NOTE *Virology*

Rapid and Sensitive Detection of Bovine Coronavirus and Group A Bovine Rotavirus from Fecal Samples by Using One-Step Duplex RT-PCR Assay

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ABSTRACT. Bovine coronavirus (BCoV) and group A bovine rotavirus (BRV) are two of major causes for neonatal calf diarrhea. In the present study, a one-step duplex RT-PCR was established to detect and differentiate BCoV and group A BRV from fecal samples. The sensitivity of this method for BCoV and group A BRV was 10 PFU/100 μl and 1 PFU/100 μl, respectively. Twenty-eight diarrhea fecal samples were detected with this method, the result showed that 2 samples were identified as co-infected with BCoV and group A BRV, 26 samples were group A BRV positive, and 2 samples were negative. It proved that this method is sensitive for clinical fecal samples and is worth applying to laboratory diagnosis for BCoV and group A BRV.

KEY WORDS: BCoV, detection, differentiation, group A BRV, one-step duplex RT-PCR.

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Neonatal calf diarrhea (NCD) is a common disease affecting the newborn calf worldwide, threatening the cattle production along with significant morbidity and mortality and inducing severe economic losses. There are numerous infectious causes for NCD, and bovine coronavirus (BCoV) and group A bovine rotavirus (BRV) are proved to be two major viral pathogens [2]. BCoV is a single-stranded, non-segmented RNA virus and belongs to the order *Nidovirales*, *Coronaviridae* family [7, 9]. BRV is a non-enveloped virus with segmented double-stranded RNA genome. Rotaviruses are classified into 7 groups (A-G) [3] and group A rotavirus is the major cause of bovine rotaviral diarrhea [3, 14]. Due to the similar signs and symptoms, it is difficult to distinguish the infection of BCoV and group A BRV according to the clinical observation.

Comparing with standard diagnostic procedures such as viral culture or serology, reverse transcription polymerase chain reaction (RT-PCR) has been proved to be extremely useful for diagnostic investigation in the detection of pathogens, especially when the detection method is time consuming, expensive or unavailable. Multiplex RT-PCR is a modification of the basic RT-PCR method, in which pairs of primers are used in the same reaction [4, 8, 12, 16]. For one-step multiplex RT-PCR, it can hold the reverse transcription from RNA to cDNA and subsequent amplification in a single tube, reducing the reaction period and reaction mixtures, and also avoiding the risk of contamination. In this study, a one-step duplex RT-PCR was established to detect and differentiate BCoV and group A BRV from fecal samples.

Based on the deposited genome sequences of BCoV and group A BRV in GenBank, two pairs of specific primer were designed according to the highly conserved regions. Primers of BCoVF (5'-CGATCAGTCCGACCAATCTA-3') and BCoVR (5'-GAGGTAGGGGTTCTGTTGCC-3') locate in N gene of BCoV and the predict product is 597 bp. Meanwhile, BRVF (5'-ATGGGTACGATGTGGCTCAA-3') and BRVR (5'-ACCGCTGGTGTCATGTTTGG-3') locate in VP6 of group A BRV and the length of predict product is 383 bp.

For virus titration and RNA extraction, BCoV Kakegawa strain [1] was titrated with plaque forming assay (PFA) on HRT-18 cells as describe previously [18]. BRV 6505 strain was isolated from group A BRV positive sample with MA-104 cells in our lab and titrated with PFA [13]. RNA was extracted with QIAamp Viral RNA Mini Kit (QIAGEN, Germantown, MD, U.S.A.) according to the manufacture's introduction. Briefly, 100 μl of virus-infected cell culture fluids was added to 560 μl of lysis buffer, followed by the addition of 560 μl of 99.5% ethanol. Each mixture was applied to a QIAamp Mini spin column. Purified RNA was then eluted in 50 μl of RNase-free water. The extracted RNA was used immediately or stored at –80°C until needed.

