Detection of Bovine Viral Diarrhea Virus (BVDV) Using Reverse Transcription Polymerase Chain Reaction Assay

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ABSTRACT. The reverse transcription polymerase chain reaction (RT-PCR) was applied to detect bovine viral diarrhea virus (BVDV) for the rapid diagnosis. The primers were selected from the p80 region of BVDV gene. The RT-PCR assay detected all of the 17 BVDV strains tested including cytopathogenic and non-cytopathogenic strains, while specific amplification was not observed from 17 bovine viruses other than BVDV. Detection limit of the assay was $10^{1.5}$ TCID₅₀/ml. Sera and organ samples were collected from four field bovine viral diarrhea-mucosal disease (BVD-MD) cases; mucosal disease, abortion, diarrhea and persistent infection. The RT-PCR assay detected BVDV from those samples more than conventional virus isolation method. — **KEY WORDS:** BVD-MD, diagnosis, RT-PCR.

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Bovine viral diarrhea-mucosal disease (BVD-MD) is caused by bovine viral diarrhea virus (BVDV) resulting in economic losses in domestic cattle herds. BVDV isolates are separated into two biotypes, cytopathogenic (cp) or non-cytopathogenic (ncp), based on cytopathic effect in cell culture. Infection of pregnant cow with a ncp BVDV strain during the first four months of gestation may result in a persistently infected (PI) calf [13]. PI cattle are a main source of virus spread. Therefore detection of PI cattle is important for control of BVD-MD [6]. Traditionally, virus isolation with confirmation by immunofluorescence has been the method used to detect BVDV in clinical samples. These techniques are time consuming and requires the use of cell culture. More than 10 reports have described the reverse transcription polymerase chain reaction (RT-PCR) technique which detects BVDV and some of them tried to apply the technique for diagnosis of BVDV [7, 9, 10, 12, 18]. In this article, we described the RT-PCR assay we developed to detect BVDV and showed its potential as a diagnosis tool for BVD-MD.

The published genome sequences of the three BVDV strains NADL [3], Osloss [16] and SD-1 [4] were used to search the highly conserved region of p80 coding region using the computer analysis program PC/GENE (IntelliGenetics). The primer sequences were selected from such the regions to amplify a 299 bp fragment. According to the NADL sequence, the 299 bp fragment is digested to 160 bp and 139 bp fragments by a restriction enzyme Hinf I, and to 249 bp and 50 bp fragments by a restriction enzyme Hap II. Their sequences (5'-3') are GAGATGGTCAAGAAGATAAC (sense) and TTTGGT TGAGGCATTTGGCA (antisense). Their position in NADL strain is 6044–6063 and 6323–6342, respectively.

RT-PCR was performed as follows. Each reverse transcription (RT) reaction mixture contained the following: 2.5 μ M antisense primer, 20 units of RNase inhibitor (Perkin Elmer), 50 units of murine leukemia virus reverse transcriptase (Perkin Elmer), 2.5 mM MgCl₂, 1 mM of each deoxyribonucleoside triphosphates (dATP, dGTP, dTTP,

dCTP; Perkin Elmer), 1 $\times$ PCR buffer (10 $\times$ PCR buffer contains 500 mM KCl, 100 mM Tris-HCl [pH 8.3]; Perkin Elmer), and 2 μ l of extracted RNA in a total volume of 20 μ l. The reaction was overlaid with 30 μ l of mineral oil (Sigma) and incubated at 42°C for 30 min, at 99°C for 5 min and at 5°C for 2 min sequentially in a thermal cycler (Perkin Elmer). After the RT reaction, 50 pmol sense primer, 4 μ l of 25 mM MgCl₂, 2.5 units of Taq DNA polymerase (Amplitaq Gold; Perkin Elmer), 8 μ l of the 10 $\times$ PCR buffer and sterile water was added to the RT reaction mixture to a total volume of 100 μ l. The reaction was initially denatured at 94°C for 2 min, then amplification was performed for 50 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. An additional incubation was done at 72°C for 7 min after the last cycle. After the RT-PCR, ten μ l of the reaction mixture was run in 3% agarose gel (NuSieve 3:1; FMC Bioproducts) in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA) at 100 V for 30 min. The gels were stained by ethidium bromide (0.5 μ g/ml) for 10 min to visualize DNA bands under UV light.

For virus titration and RNA extraction, about 30 mg of organ material samples was minced into small pieces on ice using sterile surgical scissors and then homogenized in a glass tissue grinder with 300 μ l Dulbecco's Modified Eagle's Medium (DMEM). Homogenates were stored at -80°C.

Titer of infectious BVDV was determined using microtitration plates and fetal calf muscle (FCM) cells. For ncp BVDV samples, serial dilutions of virus were inoculated onto FCM cells and then infected with a cp BVDV strain TK87-1 (+). End point was determined by observing inhibition of cytopathic effect.

