# Chapter 8

# Real-Time RT-PCR Detection of Equine Coronavirus

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## Abstract

Equine coronavirus (ECoV) is a recently identified equine virus, involved mainly in enteric infections. Since the ECoV discovery in 1999, only two real-time RT-PCRs have been developed for viral identification. In this chapter we describe a one-step real-time RT-PCR that has been routinely used in our laboratory for ECoV detection from fecal and respiratory samples.

**Key words** ECoV, Real-time RT-PCR, Molecular detection, Clinical diagnosis

## 1 Introduction

Equine coronavirus (ECoV) is a *Betacoronavirus-1* in the lineage A (betacoronavirus A1) that was identified at the end of the last century [1]. ECoV belongs to the genus *Betacoronavirus* and is closely related to human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), canine respiratory coronavirus (CRCoV), bubaline coronavirus (BuCoV), and porcine hemagglutinating encephalomyelitis virus (PHEV). ECoV was first isolated in North Carolina, USA, from the feces of a diarrheic foal in 1999 (ECoV-NC99) [2]. Multiple ECoV outbreaks have recently been reported in Japan [3, 4] and in the USA [5]. Major clinical signs observed were anorexia, fever, lethargy, leukopenia, and diarrhea, and unspecific discrete symptoms that do not lend to rapid diagnosis. ECoV was mainly detected in fecal samples from horses and less frequently in respiratory secretions [5–7]. A small number of animals with signs of encephalopathic disease have also been observed during these outbreaks [8].

The current diagnosis of ECoV infection can be performed using virus isolation, electron microscopy, serology [9]. A reverse transcription loop-mediated isothermal amplification (RT-LAMP), a non-PCR-based nucleic acid amplification assay, has been recently developed for the detection of ECoV in fecal samples [10]. ECoV was also identified by molecular methods in feces and respiratory samples of foals with and without enteric disease [2, 11, 12]. Real-time RT-PCR assays

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can enable a prompt identification of ECoV in respiratory and fecal samples of horses who were at the early stage of disease onset [5, 6].

## 2 Materials

### 2.1 Samples Preparation

#### 2.1.1 Clarification of Fecal Samples

1. Autoclaved 1× Phosphate Buffered Saline (PBS Buffer). For 1 l of 1× PBS buffer, dissolve components as described in Table 1. Dissolve all components in a distillation flask and check pH at 7.4. Then autoclave at 120 °C for 25 min (*see* **Note 1**).
2. pH meter.
3. Autoclave machine or equivalent.
4. Fecal clinical samples stored at +4 °C.
5. 1.5 ml sterile microcentrifuge tubes.
6. Laboratory tabletop centrifuge or equivalent.

#### 2.1.2 Respiratory Samples

1. Respiratory clinical samples stored at −80 °C.
2. Proteinase K Solution (Qiagen®).
3. Heating block or equivalent.

### 2.2 RNA Extraction

1. QIAsymphony® DSP Virus/Pathogen Mini Kit (Qiagen®).
2. Sample Prep Cartridges, 8-well.
3. 8-Rod Covers.
4. Filter-Tips, 200 and 1500 μl.
5. 2 ml sample tubes, with screw caps and without screw caps, from Sarstedt.
6. ATL buffer (Qiagen®).
7. 300 μl of clinical sample (clarified fecal sample or respiratory sample).
8. QIAsymphony® SP automated instrument (Qiagen®) (*see* **Note 2**).

**Table 1**
**Components for 1 l 1× PBS buffer**

| Component | Quantity/weight |
|---|---|
| Autoclaved RNase-free water or its equivalent | 1000 ml |
| Sodium chloride (NaCl) 1.5 M (VWR) | 8 g |
| Potassium chloride (KCl) 30 mM (VWR) | 0.2 g |
| Disodium phosphate ($Na_2HPO_4$) 80 mM (VWR) | 2.9 g |
| Monopotassium phosphate ($KH_2PO_4$) 20 mM (BDH Prolabo) | 0.2 g |

