A semi-nested RT-PCR assay targeted to hemagglutinin-esterase gene of Bovine Coronavirus

Reação de hemi-nested RT-PCR dirigida ao gene da hemaglutinina-esterase do Coronavírus Bovino

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Abstract

Bovine coronavirus (*BCoV*) is a non-segmented positive-sense single-stranded RNA virus whose envelope is constituted by a lipid bilayer with four structural proteins (HE, S, E and M) giving its characteristic crown-like virions appearance. Hemagglutinin-esterase (HE), is a polymorphic protein with a function of secondary receptor binder, and studies on the diversity of *HE* gene allow insights on BCoV evolution and host-parasite interactions. A semi-nested RT-PCR was developed for the amplification of a 441bp-long product of the *HE* gene of BCoV (nt 543 to 562). Optimal annealing temperatures were tested in a gradient thermocycler for the semi-nested assay and employed in the final protocol. The analytical sensitivity was determined by 10-fold serial dilutions of the BCoV Kakegawa strain (HA titer: 256) in a BCoV-free fecal suspension, with positive results up to 10^{-6} dilution, a high analytical sensitivity without PCR inhibition. The final semi-nested RT-PCR protocol was applied to 21 fecal samples of cows previously positive to BCoV and DNA sequencing of the 441bp amplicons of 14 of these resulted in highly-scored BCoV *HE* gene sequences after BLAST/n analysis. This semi-nested RT-PCR is a powerful tool for surveys of phylogenetic diversity in field strains of BCoV and for comparative studies among different genes of *Coronavirus*.

Keywords: Bovine coronavirus. RT-PCR. Hemagglutinin-esterase. Genetic diversity.

Resumo

O coronavírus bovino (BCoV) é um vírus RNA simples fita, de sentido positivo, não segmentado com envelope constituído de uma camada dupla de lipídios com quatro proteínas (HE, S, E e M) que resultam no aspecto de coroa dos vírions. Como a HE (hemaglutinina-esterase) é uma proteína polimórfica com uma função de receptor aglutinante secundária, estudos sobre a diversidade do gene *HE* podem possibilitar maiores informações sobre a evolução e interação hospedeiro-parasita do BCoV. Uma reação de hemi-*nested* RT-PCR foi desenvolvida para a amplificação de um produto de 441pb do gene *HE* do BCoV (nt 543 ao 562). Temperaturas ótimas de hibridização foram testadas em um termociclador com gradiente para a reação de hemi-*nested* e utilizada no protocolo final. A sensibilidade analítica foi determinada por meio da diluição serial na base 10 do BCoV amostra Kakegawa (título HA: 256) em uma suspensão fecal negativa para BCoV, resultando positiva até a diluição de 10^{-6}, mostrando uma alta sensibilidade analítica sem inibição na PCR. O protocolo final da hemi-*nested* RT-PCR foi aplicado a 21 amostras fecais de vacas previamente positivas para BCoV e o sequenciamento de DNA do produto de 441pb de 14 amostras resultaram em sequências com elevado escore do gene *HE* do BCoV após a análise no BLAST/n. Essa hemi-nested RT-PCR é uma ferramenta poderosa para estudos de diversidade filogenética de linhagens de campo de BCoV e para estudos comparativos entre os diferentes genes dos Coronavírus.

Palavras-chave: Coronavírus bovino. RT-PCR. Hemaglutinina-esterase. Diversidade genética.

Introduction

Bovine coronavirus (BCoV) is a member of the group 2 of the genus *Coronavirus* (*Nidovirales*: *Coronaviridae*)[1,2], with a genome formed by 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 50-60 kDa rich in basic amino-

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acids[1,3].The viral envelope of BCoV is formed by a lipidic double layer with four structural proteins (HE, S, E and M) that project from it, resulting in a spiked structure[3].

