Pesq. Vet. Bras. 29(11):869-873, novembro 2009

Rapid detection of bovine coronavirus by a semi-nested RT-PCR[1]

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ABSTRACT.- Asano K.M, Souza S.P., Silva S.O.S., Richtzenhain L.J. & Brandão P.E. 2009. **Rapid detection of bovine coronavirus by a semi-nested RT-PCR.** *Pesquisa Veterinária Brasileira 29(11):869-873.* Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: karen.asano@gmail.com

Bovine coronavirus (BCoV) is a member of the group 2 of the *Coronavirus* (*Nidovirales*: *Coronaviridae*) and the causative agent of enteritis in both calves and adult bovine, as well as respiratory disease in calves. The present study aimed to develop a semi-nested RT-PCR for the detection of BCoV based on representative up-to-date sequences of the nucleocapsid gene, a conserved region of coronavirus genome. Three primers were designed, the first round with a 463bp and the second (semi-nested) with a 306bp predicted fragment. The analytical sensitivity was determined by 10-fold serial dilutions of the BCoV Kakegawa strain (HA titre: 256) in DEPC treated ultra-pure water, in fetal bovine serum (FBS) and in a BCoV-free fecal suspension, when positive results were found up to the 10^{-2}, 10^{-3} and 10^{-7} dilutions, respectively, which suggests that the total amount of RNA in the sample influence the precipitation of pellets by the method of extraction used. When fecal samples was used, a large quantity of total RNA serves as carrier of BCoV RNA, demonstrating a high analytical sensitivity and lack of possible substances inhibiting the PCR. The final semi-nested RT-PCR protocol was applied to 25 fecal samples from adult cows, previously tested by a nested RT-PCR *RdRp* used as a reference test, resulting in 20 and 17 positives for the first and second tests, respectively, and a substantial agreement was found by *kappa* statistics (0.694). The high sensitivity and specificity of the new proposed method and the fact that primers were designed based on current BCoV sequences give basis to a more accurate diagnosis of BCoV-caused diseases, as well as to further insights on protocols for the detection of other Coronavirus representatives of both Animal and Public Health importance.

INDEX TERMS: Bovine coronavirus, diarrhea, PCR, diagnosis.

RESUMO.- [**Detecção rápida do Coronavírus Bovino (BCoV) por meio de uma semi-nested RT-PCR.**] O Coronavírus bovino (BCoV) pertence ao grupo 2 do gênero *Coronavirus* (*Nidovirales*: *Coronaviridae*) e é agente causador de enterites tanto em bezerros como em bovinos adultos, bem como de doença respiratória em bezerros. O presente estudo teve por objetivo desenvolver uma semi-nested RT-PCR para a detecção do BCoV com base em seqüências representativas e recentes do gene do nucleocapsídeo, região conservada do genoma dos coronavírus. Três *primers* foram desenhados, a primeira amplificação com um fragmento esperado de 463pb e a segunda (semi-nested) com um fragmento esperado de 306pb. A sensibilidade analítica foi determinada pela diluição do BCoV cepa Kakegawa (título HA: 256) na base de 10 em água ultra-pura tratada com DEPC, em soro fetal bovino (SFB) e em uma suspensão fecal negativa para o BCoV, onde foram encontrados resultados positivos até a diluição de 10^{-2}, 10^{-3} e 10^{-7}, respectivamente. Este resultado sugere que a quantidade total de RNA na amostra influencia na precipitação dos pellets pelo método de extração utilizado. Quando se utiliza amostra fecal, a grande quantidade de RNA total funciona como carreadora do RNA do

[1] Received on January 14, 2009.

Accepted for publication on June 1, 2009.

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BCoV, demonstrando elevada sensibilidade analítica e ausência de possíveis substâncias inibidoras da PCR. O protocolo final da semi-nested RT-PCR foi aplicado a 25 amostras fecais de vacas adultas, previamente avaliadas por uma nested RT-PCR *RdRp* utilizada como teste de referência, resultando em 20 e 17 amostras positivas para o primeiro e segundo teste, respectivamente. Os resultados dos dois sistema de diagnóstico apresentaram concordância substancial (*kappa*: 0,694). A elevada sensibilidade e especificidade do novo método proposto e o fato de que os *primers* foram desenhados baseados em sequências atuais do BCoV, oferecem bases para o diagnóstico mais acurado de infecções causadas pelo BCoV, assim como para novas perspectivas em protocolos de detecção de outros Coronavírus de importância tanto em saninade animal quanto em saúde pública.