### 2.3 Real-Time RT-PCR

1. Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen®).
2. Superscript® III RT/Platinum Taq® Mix (Invitrogen®).
3. 10 μM PCR forward primer (ECoV-M-f), 5′-GGTGGAGTT TCAACCCAGAA-3′.
4. 10 μM PCR reverse primer (ECoV-M-r), 5′-AGGTGCGAC ACCTTAGCAAC-3′.
5. 10 μM PCR probe (ECoV-M-p), 5′-(6FAM)-CCACAATAAT ACGTGGCCACCTTTA-(BHQ1)-3′ (*see* **Note 3**).
6. $MgSO_4$ (50 mM) (Invitrogen®).
7. 2× Reaction Mix (Invitrogen®).
8. Autoclaved RNase-free water or equivalent.
9. 0.1 ml strip tubes and caps (Qiagen®).
10. Loading block (Qiagen®).
11. Rotor-Gene Q® real-time PCR machine (Qiagen®) (*see* **Note 4**).

## 3 Methods

The protocol described below is routinely used for ECoV clinical diagnosis in fecal and respiratory samples. Two PCR assays are developed in our laboratory targeting partial M and N genes of the ECoV genome [6]. They were based on short RNA sequences deduced from the ECoV-NC99 strain [13]. The protocol described is based on the highest sensitive PCR targeting the M gene and proved to be a sensitive and useful tool for ECoV detection in field samples (*see* **Note 5**).

### 3.1 Samples Preparation

#### 3.1.1 Clarification of Fecal Samples

1. Transfer 10–50 % of fecal sample into a 2 ml sterile microcentrifuge tube and adjust to 1 ml with autoclaved 1× PBS buffer.
2. Centrifuge at 3,000 rpm (850×*g*) for 30 min at room temperature.
3. Transfer supernatant into a new 1.5 ml sterile microcentrifuge tube.
4. Centrifuge at 10,000 rpm (9,400×*g*) for 15 min at room temperature.
5. Collect supernatant and store at +4 °C until RNA extraction.

#### 3.1.2 Respiratory Samples

Respiratory samples (nasopharyngeal swabs) too mucous to be directly extracted with the QIAsymphony® SP automated instrument are previously treated by proteinase K.

1. Add 10 % of proteinase K to the final volume of respiratory specimen in a sterile 2 ml tube.
2. Briefly vortex tubes and incubate at 56 °C for 15 min.
3. Store at +4 °C until RNA extraction.

### 3.2 RNA Extraction

1. For a new kit, perform the following procedures before samples processing:
   (a) To prepare a carrier RNA stock solution, add 1350 μl buffer AVE to the tube containing 1350 μg lyophilized carrier RNA to obtain a solution of 1 μg/μl. Dissolve the carrier RNA thoroughly and divide it into conveniently sized aliquots. Store the buffer AVE at 2–8 °C for up to 2 weeks.
   (b) Before starting a protocol, check whether precipitate has formed in buffer ATL. If necessary, dissolve by heating at 70 °C with gentle agitation in a water bath and aspirate bubbles from the surface of buffer ATL.
2. Equilibrate all reagents and clinical samples at room temperature before starting the run.
3. Turn on the QIAsymphony® SP automated instrument (Qiagen®).
4. Load the required elution rack into the "Eluate" drawer, and load the required reagent cartridge(s) and consumables into the "Reagents and Consumables" drawer.
5. Perform an inventory scan of the "Reagents and Consumables" drawer.
   Place the samples into the appropriate sample carrier and the tubes containing the carrier RNA–Buffer AVE mixture into the tube carrier.
6. Enter the required information for each batch of samples to be processed:
   (a) Sample information.
   (b) Protocol to be run ("complex200_V6_DSP").
   (c) Elution volume (60 μl) and output position.
   (d) Tubes containing the carrier RNA–Buffer AVE mixture.
7. Run the purification procedure.
8. After the RNA purification, store the purified RNA at 2–8 °C during 24 h before the one-step real-time RT-PCR. For long-term storage of over 24 h, store purified RNA at –20 or –80 °C.

### 3.3 Real-Time RT-PCR Assay

1. Prepare a one-step RT-PCR master mix sufficient for the designated number of samples in a sterile 1.5 ml microcentrifuge tube on ice, according to Table 2. Include at least one negative control (autoclaved RNase-free water) and one positive control (*see* **Note 6**) for each run. Add additional controls (e.g., purified RNA from the studied samples) as necessary.
2. Insert the strip tubes on the loading block. Aliquot 20 μl of the master mix into separate 0.1 ml strip tubes and label the tubes accordingly.

