DOI: 10.1590/1808-1657v70p0012003

NESTED PCR ASSAY FOR DETECTION OF BOVINE CORONAVIRUS S1 GENE

P.E. Brandão*, F. Gregori, G.S. Monteleone, R.M. Soares, C.A.R. Rosales, J.A. Jerez

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, CEP 05508-900, São Paulo, SP, Brasil. E-mail: paulo@vps.fmvz.usp.br

ABSTRACT

A nested PCR targeted to the S1 ectodomain gene of the spike (S) glycoprotein of coronaviruses was developed to detect bovine coronavirus (BCoV) in stool specimens from cattle. One outer and one internal pair of primers were designed to conserved regions surrounding the hypervariable region of the S gene (GenBank accession No. M31053). Outer primers produced an 885bp-long product (nn 1204 to 2088 from S gene) and the internal primers a 488bp-long product (nn 1329 to 1816 from S gene, internal to the first PCR). Optimal annealing temperatures were tested in a gradient thermocycler and used in the final protocols. Outer primers and the final nested protocol detection limits were tested with the BCoV Kakegawa strain diluted two-fold in BCoV-free stool suspension. The nested PCR assay was tested in 22 clinical stool specimens from calves with or without diarrhea and 1 sample from a diarrheic cow, resulting in 10 positive samples both from diarrheic and non-diarrheic individuals. The nested PCR reported here is a specific and sensitive tool for bovine coronavirus diagnosis.

KEY WORDS: Coronavirus, bovine, diagnosis, PCR.

RESUMO

REAÇÃO DE "NESTED PCR" PARA DETECCÃO DO CORONAVÍRUS S1. Desenvolveu-se uma reação de "nested PCR" direcionada para o gene do ectodomínio S1 da glicoproteína S dos coronavírus para a detecção de coronavírus bovino (BCoV) em amostras fecais. Foram desenhados um par de primers externos e um par de primers internos para regiões conservadas flanqueando a região hipervariável do gene S (GenBank accession No. M31053). Os primers externos produziram um fragmento de 885pb (nn 1204 ao 2088 do gene S) e os internos um fragmento de 488bp (nn 1329 ao 1816 do gene S, interno ao produto do primeiro PCR). Temperaturas ótimas de hibridação dos primers foram testadas em um termociclador com gradiente e utilizadas no protocolo final. Os limites de detecção para os primers externos e para o protocolo "nested" final foram testados com a amostra Kakekawa de BCoV diluída em base 2, em uma suspensão de fezes livre de BCoV. O protocolo final de "nested" foi testado em 22 amostras de fezes de bezerros diarréicos e não-diarreicos e em uma amostra de uma vaca com diarréia, resultando em 10 amostras positivas entre fezes diarréicas e não-diarreicas. O nested PCR aqui relatado é uma ferramenta específica e sensível para o diagnóstico de coronavírus bovino.

PALAVRAS-CHAVE: Coronavírus, bovino, diagnóstico, PCR.

INTRODUCTION

Coronaviruses are round-shaped enveloped virus, about 120 nm in diameter, constructed by six or five structural proteins (N, M, sM, HE, S and I), depending on the viral species, classified in the order Nidovirales, family Coronaviridae, which comprises the genera *Coronavirus* and *Torovirus*. The genome is constituted by a positive-sense single-stranded RNA, which forms the helical nucleocapside in association with protein N. The viral envelope is formed by a lipidic double-layer with 4 to 5 structural proteins that project from it, giving rise to a spiked/crowned virion (Fischer et al., 1997; Lai & Cavanagh, 1997).

In cattle, the most known coronaviral disease is neonatal calf diarrhea, which affects 3- to-4-week-old calves (Pensaert et al., 1994). Older calves, about 3 months old, may also show a coronavirus-born upper respiratory tract disease, caused by the same BCoV, what raised the hypothesis that the same virus can

*Corresponding author.

cause both syndromes and an initial respiratory infection can evolve to enteritis by virus ingestion (McNulty et al., 1984; Heckert et al., 1990; Heckert et al., 1991; Tsunemitsu et al., 1991). Adult cows present a diarrheic disease named winter dysentery, first described in the USA, also caused by the bovine coronavirus similar to that found in neonatal diarrhea (Benfield & Saif, 1990; Dea et al., 1995).

As BCoV-caused diarrhea is a widespread disease that causes large economic losses in beef and dairy cattle, one needs a reliable diagnostic test to apply specific preventive and control measures and to conduct epidemiological surveys. Although BCoV diagnosis is based on ELISA kits or haemagglutination/haemagglutination inhibition tests, PCR is considered as a more advantageous method for viral diagnosis, since it puts aside the need for polyclonal hyperimmune sera or monoclonal antibodies, allowing diagnosis even if viruses are not antigenically reactive, with high sensitivity and specificity and economic viability for viral diagnosis (Forghani et al., 1994).

This article describes a nested-PCR assay targeted toc the BCoV S1 gene to be applied to stool samples from cattle suspected of BCoV infection.