One-step RT-PCR was performed with Takara One Step RNA PCR Kit (AMV) (TAKARA, Otsu, Japan). Fifty microliters of reaction mixture was prepared as follows: 5 μl of 10× One Step RNA PCR Buffer, 10 μl of 25 mM $MgCl_2$, 5 μl of 10 mM dNTP, 1 μl of RNase Inhibitor (40 U/μl), 1 μl of AMV RTase XL (5 U/μl), 1 μl of AMV-Optimized Taq (5 U/μl), 1 μl of each primer: BCoVF (20 μM), BCoVR (20 μM), BRVF (20 μM), BRVR (20 μM), and 18 μl of RNase Free water were mixed, and 5 μl of extracted RNA described above was added resulting in a final volume of 50 μl reaction. Optimal annealing temperature was determined with 11 different temperatures from 50 to 60°C as following protocol: 30 min at 50°C for reverse transcription; primary denaturation for 5 min at 94°C; 35 cycles of 50 sec at 94°C, 50 sec at gradient annealing temperatures from 50 to 60°C,

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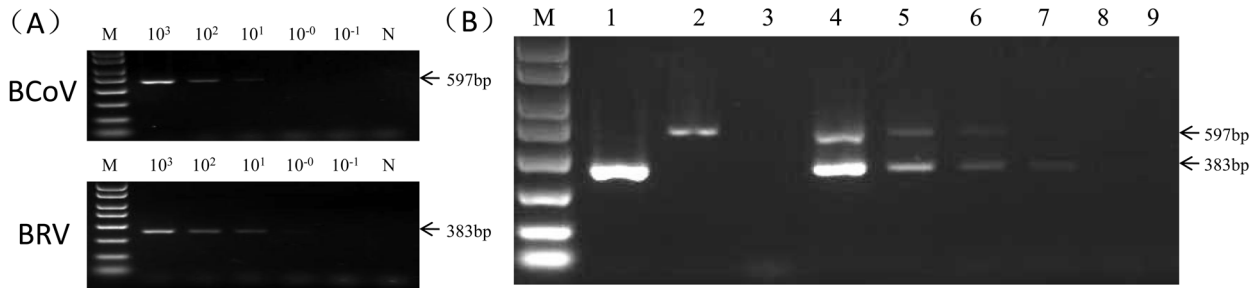

Fig. 1. Sensitivity of one-step RT-PCR for BCoV and BRV (A) and for a mixture of BCoV and BRV (B). (A) Reaction performed in 10-fold serial dilution (from 10^3 to 10^{-1} PFU/100 μl), N: negative control, (B) Lane M: PCR Marker, 50–2,000 bp. Lane 1: BRV positive control; Lane 2: BCoV positive control; Lane 3: negative control; Lane 4–9: reactions performed in 10-fold serial dilution of BRV6505 strain and BCoV Kakegawa strain in negative fecal samples (from 10^3 to 10^{-2} PFU/100 μl).