As a group-specific characteristic, most group 2 coronaviruses present the envelope protein called hemagglutinin-esterase (HE), with 65 kDa, found in the form of dimmers[4]. Despite its name, the HE has a weak hemagglutinating activity when compared to the activity of the S protein[5,6].

In bovines, BCoV replicates in absorptive cells on the villi of small intestine and in non-differentiated cells of the colon crypts region, resulting in desquamation, shortening of the villi and absorptive diarrhea[7]. Calves around three months of age may also present pathological processes affecting the upper respiratory tract caused by BCoV[8,9,10,11] . Adult cattle, mainly cows, present an enteric disease known as winter dysentery, first described in the USA, and also caused by a bovine coronavirus similar to that found in neonatal diarrhea[12,13,14]. As HE is a polymorphic protein that functions as a secondary receptor binder[3], surveys on the diversity of *HE* gene might allow insights on BCoV evolution and host-parasite interactions.

The aim of this study was to develop a semi-nested RT-PCR assay targeted to the partial amplification of the *HE* gene of BCoV

Material and Method

Primer design

The external primers were those described by Villarreal et al.[15]: CHES sense 5' TMT TTG GYG ACA GTC GTT C 3' and CHEA anti-sense 5' TTA TCM GAM TGC YTR GCA TT 3', with a predicted amplicon of 796 bp (nt 122 – 917 of Mebus strain HE gene, GenBank accession number U00735) (Figure 1).

A third, internal primer was designed using the OligoPerfect™ Designer online applicative at www.invitrogen.com for a consensus sequence for the *HE* gene obtained after alignment of sequences retrieved from the GenBank with the CLUSTAL/W algorithm using Bioedit v. 5.0.9[16]: S50936.1, U00735.2, AY184423.1, AY184422.1, AY184421.1, AY184420.1, AY184419.1, AY184418.1, AY184417.1, AY184416.1, AY184415.1, AY184414.1, AY184413.1, AY184412.1, AY184411.1, AY09770.1, AH010363.1, UO6093.2, L38963.2, M80842.1, AF058944.1, AF058943.1, AF58942.1, M84486.1 e M76372.1, resulting the anti-sense prim-

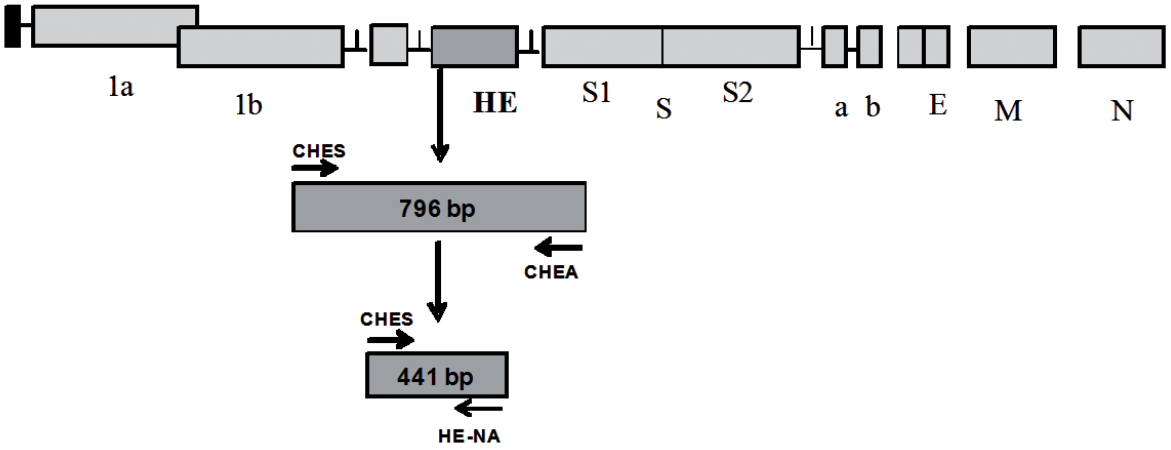

Figure 1 - Schematic representation of the genome of Bovine coronavirus and the region amplified for the gene coding for the hemagglutinin-esterase protein by RT-PCR (primers CHES + CHEA) and the Semi-nested (primers CHES + HE-NA)

