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Development of SYBR Green real-time RT-PCR for rapid detection, quantitation and diagnosis of unclassified bovine enteric calicivirus

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A B S T R A C T

Unclassified bovine enteric calicivirus (BECV) is a newly recognized bovine enteric calicivirus that differs from bovine norovirus, and which causes diarrhea in the small intestines of calves. To date, methods such as real-time reverse transcription-polymerase chain reaction (RT-PCR) have not been developed for the rapid detection, quantitation and diagnosis of BECV. Presently, a BECV-specific SYBR Green real-time RT-PCR assay was evaluated and optimized. Diarrheic specimens ($n = 118$) collected from 2004 to 2005 were subjected to RT-PCR, nested PCR and SYBR Green real-time RT-PCR. By conventional RT-PCR and nested PCR, 9 (7.6%) and 59 (50%) samples tested positive, respectively, whereas the SYBR Green assay detected BECV in 91 (77.1%) samples. Using BECV RNA standards generated by *in vitro* transcription, the SYBR Green real-time RT-PCR assay sensitively detected BECV RNA to 1.1×10^0 copies/μl (correlation coefficiency = 0.98). The detection limits of the RT-PCR and nested PCR were 1.1×10^5 and 1.1×10^2 copies/μl, respectively. These results indicate that the SYBR Green real-time RT-PCR assay is more sensitive than conventional RT-PCR and nested PCR assays, and has potential as a reliable, reproducible, specific, sensitive and rapid tool for the detection, quantitation and diagnosis of unclassified BECV.

1. Introduction

Caliciviruses are small, nonenveloped viruses that are 27–38 nm in diameter and possess a single-stranded, plus-sense RNA genome with a size of 7.3–8.3 kb, encoding a single-structural protein of 56–71 kDa (Green et al., 2000). The family *Caliciviridae* is classified into four genera: *Norovirus* (NoV), *Sapovirus* (SaV), *Vesivirus* and *Lagovirus* (Green et al., 2000). Among these genera, NoV and SaV are associated with enteric diseases in humans and animals (Bridger, 1990; Guo et al., 2001; Saif et al., 1980).

The bovine enteric calicivirus (BECV) Newbury agent-1 (NA1), which is different from bovine norovirus (BNoV), was associated with calf diarrhea in the United Kingdom in 1978 (Woode and Bridger, 1978). Data from electron microscopy, animal cross-protection experiments and solid-phase immune electron microscopy has revealed that NA1 is unrelated to BNoV (Bridger et al., 1984; Dastjerdi et al., 1999; Woode and Bridger, 1978). Recent genomic data has revealed the circulation in cows of the virus strains Bo/Nebraska/80/US (NB) and Bo/Newbury1/76/UK (NA1) (Han et al., 2004; Oliver et al., 2006; Smiley et al., 2002). NB-like BECV is genetically most similar to SaV and *Lagovirus*, and causes pathological lesions in the small intestine of gnotobiotic calves (Smiley et al., 2002). Furthermore, NB and NA1 formed a distinct clade that is independent of the four recognized genera (Oliver et al., 2006). Since this potentially new genus, which includes NB- and NA1-like unclassified BECVs, has not been named by the International Committee on Viral Taxonomy, the present study used the terms "unclassified BECVs" and "NB- and NA1-like viruses" to designate the strains.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) is relatively easy to conduct and has a high-throughput capacity. It has become the method used most widely for gene detection and quantitation (Oka et al., 2006; Yaper et al., 2005). In recent years, real-time RT-PCR has emerged as a highly reproducible and sensitive method for detection and diagnosis of NoVs and SaVs, proving to be more sensitive than methods used previously such as electron microscopy and conventional RT-PCR (Atmar and Estes, 2001; Glass et al., 2000; Trujillo et al., 2006). Various real-time RT-PCR assays have been reported for NoV and SaV detection including TaqMan assays (Chan et al., 2006; Hohne and Schreier, 2004; Jothikumar et al., 2005; Kageyama et al., 2003; Oka et al., 2006; Trujillo et al., 2006; Vainio and Myrmel, 2006) and assays

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Table 1
List of primers used for conventional RT-PCR, nested PCR, and SYBR Green real-time RT-PCR assay for the detection and quantification of unclassified bovine enteric calicivirus in the fecal specimens from the diarrheic calves.