MATERIALS AND METHODS

Primer designing

Two pairs of primers were designed for conserved regions flanking the hypervariable region (Hasoksuz et al., 2002) of the S gene (GenBank accession No. M31053): outer primers (sense S1HS 5'-CTATACCCAATGGTAGGA-3' and anti-sense S1HA 5'-CTGAAACACGACCGCTAT-3') with a predicted 885bp-long product (nn 1204 to 2088 from S gene) and internal primers (sense S1N 5'-GTTTCTGTTAGCAGGTTTAA-3' and anti-sense S1NA 5'-ATATTACACCTATCCCCTTG-3') with a predicted 488bp-long product (nn 1329 to 1816 from the S gene, internal to the first PCR product). Each primer was submitted to BLASTn to look for the most similar sequences and possible non-BCoV related similarities.

Annealing temperatures

Optimal annealing temperatures were found testing primers in a temperature gradient in an Eppendorf™ Mastercycler Gradient thermocycler with the BCoV Kakegawa strain as a sample. Reverse transcription reaction was carried out at 42º C/60' in a reaction mix containing 1 x First Strand Buffer (Invitrogen™), 1mM of each dNTP, 10mM DTT, 1pmol/µL of each primer (S1HS and S1HA or S1NS and S1NA), 7 µL of RNA extracted by the TRIzol (Invitrogen™) method and denatured at 95º C/5' and 200U M-MLV Reverse Transcriptase (Invitrogen™) for a 20µL final reaction. Next, 5 µL of c-DNA were added to the PCR mix [1 x PCR Buffer (Invitrogen™), 0.2mM of each dNTP, 0.5 pmol/µL of each primer (S1HS and S1HA or S1NS and S1NA), 1.5mM $MgCl_2$, 25.25 µL ultra-pure water and 1.25U Taq DNA polymerase for a 50µL final reaction] and submitted to 35 cycles of 94º C/1' for DNA denaturation, 53º C with 5º C gradient/1.5' for primers annealing and 72º C/1'for DNA extension and 72º C/10' for final extension. Best annealing temperatures (53.4º C for outer and 58.4º C for inner primers) were found as the temperatures yielding the strongest 885bp amplified fragment (for outer primers) or 488bp amplified fragment (for inner primers) as seen in 2% agarose gel electrophoresis stained with 0.5 mg/mL ethidium bromide.

Detection limits

Detection limit for outer primers S1HS and S1HA was found testing BCoV Kakegawa strain (HA titer = 256) diluted two-fold in a BCoV-free stool suspension (as previously diagnosed by haemagglutination/haemagglutination inhibition test according to Jerez et al., 2002) in PBS 0.01M/BSA 0.1% pH 7.2 with reverse transcription and PCR conditions as previously described except that annealing temperature was 53.4º C. Detection limit for inner primers was found diluting the BCoV Kakegawa strain two-fold in a BCoV-free stool suspension in PBS 0.01M/BSA 0.1% pH 7.2 in a nested PCR with the annealing temperature found in the gradient test and 5µL of the first round PCR product with outer primers.

Clinical samples test

The final nested PCR protocol was applied to 23 stool samples (22 from calves and 1 from a 58-month old diarrheic cow) prepared as suspensions in PBS 0.01M/BSA 0.1% pH 7.2 and clarified by centrifugation (12.000g/30'). BCoV Kakegawa strain was used as positive and PBS 0.01M/BSA 0.1% pH 7.2 as negative controls. Reverse transcription reaction was carried out at 42º C/60' in a reaction mix containing 1 x First Strand Buffer (Invitrogen™), 1mM of each dNTP, 10mM DTT, 1pmol/µL of each primer (S1HS and S1HA), 7 µL of RNA extracted with TRIzol (Invitrogen™) and 200U M-MLV Reverse Transcriptase (Invitrogen™) for a 20µL final reaction. Next, 5 µL of c-DNA were added to the PCR mix (1 x PCR Buffer (Invitrogen™), 0.2mM of each dNTP, 0.5 pmol/µL of each outer primer, 1.5mM $MgCl_2$, 25.25 µL ultra-pure water and 1.25U Taq DNA polymerase for a 50µL final reaction) and submitted to 35 cycles of 94º C/1', 53.4º C/1.5' and 72º C/1' and 72º C/10' for final extension. Second round amplification was carried out with 5 µL of first PCR product added to the PCR mix [1 x PCR Buffer (Invitrogen™), 0.2mM of each

dNTP, 0.5 pmol/µL of each S1NS and S1NA primers, 1.5mM $MgCl_2$, 25.25 µL ultra-pure water and 1.25U Taq DNA polymerase] and submitted to 25 cycles of for 94º C/1', 58.4º C /1.5' and 72º C/1' for DNA extension and 72º C/10' for final extension. An ultra-pure water-containing tube was added every three samples as nested negative control, mix was also added and it was submitted to a thermocycler.

