

Detection of Bovine Coronavirus by Conventional Reverse Transcription Polymerase Chain Reaction

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Abstract

Bovine coronavirus (BCoV) is an economically significant cause of enteric and respiratory diseases in cattle throughout the world. BCoV is a known cause of neonatal calf diarrhea, winter dysentery in adult cattle, and respiratory disorders in cattle of all ages. In this chapter, we describe a simple and efficient protocol for total nucleic acids extraction to be used in conventional RT-PCR assay. This is a technique used routinely in our virology laboratory to detect BCoV from stool and nasopharyngeal samples of cattle.

Key words Bovine coronavirus, RT-PCR, Clinical diagnosis, Stool samples, Nasopharyngeal samples

1 Introduction

Bovine coronavirus (BCoV) is a member of the order *Nidovirales*, *Coronaviridae* family, which was recently classified as member of the specie *Betacoronavirus 1* in the genus *Betacoronavirus* [1].

BCoV are frequently circulating in cattle farms worldwide, causing both enteric and respiratory disease in calves and adult cattle [2]. Because the respiratory and enteric disorders are similar to the other infectious diseases, a specific test is needed for a conclusive diagnosis of BCoV infection. Besides sensitive tests are required to detect BCoV especially at early or late stages of disease when they have low levels of viral shedding.

The current methods used for the diagnosis of BCoV consist mostly of the detection of viral RNA by conventional (RT-PCR) and real-time reverse transcription polymerase chain reaction (qRT-PCR) [3–6]. Recently, isothermal nucleic acid amplification techniques, such as recombinase polymerase amplification (RPA) assay and reverse transcription loop-mediated isothermal amplification (RT-LAMP), have been developed for rapid detection of BCoV [7, 8]. However, there are still few reports evaluating

these new assays in large-scale epidemiological studies of BCoV infections.

In this chapter, we describe a sensitive and specific conventional RT-PCR assay that has been successfully applied for diagnosis of both enteric and respiratory bovine coronaviruses [9–12].

2 Materials

2.1 RNA Extraction

1. Sodium dodecyl sulfate (SDS) 10 %.
2. Ultrapure phenol–chloroform–isoamyl alcohol (25:24:1, v/v) (Invitrogen).
3. Silicon Dioxide (SiO_2) (Sigma).
4. Guanidine isothiocyanate (GuSCn) (Invitrogen).
5. Acetone PA.
6. Ethanol solution 70 % (in water).
7. Diethylpyrocarbonate (DEPC)-treated water (Invitrogen).
8. EDTA.
9. Hydrochloric acid 32 %—HCl 32 % (Sigma).
10. Triton X-100 (Invitrogen).
11. Tris (hydroxymethyl)aminomethane (TRIS).
12. Lysis buffer L6.
13. Washing buffer L2.

2.2 Clinical Samples (See Note 1)

Fecal samples: The samples were prepared either as 10 % (w/v) suspensions of solid or semisolid feces in 0.01 M phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4 ; 15 mM KH_2PO_4) or as 50 % (v/v) suspensions of liquid feces in 0.01 M PBS and centrifuged at $3000 \times g$ for 15 min at 4 °C. The supernatant is transferred to a sterile tube. Separate an aliquot of 400 μl for RNA extraction.

Nasopharyngeal swab samples: the tip of swab containing nasopharyngeal secretions is soaked in 1 ml of sterile saline solution or 0.01 M PBS. The swab is vortex-mixed for 15 s and then discarded. Centrifuge at $3000 \times g$ for 15 min at 4 °C. The supernatant is transferred to a sterile tube. Separate an aliquot of 450 μl for RNA extraction.

2.3 Preparing Reagent and Solutions Used for RNA Extraction

Silica suspension is prepared as described by Boom et al. [13] with minor modifications.

1. Suspend six grams of silicon dioxide (SiO_2) in 50 ml of sterile distilled water in a glass graduated cylinder.
2. Slowly stir and keep at rest at room temperature for 24 h for the silica coarse particles to settle.