Primer name	Sequence (5′-3′)[a]	Region	Annealing temp (°C)	Product size (bp)	Source or references
Conventional RT-PCR[b]					
NBU-F	F: TTTCTAACYTATGGGGAYGAYG	4518–5066	52	549	Smiley et al. (2003)
NBU-R	R: GTCACTCATGTTTCCTTCTCTAAT				
Nested PCR[b]					
nF	F: CGCTCCGTGTGGGATCACGA	4788–4981	53	194	Park et al. (2006)
nR	R: GCACGGGCTTCTTCTAGAGA				
SYBR Green real-time RT-PCR					
BECF	F: CCAGCCTCAGGATTTCAAAC	4883–4981	51	99	This study
BECR[c]	R: GCACGGGCTTCTTCTAGAGA				Park et al. (2006)

[a] F: forward primer for conventional RT-PCR, nested PCR, and SYBR Green real-time RT-PCR; R: reverse primer for Conventional RT-PCR, nested PCR, and SYBR Green real-time RT-PCR.
[b] All the procedure of RNA extraction, conventional RT-PCR and nested PCR were performed as described previously (Cho et al., 2001; Park et al., 2006, 2007a,b, 2008).
[c] BECR primer sequence for SYBR Green real-time RT-PCR was same with nR primer sequence for nested PCR.

based on the SYBR Green chemistry (Pang et al., 2004; Richards et al., 2004). Although the quantitation of the four *Caliciviridae* genera including NoV, SaV, *Largovirus* and *Vesivirus* has been reported worldwide (Green et al., 2001), there are no reports of real-time RT-PCR assays for the detection and quantitation of unclassified BECV in bovine stool samples.

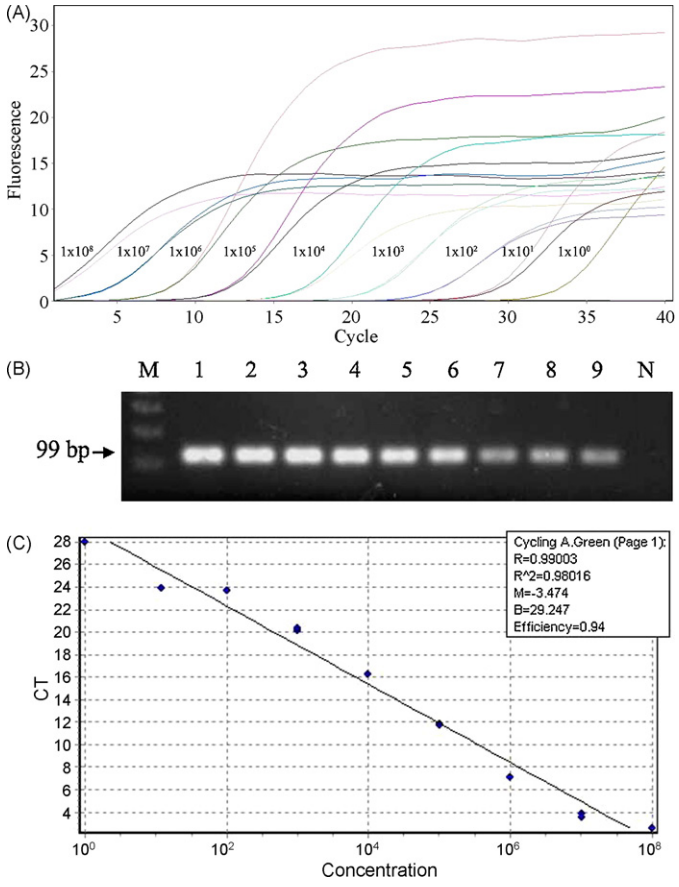

Fig. 1. SYBR green real-time RT-PCR assay for the quantitation of BECV cRNA standard. (A) Amplification of 1×10^0, 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 copies of cRNA standard used in parallel with each SYBR Green-based real-time RT-PCR assay. (B) SYBR Green real-time RT-PCR products using serially diluted *in vitro* transcripts. M: molecular marker; lanes 1–9: 1.1×10^8, 1.1×10^7, 1.1×10^6, 1.1×10^5, 1.1×10^4, 1.1×10^3, 1.1×10^2, 1.1×10^1 and 1.1×10^0 viral copies/μl; N: negative control. (C) Standard curves of the real-time RT-PCR based on serial dilutions of BECV cRNA standards. In the standard curve of these dilutions each dot represents the result of duplicate amplification of each dilution. The coefficient of determination (R^2) and the slope (s) of the regression curve are indicated.