For RNA extraction from samples, RNA extraction reagent ISOGEN (Nippon Gene) was used according to manufacturer's protocol which was based on the guanidinium thiocyanate method [2]. RNA was extracted directly from 100 μ l of cell culture fluid, homogenized organ mixture or serum and dissolved in 25 μ l of diethylpyrocarbonate-treated sterile water, and stored at

- 20°C.

To study the detection ability of the RT-PCR assay, seventeen BVDV strains as follows were used. KS86-1 (+) [17], TK87-1 (+), IS87-1 (+), AB (+), KS86-1 (-) [17], TK87-1 (-), IS87-1 (-), and AB (-), of which the first 4 strains were cp and the rest were ncp, were provided by Dr. M. Shimizu (National Institute of Animal Health, Japan). Tokachi20 [8], Nose [11], No. 12 [14] and Oregon, of which the first 2 strains were cp and the rest were ncp, were obtained from the Japanese Association of Veterinary Biologics (JAVB). Strains 3-44CP, Kaibou, Ikusei-1, Ikusei-3 and Ikusei-9, of which the first one was cp and the rest were ncp, were isolated from BVD-MD clinical cases. The titers of these viruses were equal or more than $10^{4.5}$ TCID₅₀/ml.

Seventeen bovine viruses other than BVDV and a hog cholera virus (HCV) were used to study the specificity of the RT-PCR assay: bovine rotavirus strain KK3 was provided by Dr. Y. Murakami (National Institute of Animal Health, Japan). Bovine parainfluenza 3 virus strain BN-1, bovine respiratory syncytial virus strain NM, bovine enterovirus strain 2122, bovine rhinovirus 1 strain M-17, bovine rhinovirus 2 strain EC11, bovine rhinovirus 3 strain H-1, bovine rotavirus strain Lincoln, bovine corona virus strain Kakegawa, bovine reovirus strain Lung and strain Abney, bovine adenovirus 1 strain No. 10, and bovine parvovirus 1 strain K-17 were obtained from JAVB. Ibaraki virus strain No. 12 and infectious bovine rhinotracheitis virus strain 758-43 were obtained from commercial vaccine (Nippon Institute for Biological Science and the Synthetic Feed Laboratory Co., Ltd, respectively). Akabane virus strain L+513 and HCV vaccine strain GPE⁻ was obtained from the Synthetic Feed Laboratory Co., Ltd. The titers of these viruses were equal or more than $10^{3.0}$ TCID₅₀/ml.

Our RT-PCR assay amplified an expected fragment from all the strains of BVDV tested and from the HCV, while it didn't amplify the fragment from the 17 bovine viruses at all. Figure 1 showed the data of 12 strains of the BVDVs, and other data were not shown.

To confirm the fidelity of amplification, PCR products of TK87-1 (+) were digested by Hinf I or Hap II according to manufacturer's protocol (Takara Biochemicals). Each digestion yielded the two fragments of expected sizes for each restriction enzyme.

To study the detection limit of the RT-PCR assay, BVDV strain TK87-1 (+) ($10^{5.5}$ TCID₅₀/ml) were diluted ten-fold serially with sterile water and each dilute were used for the assay. Virus was detected from the dilutes of $10^{1.5}$ TCID₅₀/ml and over.

Efficacy of the RT-PCR assay was examined using various field samples. Forty field samples (sera and organs) were collected from four clinical cases that BVD-MDV was suspected of causing. The samples were examined by virus isolation and the RT-PCR assay. The first case was a cattle, which showed the clinical signs of mucosal disease. The second case was a cattle with heavy diarrhea. The third case was a persistently infected calf, which was obtained

Fig. 1. RT-PCR products of BVDV strains electrophoresed on agarose gel. Lane a, and o, Hinf I fragments of $\phi \times 174$; lane b, KS86-1 (+); lane c, KS86-1 (-); lane d, TK87-1 (+); lane e, TK87-1 (-); lane g, IS87-1 (-); lane h, AB (+); lane i, AB (-); lane j, Tokachi 20; lane k, Nose; lane l, No. 12; lane m, Oregon; lane n, negative control.

from a beef cattle breeder at three month-old. The calf was kept in a isolated cattle pen and sera were collected 9 times for 8 months. The last case was two aborted fetal calves. Their mother cattle showed viremia. In the 1st and 2nd cases cattle were euthanized and samples were collected. As shown in Table 1, BVDV were detected from all the samples of the four clinical cases by the RT-PCR assay. On the other hand, the virus was not isolated from the samples of the aborted fetal calves.

