Protocols

Multiplex semi-nested RT-PCR with exogenous internal control for simultaneous detection of bovine coronavirus and group A rotavirus

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ABSTRACT

Neonatal calf diarrhea is a multi-etiology syndrome of cattle and direct detection of the two major agents of the syndrome, group A rotavirus and Bovine coronavirus (BCoV) is hampered by their fastidious growth in cell culture. This study aimed at developing a multiplex semi-nested RT-PCR for simultaneous detection of BCoV (*N* gene) and group A rotavirus (*VP1* gene) with the addition of an internal control (mRNA *ND5*). The assay was tested in 75 bovine feces samples tested previously for rotavirus using PAGE and for BCoV using nested RT-PCR targeted to *RdRp* gene. Agreement with reference tests was optimal for BCoV (kappa = 0.833) and substantial for rotavirus detection (kappa = 0.648). the internal control, *ND5* mRNA, was detected successfully in all reactions. Results demonstrated that this multiplex semi-nested RT-PCR was effective in the detection of BCoV and rotavirus, with high sensitivity and specificity for simultaneous detection of both viruses at a lower cost, providing an important tool for studies on the etiology of diarrhea in cattle.

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1. Introduction

Bovine neonatal diarrhea is a multi-etiology infectious disease that is endemic worldwide and has huge impact on the cattle industry due to delayed growth and mortality of calves. Bovine coronavirus (BCoV) and group A rotavirus are the most common viral pathogens found in the feces of calves with neonatal diarrhea (Athanassious et al., 1994). Besides neonatal diarrhea, BCoV is also associated with respiratory disease in cattle of any age and winter dysentery in adult bovines (Saif, 1990; Clark, 1993).

BCoV is a member of group 2 of *Coronavirus* genus (*Betacoronavirus*), *Coronaviridae* family, *Nidovirales* order (Holmes and Lai, 1996; Gonzáles et al., 2003). This enveloped, pleomorphic virus has four envelope proteins – membrane (M), envelope (E), hemagglutinin-esterase (HE), and spike (S) – and positive sense, non-segmented, single-stranded RNA genome (Holmes and Lai, 1996; Masters, 2006).

Group A rotavirus (*Reoviridae*: *Rotavirus*) is a non-enveloped virus with a triple-layered capsid formed by viral proteins VP6 (internal capsid), and VP4 and VP7 (external capsid). It has a negative-sense, segmented, double-stranded RNA (dsRNA) genome with 11 segments allocated in the core, formed by viral proteins VP1, VP2 and VP3 (Estes and Kapikian, 2007).

Detection of BCoV has been carried out using ELISA with monoclonal and polyclonal antibodies, immunohistochemistry assays (Clark, 1993; Zhang et al., 1997; Schoenthaler and Kapil, 1999), the polymerase chain reaction (PCR) (Verbeek and Tijssen, 1990; Loa et al., 2006; Takiuchi et al., 2006), and real-time PCR (Escutenaire et al., 2007; Decaro et al., 2008; Cho et al., 2010).

As for rotavirus, the most widely used diagnostic assay is polyacrylamide gel electrophoresis (PAGE) (Herring et al., 1982), which detects the 11 segments with great analytical specificity. However, the assay is lengthy and has low sensitivity (Estes and Kapikian, 2007).

This article reports the development of a novel multiplex semi-nested reverse transcription polymerase chain reaction (MSN RT-PCR) with exogenous internal control for simultaneous detection of BCoV and group A rotavirus in feces samples of cattle.

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Table 1
Oligonucleotides sequences for the detection of Bovine coronavirus, group A rotavirus and bovine NADH-dehydrogenase 5, showing the target region, melting temperatures (T_m) and amplicon sizes.

Primer	Sequence (5′–3′)	Position	T_m (°C)	Amplicon
BCOV1 (sense)	AAGAGCTCAAYCCAAGCAAACTGY	123–146[a]	60	463 bp
BCOV2 (antisense)	AGCAGACCTTCCTGAGCCTTCAAT	562–585[a]	60	
BCOV3 (antisense)	TCAATRTCGGTGCCATACTGGTCT	405–428[a]	59.9	306 bp (with BCOV1)
ROT1 (sense)	CTCTGGCAAARCTGGTGTCA	734–753[b]	59.7	492 bp
ROT2 (antisense)	CATTCGACGCTGATGACATY	1206–1225[b]	59.7	
ROT3 (antisense)	ARCAATCRACCAACCASTCCTGTA	938–961[b]	59.8	228 bp (with ROT1)
BOV-S (sense)	CAGCAGCCCTACAAGCAATGT	497–517[c]	58.1	191 bp
BOV-AS (anti sense)	GAGGCCAAATTGGGCGGATTAT	666–687[c]	58	

[a] Regarding BCoV Mebus strain (GenBank accession numbers U00735.2).
[b] Regarding rotavirus UKtc strain (GenBank accession numbers X55444.1).
[c] Regarding GenBank accession number NC_006853.