The present study evaluated, optimized and validated a SYBR Green real-time RT-PCR assay for detecting and quantitating unclassified BECVs with archived stool samples. From the same samples previously evaluated by conventional RT-PCR and nested PCR assays, SYBR Green real-time RT-PCR proved to result in more specific and sensitive quantitation.

2. Materials and methods

2.1. Specimens

Bovine stool samples ($n = 118$) were selected from archived fecal samples collected by local veterinary clinicians in Korea during 2004–2005. All bovine samples were tested for the presence of unclassified BECV by conventional one-step RT-PCR and nested PCR, and were sequenced for confirmation (Park et al., 2008). Fifty-nine samples tested positive for BECVs and 59 samples were negative for BECV by conventional RT-PCR and nested PCR, respectively. The stool specimens were stored at −80 °C.

2.2. RNA extraction, conventional RT-PCR and nested PCR

RNA was extracted from a centrifuged 200 μl starting volume of 10% fecal suspension using Trizol-LS (Gibco-BRL, Grand Island, NY). The recovered total RNA was suspended in 50 μl of RNase free water and stored at −80 °C until analysis. The oligonucleotide primers and optimal conventional RT-PCR and nested PCR conditions for the detection of unclassified BECV (Table 1) are described elsewhere (Cho et al., 2001; Park et al., 2006, 2007a,b, 2008).

2.3. Real-time RT-PCR using SYBR Green chemistry

A one-step real-time RT-PCR was developed based on SYBR Green detection. The primers were designed based on the published sequence of the BECV RdRp gene (Table 1). All reactions were performed using a Corbett Research Rotor-Gene Real-Time Amplification system (Corbett Research, Mortlake, Australia) and SensiMix one-step RT-PCR kit with SYBR Green (Quantace, London, UK). Titration of primers was achieved using RNA from positive fecal samples. Reactions were run using primer concentrations from 0.3 to 0.9 μM. A concentration of 0.5 μM of each primer gave the highest sensitivity, together with a limited formation of primer dimers. Real-time RT-PCR was performed in a final volume of 20 μl that contained 5 μl of RNA template, 10 μl SensiMix one-step mixture, 0.5 μM each of forward and reverse primers, 0.5 μl of SYBR Green solution, 0.5 μl of

RNase inhibitor and 2 μl of RNase free water. Reverse transcription was carried out at 42 °C for 30 min, followed by the activation of the hot-start DNA polymerase at 95 °C for 10 min and 45 three-step cycles: 94 °C for 15 s, 51 °C for 30 s and 72 °C for 20 s. Samples were considered positive if both an exponential increase of fluorescence and a BECV-specific melting peak were observed.

2.4. In vitro transcription of complementary RNA (cRNA) standards

The amplicon of BECV RdRp gene (594 bp) obtained from RT-PCR was used as the source of DNA for the preparation of *in vitro* RNA transcripts. After cloning the amplified PCR products (Yeastern Biotech, Taipei, Taiwan), a clone was selected based on sequencing of the insert. The plasmid DNA of the recombinant clone was digested with the restriction enzyme *Pst*I, electrophoresed using a 1% agarose gel and the purified digested plasmid was recovered from the gel using the QIAQuick gel-extraction kit (Qiagen, Valencia, CA). The gel-purified linearized DNA clone served as the template for the *in vitro* transcription using the MEGAscript kit (Ambion, Auston, TX) according to the manufacturer' instructions. After 1 h of incubation at 37 °C, the DNA template was removed by digestion with DNase treatment with a TURBO DNA Free kit (Ambion). cRNA was then purified with a RNeasy Mini kit (Qiagen). After dilution, the concentrations were calculated by measuring the absorbance at 260 nm with a NanoDropND1000 (NanoDrop Technologies, Wilmington, DE, USA). cRNA samples were stored at −80 °C until use. The regression lines between the logarithms of the input amounts of cRNAs and the corresponding mean threshold cycle (Ct) values were calculated using the Rotor-Gene software version 6.0.19 (Corbett Research).