TERMOS DE INDEXAÇÃO: Coronavírus bovino, diarréia, PCR, diagnóstico.

INTRODUCTION

Bovine coronavirus (BCoV) is a member of group 2 of the genus *Coronavirus* (Nidovirales: Coronaviridae) (Holmes & Lai 1996, Gonzáles et al. 2003), with a genome formed by a single-stranded non-segmented positive-sense RNA with 32 kb, arranged in a nucleocapsid of helical symmetry in association with the *N* nucleoprotein, a conserved phosphoprotein with 50-60kDa rich in basic amino acids (Holmes & Lai 1996, Masters 2006).

The viral envelope of BCoV is formed by a lipidic double layer with five structural proteins (M, sM, HE, S, and I) that project from it, resulting in a spiked structure (Masters 2006).

BCoV replicates in absorptive cells on the villi of small intestine and in non-differentiated cells of the crypts region of the colon, resulting in desquamation, shortening of the villi and absorptive diarrhea (Pensaert et al. 1994). Calves around 3 months of age may also present pathological processes of upper respiratory tract caused by BCoV (Mcnulty et al. 1984, Heckert et al. 1990, Heckert et al. 1991, Tsunemitsu et al. 1991).

Adult cattle, mainly cows, present an enteric disease known as winter dysentery, first described in the USA, also caused by a bovine coronavirus similar to that found in neonatal diarrhea (Benfield & Saif 1990, Dea et al 1995, Brandão et al. 2002).

A simple method for the direct diagnosis of BCoV is the hemagglutination inhibition, which uses the property of the S and HE proteins to connect the sialic acids, such as the 9-O-acetyl-neuraminic acid, present on the surface of the membrane of red blood cells (Sato et al. 1977).

ELISA methods with polyclonal and monoclonal antibodies and immunohistochemistry (Clark 1993, Zhang et al. 1997) can also be used to direct diagnosis of BCoV, as well as imunoeletromicroscopy (Athanassious et al. 1994).

A large number of methods based on the polymerase chain reaction (PCR) for detecting BCoV can be found in the relevant literature (Brandão et al. 2003, Brandão et al. 2005, Loa et al. 2006, Takiuchi et al. 2006, Verbeek et al. 1990.) as well as variations based on quantitative polymerase chain reaction (Escutenaire et al. 2007).

However, taking into account the high rates of coronavirus RNA mutation (10^{-4}) and recombination (20%), (Baric et al. 1997, Rottier 1999, Moya et al. 2000), the development of genetic sequences-based diagnostic methods must be based on the most up-to-date sequences available in order to avoid inaccurate results due to inappropriate primers or probes annealing.

The aim of this study was to standardize and test a semi-nested RT-PCR protocol, from now on named PCR *N,* for a fast detection of BCoV in fecal samples, based on representative up-to-date sequences of the nucleocapsid gene.

MATERIALS AND METHODS

Primers designing

Three primers were designed for a conserved region of the nucleocapsid protein (N) gene of BCoV using the OligoPerfect ™ Designer online applicative at http://tools.invitrogen.com/content.cfm?pageid=9716 for a consensus sequence for the *N* gene obtained after alignment of sequences retrieved from the GenBank and published from 2006 to 2008: AB354579, NC_003045, EF424620, EF424617, EF424616, EF424615, EF193074, EF193073, and DQ811784; beside, sequences from reference, classic BCoV strains were also included: U00735 (Mebus strain) AF220295 (Quebec strain), AF391542 (BCoV-LUN strain), AF391541 (BCoV-ENT strain), and M36656 (F15 strain).