2.3.1 Silica Hydration Process

3. Remove and discard 43 ml of the supernatant from the top with vacuum suction or pipette. Then, resuspend the silica by adding 50 ml of sterile distilled water.
4. Slowly stir and leave for 5 h.
5. Remove and discard 44 ml of the supernatant from the top with vacuum suction or pipette.
6. Add 60 μ l of concentrated HCl (32 % w/v) to adjust pH (pH=2.0).
7. Mix the final content (resulting suspension of silica coarse) and divide into small aliquots (4 ml) into glass bottles with autoclavable cap, tightly closed.
8. Sterilize in an autoclave at 121 °C for 20 min. Store at 4 °C.

2.4 Prepare of Lysis Buffer L6 and Washing Buffer L2

2.4.1 0.1 M Tris-HCl (pH 6.4)

Prepare the following fresh solutions before preparing lysis buffer:

Dissolve 2.67 g of TRIS in 180 ml of distilled water. Add HCl 32 % to adjust the pH to 6.4. Stir vigorously on a magnetic stirrer. Fill up to volume 220 ml with distilled water and set aside at room temperature until use.

2.4.2 0.2 M EDTA (pH 8.0)

Add 1.86 g of EDTA in 15 ml of distilled water. Adjust the pH to 8.0 with NaOH. Fill up to volume 25 ml with distilled water and set aside at room temperature until use (*see Note 2*).

2.4.3 Lysis Buffer L6

1. Dissolve 120 g of GuSCN in 100 ml of 0.1 M Tris-HCl (pH 6.4) in a beaker.
2. Add 22 ml of a 0.2 M EDTA solution (pH 8.0).
3. Add 2.6 g of Triton X-100.
4. Homogenize vigorously the solution (*see Note 3*).
5. Transfer the solution into a glass bottle with autoclavable cap.
6. Sterilize in an autoclave at 121 °C for 20 min. Store at 4 °C (*see Note 4*).

2.4.4 Washing Buffer L2

1. Dissolve 120 g of GuSCN in 100 ml of 0.1 M Tris-HCl (pH 6.4) in a beaker.
2. Homogenize vigorously the solution (*see Note 3*).
3. Transfer the solution into a glass bottle with autoclavable cap.
4. Sterilize in an autoclave at 121 °C for 20 min. Store at 4 °C.

2.5 Reverse Transcription

1. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 200 U/ μ l (Invitrogen).
2. 5 \times First-Strand Buffer: 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂ (Invitrogen).

3. 0.1 M dithiothreitol (DTT) (Invitrogen).
4. 20 μ M reverse primer BCoV2: 5'-TGTGGGTGCGAGTTCTGC-3' (nt 940–957).
5. Deoxynucleotide triphosphates (dNTP) mix, 10 mM each (Invitrogen).
6. DEPC-treated water (Invitrogen).
7. Thermocycler (Applied Biosystems).

2.6 Polymerase Chain Reaction

1. Platinum *Taq*DNA polymerase, 5 U/ μ l (Invitrogen).
2. 10 \times PCR buffer (Invitrogen).
3. 50 mM MgCl₂ solution (Invitrogen).
4. Deoxynucleotide triphosphates (dNTP) mix, 10 mM each.
5. 20 μ M PCR forward primer, BCoV1: 5'-CGATGAGGCTATTCCGAC-3' (nt 504–521).
6. 20 μ M PCR reverse primer, BCoV2: 5'-TGTGGGTGCGAGTTCTGC-3' (nt 940–957).
7. Thermocycler (Applied Biosystems).

2.7 Semi-nested Polymerase Chain Reaction

1. Platinum *Taq*DNA polymerase, 5 U/ μ l (Invitrogen).
2. 10 \times PCR buffer (Invitrogen).
3. 50 mM MgCl₂ solution (Invitrogen).
4. Deoxynucleotide triphosphates (dNTP) mix, 10 mM each.
5. 20 μ M PCR forward primer, BCoV3: 5'-TTGCTAGTCTTGTCTGGC-3' (nt 707–725).
6. 20 μ M PCR reverse primer, BCoV2: 5'-TGTGGGTGCGAGTTCTGC-3' (nt 940–957).
7. Thermocycler (Applied Biosystems).

