A NESTED POLYMERASE CHAIN REACTION TEST FOR BOVINE CORONAVIRUS DIAGNOSIS BASED ON THE RNA-DEPENDENT RNA-POLYMERASE GENE

ABSTRACT

Bovine coronavirus (BCoV) causes enteritis and respiratory illness in calves and dysentery in cows. Difficulties found in the production of large amounts of BCoV in cell culture and those regarding envelope integrity pose a major problem to antigen, hyperimmune sera and monoclonal antibodies production. This study aimed at the development of a polymerase chain reaction for BCoV detection in fecal samples of calves targeted to the gene of the RNA-dependent RNA-polymerase, and the comparison of this test with the hemagglutination/ hemagglutination inhibition test (HA/HI). Both tests were applied to 203 fecal samples. HA/HI resulted in an individual-level BCoV frequency of 35.47% and a farm-level frequency of 73.68%, while the proposed PCR resulted in an individual-level BCoV frequency of 25.12% and a farm-level frequency of 52.63%. Both kappa and Youden’s tests revealed a low agreement between PCR and HA/HI. Hypotheses are suggested to explain such a disagreement. The PCR proposed is a useful tool for the detection of BCoV, for epidemiological surveys and as a screening test for studies focused on other BCoV genes, such as the spike gene.

INTRODUCTION

Coronaviruses are classified in the order *Nidovirales*, family *Coronaviridae*, which comprises the genera *Coronavirus* and *Torovirus*. In this same order, one can also find the families *Arteriviridae* and *Roniviridae* (Van Regenmortel et al. 2000, González et al. 2003). The genus *Coronavirus* is still sub-divided into three groups (1, 2 and 3) according to epitopes and nucleotide sequences of envelope and non-structural proteins and natural hosts (Holmes & Lai 1996).

Bovine coronavirus (BCoV), with a diameter of 220nm, is a Group 2 species that causes enteritis and respiratory illness in calves and dysentery in adult cows (McNulty et al. 1984, Snodgrass et al.

Corresponding author: Paulo Eduardo Brandão, Instituto Pasteur de São Paulo, Avenida Paulista 393, 01311-000 Cerqueira César, São Paulo, SP, Brazil. Tel.: +55 11 3288-0088 ext. 116 E-mail: paulo7926@yahoo.com

Key words: coronavirus, diagnosis, PCR
The viral envelope is formed by a double lipid with five structural proteins (HE, S, sM, M and I) that produce the crown-like appearance of the virions under the electron microscope. The BCoV genome is a non-segmented positive sense single-stranded RNA with 32 kb that constitutes an helicoidal nucleocapsid in association with nucleoprotein (N) (Lai & Cavanagh 1997).

Direct diagnosis of BCoV-caused diarrheas began with electron microscopy by negative staining, but an accurate identification is difficult due to the high degree of pleomorphism shown by the virion and to the presence of particles and debris in fecal samples similar to the fringed appearance of coronaviruses, leading to misdiagnosis. The development of specific antibodies and gold-conjugates has made the visual identification of BCoV by immunoelectron microscopy easier (Clark 1993, McIntosh 1996). Nonetheless, regarding the high costs of implementation and costs per sample analyzed, the long time required and the need for skilled personnel, electron microscopy and immunoelectron microscopy became economically incompatible with laboratories with a high influx of samples.

ELISA techniques based on polyclonal sera and, currently, monoclonal-based ones, are largely employed to detect BCoV (Clark 1993). Difficulties found in the production of large amounts of BCoV in cell culture and those regarding envelope integrity, an essential feature of coronavirus immunogenicity, pose a major problem to antigen, hyperimmune serum and monoclonal antibody production. The simplest technique for the direct diagnosis of bovine coronavirus, once hyperimmune sera are available, is the hemagglutination/hemagglutination inhibition test as described by Sato et al. (1977), but the problems mentioned above remain unsolved. Thus, the development of fast, accurate, cheap and easy methods for BCoV diagnosis in stool samples of calves are a key factor in studies on the occurrence, transmission chain and control measures of bovine diarrheas.

The polymerase chain reaction is a highly applicable alternative, putting aside the need for sera and antibodies, allowing BCoV detection even if the virion is not intact, presenting high sensitivity and feasibility (Forghani et al. 1994).

This study aimed to develop of a polymerase chain reaction for bovine coronavirus detection in stool samples of calves and the comparison of this test with the hemagglutination/hemagglutination inhibition test.

**MATERIALS AND METHODS**

**Stool samples.**

Two hundred-three stool samples from 1 day to adult-aged cattle, both males and females, with or without diarrhea, were collected from 2000 through 2002 in 19 dairy farms located in Sào Paulo and Minas Gerais.

