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Simultaneous Detection of Major Pathogens Causing Bovine Diarrhea by Multiplex Real-time PCR Panel

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Abstract : Bovine diarrhea is a major economical burden to the bovine industry in Korea. Since multiple infectious agents can be involved in bovine diarrhea, differential diagnosis is essential for effective treatment. Therefore, a panel of two multiplex real-time PCR assays which can simultaneously detect six major bovine enteric pathogens [i.e., bovine viral diarrhea virus (BVDV), bovine coronavirus (BCoV), group A bovine rotavirus (BRV), *Salmonella* spp., *Escherichia coli* (*E. coli*) K99$^+$, and *Cryptosporidium parvum*] was developed and applied to test 97 fecal samples collected from cattle farms in Korea. In addition, microscopic examination was also preformed on the samples to detect *Coccidium* oocyst. The estimated sensitivity of the multiplex PCR was 0.1 $TCID_{50}$ for BVDV, BCoV and group A BRV, 5 and 0.5 CFU for *E. coli* K99$^+$ and *Salmonella*, respectively, and 50 oocysts for *Cryptosporidium*. The amplification efficiency of the multiplex PCR ranged between 0.97 and 0.99 for each pathogen. Among 97 samples, 36 samples were positive for at least one of the 6 major pathogens and 6 samples were simultaneously positive for 2 pathogens by the multiplex PCR assay. *Coccidium* oocysts were also detected in 48 samples, which were all collected from over 1 month old calves. In conclusion, the multiplex real-time PCR panel can be a useful tool for fast and accurate diagnosis of calf diarrhea associated with BVDV, BCoV, group A BRV, *E. coli* K99$^+$, *Salmonella*, and/or *Cryptosporidium* and *Coccidium* may be an important target which needs to be included in the multiplex PCR panel in the future.

Key words **:** Multiplex real-time PCR, Bovine viral diarrhea virus, Bovine coronavirus, Group A Bovine rotavirus, *Salmonella*, *E. coli* K99, *Cryptosporidium.*

Introduction

Bovine diarrhea causes major economical losses to the Korean bovine industry due to high mortality and morbidity. It has been reported that gastrointestinal disorder was the most frequent diagnosis in calves and caused the most economically significant problem in Korean cattle farms (5). The cause of bovine diarrhea could be noninfectious factors, such as insufficient uptake of colostrum, poor sanitation, stress, and cold weather. Various viral, bacterial and protozoan agents such as bovine coronavirus (BCoV), bovine rotavirus (BRV), bovine viral diarrhea virus (BVDV), *Salmonella* spp., *E. coli* K99$^+$, *Clostridium spp Cryptosporidium* and *Coccidium* could also be implicated in calf diarrhea (1,11,12).

Since various pathogens can cause calf diarrhea individually or concurrently, accurate and fast differential diagnosis is essential to implement appropriate treatment and preventive practice and contributes to minimizing the spread of infection and increasing treatment efficiency (13). However, the common differential diagnostic methods such as virus isolation, electron microscopy, bacterial culture, fecal flotation method, antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA), latex agglutination test (LAT), and/or polymerase chain reaction (PCR) are laborious and slow in turnaround. Therefore, a multiplex PCR assay which can simultaneously detect major pathogens involved in bovine diarrhea may be a very convenient and effective tool for differential diagnosis on bovine diarrhea. Based on the practical needs, multiplex real-time PCR that can simultaneously detect 5 pathogens (i.e., BCoV, group A BRV, *Salmonella spp.*, *E. coli* K99, and *Cryptosporidium*) has been developed and evaluated with field and experiment samples in a previous study (2). Because the multiplex PCR was focused on new born calves less than 1 month old, BVDV was not considered in the PCR. However, as diarrhea is a consistent problem in all ages of cattle in Korea and BVDV is an important pathogen which causes severe diarrhea in over 1 month old calves (9,10), BVDV should be included in the multiplex PCR panel. In the current study, a new multiplex real-time PCR panel which can detect BVDV in addition to the other 5 pathogens has been developed to diagnose general diarrhea in all ages of cattle.