2.8 Gel electrophoresis

1. 1 \times TRIS–borate–EDTA buffer pH 8.0 (0.89 M TRIS; 0.89 M boric acid; 0.02 M EDTA).
2. UltraPure agarose (Invitrogen).
3. 123 bp DNA ladder markers (Invitrogen).
4. Ethidium bromide, 10 mg/ml.
5. Agarose gel electrophoresis system (Apelex).
6. Power supply (Apelex).
7. Gel Documentation and Analysis System (Kodak).
8. Gel loading buffer (6 \times): 10 mM Tris–HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol, 60 % glycerol, and 60 mM EDTA.

3 Methods

The protocols described below are routinely used for clinical diagnosis of BCoV in cases of neonatal diarrhea, winter dysentery in adult cattle, and respiratory syndrome in cattle of all ages [9, 10].

Firstly, we describe how to obtain high quality nucleic acid total for routine molecular biology applications such as PCR and sequencing. We present an efficient and reproducible protocol for extracting RNA from feces and nasopharyngeal swab samples for BCoV diagnosis. This protocol, which we call phenol chloroform silica method, is based on cellular lysis and protein denaturation by SDS and phenol–chloroform–isoamyl alcohol treatment followed by nucleic acid purification by the guanidinium thiocyanate and silica [14]. Guanidinium thiocyanate is an excellent protein denaturant and hence very effective in inactivating nucleases. Using this protocol extraction it is possible to obtain high quality of RNA in the laboratory on a routine basis without the need for expensive commercial kits extractions.

For RT-PCR assay we chose the N gene because it is highly conserved among BCoV strains (*see Note 5*). Also it is known that the N protein is the most abundant antigen in coronavirus-infected cells because its RNA template is the smallest and it has the most abundant sgRNA (subgenomic RNA) during transcription [15]. This indicates that there is more available RNA for the N gene than for the other BCoV protein genes. Consequently, detection of the N gene RNA might be advantageous due to its high abundance in cells, facilitating a high sensitivity of the diagnostic technique.

3.1 RNA Extraction

1. Transfer 450 μ l of the fecal or nasopharyngeal suspensions into a 1.5 ml polypropylene microtube. Include at least one positive control (HRT-18 cell culture-adapted BCoV Mebus strain or BCoV wild type strains) and one negative control (DEPC-treated water) in all the RNA extraction procedures.
2. Add 50 μ l of SDS 10 % to a final concentration of 1 % (v/v).
3. Vortex for 5 s and incubate in a water bath at 56 °C for 30 min.
4. Add an equal volume (500 μ l) of ultrapure phenol–chloroform–isoamyl alcohol to the microtube (*see Note 6*). For nasopharyngeal swab samples the treatment with phenol–chloroform–isoamyl alcohol (**steps 2–5**) are not performed.
5. Vortex vigorously for 20 s to mix the phases and incubate in a water bath at 56 °C for 15 min.
6. Centrifuge at 10,000 $\times g$ for 10 min to separate the phases (upper aqueous phase, interface, and lower organic phase).
7. Use a micropipette to remove the aqueous phase (upper aqueous phase) into a new 1.5 ml polypropylene microtube. Discard the