2.5. Statistical validation

Statistical analyses were performed by SPSS version 11.5.1 for Windows (SPSS, Chicago, IL). The two-tailed Fisher's exact test was used to assess the statistical significance of the detection rate between real-time RT-PCR, conventional RT-PCR and nested PCR. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Validation of SYBR Green real-time RT-PCR assay for sensitivity, reproducibility and specificity with in vitro transcripts of BECV RNA

Forward and reverse BECV primers for real-time RT-PCR were designed in conserved stretches of a portion of the RdRp gene to allow a broad reactivity to unclassified BECV. The standard RNA was diluted in a 10-fold dilution series ranging from undiluted to 1.0×10^{-8}, and was tested in duplicate in each run in the SYBR Green real-time RT-PCR assay. Quantities used for each dilution corresponded to 1.1×10^{0}–1.1×10^{8} viral copy numbers of stranded BECV RNA (Fig. 1). The SYBR Green real-time RT-PCR assay detected as little as 1.1×10^{0} copies of BECV *in vitro* transcripts per reaction and displayed linearity over a wide dynamic range of copy number (1.1×10^{8}–1.1×10^{0} copies) (Fig. 1A). Amplicons of expected size by SYBR Green real-time RT-PCR were visualized by gel electrophoresis (Fig. 1B). Standard curves with a higher correlation coefficient ($R^2 > 0.98$) were generated using a serial dilution of *in vitro* transcripts of BECV RNA from 1.1×10^{0} to 1.1×10^{8}, and the slope value of the standard curves with these transcripts was 3.474 (Fig. 1C). The standard RNA subjected to SYBR Green real-time RT-PCR presented a specific fluorescence signal and Ct values between 8.9 and 33.6 (data not shown). Real-time RT-PCR was successful in detecting positive samples and quantifying the unclassified BECV viral load.

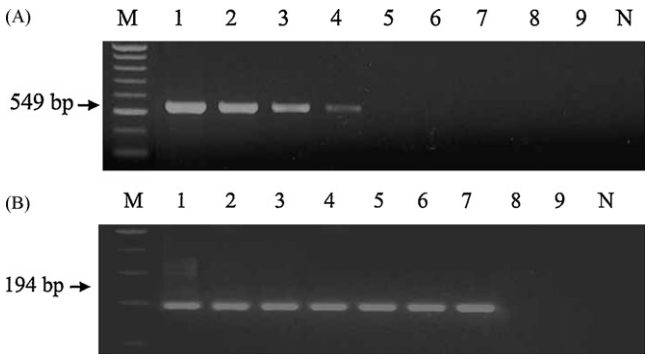

Fig. 2. Sensitivity of conventional RT-PCR and nested PCR with *in vitro* transcripts. (A) One-step conventional RT-PCR was performed in the same tube with serially diluted *in vitro* transcripts. (B) Nested PCR products with one-step conventional RT-PCR products. M: molecular marker; lanes 1–9: 1.1×10^{8}, 1.1×10^{7}, 1.1×10^{6}, 1.1×10^{5}, 1.1×10^{4}, 1.1×10^{3}, 1.1×10^{2}, 1.1×10^{1} and 1.1×10^{0} viral copies/μl, respectively; N: negative control.

3.2. Comparison of the detection limits with in vitro transcripts of BECV RNA by conventional RT-PCR, nested PCR and SYBR Green real-time RT-PCR

To assess the sensitivity and end point of SYBR Green real-time RT-PCR as compared to conventional RT-PCR and nested PCR assay, 10-fold serial dilutions of the *in vitro* transcripts described above were tested. Conventional RT-PCR was performed in the same tube. Nested PCR was also performed using the amplified products from the conventional RT-PCR. The lowest detection limits of the conventional RT-PCR and nested PCR as determined using serial dilutions were 1.1×10^{5} and 1.1×10^{2} viral copies/μl, respectively (Fig. 2A and B). Analysis of the dilution series showed that the SYBR Green real-time RT-PCR assay could detect a quantity 10,000 and 100 times lower than conventional RT-PCR and nested PCR, respectively. Nonspecific reactions were not evident with any of the three PCR approaches (data not shown).