Material and Methods

Specimens

A total of 243 feces or intestinal contents which were used in a previous study (2) were used to evaluate the performance

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of the multiplex real-time PCR panel in comparison to other laboratory procedures for the same target agents (i.e., real-time RT-PCR for BVDV and BCoV, Ag-ELISA for rotavirus group A, bacterial culture and LAT for *E. coli* K99$^+$, bacterial culture and serotyping for *Salmonella*, and microscopic observation with acid-fast staining *Cryptosporidia*). In addition, 97 fecal samples submitted to Chonbuk National University-Veterinary Diagnostic Center (CBNU-VDC) from Korean farms between 2010 and 2011 were tested by the multiplex real-time PCR panel.

Nucleic acid extraction

Nucleic acids of all target agents were simultaneously extracted from specimens by use of MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA) as described in the manufacturer's manual. Briefly, 0.01 M phosphate buffered saline (PBS, pH 7.4) was added to each sample to make 30% fecal homogenates. After centrifugation for 1 min at 100 *x g* to pellet larger-size particles, 175 μl of the supernatant of each sample was added to a tube containing 235 μl of lysis/binding solution. The bead tube was beaten at maximum speed for 5 min with Bullet Blender® (Next Advance, Averill Park, NY, USA). After the beating process, the bead tubes were centrifuged at 16,000 *x g* for 3 min, and the supernatant was carefully transferred into clean microcentrifuge tubes. After another centrifugation at 16,000 *x g* for 6 min, 115 μl of the supernatant was transferred to a 96-well, microplate along with washing and elution buffers as suggested by manufacturer's manual. The automated process consisted of lysis/binding step for 5 min; two-time first washing step each for 90 sec; two-time second washing step each for 2 min and 30 sec respectively; dry step for 1 min; and, finally, the elution step for 3 min. The extracted total nucleic acids in the elution plate were stored in –80°C until used for PCR reaction.

Real-time RT-PCR for bovine viral diarrhea virus and bovine Coronavirus

BVDV and BCoV real-time PCR which are routinely used to diagnose calf diarrheic cases at the CBNU-VDC was employed. RNA was extracted from specimens with MagMAX™ Viral RNA Isolation Kit (Applied Biosystems) as described in the manufacturer's manual. The PCR was performed with AgPath-ID™ One-step RT-PCR Kit (Applied Biosystems) in a 25-μl reaction volume using 5-μl extracted template. Primers and probe were the same as employed in the multiplex PCR (Table 1) and the final concentration of primers or probe was 0.4 or 0.2 μM, respectively. The PCR

Table 1. The nucleotide information of primers and probes used for multiplex PCR

Agent (target gene)	Primer or probe (5'/3' labels)	Sequence (5'-3')	Product size (bp)	Reference no.
BVDV (M)	BVDV-F (fwd)	GGGNAGTCGTCARTGGTTCG	190	(10)
	BVDV-R (rev)	GTGCCATGTACAGCAGAGWTTTT		
	BVDV probe (Cy5/BHQ2)	CCAYGTGGACGAGGGCAYGC		
BCoV (N)	BCoV-fwd	CTAGTAACCAGGCTGATGTCAATACC	87	(6)
	BCoV-rev	GGCGGAAACCTAGTCGGAATA		
	BCoV-probe (FAM/MGB)	CGGCTGACATTCTCGATC		
BRV group A (VP6)	BRV-fwd1	TCAACATGGATGTCCTGTACTCCT	155	(6)
	BRV-fwd2	TCAACATGGATGTCCTGTATTCCT		
	BRV-fwd3	TCAACATGGATGTCCTTTATTCCT		
	BRV-rev1	TCCTCCAGTTTGGAACTCATT		
	BRV-rev2	TCCCCCAGTTTGGAATTCATT		
	BRV-rev3	CCCTCCAGTTTGGAATTCATT		
	BRV-probe1 (VIC/MGB)	TCAAAAACTCTTAAAGATGCTAG		
	BRV-probe2 (VIC/MGB)	TCAAAAACTCTTAAAGATGCAAG		
E. coli K99$^+$ (K99)	K99-fwd	GCTATTAGTGGTCATGGCACTGTAG	80	(12)
	K99-rev	TTTGTTTTCGCTAGGCAGTCATTA		
	K99-Probe (FAM/BHQ)	ATTTTAAACTAAAACCAGCGCCCGGCA		
Salmonella (Stn)	Stn-fwd	GCCATGCTGTTCGATGAT	129	(13)
	Stn-rev	GTTACCGATAGCGGGAAAGG		
	Stn-probe (Cy5/BHQ)	TTTTGCACCACMGCCAGCCC		
Cryptosporidium (COWP)	Crypto-fwd	CAAATTGATACCGTTTGTCCTTCTG T	151	(14)
	Crypto-rev	GGCATGTCGATTCTAATTCAGCT		
	Crypto-probe (JOE/BHQ)	TGCCATACATTGTTGTCCTGACAAATTGAA		