**Hemagglutination/hemagglutination inhibition test.**

The hemagglutination/hemagglutination inhibition test was carried out according to procedures described by Jerez et al. (2002). Stool samples were prepared as suspensions in PBS 0.01M / BSA 0.1% pH 7.2 (PBS) to a 1:4 final dilution and clarified by centrifugation (12,000xg/30’ at 4°C). Supernatants were diluted 2-fold in PBS in 96-well U-bottomed plates to a 25μL final volume/dilution, adding 25μL of a 0.4% hamster red cells suspension in PBS to each well. BCoV Kakegawa strain, kindly provided by Dr. Takeo Sakai, Nihon University, Japan, grown in HmLu-1 (hamster lung) cells was used as positive control and PBS as negative control. After 2 hours at room temperature, end point titers were read as the inverse of the last dilution in which hemagglutination was found. Samples with HA titers higher than 4 were tested in the hemagglutination inhibition test (HI) in 96-well V-bottomed plates, diluting stool suspensions 2-fold in PBS to a 25μL final volume/dilution; next, 25μL of a kaolin treated hyperimmune anti-BCoV sheep serum with 8 hemagglutination inhibition units were added. After an 1-hour incubation at 37 °C, 25μL of a 0.4% hamster red cell suspension in PBS were added to each well and plates were incubated for 2 hours at room temperature, when end point titers were read as the inverse of the last dilution in which hemagglutination could be found. Samples were considered positive if an at least 4-fold fall in HA titer was found.

**Polymerase chain reaction to the RNA-dependent RNA-polymerase gene (PCR pol).**

**Primer design** - A nested RT-PCR was developed internal to that described by Stephensen et al. (1999) targeting the RNA-dependent RNA-polymerase gene (RdRp gene). A pair of primers
(lower CV2L 5’ AACATCTTTAATAAGGCCGRCGTATC and upper CV2U 5’ TACTATGACTGCGCAAGATGTCTTCA 3’) was designed with Oligo software (©1999-2003 Molecular Biology Insights, Inc), with a 136bp-long predicted product based on sequences retrieved from GenBank related to the RdRp gene of bovine coronavirus, human coronavirus OC-43, porcine haemagglutinating encephalitis virus and rat sylodacryoadenitis virus (accession numbers AF124985, AF124989, AF124988 and AF124990).

c-DNA synthesis - Total RNA from stool samples was extracted with TRIzol reagent™ (Invitrogen™) and 7μL of each extracted RNA re-suspended in DEPC-treated water was denatured at 95℃ for 5 minutes and added to c-DNA mix containing 1x First Strand Buffer™ (Invitrogen™), 1mM of each dNTP, 10mM DTT, 1pmol/μL of each primer (4Bm and 2Bp as described by Stephensen et al. 1999) and 200U M-MLV Reverse Transcriptase to a 20μL final reaction. Reverse transcription was carried out at 42℃/10’. First round amplification and nested PCR - Next, 5μL of c-DNA were added to the PCR mix [1x PCR Buffer™ (Invitrogen™), 0.2mM of each dNTP, 0.5pmol/μL of each primer (4Bm and 2Bp), 1.5mM MgCl₂, 25.25μL ultra-pure water and 1.25U Taq DNA polymerase to a 50μL final reaction] and submitted to 94℃/5’, 6 cycles of 94℃/1’, 40℃/2’ and 72℃/1’, 36 cycles of 94℃/1’, 50℃/1.5’ and 72℃/1’, followed by 72℃/10’ for final extension. Second round (nested) amplification was carried out with 5μL of first PCR product added to the PCR mix [1x PCR Buffer™ (Invitrogen™), 0.2mM of each dNTP, 0.5pmol/μL of each CV2L and CV2U primers, 1.5mM MgCl₂, 25.25μL ultra-pure water and 1.25U Taq DNA polymerase] and submitted to 94℃/5’, 26 cycles of 94℃/1’, 54.8℃/1.5’ and 72℃/1’ followed by 72℃/10’ for final extension.

The Kakegawa strain of BCoV was used as the positive control sample and PBS was used as a negative control sample from the RNA extraction to the nested step. Each step (RNA extraction, c-DNA synthesis and PCR, nested and, finally, electrophoresis) was carried out in different rooms with exclusive equipment Additionally, an ultra-pure water-containing tube was added every three samples as an extra “nested-negative control” exclusively in the nested step, also added mix and submitted to thermocycler, in order to monitor amplicon contamination. Next, ten μL of the nested product were analyzed in 1.5% agarose gel electrophoresis stained with 0.5μg/mL ethidium bromide, positive samples being those producing the 136-bp predicted fragment.

Comparison between PCR and the hemagglutination/hemagglutination inhibition test.

Dichotomous results produced by the tests expressed as positive and negative were analyzed with Kappa statistics and Youden’s J test with 99.5% confidence level with WinEpiscope 2.0 software.

RESULTS

The hemagglutination/hemagglutination inhibition test in the 203 stool samples resulted in an individual-level BCoV frequency of 35.47% (72/203) and a farm-level frequency of 73.68% (14/19) (Tables 1 and 2), positive farms being those with at least one positive sample.

The proposed PCR resulted in an individual-level BCoV frequency of 25.12% (51/203) and a farm-level frequency of 52.63% (10/19) (Tables 1 and 2), positive farms being those with at least one positive sample. No bands were found either in the reactions correspondent to the PBS included as negative control, nor in the nested negative controls.