The alignment was carried out with the CLUSTAL/W algorithm using Bioedit 7.0.5.3 (HALL 1999), being the external primers BCOV1 sense 5' AGAGCTCAAYCCAAGCAAACTGY 3' (nt 123-146) and BCOV2 anti-sense 5' AGCAGACCTTCCT GAGCCTTCAAT 3' (nt 562-585) and the internal primer BCOV3 anti-sense 5' TCAATRTCGGTGCCATACTGGTCT 3'(nt 405-428).

Each primer was submitted to BLAST/n at http://www.ncbi.nlm.nih.gov/BLAST in order to assess non-BCoV *N* gene identities.

The predicted PCR and semi-nested amplicons for PCR *N* are 463bp and 306bp, respectively. Nucleotides positions are relative to the Mebus strain (Genbank accession number U00735).

Annealing temperatures

Optimal annealing temperatures for the combinations of primers BCOV1 + BCOV2 and BCOV1 + BCOV3 were found by temperature gradient in an Eppendorf™ Mastercycler Gradient thermocycler with the BCoV Kakegawa strain (kindly provided by Dr. José Antonio Jerez, College of Veterinary Medicine, University of São Paulo) as a positive control, with an hemagglutination titre of 256.

Reverse transcription reaction was carried out at 42°C for 60 min in a reaction mix containing 1x First Strand Buffer (Invitrogen™), 1mM of each dNTP, 10mM DDT, 1μM of each primer (BCOV1 and BCOV2 or BCOV1 and BCOV3), 3.5μL of RNA extracted by the TRIzol reagent (Invitrogen™) method previously denatured at 94°C for 5 minutes and 100U M-MLV Reverse Transcriptase (Invitrogen™) for a 10μL final reaction. The choice for the inclusion of sense and anti-sense primers in the reverse transcription step was based on the elementary knowledge of the *Coronaviridade*

replication that the positive-sense genomic RNA is not the only coronavirus-associated RNA that occurs during infection, as genomic and also sub-genomic negative-sense RNAs also occur as intermediates during viral transcription and replication (Holmes & Lai 1996), aiming a higher cDNA yield.

For PCR, 2.5μL of c-DNA were added to the PCR mix, containing 1x PCR Buffer (Invitrogen™), 0.2mM of each dNTP, 0.25ìM of each primer (BCOV1 and BCOV2 or BCOV1 and BCOV3), 1.5mM $MgCl_2$, 13.9μL of DEPC-treated ultra-pure water and 0.5U of Platinum Taq DNA Polymerase (Invitrogen™) for a 25μL final reaction, and submitted to 94°C/4min, followed by 35 cycles of 94°C/30sec for DNA denaturation, 55°C with 5°C gradient/30sec for primers annealing and 72°C/45sec for DNA extension and 72°C/ 5min for final extension.

Detection limits

Detection limits of the semi-nested reaction (primers BCOV1 +BCOV2 followed by BCOV1+BCOV3) were assessed using ten-fold dilutions of Kakegawa strain in DEPC-treated ultra-pure water, fetal bovine serum (FBS) and also in a BCoV-free bovine fecal sample with reverse transcription and PCR conditions as previously described using 50°C and 55°C annealing temperatures, respectively. Each of the three diluents was also tested as negative controls.

For the second round (semi-nested) amplification, 2.5μL of the first amplification product were added to the semi-nested PCR mix, containing 1x PCR Buffer (Invitrogen™), 0.2mM of each dNTP, 0.25μM of each primer (BCOV1 and BCOV3), 1.5mM $MgCl_2$, 13.9μL of DEPC-treated ultra-pure water and 0.5U of Platinum Taq DNA Polymerase (Invitrogen™) for a 25μL final reaction, and submitted to 94°C/4min, followed by 25 cycles of 94°C/30sec for DNA denaturation, annealing at 55°C for 30sec and 72°C/45sec for DNA extension and 72°C/5min for final extension.

DNA sequencing

The amplicons obtained for the Kakegawa strain (463bp and 306bp) were purified from the agarose gels using Ilustra (GE Healthcare™) and submitted to bi-directional DNA sequencing with BigDye 3.1 (Applied Biosystems™) accordings to manufacturer's instructions and the sequences were resolved in a ABI-377 automatic sequencer (Applied Biosystems™).