amplification was performed on ABI 7500 Fast Real-Time PCR System (Applied Biosystems) as follows: 45°C for 10 min, 95°C for 10 min, and 35 cycles of 95°C for 15 sec and 60°C for 45 sec. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive.

Rotavirus group A antigen-capture ELISA

Samples were assayed by Premier™ Rotaclone® Kit (Meridian Bioscience, Cincinnati, OH, USA) following the procedures recommended by the manufacturer. Samples with optical density ≥ 0.3 at 450 nm were considered positive for rotavirus.

Isolation and identification of *Salmonella* spp.

Samples were inoculated in Tetrathionate Broth (Difco, Sparks, MD, USA) and incubated for 24 hr at 42°C for enrichment. The enriched samples were plated on Brilliant Green Agar with Novobiocin (Difco), Hektoen Enteric Agar (Remel, Lenexa, KS, USA) and/or XLT4 Agar (Difco), and then incubated at 35-37°C for 24 hr. Suspect *Salmonella* were subcultured from Brilliant green (pink colonies), Hektoen-enteric (green or black colonies), or XLT4 (black colonies) and confirmed as *Salmonella* in Kliglers, Urea, Sims, and Lysine agars (Difco) and in the Trek Sensititre® Gram-Negative Identification panel (Trek Diagnostic Systems, Cleveland, OH, USA).

Isolation and identification of *E. coli* K99$^+$

Samples were plated directly onto Tergitol 7 Agar with triphenyltetrazolium chloride (Remel, Lenexa) and incubated aerobically at 35-37°C for 24 hr. *E. coli* colonies with rough, intermediate, smooth, or mucoid morphology were subcultured to conventional biochemical tube media for identification. Kligler's Iron Agar, Sims Agar, and Urea agar slants (Difco) were inoculated and incubated aerobically at 35-37°C for 24 hr. The reactions were read and then compared to charts to confirm the identification. *E. coli* growth from direct plating or from pure culture plating to E-agar (Difco) was tested for the presence of K99 pilus antigen using the Pilitest™ kit (VMRD, Pullman, WA, USA).

Identification of *Cryptosporidium* by acid-fast staining

Each sample was smeared on a glass slide, and the smear was dried briefly in ambient temperature. The air-dried smear was fixed in methanol for 10 min and then placed in Carbol Fuchsin (VWR, West Chester, PA, USA) for 1-2 hr. The smear was washed with tap water for 1 min and placed in 1% acid alcohol until no more red color ran off. After washed briefly with tap water, the smear was counterstained in 0.5% Fast Green for 1 min. The slide was read under a light microscope after brief steps of washing and drying. Red and halo-shaped oocysts were identified as cryptosporidia (4).

Identification of *Coccidium* by fecal floatation method

Two to three gram of each fecal sample was prepared in 10 ml of 5.32M sodium chloride solution. The prepared sample was passed through filter to remove large fecal debris and the filtrate was transferred into a narrow tube until a sligt, bulging meniscus forms above the rim of the tube. After leaving the tube for 15 minutes for oocysts to rise to the surface, microscope slide was placed on the top of the meniscus to collect floating parasitic oocysts. The slide was examined under a light microscope after placing a cover glass on the droplets of sample adhering to the slide.