RESULTS AND DISCUSSION

Both outer and inner primers were able to detect BCoV Kakegawa strain until 1:2 dilution in the detection limit assay. Ten out of the 23 samples tested in the final nested protocol resulted positive to bovine coronavirus, including that from the diarrheic cow, according to the appearance of the 488bp-long predicted fragment in 2% agarose gel electrophoresis stained with 0.5 mg/mL ethidium bromide, as seen for the BCoV Kakegawa positive control. Neither the nested negative controls nor the PBS 0.01 M BSA 0.1% pH 7.2 showed bands. No non-specific band was found in agarose gels. BLASTn showed BCoV S gene sequence as the highest ranked sequence, with no other important sequence related to the primers tested.

Test with clinical samples showed that this protocol is applicable to BCoV diagnosis directly from calf stool specimens, since it allowed for evidencing of 10 positive samples with no non-specific reactions. It was possible to detect BCoV in clinically normal individuals, an essential approach concerning epidemiological surveillance in cattle breeding as it allows one to apply preventive measures prior to the emergence of diarrhea on a farm. Also, it may be useful for to the diagnosis of winter dysentery, a disease newly reported in Brazil (Brandão et al., 2002) that affects adult cows, as evidenced by the PCR-positive diarrheic cow found in the clinical samples test. Besides, since both pairs of primers used are targeted to a gene specific to bovine coronavirus and PCR conditions were the most stringent possible, one can expect a high specificity related to the present protocol.

The protocol described allows for a fast and reliable method for bovine coronavirus detection in stool samples and may be carried out by any laboratory with minimal conditions for molecular biology procedures without the need for the production of hyperimmune sera, monoclonal antibodies and the purchase of diagnostic kits.

References

Benfield, D.A. & Saif, L.J. Cell culture propagation of a bovine coronavirus isolated from cows with winter dysentery. *J. Clin. Microbiol.*, v.28, n.6, p.1454-1457, 1990.

Brandão, P.E.; Birgel JR., E.H.; Gregori, F.; Rosales, C.A.R.; Ruiz, V.L.A.; Jerez, J.A. Bovine coronavirus detection in adult cows in Brazil. *Arq. Inst. Biol.*, São Paulo, v.69, n.2, p.103-104, 2002.

Dea, S.; Michaud, L.; Milane, G. Comparison of bovine coronavirus isolates associated with neonatal calf diarrhoea and winter dysentery in adult dairy cattle in Québec. *J. Gen. Virol.*, v.76, p.1263-1270, 1995.

Fischer, F.; Ding, P.; Hingley, S.T.; Weiss, S.R.; Masters, P.S. The internal open reading frame within the nucleocapsid gene of mouse hepatitis virus encodes a structural protein that is not essential for viral replication. *J. Virol.*, v.71, n.2, p.996-1003, 1997.

Forghani. B. & Erdman, D.D. Amplification and detection of viral nucleic acids. In: Schmidt, J.N. & Emmons, R.W. *Diagnostic procedures for viral, rickettsial and chlamydial infections.* Washington, DC: American Public Health Association, 1994. p.97-120.

Hasoksuz, M.; Sreevatsan, S.; Kyoung-Oh, C.; Hoet, A. E.; Saif, L.J. Molecular analysis of the S1 subunit of the spike glycoprotein of respiratory and enteric bovine coronavirus isolates. *Virus Res.*, v.84, p.101-109, 2002.

Heckert, R.A.; Saif, L.J.; Hoblet, K.H.; Agnes, A.G. A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. *Vet. Microbiol.*, v. 22, p. 187-201, 1990.

Heckert, R.A.; Saif, L.J.; Myers, G.W.; Agnes, A.G. Epidemiologic factors and isotype-specific antibody responses in serum and mucosal secretions of dairy calves with bovine coronavirus respiratory tract and enteric tract infections. *Am. J. Vet. Res.*, v.52, n.6, p.845-851, 1991.

Jerez, J.A.; Brandão, P.E.; Buzinaro, M.G.; Gregori, F.; Rosales, C.A.R.; Ito, F.H.; Sakai, T. Detecção de rotavírus e coronavírus em fezes de bezerros neonatos com diarréia criados em vários municípios do Estado de São Paulo, Brasil. *Arq. Inst. Biol.*, São Paulo, v.69, n.2, p.19-23, 2002.

Lai, M.C.M. & Cavanagh, D. The molecular biology of coronaviruses. *Adv. Virus. Res.*, v.48, p.1-100, 1997.

McNulty, M.S.; Bryson, D.G.; Alan, G.M.; Logan, E.F. Coronavirus infection of the bovine respiratory tract. *Vet. Microbiol.*, v.9, p.425-434, 1984.

Pensaert, M.; Callebaut, P.; Cox, E. Enteric coronaviruses of animals. In: Kapikian, A.Z. *Viral Infections of the Gastrointestinal tract.* 2.ed. New York: Marcel-Dekker, 1994. p.627-696.

Tsunemitsu, H.; Yonemichi, H.; Hirai, T.; Kudo, T.; Onoe, S.; Mori, K.; Shimizu. M. Isolation of bovine coronavirus from feces and nasal swabs of calves with diarrhea. *J. Vet. Med. Sci.*, v.53, n.3, p.433-437, 1991.

Received on 23/9/02
Accepted on 3/1/03