Table 1. The detection result of pathogens in fecal samples

SN[1]	Breed[2]	Age (days)	Sex[3]	Fecal trait character	*E.coli*						Sal[4]	CP[5]	AdV[6]	CPSD[7]	*Coccidia*[8]			BRV (Kit)	This study	
					stx1	stx2	eaeA	K99	LT	ST					E.b.	E.z.	E.e.		BRV	BCoV
1	U	10	F	yellowish-white soft	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
2	JB	8	F	yellowish-white watery	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
3	U	7	F	yellowish-white sticky	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
4	F1	7	F	yellowish solid	–	–	+	–	–	–	–	–	–	–	–	–	–	+	+	–
5	F1	9	M	yellowish-white watery	–	–	–	–	–	–	–	–	–	+++	–	–	–	+	+	–
6	JB	8	M	yellowish watery	–	–	–	–	–	+	–	–	–	∞	–	–	–	+	+	–
7	F1	10	M	yellowish watery	–	–	–	–	–	–	–	–	–	∞	–	–	–	+	+	+
8	F1	8	F	yellowish-white watery	–	–	–	–	–	–	–	–	–	+++	–	–	–	+	+	–
9	F1	25	M	yellowish-white sticky	–	–	+	–	–	+	–	–	–	–	–	–	–	+	+	–
10	ETJB	7	M	yellowish lutose	–	–	–	–	–	+	–	+	–	–	–	–	–	+	+	–
11	F1	10	M	pale yellowish lutose	–	–	–	–	–	–	–	–	–	∞	–	–	–	+	+	+
12	F1	6	M	yellowish-white watery	–	–	–	–	–	–	–	–	–	+++	–	–	–	+	+	–
13	ETJB	5	F	yellowish watery	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
14	F1	5	M	pale yellowish sticky	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
15	ETJB	15	M	oyster white sticky	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
16	HOL	7	F	pale yellowish watery	–	–	–	–	–	+	–	–	–	+++	–	–	–	+	+	–
17	ETJB	12	M	brown lutose	–	+	+	–	–	+	–	–	–	–	–	–	–	–	+	–
18	JB	11	F	yellowish-white lutose	–	–	–	–	–	+	–	–	–	++	–	–	–	+	+	–
19	F1	10	F	flavo-green watery	–	–	–	–	–	+	–	+	–	++	–	–	–	–	+	–
20	F1	10	M	yellowish-white watery	+	–	–	–	–	+	–	–	–	+++	–	–	–	+	+	–
21	F1	9	M	yellowish-white mucoid	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–
22	ETJB	5	F	green lutose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
23	F1	7	F	greenish-brown sticky	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	F1	6	M	brown sticky	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	ETJB	8	M	yellowish-brown lutose	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
26	JB	14	M	yellowish-white watery	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
27	F1	17	F	yellowish-white watery	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
28	U	7	F	yellowish watery	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–

1) SN: serial number. 2) JB: Japanese black cattle. F1: cross bred F1 generation of Japanese black and Holstein. ETJB: fertilized eggs transplanted Japanese black cattle. HOL: Holstein. U: unknown. 3) M: male; F: female. 4) Sal: *Salmonella*. 5) CP: *Clostridium perfringens*. 6) AdV: Adenovirus. 7) CPSD: *Cryptosporidium*. "–"<"+"≤"10^3"<"++"≤"10^4"<"+++" ≤"10^5"<"∞" (OPG). 8) E.b.: Eimeria bovis; E.z.: Eimeria zuernii; E.e.: Eimeria ellipsoidalis.

and 1 min at 72°C; and final elongation of 10 min at 72°C. PCR products were electrophoresed on 1.5% agarose gel containing 1 × GelRed (BIOTIUM, Hayward, CA, U.S.A.) and then analyzed with the software CS Analyzer Ver 3.0 (ATTO, Tokyo, Japan). The result showed that the optimal annealing temperature is 55°C. Meanwhile, the RT-PCR products were sequenced and analyzed. The result showed that they are BCoV and group A BRV sequences respectively, illustrating that this method successfully amplified the target segments from BCoV and group A BRV.

To examine the analytical sensitivity, BCoV Kakegawa strain and BRV 6505 strain were serially diluted 10-fold in bovine fecal samples [supernatant of 20% v/v fecal suspension in phosphate-buffered saline (PBS), free from BCoV and group A BRV] from 10^3 to 10^{-2} PFU/100 μl. One hundred microliters of the samples were subjected to RNA extraction. One-step RT-PCRs for single virus and both virus were carried out with optimized protocol described above. As can be seen from Fig.1, the detection limits of