3.3. Comparison of BECV detection rate in stool samples by SYBR Green real-time RT-PCR, conventional RT-PCR and nested PCR

The detection rates of BECV by real-time RT-PCR, conventional RT-PCR and nested PCR were compared using 118 stool samples (Table 2). Nine samples (7.6%) were positive by both conventional RT-PCR and real-time RT-PCR, and 82 samples (69.5%) were positive by real-time RT-PCR and negative by conventional RT-PCR. No samples that were negative by real-time RT-PCR were positive by conventional RT-PCR, and 27 samples (22.9%) were negative by both tests (Table 2(A)). The percentage of agreement between these two assays was 30.5%. The sensitivity and specificity of real-time RT-PCR compared with conventional RT-PCR were 100.0% and 24.8%, respectively. The agreement beyond chance was calculated with a Kappa statistic, which was 0.048. The detection rate of real-time RT-PCR was slightly higher than that of conventional RT-PCR ($P > 0.05$). Comparing nested PCR and real-time RT-PCR, 58 samples (49.2%) were positive by both approaches, 33 samples (28.0%) were positive by real-time RT-PCR and negative by nested PCR, one (0.8%) sample was negative by real-time RT-PCR and positive by nested PCR and 26 (22.0%) were negative by both tests (Table 2(B)). The percentage of agreement between the two assays was 71.2%. The sensitivity and specificity of real-time RT-PCR compared with nested PCR were 98.3% and 44.1%, respectively. The agreement beyond chance calculated with a Kappa statistic, which was 0.424. The detection rate

Table 2
Comparison of the detection of unclassified BECs by the SYBR Green real-time RT-PCR, conventional RT-PCR, and nested PCR assay.

		RT-PCR		Total
		+	–	
(A)[a]				
Real-time	+	9[b]	82	91
RT-PCR	–	0	27	27
	Total	9	109	118
		Nested PCR		Total
		+	–	
(B)[c]				
Real-time	+	58	33	91
RT-PCR	–	1	26	27
	Total	59	59	118

[a] Percent observed agreement (Po) = (9 + 27)/118 = 31%. Sensitivity = 9/91 = 10%. Specificity = 109/118 = 92.4%. Kappa = 0.048.
[b] Numbers indicate the samples positive (+) or negative (–) for unclassified BEC.
[c] Po = (58 + 26)/118 = 71.2%. Sensitivity = 58/91 = 63.7%. Specificity = 109/118 = 96.3%. Kappa = 0.424.

of real-time RT-PCR was moderately higher than that of nested PCR ($P < 0.05$).

3.4. Evaluation of the SYBR Green real-time RT-PCR with BECV from stool samples

The efficacy of SYBR Green real-time RT-PCR for the quantitation of BECV in 118 stool samples was evaluated. All positive samples subjected to SYBR Green real-time RT-PCR presented a specific fluorescence signal and Ct values between 9.8 and 32.8 (data not shown).

BECV amplicons displayed a melting temperature (T_m) between 79.5 and 83.5 °C (data not shown). Range of T_m values for unclassified BECV using real-time RT-PCR using SYBR Green chemistry *in vitro* transcripts for previously positive and negative fecal samples tested by conventional RT-PCR and nested PCR, was 82–83.5, 81–82 and 79.5–81 °C, respectively. T_m values depend on different factors including the initial concentration and size of the template, GC content and the sequence of the amplified fragment (Ririe et al., 1997). The viral copy numbers in SYBR Green real-time RT-PCR positive samples ranged from 1.3×10^0 to 5.7×10^8. Nine samples that tested positive in real-time RT-PCR and conventional RT-PCR contained 3.4×10^4–5.7×10^8 viral copies, whereas 58 samples that tested positive by real-time RT-PCR and nested PCR had 2.8×10^2–3.8×10^4 viral copies. However, the 33 fecal samples that tested positive only by real-time RT-PCR possessed comparatively lower viral copies, ranging from 1.3×10^0 to 3.4×10^4 viral copies. The specificity of real-time RT-PCR was assessed using fecal samples that had tested positive for other enteric pathogens including groups A, B and C bovine rotaviruses (BRV A–C), bovine torovirus (BToV), bovine coronavirus (BCoV), BNoV, bovine viral diarrhea virus (BVDV), *Salmonella* spp., *Clostridium* spp., *Campylobacter* spp., shiga-toxin-producing *Escherichia coli*, *Coccidium* spp. and *Cryptosporidium* spp. (Asakura et al., 1998; Park et al., 2007a, b). No positive signal was recorded for any of these pathogens.

