

## Reverse Transcription-Nested Polymerase Chain Reaction for Detecting p40 RNA of Borna Disease Virus, without Risk of Plasmid Contamination

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**ABSTRACT.** Several methods for the detection of Borna disease virus (BDV) RNA have been reported, one being the reverse transcription-nested polymerase chain reaction (RT-nested PCR) method. However, due to the possibility of contamination of the cloned DNA in a reaction tube, false-positive results might be obtained by RT-nested PCR. To detect only BDV RNA without anxiety of contamination, we developed an RT-nested PCR system using "mRNA selective PCR kit". Using this system, cDNA of BDV p40 in the plasmid (up to  $5 \times 10^7$  molecules) was not amplified. BDV specific sequence was amplified from total RNA (more than 50 pg) of MDCK/BDV cells, which were persistently infected with BDV. These results indicate that this mRNA selective RT-nested PCR system can specifically amplify target RNA as distinguished from plasmid contaminated.—**KEY WORDS:** Borna disease virus, mRNA selective PCR, plasmid contamination.

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Borna disease virus (BDV) causes central nervous system disease in several vertebrate species and is manifested by behavioral abnormalities and diverse pathology [5, 9, 14]. BDV contains a nonsegment negative-sense 8.9 kilobase, single-stranded RNA genome, containing at least six open reading frame encoding proteins of 40 kDa (p40), 23 kDa (p24), 10 kDa (p10), 16 kDa (gp18), 57 kDa (G) and 190 kDa (pol) (Fig. 1A) [2, 4, 16–18]. The p40 protein, which is a candidate for nucleocapsid protein, is detected in high concentrations in the brain of BDV-infected animals and cultured cells [1, 13], suggesting that p40 encoding mRNA is suitable as a viral mRNA target for diagnostic detection *in vivo* and *in vitro*.

RT-nested PCR is performed to demonstrate BDV infection *in vivo* and *in vitro* at many laboratories [6, 8, 11, 12, 15]. Because RT-nested PCR is thought to be the most sensitive method for the detection of RNA, contamination of cloned DNAs originated from BDV sequences results in false positives. It is thought that contamination of DNA in a reaction tube originates from cloned aerosoled DNA, which is amplified in transformed bacteria and extracted in the laboratory. The other possibility of contamination is incorrect handling while transferring the first PCR product to the second PCR solution. The latter case can be solved by using a filtered tip. However, it is very difficult to solve the former case unless the two experiments, cloning of cDNA and RT-nested PCR, are performed in physically separated laboratories. In our laboratory, some plasmids containing cDNA of BDV p40 were transformed into bacteria and high amounts of plasmids were extracted from bacteria in routine work. Therefore, we had to develop an RT-nested PCR method which can amplify only target BDV RNA, even if plasmid DNA is mixed in the reaction tube.

In this study, we used "mRNA selective PCR kit" (TaKaRa Shuzo Co., Ltd.) that can only detect target mRNA as distinguished from genomic DNA of host cells, by using dNTP analogs. The dNTP/analog are incorporated into cDNA formed with mRNA as a template at the reverse

transcription step. The cDNA/mRNA hybrid is denatured at about 85°C, but genomic DNA is not. The dNTP/analog incorporated into cDNA is selectively amplified at the next PCR step. Using this system, there is the possibility that only p40 RNA of BDV is detected, even if there is contamination by plasmid DNA. The one-step RT-PCR system is used in this kit, but the sensitivity is very low (data not shown). Therefore, we performed nested PCR in the presence of dNTP/analog. Based upon our findings, we obtained the appropriate conditions as follows (Fig. 1C). Primers used in this study are slightly modified from those of ref 19. RNA and/or DNA solution was added to the reaction mixture containing 1 × buffer I, 5 mM MgCl<sub>2</sub>, 1 mM dNTP/analog mixture, 4U RNase inhibitor, 5U AMV RTase XL, 5U AMV-OptimizedTaq, 0.4 μM sense primer, A1 [5'-GTCACGGCGCGATATGTTTC-3', corresponding to positions 242 to 261 of BDV strain V (2)] and 0.4 μM antisense primer, D1 [5'-CTTCTTACTCCAGTAAAACGC-3', corresponding to positions 969 to 989 of BDV strain V (2)]. The constituents of buffer I and dNTP/analog mixture are not known in detail because TaKaRa Corp. Ltd. is applying for a patent. The reaction mixture was incubated at 42°C for 30 min to perform reverse transcription, followed by 30 cycles of amplification. The PCR cycle consisted of denaturation at 80°C for 30 sec, annealing at 50°C for 30 sec and primer extension at 72°C for 1 min. For the second round, 1 μl of first PCR product was added to the reaction mixture containing 1 × buffer I, 5 mM MgCl<sub>2</sub>, 1 mM dNTP/analog mixture, 5U AMV-OptimizedTaq and each 0.4 μM primer D2 [5'-GCCTTGTGTTTCTATGTTTG-3', corresponding to positions 277 to 296 of BDV strain V (2)] and primer A2 [5'-ATTCTTTACCTGGGGACTCA-3', corresponding to positions 698 to 717 of BDV strain V (2)]. Conditions of amplification were the same as the first.