Chromatograms were analyzed with Phred at http://asparagin.cenargen.embrapa.br/phph/ and positions with scores of at least 20 were used to generate contig sequences with Cap-Contig implemented in the software Bioedit v. 5.0.9 (Hall, 1999) which were then submitted to BLAST/n at http://www.ncbi.nlm.nih.gov/BLAST.

Clinical sample tests

The final semi-nested PCR protocol was applied to 25 fecal samples from adult cows, being 17 positive and 8 negative to BCoV as previously tested by a nested-RT-PCR described by Brandão et al. (2005), targeted to the RNA-dependent RNA-polymerase gene (*RdRp*), from now on named PCR *RdRp*.

Fecal suspensions were prepared in DEPC-treated ultra-pure water to a 1:4 final dilution, clarified at 5,000xg/15min at 4ºC and the supernatant submitted to RNA extraction with TRIzol (Invitrogen™). BCoV Kakegawa strain was used as positive and DEPC-treated ultra-pure water as negative controls, respectively, also submitted to RNA extraction with TRIzol (Invitrogen™).

Reverse transcription, first-round and semi-nested PCR were carried out as described in the sections above. At the semi-nested step, a tube containing DEPC-treated ultra-pure water as a sample was included every three samples, also added mix and submitted to thermocycler in order to monitor contamination.

Positive samples were those for which the 306bp amplicon was detected after electrophoresis in 1.5% agarose gel stained with 0.5μg/mL ethidium bromide.

Each step (RNA extraction, preparation of reaction mixes, reverse transcription and PCR, semi-nested PCR and DNA sequencing and electrophoresis) was carried out in different rooms with exclusive materials in order to avoid contamination.

Comparison between semi-nested RT-PCR and the nested RT-PCR assay

Dichotomous results from PCR *N* and those derived from the nested RT-PCR described by Brandão et al (2005), named PCR *RdRp* applied to the clinical stool samples expressed as positive and negative were analyzed with *Kappa* statistics with 99.5% confidence level using the WinEpiscope 2.0 software.

RESULTS

BLAST/n analysis of primers BCOV1, BCOV2, and BCOV3 revealed no non-BCoV *N* gene significant identities.

Using these annealing temperatures, PCR *N* was able to detect BCoV Kakegawa strain until 10^{-2} dilution in DEPC-treated ultra-pure water, 10^{-3} in FBS and 10^{-7} in the BCoV-free bovine fecal sample. None of these diluents produced bands after tested in parallel with the dilutions.

DNA sequencing of the 463bp (first round PCR) and 306bp (semi-nested step) amplicons confirmed the homology with BCoV *N* gene. The sequences have been assigned GenBank Accession numbers FJ218459 and FJ218460.

Twenty out of the 25 samples tested in the final semi-nested protocol of PCR-*N* resulted positive to BCoV, according to the appearance of the 306bp-long predicted fragment. The negative controls, including the nested negative-controls, produced no bands (Fig.1).

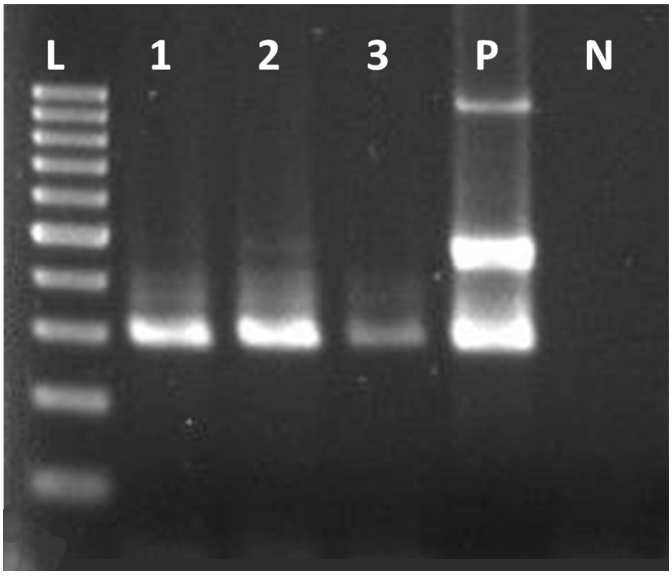

Fig.1. Agarose gel electrophoresis of semi-nested RT-PCR for the N gene of bovine coronavirus (BCoV). L = 100bp DNA ladder; 1-3: examples of bovine fecal positive samples; P = positive control; N = negative control (DEPC-treated ultra-pure water). Specific amplicons are 306-base-pairs long.