3. Add 5 μl of each sample and positive control to these tubes. For the negative control, add 5 μl of autoclaved RNase-free water.
4. Close the strip tubes with caps. Insert the strip tubes into the 72-well rotor and lock the rotor into place on the rotor hub of the Rotor-Gene Q® PCR machine.
5. Turn on the real-time PCR machine (Rotor-Gene Q®). Open the "Rotor-Gene Q® series software".
6. Check the "Locking Ring Attached" checkbox and then click "Next".
7. Set the thermal cycle conditions according to Table 3.
8. Run the real-time RT-PCR under the conditions shown.
9. In the "Edit Samples" window, input the necessary information for the corresponding samples (e.g., name of the clinical specimen, positive and negative controls).

**Table 2**
**Components of one-step real-time RT-PCR assay**

| Reagent | Volume per reaction (μl) | Volume mix for *N* reactions[a] (μl) | Final concentration |
|---|---|---|---|
| 2× Reaction Mix | 12.5 | 12.5 × *N* | 1× |
| $MgSO_4$ (50 mM) | 1.5 | 1.5 × *N* | 3.0 mM |
| forward primer (ECoV-M-f) (10 μM) | 1.5 | 1.5 × *N* | 0.6 μM |
| PCR reverse primer (ECoV-M-r) (10 μM) | 1.5 | 1.5 × *N* | 0.6 μM |
| PCR probe (ECoV-M-p) (10 μM) | 0.5 | 0.5 × *N* | 0.2 μM |
| Superscript® III RT/Platinum Taq® Mix | 0.5 | 0.5 × *N* | – |
| Autoclaved RNase-free water or equivalent | 2 | 2 × *N* | – |
| Total | 20 | 20 × *N* | – |

[a] *N* = number of 0.1 ml strip tubes

**Table 3**
**Conditions for the one-step real-time RT-PCR assay**

| Step | Temperature (°C) | Time |
|---|---|---|
| 1. Reverse transcription | 50 | 15 min |
| 2. Initial PCR activation step | 95 | 2 min |
| 3. Thermal cycling (45 cycles) | | |
| Denaturation | 95 | 15 s |
| Annealing and extension | 60 | 60 s |

10. After performing the RT-PCR, examine the amplification curves of the reactions and the corresponding threshold cycles (Ct). Positive clinical samples will generate amplification curves above the threshold line, and negative samples and water control will be, by contrast, below the threshold line (Fig. 1a). Based on the Ct values from tenfold serial dilutions of a reference standard, the RT-PCR can be used to quantify the amount of input target in the positive samples by comparison with the reference (Fig. 1b). This amount can be automatically calculated by the software.