In this study, plasmid pCRII-p40 was kindly provided by Dr. Nakaya (Institute of Immunological Science, Hokkaido University) (Fig. 1B and ref. 10). The pCRII-p40 was generated by insertion of the full-length cDNA of p40 into

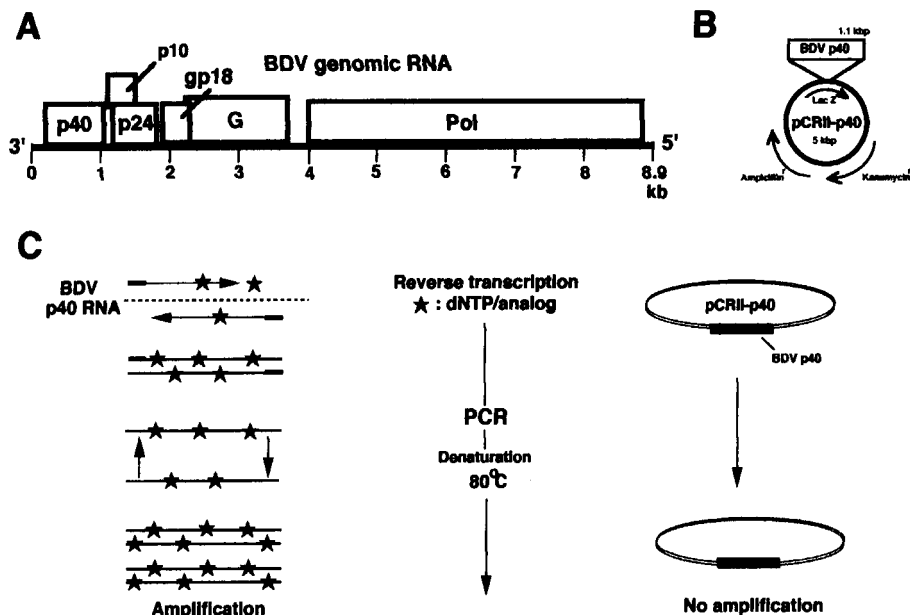


Fig. 1. (A) Genomic organization of BDV, (B) construction of pCRII-p40 and (C) schematic drawing depicting the design of the RT-nested PCR assay.

plasmid pCR2.1 (Invitrogen, USA) using TA cloning technique. We obtained purified pCRII-p40 using FlexiPrep Kit (Pharmacia Biotech, USA). After the serially dilution of the pCRII-p40 solution from  $5 \times 10^7$  to  $5 \times 10^1$  molecules, we performed selective RT-nested PCR method and the p40 cDNA in the plasmid DNA was not amplified at either the first or second round of PCR (data not shown). To confirm whether the primer sets in this study were suitable for the amplification of pCRII-p40, standard nested PCR was performed. As shown in Fig. 2, we were able to detect at least 50 molecules of the plasmid. These results indicate that cloned p40 DNA was not amplified by this system, even if contamination occurred in a reaction tube.

RNA extracted from persistently BDV-infected Madin-Darby canine kidney (MDCK/BDV) cells [7] using RNA extraction kit (ISOGEN, Nippon Gene Co., Ltd.) was serially diluted and applied to this system. As shown in Fig. 3, amplified product was detectable from 50 pg of MDCK/BDV RNA. To confirm whether only p40 RNA can be detected in the presence of plasmid DNA, 500 pg of MDCK/BDV RNA was assayed in a reaction tube containing  $5 \times 10^6$  molecules of plasmid pCRII-p40. As shown in Fig. 4, bands in the length of approximately 440 bp were detected in both samples containing MDCK/BDV RNA with and without pCRII-p40. However, we were not able to detect pCRII-p40 in the absence of MDCK/BDV RNA. This result indicated that only RNA was amplified in the solution containing RNA and plasmid DNA.