Because of high mutation frequencies among pestivirus isolates [5], the selection of appropriate primers is important to detect a broad range of BVDV strains. Therefore, in this study a set of primers was chosen in the highly conserved region among the p80 coding region of the BVDV strain NADL, Osloss and SD-1, because p80 is encoded by one of the most conserved pestivirus sequence and shows the highest degree of homology between NADL and Osloss [4]. The primer set amplified cDNA derived from RNA of all BVDV isolates tested including both cp and ncp viruses. Those results proved that the strategy of the primer design was reasonable. Recently, two antigenically and genomically distinct subgroups of BVDV, type 1 and type 2, have been reported [15]. Among the 17 BVDV strains which we tested, Oregon had been found to be in Type 1 group [15]. As any apparent type 2 virus was not used in this article, further studies with the type 2 virus will help us to evaluate the primers furthermore. A primer set is required high specificity because PCR tends to amplify non-specific DNA from contaminated cells or other microorganisms in sample specimen. Using 17 bovine viruses other than BVDV, our primer set didn't amplify non-specific DNA fragments of similar size to the specific DNA fragments. The primer set amplified the DNA fragments from HCV strain GPE⁻ and the fragments was the same size as those of BVDV. This result suggested that the primer sequences were also conserved in the HCV p80 coding region. This character doesn't seem to be a defect for diagnosis of BVD-MD because differentiation of BVDV and HCV is not required in general.

Table 1. Detection of BVDV (genome) from field samples

Case No.	Source	Symptoms of the source	Specimen	Virus isolation ^{a)}	PCR results ^{b)}
1	Cattle	Mucosal disease	Serum	2.0	NT
			Heart	3.0	+
			Lung	2.5	+
			Liver	2.0	+
			Kidney	2.5	+
			Spleen	2.0	+
			Cecal lymph node	1.5	+
2	Cattle	Diarrhea	Brain	1.5	+
			Lung	4.0	+
			Heart	4.0	+
			Liver	4.0	+
			Spleen	4.5	+
			Kidney	2.0	+
			Duodenum	4.0	+
			Cecum	3.5	+
			Colon	4.5	+
			Mandibular lymph node	4.0	+
			Superficial inguinal lymph node	3.5	+
			Mesenteric lymph node	3.5	+
			Serum	2.5	+
3	Calf	Persistently infection	Serum 1	5.55	+
			Serum 2 (3 weeks) ^{c)}	4.30	+
			Serum 3 (1 month)	4.30	+
			Serum 4 (2 months)	5.05	+
			Serum 5 (3 months)	5.80	+
			Serum 6 (4 months)	3.80	+
			Serum 7 (5 months)	4.30	+
			Serum 8 (6 months)	4.80	+
			Serum 9 (10 months)	4.30	+
4	Fetal calf (male)	Aborted	Heart	—	+
			Lung	—	+
			Liver	—	+
			Spleen	—	+
			Ascites	—	+
	Fetal calf (female)	Aborted	Heart	—	+
			Lung	—	+
			Liver	—	+
			Spleen	—	+
			Ascites	—	+
	Dam		Serum	2.0	+

a) Each number means virus titer of samples (log TCID₅₀/ml). — = virus was not isolated. NT=not tested.

b) + = detected, — = not detected.

c) Time of collection after the serum 1 was collected.

Our RT-PCR assay was able to detect BVDV from 10^{1.5} TCID₅₀/ml virus fluid. The amount of virus RNA applied to the RT-PCR was equivalent to 0.25 TCID₅₀ calculated from the viral RNA extraction and concentration ratio. This result agreed with the former reports [7, 9, 10, 12]. Because the virus titer of the field samples are usually higher than 10³ TCID₅₀ [17], our RT-PCR assay appears to be sensitive enough to diagnose BVD-MD.

We detected BVDV from all the samples of diarrhea, mucosal disease, abortion and persistent infection cases

using the RT-PCR assay. In contrast to it, the 10 specimens of aborted fetal calves were negative for virus isolation. Because of the negative results, we couldn't decide that the abortion was caused by BVD-MDV. By nature the RT-PCR assay can detect the inactivated virus, therefore the assay appeared to detect the virus from the samples from which virus was not isolated. This property of the RT-PCR assay seems to be a great advantage for diagnosis of BVDV because it is not always easy to take specimens fresh enough for virus isolation. Rapid method is another advantage of

the RT-PCR assay. The viral RNA was extracted directly from serum or organ homogenates, therefore the RT-PCR assay required only one day. High frequency of BVDV contamination into commercially supplied FCS has been a serious problem [1]. In this study, nineteen lots of fetal calf sera for cell culture were obtained from 6 different commercial suppliers and BVDV was detected from 17 lots from 6 commercial suppliers by the RT-PCR assay (data not shown). These results showed the efficacy of the RT-PCR assay for screening BVDV contamination in FCS.

We developed the RT-PCR assay for diagnosis of BVD-MD and demonstrated that the assay had enough sensitivity and specificity for detection of BVDV from various samples. The RT-PCR assay seems to have a potential to help cattle to be free from BVDV.

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