- microtube containing the interface (between the organic and aqueous phases) and organic phase (lower) (*see* **Notes 7 and 8**).
8. Add 30 μl of hydrated silica (*see* **Note 9**).
 9. Add 900 μl of lysis buffer L6.
 10. Vortex vigorously for 20 s.
 11. Place the microtubes in a rack and then incubate on an orbital shaker for 30 min at room temperature.
 12. Briefly centrifuge the microtubes at $10,000 \times g$ for 30 s to pellet the silica resin.
 13. Gently discard the supernatant by inversion (*see* **Note 10**).
 14. Add 500 μl of ice-cold washing buffer L2 to the pellet. Vortex for 5 s to resuspend the silica pellet.
 15. Briefly centrifuge the microtubes at $10,000 \times g$ for 30 s to pellet the silica resin.
 16. Gently discard the supernatant by inversion (*see* **Note 10**).
 17. Once again, add 500 μl of ice cold washing buffer L2 to the pellet. Vortex for 5 s to resuspend the silica resin. Repeat **steps 13 and 14**.
 18. Add 1 ml of ice-cold ethanol 70 % to the silica pellet. Vortex for 5 s to resuspend the silica pellet.
 19. Briefly centrifuge the microtubes at $10,000 \times g$ for 30 s to pellet the silica resin.
 20. Gently discard the supernatant by inversion (*see* **Note 10**).
 21. Once again add 1 ml of ice cold ethanol 70 % to the pellet. Vortex for 5 s to resuspend the silica resin. Repeat **steps 17 and 18**.
 22. Add 1 ml of ice-cold acetone PA to the silica pellet. Vortex for 5 s to resuspend the silica pellet.
 23. Centrifuge the microtubes at $10,000 \times g$ for 60 s to pellet the silica resin.
 24. Gently discard the supernatant by inversion (*see* **Note 10**).
 25. Place the microtubes with open lids in an inverted position over a clean filter paper to drain off the acetone excess.
 26. Return the tubes to their normal position and keep them with lids open in a thermoblock (dry block heater) at 56 °C for 15 min to evaporate the acetone and dry the silica pellet.
 27. Add 50 μl of DEPC water. Vortex for 5 s to resuspend the silica pellet.
 28. Incubate the microtubes with the lids closed in a water bath at 56 °C for 15 min to elute nucleic acid from the silica resin.

29. Centrifuge the microtubes at $10,000 \times g$ for 2 min to pellet the silica resin.
30. Remove the supernatant (nucleic acid eluted) with a micropipette into a new 0.5 ml polypropylene microtube. Discard the microtube containing the silica pellet.
31. Store the eluted nucleic acid at -20 or -80 °C until use (*see Note 11*).

3.2 Reverse Transcription (See Note 12)

1. Prepare a reverse transcription (RT) master mix in a volume sufficient for the number of reactions plus 1 in a sterile 1.5 ml polypropylene microtube (label it “RT mix”). The volume of each RT reagent per reaction and the initial and final concentrations are shown in Table 1.
2. Vortex and centrifuge the tube briefly. Keep the microtube “RT mix” on an ice bath until use.
3. Label appropriately the 0.5 μ l polypropylene microtubes with the sample identification.
4. Add to each microtube 5 μ l of the corresponding sample (eluted RNA), 1 μ l of reverse primer BCoV2 and 4 μ l of DEPC-treated water.
5. Incubate the microtubes at 97 °C in a heat block for 4 min.
6. Immediately after denaturing place on ice for at least 5 min.
7. Add 10 μ l of “RT mix” solution into each microtube.
8. Vortex and centrifuge the tubes briefly.
9. In a thermocycler, incubate the microtubes at 42 °C for 30 min, inactivate the transcription reaction at 95 °C for 5 min and then chill the samples on ice bath.
10. Store the cDNA samples at -20 °C until use.

Table 1
Components of reverse transcription reaction

Reagent	Volume per reaction (μ l)	Volume mix for N reactions (μ l)	Final concentration
5 \times First strand buffer	4	$4 \times N$	1 \times
0.1 mM DTT	2	$2 \times N$	0.01 mM
10 mM dNTP	1	N	0.5 mM
MMLV reverse transcriptase (200 U/ μ l)	0.5	$0.5 \times N$	100 U/reaction
Water	2.5	$2.5 \times N$	–
Total volume of RT master mix	10	$10 \times N$	–

3.3 PCR Assay

1. Prepare a PCR master mix in a volume sufficient for the number of reactions plus 1 in a sterile 1.5 ml polypropylene microtube and label it “PCR mix.” The volume of each PCR reagent per reaction and the initial and final concentrations are shown in Table 2.
2. Vortex and centrifuge the tube briefly. Keep the “PCR mix” on ice bath.
3. Dispense 42 μl of the “PCR mix” into separate 0.5 polypropylene microtube and label the tubes accordingly.
4. Add 8 μl of cDNA generated from the reverse transcription reactions into the correspondent tubes.
5. Vortex and centrifuge the tubes briefly.
6. Run the PCR under the conditions shown in Table 3.