The global agreement test resulted in a *kappa* value of 0.694, indicating a substantial agreement between PCR *N* and PCR *RdRp*.

DISCUSSION

As BLAST/n revealed no significant non-BCoV *N* gene identities, a high analytical specificity can be inferred for the use of primers BCOV1, BCOV2, and BCOV3, once non-specific amplifications due to partial primer matching would not be expected. Also, as BLAST/n returned a large and diverse amount of BCoV gene sequences (data not shown), these primers shall allow the detection of a broad range of BCoV lineages.

DNA sequencing of first-round and semi-nested amplifications substantiate the analytical specificity of the test, as analysis of the sequences produced (FJ218459 and FJ218460) reveals that the amplicons not only are homologous to BCoV *N* gene, but are also of the predicted size (463 and 306bp, respectively).

Regarding the detection limits assay, an increasing analytical sensitivity was found according to the type of diluent used, *i.e.*, 10^{-2} dilution of BCoV in DEPC-treated ultra-pure water, 10^{-3} in FBS, and 10^{-7} in a suspension of a BCoV-negative fecal suspension.

A major explanation for such a result can be found in the total RNA content of each dilution in each of the three diluents, as this amount is theoretically zero in DEPC-treated ultra-pure water, high in the fecal suspension and intermediate in FBS.

Taking into account that the RNA extraction method used in the present research is intended to extract total RNA, the higher the total RNA content of a dilution, including viral and non-viral RNA, the higher the carrier effect, *i.e.*, a higher concentration of total RNA in a sample/ dilution would allow a more efficient RNA extraction as a higher amount of total RNA would help in the formation of pellets during precipitation with propanol (Sambrook 1989). It must be pointed out, though, that, if the method of RNA extraction was changed by, for instance, silica extraction protocols, the results for each diluent could be putatively different.

As the limit detection using the fecal suspension as a diluent was 10^5 times lower, *i.e.*, better, than in DEPC-treated ultra-pure water, one can infer that PCR inhibition by substances common to fecal samples will not hinder the transcription or amplification steps leading to false-negative results.

The detection limit of PCR *RdRp* used as reference test was reported as being the dilution 10^{-2} of the same Kakegawa strain at the same titre diluted in fecal suspension (Brandão et al., 2005), which is 10^5 times less sensitive than PCR *N*.

It can thus be argued that PCR *N* is a more sensitive test for the detection of BCoV at low titres as, for instance, in cases of animals during the incubation or convalescence periods or health carriers of the virus.

PCR assays for the detection of the *N* gene of BCoV are quite rare. One of the best examples so far is the paper by Takiuchi et al. (2006), which have reported a similar detection limit (10^{-7}) as the one found here; but, despite this agreement, testing the detection limit in the same kind of clinical sample the PCR is directed to, *i.e.*, fecal samples, as carried out in the present research, is fundamental to assess its efficiency, mainly regarding polymerase or RT inhibitors.

The application of the PCR *N* to 25 fecal samples of cows allowed to 20 of them as positive to BCoV, with substantial agreement (*kappa* value 0.694) with the PCR *RdRp* used as reference test.

Taking into account that the RdRp-coding region is more conserved amongst strains of BCoV when compared to the *N* gene (Holmes & Lai 1996), the most probable explanation for the disagreements between both tests is that the optimal reaction conditions of PCR *N* where obtained after a more extensive standardization assay, leading to a higher analytical sensitivity.

A further pro point regarding PCR *N* is that the total time consumed for reverse transcription, first and second round amplifications for PCR *N* was 4.5 h, in contrast to 7 h necessary for PCR *RdRp*.

As a conclusion, a fast, low-reagent consuming RT-PCR assay with high analytical sensitivity and specificity and low interference of inhibitors was standardized for the detection of BCoV in cases of enteric disease of cattle.

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