The comparison between the results produced by both tests resulted in a Kappa value of -0.048 and -0.08 to the individual and per farm results, respectively and Youden’s J values of -0.045 and -0.1 to the individual and per farm results, respectively.

Table 1 – Individual results of PCR and HA/HI tests for BCoV detection in fecal samples from calves.

<table>
<thead>
<tr>
<th></th>
<th>PCR pol</th>
<th>HA/HI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>56</td>
<td>96</td>
</tr>
<tr>
<td>TOTAL</td>
<td>72</td>
<td>131</td>
</tr>
</tbody>
</table>
Table 2 – Per farm results of PCR and HA/HI tests for BCoV detection in fecal samples from calves.

<table>
<thead>
<tr>
<th>PCR pol</th>
<th>HA/HI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>7</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
</tr>
</tbody>
</table>

DISCUSSION

Kappa statistics express the proportion of agreement not due to chance. A kappa of zero indicates no agreement beyond chance, a kappa from 0.4 to 0.5 indicates moderate agreement and a kappa of 1 indicates perfect agreement (Martin et al. 1987). As kappa values to the comparison between HA/HI and PCR pol were lower than zero both regarding individuals and farms, there was no agreement between these two tests.

Youden’s J test combines information about sensitivity and specificity into a single number and can take values between 1 and -1. As sensitivity and specificity increase, the value of Youden’s J increases. When the sum of sensitivity and specificity is 200%, J = 1, when it is 100%, J = 0 and when it is 0%, J = -1 (Armitage & Berry, 1987). Thus, as the Youden’s J for comparison between HA/HI and PCR pol was lower than zero both individuals and farms, the low agreement between these two tests is confirmed.

Two reasons for disagreement between HA/HI and PCR pol are possible:

Samples positive in HA/HI and negative in PCR: hypothesis linked to the stool samples, to the reactions conditions and to BCoV itself may help to explain such disagreement. Although hemagglutination inhibition is a specific test, in the present survey it was carried out with a polyclonal hyperimmune serum, which might have raised false-positive results in the HA/HI test by nonspecific reactions between this serum and other possible viruses, bacteria or other hemagglutinating elements present in the samples, once the sheep used to produce this serum was not specific pathogen free and might have had contact with such agents, ending up in hemagglutination inhibition in its serum. Still, variations in red cell suspension concentration may have contributed to variations in the hemagglutination titer readings, inserting here a tendency to generate false positive results.

However, it should be taken into account that conditions linked to the samples, such as storage time, since some of the samples were tested at different times by to HA/HI and PCR, and the presence of RNAses and PCR inhibitors in the fecal samples might have contributed to PCR false-negative results in samples that were truly positive in HA/HI.

Besides, it is possible that polymorphisms in the RdRp gene might have avoided primer annealing and amplification of the target segment, also raising false-negative results, although this gene is highly conserved in the genus Coronavirus (Stephensen et al. 1999).

Samples negative in the HA/HI test and positive in PCR: This kind of disagreement is most probably due to the known low detection limit of PCR assays, that allows the detection of viruses even if these are present in low concentration, such concentration being unreachable by the HA/HI detection. So, a BCoV-containing sample might have been classified as negative in HA/HI by a viral titer not high enough to allow the detection by this method, but high enough for PCR detection.

The PCR here proposed and the traditional HA/HI test detected BCoV both in samples from calves with and without diarrhea (data not shown). Nevertheless, as the present study was not aimed to investigate the causality of diarrhea regarding bovine coronavirus, which has already been done (Snodgrass et al., 1986), but rather to propose a diagnostic tool to detect this virus in diarrheic calves as well as in non-diarrheic ones, this issue will not be discussed herein.

The hemagglutination/hemagglutination inhibition test permits bovine coronavirus diagnosis at low cost and easily, but requires the use of laboratory animals, besides the possibility of false positive results when a polyclonal serum is used.

Nucleotide-based diagnosis is an alternative to serological reactions, since they allow a large number of samples to be processed and avoid the need for laboratory animals, allowing also higher inter-test and inter-laboratory precision, eliminating variations due to red cells and the quality of hyperimmune sera, presenting also a lower detec-
tion limit and a higher theoretical specificity when compared to HA/HI.

The per farm rather than per animal diagnosis is a powerful approach in preventive veterinary medicine, since it allows one to treat the whole farm as an individual both during surveys for diseases and intervention programs, i.e. the detection of a single positive or infected individual is enough to consider the whole farm as positive or "infected", saving money and time during sanitary interventions.

The polymerase chain reaction here proposed is a useful tool for the detection of bovine coronavirus, as well as for more comprehensive epidemiological surveys of BCoV-caused diseases, still scarce in Brazil, and as a screening test for studies focused on other BCoV genes, such as the spike gene, targeting genealogic studies on this virus.

ACKNOWLEDGEMENTS

The authors are grateful to FAPESP for the financial support, Laboratório Biovet for the cell culture medium and to Mr. Alexandre Abelardo Sanches for his technical support.

REFERENCES