2. Materials and methods

2.1. Reference viruses and cells

Bovine coronavirus: BCoV Kakegawa strain (Akashi et al., 1980) grown in HmLu (hamster lung) cells, with hemagglutination titer (HA) of 256 (40 hemagglutinating units (HAU)/mL).

Rotavirus: Group A bovine rotavirus strain 8209 (Rodriguez et al., 2004) grown in MA-104 (green monkey fetal kidney) cells, with titer equal to $10^{2.36}$ TCID$_{50\%}$/mL.

Exogenous internal control: MDBK (Madin Darby Bovine Kidney) cells grown in 25 cm^2 flasks with MEM added of 5% fetal bovine serum.

2.2. Field samples

A total of 75 feces samples from dairy cattle (53 calves and 22 cows) collected in Brazilian farms were tested. Animals with and without signs of enteritis were both included in the study.

All samples were tested beforehand for BCoV using nested RT-PCR targeted to RNA-dependent RNA-polymerase (*RdRp*) gene (Brandão et al., 2005), and for rotavirus using polyacrylamide gel electrophoresis (PAGE) (Herring et al., 1982). From the 75 samples, 15 were positive for BCoV and three were positive for rotavirus in these references tests.

Feces suspensions (v/v; 50%, watery feces; 20%, loose feces; or 10%, normal feces) were prepared with ultra-pure water treated with 0.1% diethyl-pyrocarbonate (DEPC water). Suspensions were clarified at $5000 \times g$/15 min at 4 °C and only the supernatants were used in the assays.

2.3. RNA extraction

Extraction of total RNA from reference viruses and cells, and from the supernatants of the field samples was carried out with TRIzol Reagent^{TM} (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Primers

BCoV: A set of three primers described by Asano et al. (2009) and targeted to the nucleocapsid (*N*) gene of the virus was used to amplify a 306 bp fragment of nucleotides 123–428 of Mebus strain (GenBank accession number U00735.2) (Table 1).

Group A rotavirus: Three new primers were designed to detect VP1 (RNA-dependent RNA-polymerase) gene, a highly conserved gene in different genotypes of group A rotavirus (Estes and Kapikian, 2007).

Alignment of the sequences of GenBank accession number X55444.1, J04346.1, DQ494405.1 and DQ494406.1 generated a consensus sequence using the CLUSTAL/W algorithm in Bioedit 7.0.9.0

(Hall, 1999). This sequence was submitted to the OligoPerfect^{TM} Designer application (www.invitrogen.com), with a final amplicon of 228 bp, from nucleotides 734–961 of UKtc strain (GenBank accession number X55444.1).

Exogenous internal control: A pair of primers described by Caldwell et al. (2007) for detection of NADH-dehydrogenase 5 (*ND5*) mitochondrial gene was used, with an amplicon of 191 bp from nucleotides 497 to 687 of *Bos taurus* mitochondrial genome (GenBank accession number NC_006853).

All primers were submitted to BLAST/n and analyzed for the formation of homo- and heterodimers and hairpins, as well as for theoretical melting temperatures (T_m) using the OligoAnalyser 3.0^{TM} application (www.idtdna.com).

2.5. Determining annealing temperatures

First, 14 μL of total RNA extracted from each positive control (BCoV, group A rotavirus, and MDBK cells) were denatured at 95 °C for 5 min and reverse transcribed with 1× First Strand Buffer^{TM} (Invitrogen, Carlsbad, CA, USA), 1 mM of each dNTP, 10 mM DTT, 1 μM of each primer (BCOV1 + BCOV2 for BCoV, ROT1 + ROT2 for rotavirus, and BOV-S + BOV-AS for *ND5* mRNA) and 400 U of M-MLV Reverse Transcriptase^{TM} (Invitrogen, Carlsbad, CA, USA) to a final volume of 40 μL, incubated at 42 °C/60 min.