Statistical analysis

The performance of the multiplex PCR was compared by calculating the percent agreement with other test results and the κ value as follows:

$$\% \text{ Agreement} = [\text{Agreed Pos} + \text{Neg} / \text{Total tests } (n = 243)] \times 100;$$

$$\kappa = [\Pr(a) - \Pr(e)] / [1 - \Pr(e)]$$

where Pr(a) is the relative observed agreement between tests, and Pr(e) is the probability that agreement is due to chance. If the tests are in complete agreement, $\kappa = 1$. If there is no agreement between the tests, then $\kappa \leq 0$. The interpretation of κ value was based on the guide provided by Landis and Koch:[16] Poor agreement ($\kappa = 0.00$); slight ($0.01 < \kappa$ 0.20); Fair ($0.21 < \kappa < 0.40$); Moderate ($0.41 < \kappa < 0.60$); Substantial ($0.61 < \kappa < 0.80$); Almost perfect ($0.81 < \kappa < 1.00$).

Results

Sensitivity, specificity and amplification efficiency of multiplex real-time PCR panel

All reference strains of the six agents with known virus

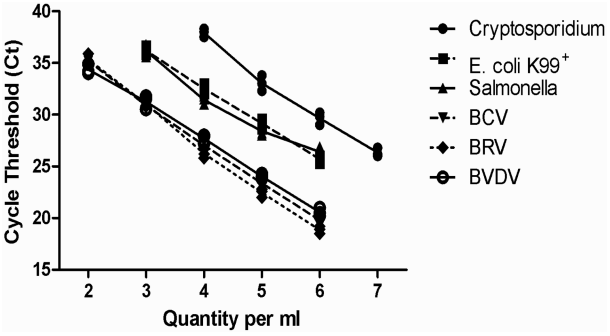

Fig 1. Multiplex detection of Bovine viral diarrhea virus (BVDV), Bovine coronavirus (BCoV), group A Bovine rotavirus (BRV), *Salmonella* sp., *E. coli* K99$^+$, and *Cryptosporidium parvum*. All of the six agents with known virus titer ($TCID_{50}$/ml), number of bacterial colony (CFU/ml), or number of oocysts (per ml) were mixed and serially diluted 10-fold for simultaneous detection by multiplex PCR. Y axis indicates cycle threshold (Ct) values. Each regression line was constructed based on three repeated measurements. Statistics for each regression line is $Y = -3.48*\text{Log}_{10}(X) + 41.52$, $R^2 = 0.99$ for BVDV, $Y = -3.83*\text{Log}_{10}(X) + 42.59$, $R^2 = 0.99$ for BCV, $Y = -4.15*\text{Log}_{10}(X) + 43.39$, $R^2 = 0.99$ for BRV, $Y = -3.23*\text{Log}_{10}(X) + 45.15$, $R^2 = 0.97$ for salmonella, $Y = -3.48*\text{Log}_{10}(X) + 46.57$, $R^2 = 0.99$ for E. coli K99$^+$, or $Y = -3.82*\text{Log}_{10}(X) + 52.73$, $R^2 = 0.99$ for cryptosporidium.

titer ($TCID_{50}$/ml), number of bacterial colony (CFU/ml), or number of oocysts (per ml) were serially diluted 10-fold and used to optimize the multiplex PCR. The multiplex PCR panel simultaneously detected all of those reference strains, yet only specific target agents without any false-positive result. Standard curves were generated using the 10-fold serial dilutions with correlation coefficients ranging from 0.97 to 0.99 and slopes of 3.23 to 4.15 (Fig 1). The limits of detection (i.e., analytic sensitivity) for each agent are 0.1 $TCID_{50}$ for BVDV, BCoV and BRV, 5 and 0.5 CFU for *E. coli* $K99^+$ and *Salmonella*, respectively, and 50 oocysts for *Cryptosporidium* per reaction.

