNA was expected. The optimal working temperature was 55°C. Sixty minutes heat inactivation of reverse transcriptase was suitable. DNA polymerases including AmpliTaq Gold were tested. Although the hot start method is important for avoiding non-specific primer annealing and extension, opening the cover of tubes at high temperatures will permit contamination of DNA from the air. Because AmpliTaq Gold, which is inactive at room temperature, is activated in a pre-PCR heat step at 95°C, hot start PCR is possible. We tested pre-heating time from 0 to 12 min and obtained efficient results at 12 min.

Using St RT-PCR method developed in order to detect negative-stranded RNA of BDV in this study, we could detect more than 10 copies of synthetic BDV RNA. In the case of HCV and BDV, the detection limit of synthetic RNA by nested PCR method which is most sensitive among PCR methods, is 10 copies, indicating that St RT-PCR method has the same sensitivity as RT-nested PCR. By St RT-PCR method, we could detect more than 1 pg of total RNA from MDCK/BDV cells. Although a tagged primer for reverse transcription raises specificity to target sequence, sensitivity is decreased. Because a tagged primer is not used for the St RT-PCR method, the method is 10-fold more sensitive as compared with the method for HCV. In case of BDV, the tagged primer is not necessary for strand specific detection. When RT-nested PCR was performed using complementary primer against p24 mRNA at reverse transcription, BDV RNA was detected from 10 fg of total MDCK/BDV RNA (data not shown), indicating that the PCR product mainly originates from p24 mRNA. Thus, we can distinguish negative-stranded from positive-stranded BDV RNA using each method without a tagged primer. If large amounts of cellular RNA exist in the tube,



non-specific reactions to cellular RNA sometimes occur. As shown in Fig. 4B and 5B, all PCR products should be confirmed by Southern blot hybridization.

As shown in Fig. 6, we could detect BDV genomic RNA from the *in vivo* RNA samples using St RT-PCR method, indicating that this method is useful for analysing epidemiological samples. It is unknown how BDV is transmitted between horses or among animals and humans. Detection of BDV genomic RNA from the infected cells is an important key to examine the possibility of cell-free and/or cell-to-cell viral transmission. This St RT-PCR method will help attain this object.

#### Acknowledgements

We thank Drs. N. Kato (National Cancer Research Institute, Tokyo, Japan), K. Ikuta, M. Kishi and T. Nakaya (Immunological Science, Hokkaido University, Sapporo, Japan) for their useful advice. We also thank Drs. R. Rott and S. Herzog (Justus-Liebig-Universitat Giessen, Giessen, Germany) for providing MDCK cells persistently infected with BDV.

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