For the first-round PCR, 2.5 μL of cDNA were added to 1× PCR Buffer^{TM} (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP, 0.5 μM of each primer pair (BCOV1 + BCOV2 and BCOV1 + BCOV3 for BCoV, ROT1 + ROT2 and ROT1 + ROT3 for rotavirus, and BOV-S + BOV-AS for *ND5* mRNA in separate reactions), 1.5 mM MgCl$_2$ and 0.625 U of Platinum Taq DNA Polymerase^{TM} (Invitrogen, Carlsbad, CA, USA) to a final volume of 25 μL completed with DEPC water. Twelve tubes of each primer combination were placed in a Mastercycler^{TM} Gradient Thermocycler (Eppendorf, Hamburg, Germany) for initial denaturation at 94 °C/4 min followed 35 cycles of 94 °C/30 s, 55 °C/30 s with a 5 °C gradient to 12 different temperatures (50.0–60.4 °C for hybridization), and 72 °C/45 s, followed by a final extension at 72 °C/5 min.

Optimal annealing temperatures were determined after electrophoresis in 1.5% agarose gel stained with 0.5 μg/mL of ethidium bromide and observed under ultraviolet light. Optimal annealing temperatures resulted in bands of expected sizes and great intensity, and few unspecific bands.

2.6. Determining the combination of reagents to be used in MSN RT-PCR

Total RNA was extracted from 10-fold dilutions (up to 10^{-5}) of the two viral controls (BCoV and rotavirus) in 10% bovine feces suspension free of BCoV and rotavirus added of 10% MDBK cell suspension as the exogenous internal control.

For RT, 3.5 μL of total RNA extracted was denatured at 95 °C for 5 min and added to the combination of reagents containing 1× First Strand Buffer[TM] (Invitrogen, Carlsbad, USA), 1 mM of each dNTP, 10 mM DTT, 1 μM of each primer (BCOV1, BCOV2, ROT1, ROT2, BOV-BOV-S and AS, simultaneously) and 100 U M-MLV Reverse Transcriptase[TM] (Invitrogen, Carlsbad, USA) for a final volume of 10 μL, incubated at 42 °C/60 min. In parallel, a RT mix containing 2.5 ng of random primers (Invitrogen, Carlsbad, USA) was tested under the same conditions.

Twelve mixes were tested for PCR and semi-nested PCR with different concentrations of DNA polymerase, MgCl$_2$, primers and DNA. The final protocol was selected based on the detection threshold and the amount of reagents used.

The best-performing combination of reagents was determined after electrophoresis of PCR and semi-nested PCR products. The combination chosen for the final protocol had the greatest analytical sensitivity with the least use of reagents and absence of unspecific amplification.

2.7. Determining analytical sensitivity of MSN RT-PCR

Ten-fold dilutions (up to 10^{-10}) of Kakegawa and 8209 strains in DEPC water, fetal bovine serum, and bovine feces suspension free of BCoV and rotavirus (the latter two suspensions added of 10% (v/v) MDBK cell suspensions as exogenous internal control) were submitted to the final protocol (as determined in Section 2.6). Detection threshold of MSN RT-PCR was determined as the highest dilution in which the amplicons for each of the two targets were detected in 1.5% agarose gel electrophoresis stained with 0.5 μg/mL of ethidium bromide.

2.8. Using MSN RT-PCR in the analysis of different concentrations of each virus

Different combinations of the two targets were analyzed in order to evaluate the interference of excess of one target in the detection of the other target virus.

Six different proportions (v/v) of Kakegawa BCoV and 8209 group A rotavirus strains (1:100, 1:10 and 1:5) were tested from extraction of RNA to semi-nested RT-PCR, in the conditions described in Section 2.7.

2.9. Simultaneous detection of rotavirus and BCoV in bovine feces samples using MSN RT-PCR

Final semi-nested RT-PCR protocol was carried out in 75 field samples (250 μL) added of 10 μL of MDBK cell suspension.

Samples were considered positive if at least the 306 bp BCoV amplicon and/or the 228 bp group A rotavirus amplicon were detected after electrophoresis. Samples in which the 191 bp amplicon of the exogenous internal control was not detected were considered to be possible false-negative results.

Each step of the study (RNA extraction, RT-PCR, semi-nested PCR, electrophoresis, and DNA sequencing) was carried out in a different room with materials and reagents exclusive for that specific step in order to prevent DNA carryover. In the second amplification, a negative control (DEPC water) was used as a mock sample every other three samples and it was added to the mix and placed in the thermocycler in order to monitor contamination by DNA amplicons.