er HE-NA 5' CCC CAA AAT TAG CTT CAC GA 3', with a predicted amplicon of 441bp (nt 121 - 562 of Mebus strain *HE* gene, GenBank accession number U00735) (Figure 1). The internal primer was submitted to BLAST/n at http://www.ncbi.nlm.nih.gov/BLAST in order to assess non-BCoV *HE* gene identities.

Annealing temperatures

Optimal annealing temperature for primer combinations of CHES and HE-NA were determined by temperature gradient in an Eppendorf ™ Mastercycler Gradient thermocycler with a BCoV-positive cow fecal sample (sample 96)[17]. Reverse transcription reaction was carried out at 42 °C for 60 minutes in a reaction mix containing 1x First Strand Buffer (Invitrogen™), 1 mM of each dNTP, 10 mM DDT, 1 µM of each primer (CHES and HE-NA), 3.5 µL of RNA extracted by the TRIzol reagent (Invitrogen™) method previously denatured at 94 °C for five minutes and 100U M-MLV Reverse Transcriptase (Invitrogen™) for a 10 µL final reaction.

For PCR, 2.5 µL of c-DNA were added to the PCR mix, containing 1x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5 µM of each primer (CHES and HE-NA), 1.5 mM $MgCl_2$, 12.625 µL of DEPC-treated ultra-pure water and 0.625 of Platinum Taq DNA Polymerase (Invitrogen™) for a 25 µL final reaction, and submitted to 94 °C/4 min, followed by 25 cycles of 94 °C/1min for DNA denaturation, 53 °C with 5 °C gradient/1.5 min to 12 different temperatures (48 °C, 48.2 °C, 48.7 °C, 49.6 °C, 50.7 °C, 52 °C, 53.4 °C, 56.1 °C, 57.1 °C, 58.8 °C e 58.4 °C) for primers annealing and 72 °C/1 min for DNA extension and 72 °C/ 10 min for final extension. Optimal annealing temperature was determined as that yielding the most intense 441-bp amplicon as seen after electrophoresis in 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide.

Detection limits

Detection limits of the semi-nested reaction (primers CHES and HE-NA) were assessed using ten-fold dilutions of strain Kakegawa in 20% BCoV-free bovine fecal sample suspension previously tested by a nested RT-PCR targeted to *RdRp* gene[18]. This sample was also tested with primers CHES and HE-NA in order to ensure it was BCoV negative.

Reverse transcription was carried out at 42 °C for 60 minutes in a reaction mix containing 1x First Strand Buffer (Invitrogen™), 1 mM of each dNTP, 10 mM DDT, 1 µM of each primer (CHES and CHEA), 3.5 µL of RNA extracted by the TRIzol reagent (Invitrogen™) method previously denatured at 94 °C for five minutes and 100 U M-MLV Reverse Transcriptase (Invitrogen™) for a 10 µL final reaction.

For the first round amplification (PCR), 2.5 µL of c-DNA were added to the PCR mix, containing 1x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5 µM of each primer (CHES and CHEA), 1.5 mM $MgCl_2$, 12.625 µL of DEPC-treated ultra-pure water and 0,625 Uof Platinum Taq DNA Polymerase (Invitrogen™) for a 25 µL final reaction, and submitted to 94 °C/4 min, followed by 35 cycles of 94 °C/1 min, 50 °C/1.5 min and 72 °C/1 min, followed by 72 °C/ 10 min for final extension.