4. Discussion

There are no reports concerning the use of real-time RT-PCR for the detection and quantitation of unclassified BECVs in bovine stool samples. In this study, a one-step SYBR Green real-time RT-PCR is described. This method allows the detection and quantitation of unclassified BECVs in a shorter period of time than has previously been obtained. In addition, the sensitivity and specificity of the developed SYBR Green real-time RT-PCR was evaluated and compared to conventional RT-PCR and nested PCR assays reported previously (Park et al., 2008).

The ideal method for detection of BECVs in stool samples should have a high degree of sensitivity and consistency of performance in the laboratory. Presently, the one-step SYBR Green quantitative real-time RT-PCR assay was significantly superior to the other PCR assays in terms of sensitivity, specificity and quantitative linearity. RT-PCR and nested PCR using serially diluted BECV cRNA could detect 1.1×10^5 and 1.1×10^2 viral copies/μl, respectively, whereas the lower detection limit of SYBR Green real-time RT-PCR was 1.1×10^0 viral copies/μl. Therefore, the SYBR Green RT-PCR assay is 100 and 10,000 times more sensitive than conventional RT-PCR and nested PCR, respectively. The short amplicons in the real-time PCR assays used in this study likely resulted in more efficient amplification and higher sensitivity. Taken together, the observations indicate the utility of the SYBR Green-based RT-PCR assay in the detection of BECVs.

In this study, a primer pair for SYBR Green-based real-time RT-PCR assay was designed to target sequences centered on the conserved RdRp gene. Since NoV, SaV and BECV belong to family *Caliciviridae*, the RdRp gene appears to be highly conserved within the family and is a region of choice in the design of new detection methods. In addition, the new SYBR Green real-time RT-PCR assay could be used to screen human fecal samples to ascertain further the role of unclassified BECVs in human diarrhea outbreaks, with a particular emphasis on human diarrhea cases from developing countries where cattle-human contact is more common (Park et al., 2006; Smiley et al., 2003).

To date, the reported fecal prevalence of BECVs in calf diarrhea by conventional RT-PCR or nested PCR has ranged from 8.4% to 9.2% in UK (Oliver et al., 2006) and South Korea (Park et al., 2008) to 28.0% in United States (Smiley et al., 2003). In the present study, 118 bovine stool samples were selected from archival diarrheic fecal samples. Fifty-nine tested positive for BECV by both conventional RT-PCR and nested PCR, and the remaining 59 samples tested negative by conventional RT-PCR and nested PCR (Park et al., 2008). Among these 118 diarrheic stool samples, one-step SYBR Green real-time RT-PCR not only detected BECVs in all 59 fecal samples that had tested positive by nested PCR but also detected BECVs in 32 fecal samples that had tested negative by nested PCR. These findings support the above suggestion that SYBR Green real-time RT-PCR is more sensitive than conventional RT-PCR and nested PCR. In addition, these observations indicate that the fecal prevalence of the BECVs in calf diarrhea might be higher than that reported previously in Korea (Park et al., 2008).

BECV is more pathogenic than BNoVs, inducing anorexia, diarrhea and xylose malabsorption (Bridger et al., 1984) and severe pathology in the small intestine (Hall et al., 1984; Smiley et al., 2002). Interestingly, 32 fecal samples that tested positive only by SYBR Green real-time RT-PCR contained lower numbers of BECV RNA (1.3×10^0–3.4×10^4 copies/μl). These fecal samples of diarrhea with lower number of BECVs might be coinfected with other enteric pathogens. Indeed, these samples positive for other enteric pathogens including BCoV, BRVA and BVDV (data not shown). Therefore, it is presumed that BECV alone can induce diarrhea in calves or can play a role in accelerating the clinical and pathological presentation of diarrhea in the field calves.

In conclusion, this study reports the development of a rapid, sensitive, specific and reproducible one-step SYBR Green real-time RT-PCR assay for detection, diagnosis and quantitation of unclassified BECV in stool samples. This high-throughput assay may be

useful in the investigation of possible sporadic and nosocomial gastroenteritis outbreaks in humans, in epidemiological and etiological studies and even for routine surveillance.

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