As a next step, the performance of the multiplex PCR and simplex PCR for each of 6 agents was directly compared on the same 96 extracts to determine any negative effect of multiplexing on the PCR detection. The test results by both simplex and multiplex PCR were completely matched and Ct differences between the both PCR reactions were not statistically significant ($P > 0.1$), demonstrating that multiplexing did not cause significant negative effect on the sensitivity or specificity of the PCR reactions.

Performance of multiplex PCR panel in comparison with other tests

Comparisons of test results on 243 scour samples between the multiplex PCR panel and other laboratory tests for the 6 target agents are summarized in Table 2. As compared to BVDV real-time RT-PCR, the multiplex PCR identified 2 more samples as positive for BVDV and all of the other samples (n = 231) were negative by both of the PCR assay. The test agreement between two tests was 99% (241/243), and κ value was 0.904. The multiplex PCR panel also detected the BCoV genome in 54 samples, which was 12 more than the number of positive samples identified by BCoV real-time RT-PCR (n = 42), whereas all the other samples (n = 189) were negative for BCoV by both the PCR tests. Accordingly, the test agreement between BCoV RT-PCR and multiplex PCR was 95% (231/243), and κ value was 0.844. In the case of BRV detection, the multiplex PCR identified 23 more samples as positive for BRV compared with the rotavirus Ag-ELISA, whereas 2 positive samples by the ELISA were negative for BRV by the multiplex PCR. The test agreement between two tests was 89% (218/243), and κ value was 0.733.

In comparison with bacterial culture methods for two bacterial pathogens (i.e., *Salmonella* and *E. coli* $K99^+$), the multiplex PCR identified four more positive samples for *Salmonella* sp. compared with the culture results, whereas 3 positive samples by culture were negative by the multiplex PCR. The agreement between the multiplex PCR and *Salmonella* culture was 97% (236/243), and κ value was 0.887. In the case of *E. coli* $K99^+$, the multiplex PCR detected 39 positive samples, which were 9 more than the number of positive samples identified by culture and LAT. However, 5 positive samples by the culture method followed by LAT were nega-

Table 2. Comparative performance of multiplex PCR and traditional diagnostic assays in detecting Bovine viral diarrhea virus (BVDV), Bovine coronavirus (BCoV), group A Bovine rotavirus (BRV), *Salmonella* spp., *Escherichia coli* $K99^+$, or *Cryptosporidium parvum* from fecal samples

Traditional tests		Multiplex PCR		Total	% agreement (κ value)
		Positive	Negative		
BVDV real-time RT-PCR	Positive	10	0	10	99% (κ = 0.904)
	Negative	2	231	233	
	Total	12	231	243	
BCoV real-time RT-PCR	Positive	42	0	42	95% (κ = 0.844)
	Negative	12	189	201	
	Total	54	189	243	
Rotavirus group A antigen-capturing ELISA	Positive	50	2	52	89% (κ = 0.733)
	Negative	23	168	191	
	Total	73	170	243	
Salmonella culture and serotyping	Positive	33	3	36	97% (κ = 0.887)
	Negative	4	203	207	
	Total	37	206	243	
E. coli culture and latex agglutination for K99	Positive	30	5	35	94% (κ = 0.776)
	Negative	9	199	208	
	Total	39	204	243	
Microscopic observation with acid fast staining for *Cryptosporidium*	Positive	31	2	33	93% (κ = 0.756)
	Negative	14	196	210	
	Total	45	198	243	

tive by the multiplex PCR. The agreement of the two tests was 94% (229/243), and κ value was 0.776. The multiplex PCR was also compared with microscopic observation with acid-fast staining for the detection of *Cryptosporidium* spp. in the samples. The PCR detected 14 more positive samples than those by the microscopic observation, while two samples identified as positive for *Cryptosporidium* spp. by the microscopic observation were negative by the PCR for *Cryptosporidium parvum*. The test agreement between the multiplex PCR and microscopic observation was 93% (227/243), and κ value was 0.756.