Kappa statistics (Thrusfield, 2004) in Microsoft Excel[TM] 2007 (© Microsoft Corporation, 2007) were used in the comparison between MSN RT-PCR and nested RT-PCR targeted to RdRp gene for BCoV detection (Brandão et al., 2005), and between MSN RT-PCR and PAGE for rotavirus detection (Herring et al., 1982).

2.10. Determining amplicon identity

BCoV and rotavirus amplicons were purified from agarose gels with Ilustra[TM] (GE, UK) and submitted to bi-directional sequencing with BigDye 3.1[TM] (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequences were resolved in the automatic sequencer ABI-377[TM] (Applied Biosystems, Carlsbad, CA, USA).

Chromatograms were submitted to Phred analysis at http://asparagin.cenargen.embrapa.br/phph/. The final consensus sequence of each sample was assembled with CAP-Conting in Bioedit 7.0.9.0 (Hall, 1999), and submitted to BLAST/n.

3. Results

3.1. Determining primer compatibility and optimal annealing temperatures

No significantly scored unspecific sequences were found after BLAST/n analysis of BCoV, group A rotavirus and ND5 gene primers. No theoretical dimeric, secondary structures or melting temperature incompatibilities were detected, either.

In the annealing temperature gradient assay using specific primers with their specific targets, amplicons were detected with the same efficiency in all temperature ranges tested, as evidenced by the intensity of the bands after agarose gel electrophoresis.

Therefore, in order to optimize sensitivity and specificity in the first-round amplification and nested step, annealing temperatures were arbitrarily set to 50 and 55 °C, respectively, for all subsequent assays.

3.2. Determining the combination of reagents to be used in MSN RT-PCR

Combination of specific primers (Table 1) in the RT step led to expected amplicons only for the positive controls; all dilutions were negative. The use of random primers in the RT step enabled a detection threshold of $10^{0.36}$ TCID$_{50\%}$/mL for group A rotavirus, and 4×10^{-1} AU/mL for BCoV.

From the 12 mixes tested, only four showed detection threshold of $10^{-2.64}$ TCID$_{50\%}$/mL for group A rotavirus and 4×10^{-4} HAU for BCoV in feces suspension. From these four mixes, the one chosen for the final MSN RT-PCR protocol required the least amount of DNA polymerase.

For the first and second round amplifications, 2.5 μL of cDNA were added to 1.2× PCR Buffer[TM] (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP, 0.4 μM of each BCoV primer pair (BCOV1 + BCOV2 and BCOV1 + BCOV3), 0.48 μM of each rotavirus primer pair (ROT1 + ROT2 and ROT1 + ROT3) and 0.32 μM of ND5 mRNA primer pair (BOV-S + BOV-AS), 2 mM MgCl$_2$ and 1.25 U of Platinum Taq DNA Polymerase[TM] (Invitrogen, Carlsbad, CA, USA) for a final volume of 25 μL completed with DEPC water.

3.3. Determining analytical sensitivity of MSN RT-PCR

Analytical sensitivity of the multiplex reaction applied to v/v dilutions of BCoV and group A rotavirus reference strains when random primers were used in the RT step and in the final protocol for first-round and nested amplifications was equal to: $10^{0.36}$ TCID$_{50\%}$/mL and 4×10^{-1} HAU/mL in DEPC water, $10^{-3.64}$ TCID$_{50\%}$/mL and 4×10^{-5} HAU/mL in bovine fetal serum, and $10^{-4.64}$ TCID$_{50\%}$/mL and 4×10^{-6} HAU/mL in bovine feces suspension free of BCoV and rotavirus.

The six different proportions (v/v) of Kakegawa BCoV and 8209 group A rotavirus strains (1:100, 1:10 and 1:5) tested by MSN

Table 2
Results of the BCoV/group A rotavirus multiplex semi-nested RT-PCR and by nested RT-PCR target to *RdRp* for BCoV detection used as BCoV reference test.

Multiplex semi-nested RT-PCR	Nested RT-PCR (*RdRp* gene)		
	Positive	Negative	Total
Positive	13	2	15
Negative	2	58	60
Total	15	60	75

Table 3
Results of the BCoV/group A rotavirus multiplex semi-nested RT-PCR and by PAGE used as rotavirus reference test.

Multiplex semi-nested RT-PCR	PAGE		
	Positive	Negative	Total
Positive	3	3	6
Negative	0	69	69
Total	3	72	75

RT-PCR resulted in the expected amplicons for both viruses and internal control.