Table 2
Components of the PCR assay

Reagent	Volume per reaction (μl)	Volume mix for N reactions (μl)	Final concentration
10 \times First strand buffer	7.5	$7.5 \times N$	1.5 \times
MgCl ₂ , 50 mM	2	$2 \times N$	2 mM
dNTP, 10 mM	4	$4 \times N$	0.8 mM
Reverse primer BCoV2 (20 μM)	1	N	0.4 μM
Forward primer BCoV1 (20 μM)	1	N	0.4 μM
DNA polymerase (5 U/ μl)	0.5	$0.5 \times N$	2.5 U/reaction
Water	26	$26 \times N$	–
Total	42	$42 \times N$	–

N = number of 0.2 ml tubes

Table 3
Conditions for PCR assay

Step	Temperature ($^{\circ}\text{C}$)	Time
1. Heat activation	94	4 min
2. Thermal cycling (40 cycles)		
Denaturing step	94	1 min
Annealing step	55	1 min
Extension	72	1 min
3. Final extension	72	7 min
4. Soak	4	∞

3.4 Semi-nested PCR Assay

1. Prepare a SN-PCR master mix in a volume sufficient for the number of reactions plus 1 in a sterile 1.5 ml polypropylene microtube and label it “SN-PCR mix”. The volume of each PCR reagent per reaction and the initial and final concentration are shown in Table 4. Vortex and centrifuge the tube briefly. Keep the “SN-PCR mix” on ice bath.
2. Dispense 47 μl of the “SN-PCR mix” into separate 0.5 polypropylene microtube and label the tubes accordingly.
3. Add 3 μl of the first amplification product (PCR assay) into the correspondent tubes.
4. Vortex and centrifuge the tubes briefly.
5. Run the SN-PCR under the conditions shown in Table 5.

Table 4
Components of the semi-nested PCR assay

Reagent	Volume per reaction (μl)	Volume mix for N reactions (μl)	Final concentration
10 \times First strand buffer	5	$5 \times N$	1 \times
MgCl ₂ , 50 mM	2	$2 \times N$	2 mM
dNTP, 10 mM	4	$4 \times N$	0.8 mM
Reverse primer BCoV2 (20 μM)	1	N	0.4 μM
Forward primer BCoV3 (20 μM)	1	N	0.4 μM
DNA polymerase (5 U/ μl)	0.5	$0.5 \times N$	2.5 U/reaction
Water	33.5	$33.5 \times N$	–
Total	47	$47 \times N$	–

N = number of 0.2 ml tubes

Table 5
Conditions for the semi-nested PCR assay

Step	Temperature ($^{\circ}\text{C}$)	Time
1. Heat activation	94	4 min
2. Thermal cycling (30 cycles)		
Denaturing step	94	1 min
Annealing step	55	1 min
Extension	72	1 min
3. Final extension	72	7 min
4. Soak	4	∞

6. After the run, analyze the SN-PCR products by gel electrophoresis. Alternatively, the products can be kept at -20°C for short-term storage.