For the second round amplification (semi-nested), 2.5 µL of first amplification product (PCR) were added to the PCR mix, containing 1x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5 µM of each primer (CHES and HENA), 1.5 mM $MgCl_2$, 12.625 µL of DEPC-treated ultra-pure water and 0.75U of Platinum Taq DNA Polymerase (Invitrogen™) for a 25 µL final reaction, and submitted to 94 °C/4 min, followed by 25 cycles of 94 °C/1 min, optimal annealing temperature/1.5 min and 72 °C/1 min and 72 °C/ 10 min for final extension.

Clinical sample tests

The final semi-nested RT-PCR protocol was applied to 21 BCoV positive fecal samples from adult cows by a nested RT-PCR[17]. Fecal suspensions were prepared in DEPC-treated ultra-pure water to a 1:4 final dilution, clarified at 12,000 x g/ 30 min at 4 °C recovering the supernatant. BCoV Kakegawa strain was used as positive and DEPC-treated ultra-pure water as negative controls, respectively.

Reverse transcription, first-round and second-round amplification were carried out as described in the sections above. At the semi-nested step, in order to monitor contamination, a tube containing DEPC-treated ultra-pure water also containing the reagents mix was included at every three samples and submitted to amplification in the thermocycler. Positive samples yielded a 441 bp amplicon after electrophoresis in 1.5% agarose gel stained with 0.5 µg/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 for each step in order to avoid contamination.

DNA sequencing

Resulting amplicons were purified from the agarose gels using Ilustra (GE Healthcare™) DNA purifying kit and submitted to bi-directional DNA sequencing with BigDye 3.1 (Applied Biosystems™) according to manufacturer's instructions. Sequences were resolved in an ABI-377 automatic sequencer (Applied Biosystems™). Chromatograms were analyzed with Phred at http://asparagin.cenargen.embrapa.br/phph/ and positions with scores > 20 were used to generate contig sequences with Cap-Contig implemented in the software Bioedit v. 5.0.9[16] which were then submitted to BLAST/n at http://www.ncbi.nlm.nih.gov/BLAST.

Results

BLAST/n analysis of internal primer HE-NA did not reveal non-BCoV *HE* gene significant identities.

Using the sample 96 as reference strain, the temperature of 53.4 ºC was chosen as the optimum annealing temperature for the semi-nested step.

The semi-nested RT-PCR assay allowed detection of the BCoV Kakegawa strain up to the 10^{-6} dilution in the BCoV-free bovine fecal sample, which previously produced no bands when tested by the same reaction protocol (Figure 2).

The semi-nested RT-PCR targeted to HE gene resulted in sixteen positives samples according to the observed 441 bp amplicon. Fourteen sequences out of sixteen positives samples resulted in sequences of DNA with Phred score ≥ 20, while sequences obtained of two samples were of low quality. For each obtained sequence, BLAST/n analysis confirmed the homology with the *HE* gene of BCoV with significant scores. The sequences were registered in GenBank with the following access numbers: GU214757, GU214758,