This system lacks strand specificity due to the presence of reverse and forward primers at the reverse transcription step. Since there is a high amount of p40-mRNA in BDV-infected cultured cells [3], PCR products might originate

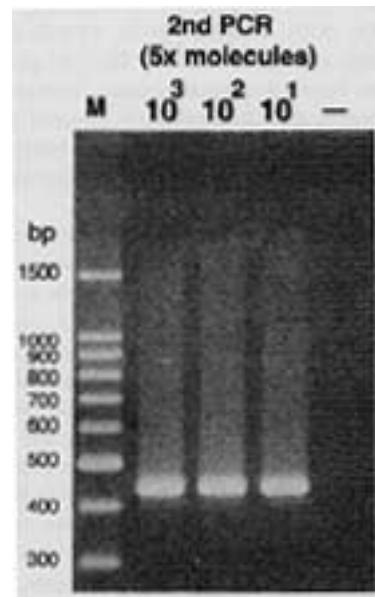


Fig. 2. Detection of plasmid DNA by nested PCR. Serially diluted plasmid DNA solution was added to the reaction mixture containing  $1 \times$  PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM  $MgCl_2$ ], each 0.4  $\mu$ M primers A1 and D1, 200  $\mu$ M dNTP mixture and 3U *Taq* DNA polymerase (TaKaRa Shuzo Co. Ltd.). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and primer extension at 72°C for 1 min. This reaction was performed for 35 cycles. We used D2 and A2 primers as an internal primer pair for the second round. Conditions for the second PCR are the same as the first. PCR product was detected by ethidium bromide staining after separation by 3% agarose gel electrophoresis.

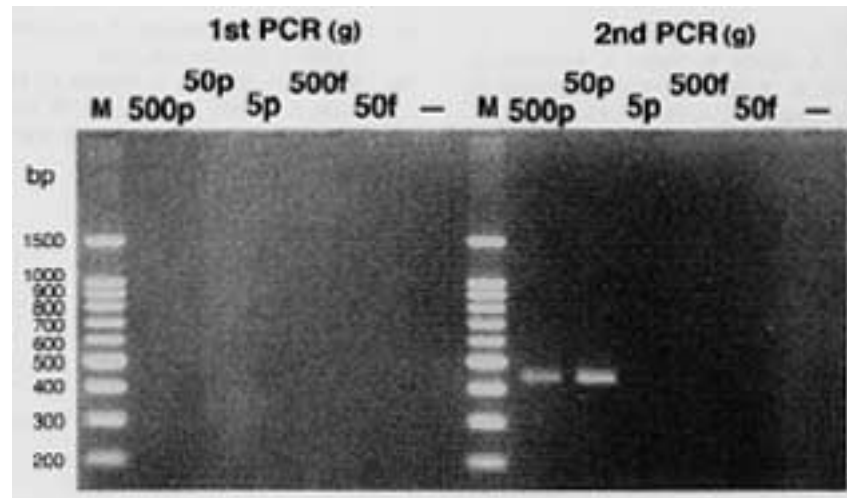


Fig. 3. Detection of BDV RNA by p40-RNA selective RT-nested PCR. BDV RNA extracted from MDCK/BDV cells was serially diluted 10-fold and amplified.

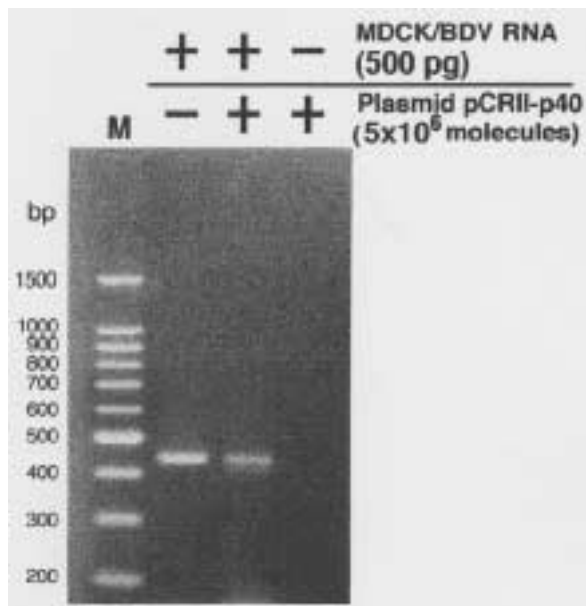


Fig. 4. Detection of BDV RNA distinguished from plasmid DNA. BDV RNA (500 pg) was amplified using p40-RNA selective RT-nested PCR in the presence or absence of  $5 \times 10^6$  plasmid DNA.

primarily from the mRNA of p40. This method is anticipated to gain acceptance as a powerful means of detecting BDV mRNA without risk of contamination in the future epidemiological study of BDV.

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providing MDCK cells persistently infected with BDV.

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