3.5 Agarose Gel Electrophoresis

1. Prepare 2 % agarose gel by weighing out 2 g de agarose powder (for gel-tray dimension $14 \times 10 \times 0.7$ cm). Add it into a 250 ml Erlenmeyer flask containing 100 ml $1 \times$ TBE buffer.
2. Heat the agarose powder and electrophoresis buffer in a microwave oven until the agarose is completely melted.
3. Let agarose solution cool down to approximately 55°C .
4. Add $5 \mu\text{l}$ of ethidium bromide (10 mg/ml) to a final concentration of approximately $0.5 \mu\text{g/ml}$ (*see Notes 13–15*).
5. Pour the solution into a sealed gel casting tray containing a gel comb.
6. Let sit for at least 30 min to solidify at room temperature (the solidified gel is opaque in appearance).
7. Remove the seal and comb. Place the gel and the plastic tray horizontally into the electrophoresis chamber with the wells at the cathode side.
8. Cover the gel with $1 \times$ TBE buffer.
9. Pipet $0.5 \mu\text{l}$ of the DNA markers, $2 \mu\text{l}$ of $6 \times$ gel loading dye and $8 \mu\text{l}$ de water on a Parafilm sheet and mix well (pipetting up and down).
10. Pipet $10 \mu\text{l}$ of the SN-PCR products and $2 \mu\text{l}$ of $6 \times$ gel loading dye on a Parafilm sheet and mix well (pipetting up and down).
11. Pipet the samples into the wells.
12. Close the lid and connect the power leads on the electrophoresis apparatus.
13. Turn on power supply and apply a voltage of 80–100 V. Run the gel for approximately 40 min.
14. Turn off the power supply when the tracking dye has migrated a sufficient distance.
15. Remove the cover and retrieve the gel (*see Note 16*).
16. Place the gel on an ultraviolet transilluminator. Switch on the power of the gel photo-documentation machine (*see Note 17*).
17. Adjust the position of the gel and record the results. The size of the expected product for BCoV is 454 bp and 251 bp for the first and second round of amplification, respectively.

4 Notes

1. Clinical samples must be stored at 4 °C for up to 48 h or frozen at -80 °C for longer periods of time. The sensitivity of the PCR could be reduced if you repeat freezing and thawing of fecal samples. Store samples in multiple small aliquots to avoid repeated freeze-thaw cycles which accelerate degradation of nucleic acids, especially RNA.
2. The disodium salt of EDTA will not dissolve until the pH of the solution is adjusted to 8.0 by the addition of NaOH.
3. Dissolution of GuSCN is facilitated by heating in a 60–65 °C water bath under continuous shaking.
4. Buffers L6 and L2 are stable for at least 3 weeks at room temperature in the dark [13].
5. The three primers used in SN-PCR were designed from the highly conserved region of the N gene of the Mebus strain (GenBank accession number U00735).
6. Phenol is highly corrosive and can cause severe burns to skin. Personal protective equipment such as gloves, safety glasses, and a lab coat are essentials whenever working with phenol.
7. Be careful not to transfer any of the protein at the interphase.
8. Phenol and chloroform are considered hazardous chemicals by waste environment protection agencies and similar bodies around the world. Discard phenol-chloroform-isoamyl alcohol waste in a properly labeled container. For more information regarding hazardous waste management, contact your state hazardous waste agency.
9. Be careful to stir the tube containing hydrated silica before use to resuspend settled silica.
10. Be careful not to disturb the silica pellet when discarding the supernatant. Attention: GuSCN-containing waste should be collected in a strong alkaline solution (10 N NaOH).
11. If you perform the RT-PCR on the same day, RNA extraction can be conserved at 4 °C. For a longer conservation period, RNA must be stored at -80 °C.
12. It is recommended to carry out the cDNA synthesis immediately after the RNA preparation. Being more stable than RNA, the storage of a first-strand cDNA is less critical.
13. Alternatively, the gel can be stained after DNA separation by electrophoresis. Place the gel into a container filled with 100 ml of TBE running buffer and 5 µl of ethidium bromide. Let the gel soak in this solution for 20–30 min. Wash the gel briefly with water before visualization under UV light.

14. Ethidium bromide is mutagenic and moderately toxic and must be handled with care. Wear a lab coat, eye protection, and gloves when working with this chemical.
15. Liquid (running buffer) and solid (agarose gel, gloves, tips) waste contaminated with ethidium bromide must be managed as a hazardous waste. No liquids should be placed in the containers with the solid wastes. For more information regarding hazardous waste management, contact your state hazardous waste agency.
16. DNA will diffuse within the gel over time. Therefore, examination or photography should take place shortly after cessation of electrophoresis.
17. Exposure to UV light can cause severe skin and eye damage. Wear safety glasses and close the photography hood before turning on the UV transilluminator. Be certain to turn off UV light immediately after gel image is captured.

Acknowledgments

Alfieri, A.A. and Alfieri A.F. are recipients of the CNPq fellowships.

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