Evaluation of multiplex PCR panel on clinical samples collected from Korean farms

The multiplex PCR was performed on 97 fecal samples submitted to CBNU-VDC during 2010-2011. As summarized in Table 3, 36 samples were positive for at least one of 6 agents. For viral agents, 2, 10, 14 samples were positive for BVDV, BCoV, BRV, respectively, while no samples were positive for bacterial agents, *E. coli* K99$^+$ and *Salmonella*. In addition, 10 samples were positive for *Cryptosporidium parvum*. Among those 36 samples, 6 samples were also positive for 2 agents. Since 61 samples were negative for the 6 agents by the multiplex PCR panel, those samples were tested for the presence of coccidium oocysts by microscopic exam. Based on the test results, coccidium oocysts were detected in 48 samples in total; more than 100,000 oocysts per gram were detected in 8 samples; 10,000-100,000 in 18 samples; 1,000-10,000 in 16 samples; and 100-1000 in 6 samples (Fig 2).

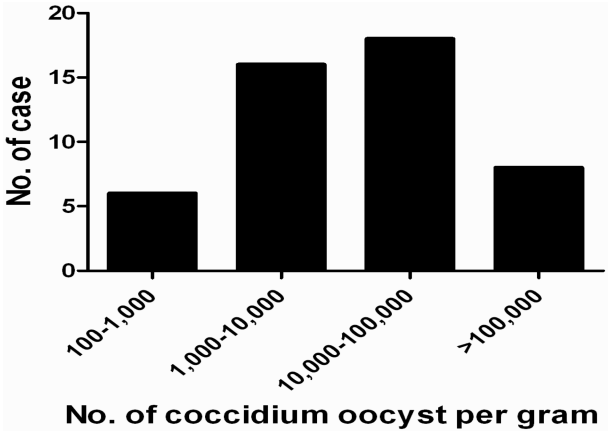

Fig 2. Number of Coccidium oocysts observed in clinical fecal samples.

Discussion

A multiplex real-time PCR panel that can simultaneously detect six major causative agents of calf diarrhea (i.e., BVDV, BCoV, BRV, *Salmonella*, *E. coli* K99$^+$, and *Cryptosporidium parvum*) was developed in this study. The new multiplex PCR panel and the traditional diagnostic methods, such as individual PCR, bacterial culture, serotyping, LAT, microscopic examination, and Ag-ELISA were evaluated and compared for their test performance on 243 clinical samples and good agreement was observed, ranging between 89% and 99%. However, the test results of 76 samples were opposite between the multiplex PCR and the traditional tests. Sixty-four out of the 76 discrepant samples were due to the higher sensitivity of the multiplex PCR panel because the positive results of 64 samples by the multiplex PCR panel could be confirmed with sequencing of the PCR products. The other 12 discordant samples, which were positive by the traditional tests, but negative by the multiplex PCR panel, should be considered as false-positive results by the traditional tests because those positive results couldn't be reproducible by other available methods. In addition, BVDV, BCoV, or *E. coli* K99$^+$ spiked in those samples were successfully detected by the PCR panel, ensuring that the negative PCR results were not due to gene degradation or PCR inhibition during the extraction or amplification procedures.

In a previous study (2), a multiplex real-time PCR which can simultaneously detect 5 major pathogens (i.e., BCoV, BRV, *Salmonella*, *E. coli* K99$^+$, and *Cryptosporidium parvum*) except BVDV has been developed and successfully applied to diagnose diarrhea in new born calves, which are less than 1 month old. Since BVDV is an important causative pathogen for bovine diarrhea in Korea (9,10), BVDV was included in the previous multiplex real-time PCR format. BVDV real-time PCR employed in the multiplex PCR panel has been developed by Mahlum *et al.* (6) and being routinely used to detect BVDV from bovine cases submitted to CBNU-VDC. The test results of the real-time PCR have also shown a good agreement with a commercial BVDV antigen-capture ELISA kit (IDEXX, Westbrook, Maine, USA). The new multiplex PCR panel was applied to 97 clinical samples collected from Korean farms. Although 36 samples were positive for one of the 6 major pathogens, 61 samples