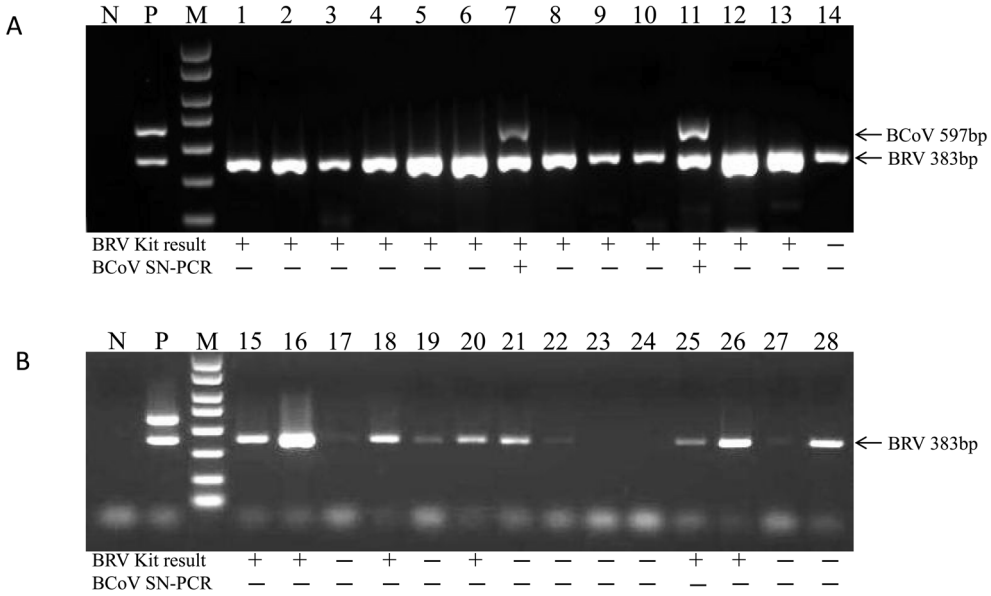

Fig. 2. Visualization by gel electrophoresis of duplex one-step RT-PCR products on diarrhea fecal samples. Lane N: negative control; Lane P: positive control; Lane M: PCR Marker, 50–2,000 bp; Lane 1–28: diarrhea fecal samples. BRV Kit result: result of commercial immunochromatography kit for BRV; BCoV SN-PCR: result of SN-PCR (semi-nested PCR) for BCoV.

BCoV and group A BRV were 10 and 1 PFU/100 μl, respectively. It indicated that the sensitivity of duplex RT-PCR was same with single PCR. Compared with the reported RT-PCR methods [5, 6, 10, 11], our method appears to be sensitive enough for the detection of clinical samples.

To evaluate the diagnostic efficacy of the one-step duplex RT-PCR assay, a total of 28 diarrhea fecal samples (detail was shown in Table 1) were collected from dairy herds, and then were diluted with PBS to 20% suspensions (v/v) and clarified by centrifugation at 750 × g for 10 min, 100 μl of the supernatant were subjected to RNA extraction following the procedures described above. Subsequently, the presence of BCoV was tested by using the one-step duplex RT-PCR in comparison with a semi-nested PCR assay described previously [15]. It indicated that two samples were identified as BCoV positive with the one-step duplex RT-PCR, the result was compatible with that from the semi-nested PCR assay (Fig. 2). Meanwhile, the presence of group A BRV was detected by using both the one-step duplex RT-PCR and BRV commercial immunochromatography kit (Rapidtesta Rota-Adeno: Orion Diagnostica, Espoo, Finland). The detection rate was 92.86% (26/28) by using the one-step duplex RT-PCR, while that of commercial immunochromatography kit was 67.86% (19/28) (Fig. 2). No RT-PCR negative sample was positive with immunochromatography kit, indicating that the one-step duplex RT-PCR assay possesses higher sensitivity than the commercial kit. Additionally, two BCoV positive samples were identified as co-infection with group A BRV. The amplified products from 2 BCoV positive samples were sequenced and they possessed more than 97.2% homology with Kakegawa strain and 99.3% homology each other. Meanwhile, products from 5 BRV positive samples were also sequenced and the blast result showed that they possessed high homology with the group A BRV sequences deposited in Genbank (more than 93%). Therefore, this method can amplify the target gene segments from wild type viruses.

In diagnostic laboratories, multiplex RT-PCR assay was proved to be very useful for the simultaneous detection of different RNA targets in the same sample, especially when the quantity of the clinical samples is low and the panel of potential pathogens is wide [4, 12, 16, 17]. On the other hand, multiplex detection method was achieved in less time and at less expense due to reduced use of reagents and disposable materials. Additionally, it can reduce the manipulation of the samples and avoid the risk of contamination. In the present study, a one-step duplex RT-PCR was established to detect and differentiate BCoV and group A BRV from fecal samples, and it proved that this method is sensitive for clinical fecal samples. It is worth applying to laboratory diagnosis for BCoV and group A BRV.

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