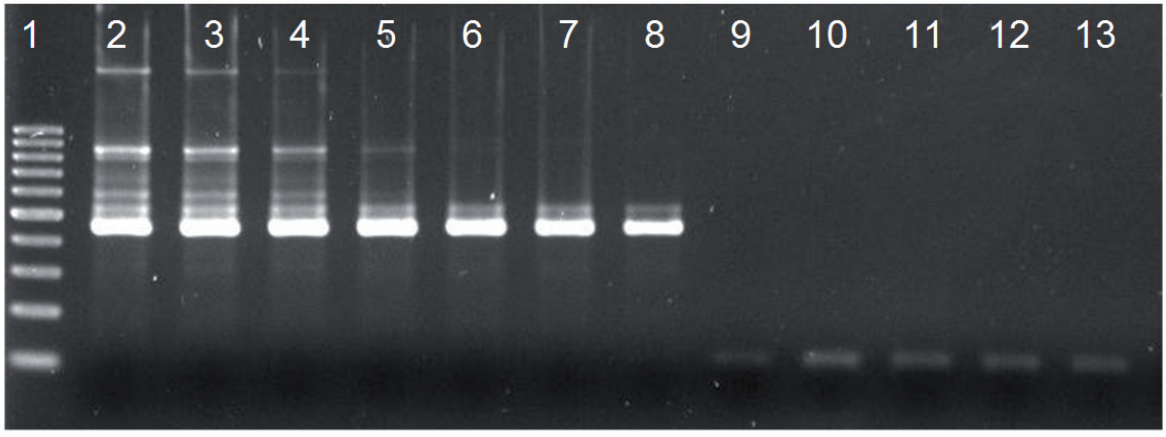

Figure 2 - Agarose gel electrophoresis of semi-nested RT-PCR for the *HE* gene of bovine coronavirus (BCoV). 1: 100 bp DNA ladder; 2:– 12: 10^{0} – 10^{-10} dilution of the BCoV Kakegawa strain in BCoV-free fecal suspension; 13: negative control (fecal suspension). Specific amplicons are 441-base-pairs long

GU214759, GU214760, GU214761, GU214762, GU214763, GU214764, GU214765, GU214766, GU214767, GU214768, GU214769, GU214770.

Discussion

In the present study, a newly developed semi-nested RT-PCR assay allowed the partial amplification of the *HE* gene of BCoV strain involved in clinical cases of winter dysentery and DNA analysis of the resulting amplicons showed the specificity of this assay.

The RT-PCR described by Villarreal et al.[15] has not detected *HE* amplicons for most of the samples include in the present study (data not shown), probably due to its low sensitivity.

The optimal temperature of hybridization using primers HE-NA in the semi-nested assay with the primer CHES was 53.4 ºC, higher than the one employed for the first amplification (50 °C) with primers CHES and CHEA. This result gives the semi-nested assay greater analytical specificity, since the higher the temperature of hybridization of primers, the lower the occurrence of non-specific amplifications[19], a fact which is primordial for the efficiency of amplification reactions and DNA sequencing.

Though the semi-nested protocol has shown a high analytical sensitivity, with a detection limit at 10^{-6} dilution of the Kakegawa strain, this sensitivity was lower when compared to those described by Asano et al.[20] and Takiuchi et al.[21], who found a detection limit at the 10^{-7} dilution of the Kakegawa strain of the same HA titer. Such dissimilarity may be due to the genomic region chosen as the target of the coding region of HE protein, which presents a higher diversity if compared to the coding region of N protein, a highly conserved region[22], targeted by the authors of the aforementioned studies.

Nevertheless, the semi-nested RT-PCR described in the present report, unlike the techniques previously mentioned, has no purpose of diagnosis, but rather the generation of amplicons of the *HE* gene for DNA sequencing for continued studies on molecular diversity.

The application of the semi-nested RT-PCR resulted in the 441 bp expected amplicons for 16 out of the 21 samples previously known as positive for BCoV, while for the five remaining samples no amplification was obtained.

Polymorphisms in primer annealing sites at the sequences in the *HE* gene could theoretically avoid efficient hybridization of primers, thus impeding the detection of the expected fragment. Although, given the high region conservation chosen for the primers design[23], it can be argued that this is not a probable cause for the failures, which can be primarily assigned to different biochemical efficiency of each reaction.

Sequence analysis of 14 out of the *HE* 16 amplicons revealed highly scored bases with high identity with GenBank BCoV *HE* sequences, showing that the assay described in the present study is a reliable way to obtain data for sequence-dependent surveys.

Even though the internal primer HE-NA has been designed specifically based on sequences derived from the *HE* gene of BCoV, the genetic proximity between this specie and other species of coronavirus belonging to Group 2, such as human coronavirus OC43[24], could putatively allow its application to studies on molecular diversity in a wider range of coronaviruses.

As a conclusion, a semi-nested RT-PCR assay was developed for a rapid, effective generation of amplicons for further applications on the fields of molecular epidemiology of BCoV-caused diseases and viral molecular evolution.

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