3.4. Using MSN RT-PCR in the analysis of bovine feces samples

The use of the multiplex reaction with random primers in the RT step and final protocol in the first-round and nested steps with the addition of MDBK cells to each sample as an exogenous internal control resulted in 15 samples positive for BCoV and 6 samples positive for group A rotavirus. In all samples, including those in which BCoV and rotavirus amplicons were not detected, the 191 bp amplicon of the internal control was successfully amplified.

Total agreement between multiplex assay and nested RT-PCR targeted to RdRp gene, used as the reference test for BCoV detection, resulted in kappa value equal to 0.833, indicating optimal agreement (Table 2).

Total agreement between multiplex reaction and PAGE, used as the reference test for rotavirus detection, resulted in a kappa value of 0.648, or substantial agreement (Table 3).

DNA sequencing of four rotavirus and 12 BCoV amplicons confirmed homology with rotavirus VP1 gene and BCoV *N* gene, respectively. The remaining amplicons resulted in low Phred scores and, thus, were not included in the analysis. The following GenBank accession numbers were assigned to the sequences in this study: GU808341, GU808342, GU808343, GU808344, GU808345, GU808346, GU808347, GU808348, GU808349, GU808350, GU808351, GU808352, GU808353, GU808354, GU808355, GU808356.
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4. Discussion

The use of random primers rather than a combination of specific primers was an important modification in the RT reaction carried out in this study. This change increased the analytical sensitivity of the assay possibly due to greater annealing efficiency of polymorphic regions in the target sequences (Ståhlberg et al., 2004; Nardon et al., 2009).

After comparing different first-round PCR and nested amplifications, the final protocol was applied to 10-fold dilutions of BCoV and rotavirus reference strains (1:1) in DEPC water, bovine fetal serum, and feces suspension free of BCoV and rotavirus. Results showed increased analytical sensitivity. A possible explanation for these results is the difference in concentration of total RNA in each diluent, which would theoretically be zero in DEPC water and higher in bovine fetal serum and feces suspension. Besides, greater amounts

of total RNA improve pellet formation during precipitation with propanol in the extraction protocol (Sambrook and Russel, 2001; Ståhlberg et al., 2004).

Furthermore, as analytical sensitivity was greater when viruses were diluted in fecal suspension than in DEPC water or bovine fetal serum, it can be inferred that inhibitory substances commonly present in feces did not prevent RT or amplification, which would lead to false-negative results.

The choice if an exogenous instead of an endogenous internal control was based on the fact that most cells found in stool samples come from physiological or pathological sloughing and are, therefore, dead cells with no mRNA transcription activity. Thus, if no external source were provided, the amounts of natural mRNA would be too small for the *ND5* gene to be detected.

The use of six different proportions of the two viruses showed that high concentrations of one target would not interfere with detection of the other target due to competition during RT or amplification steps.

The 191 bp amplicon of the exogenous internal control was detected in all 75 feces samples analyzed by MSN RT-PCR, demonstrating that this kind of control enabled more efficient detection of false-negative results, with the advantage of providing constant amount of target RNA in all samples.

Takiuchi et al. (2006) and Cho et al. (2010) used internal controls in their reactions, but only for the PCR amplification step. The use of an internal control in the RNA extraction and RT steps, as described in the present protocol, enabled accurate monitoring of these steps, which are critical for the detection of RNA viruses.

Agreement between this new proposed multiplex semi-nested RT-PCR and PAGE for rotavirus diagnosis was only substantial. This may be due to the fact that PAGE is based on the detection of electrophorotype of rotavirus genomic RNA, without prior amplification steps. Although this is a low-cost technique that distinguishes between groups of rotaviruses, it depends on high concentrations of viral particles: while some RT-PCRs can detect approximately 2 ng of viral RNA, PAGE requires at least 10 ng of viral RNA (Herring et al., 1982; Gouvea et al., 1990).

Optimal agreement between the new multiplex semi-nested RT-PCR and nested RT-PCR targeted to RdRp gene for BCoV detection showed that *N* gene is a good target for sensitive and specific detection of BCoV.

DNA sequencing of PCR and semi-nested PCR amplicons showed homology with expected target sequences after BLAST/n analysis. These results are practical demonstrations of the specificity of the primers and accuracy of the reactions.

In conclusion, it was demonstrated that, when used according to the methodology described here, this novel multiplex semi-nested RT-PCR with primers based on sequences presently found in GenBank is an economical and accurate method for diagnosis of BCoV and group A rotavirus in cattle.

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