Table 3. Test results on fecal samples collected from Korean farms between 2010 and 2011

	BVDV*	BCoV	BRV	Sal	K99$^+$	Crypto	Cocci	Unknown
< 1 month	2	2	8	0	0	10	0	6
> 1 month	0	8	6	0	0	0	48	7
Total	2	10	14	0	0	10	48	13
Percent (%)	2.1	10.3	14.4	0	0	10.3	49.5	13.4

*BVDV: Bovine viral diarrhea virus, BCoV: Bovine coronavirus, BRV: group A Bovine rotavirus, Sal: *Salmonella* spp., K99$^+$: *Escherichia coli* K99$^+$, Crypto: *Cryptosporidium parvum*, Cocci: *Coccidium spp.*

were negative by the multiplex PCR panel. Because 55 samples were collected from over 1 month old calves, microscopic examination with fecal floatation method was conducted on all of the clinical samples to find any causative parasitic oocysts (8). *Coccidium* oocysts were observed in 48 samples which were all collected from over 1 month old calves. Therefore, addition of *Coccidium* to the multiplex PCR panel should be considered in the future.

In conclusion, the new multiplex PCR panel could simultaneously detect 6 major viral, bacterial and protozoan pathogens with higher sensitivity and specificity as compared to other traditional diagnostic methods. Therefore, the new PCR panel would be useful in making fast and accurate differential diagnosis for bovine diarrhea in the early stages of disease and could be used for the diagnosis of diarrheic samples collected from all ages of cattle.

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Multiplex real-time PCR을 이용한 송아지 설사병 원인 주요 병원체의 동시검출

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요 약 : 송아지 설사병은 국내 축산산업에 큰 피해를 주는 중요한 질병이다. 다양한 감염성 원인체들이 송아지 설사병에 관련될 수가 있기 때문에 효과적인 치료를 위해서는 신속한 감별진단이 필수적이다. 따라서 소설사병 바이러스(BVDV), 소 코로나바이러스(BCoV), A형 소 로타바이러스(BRV), 살모넬라, K99$^+$ 대장균, *Cryptosporidium parvum* 등의 6개의 주요 병원체들을 동시에 검출하는 두 개의 multiplex real-time PCR으로 구성된 PCR 패널을 개발하여 국내 농가에서 전북대학교 동물질병진단센터로 접수된 97개의 설사 분변을 검사하였다. 또한 현미경 검사법을 이용하여 97개의 분변에서 *Coccidium* 충란을 검사하였다. 개발된 multiplex PCR의 민감도는 BVDV, BCoV와 BRV의 경우는 0.1 $TCID_{50}$, K99$^+$ 대장균은 5 CFU, *Salmonella*는 0.5 CFU, *Cryptosporidium*는50 oocysts로 측정되었다. 또한 multiplex PCR의 증폭효율은 병원체에 따라0.97에서 0.99였다. 국내에서 접수된97개의 분변 중 36개의 분변은 multiplex PCR에 의해 최소 하나의 병원체에 대해 양성으로 판정되었고, 6개의 샘플은 2개의 병원체에 동시에 양성반응을 보였다. 또한 1달 이상 연령의 송아지 분변48개에서는 *Coccidium* 충란이 발견되었다. 결과적으로, 새로이 개발된 multiplex real-time PCR은 BVDV, BCoV, BRV, K99$^+$ 대장균, *Salmonella*와 *Cryptosporidium*과 관련된 송아지 설사병을 신속하고 정확하게 진단할 수 있는 유용한 검사법이 될 수 있을 것으로 기대되며 향후 *Coccidium*까지 검출할 수 있는 동시 진단법이 개발될 필요가 있을 것으로 생각된다.

주요어 : Multiplex real-time PCR, 소 설사병 바이러스, 소 코로나바이러스, 소 로타바이러스, 살모넬라, K99$^+$ 대장균, *Cryptosporidium*