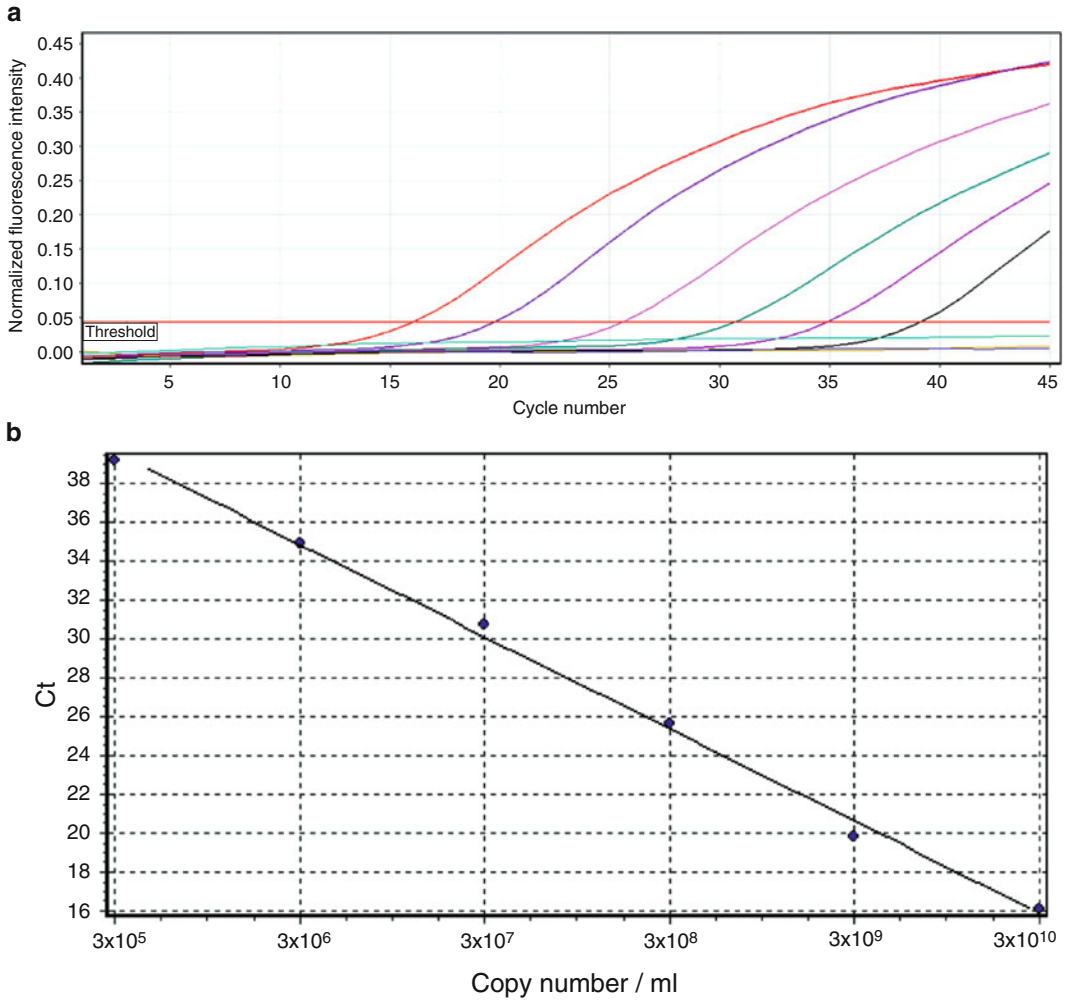


**Fig. 1** Real-time RT-PCR assay for ECoV detection used as a quantitative RT-PCR (qRT-PCR): partial M gene amplification of tenfold serial dilution of RNA transcript from ECoV-NC99 strain. (a) Amplification plot of normalized fluorescence intensity versus number of PCR cycles. The *X*-axis represents the cycle number of the quantitative "M qRT-PCR" assay and the *Y*-axis, the normalized fluorescence intensity. (b) Standard curve for quantitative analysis of ECoV. The threshold cycle (Ct) is the number of PCR cycles requires for the fluorescent intensity of the reaction to predefine the threshold. The Ct is inversely proportional to the concentration of the input target (from $3 \times 10^{10}$ to $3 \times 10^{5}$ copy per ml). A linear quantitative detection range with a correlation coefficient ($R^2$) of 0.997 was obtained with an efficiency of 63 %

## 4 Notes

1. 1× PBS buffer can be stored at room temperature for 6 months.
2. Two other kits have also been used for RNA extraction from clinical fecal and respiratory samples: the High Pure RNA isolation Kit (Roche®) with 200 μl of clinical sample and a 50 μl final elution volume; and the Mag Attract Viral RNA M48 Kit (Qiagen®), using the BioRobot M48 automated machine (Qiagen®) with 300 μl of clinical sample and a 65 μl final elution volume.
3. Primers and probe used in these assays are perfectly matched with the sequences deduced from the original ECoV-NC99 strain (EF446615) [13].
4. The RT-PCR has also been validated on the SmartCycler II® real-time PCR system (Cepheid®) with the same RT-PCR thermal cycling conditions.
5. The "M qRT-PCR" should be tested with an internal quality control for viral diagnosis in order to exclude false negatives due to possible inhibition.
6. A RNA transcript ($10^5$ copy/μl) deduced from M gene sequence of ECoV-NC99 strain has been used as positive control.

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