

## RT-PCR Detection of Avian Coronaviruses of Galliform Birds (Chicken, Turkey, Pheasant) and in a Parrot

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### Abstract

Of the many primer combinations that we have investigated for the detection of avian coronaviruses, two have worked better than any of the others: they worked with the largest number of strains/samples of a given coronavirus and the most species of avian coronavirus, and they also produced the most sensitive detection tests. The primer combinations were: oligonucleotide pair 2Bp/4Bm, which is in a region of gene 1 that is moderately conserved among all species of coronavirus (1); and UTR11-/UTR41+, which are in a highly conserved part of the 3' untranslated region of avian coronaviruses related to infectious bronchitis virus (2). The gene 1 primer pair enabled the detection of a new coronavirus in a green-cheeked Amazon parrot (*Amazon viridigenalis* Cassin). In this chapter we describe the use of these oligonucleotides in a one-step (single-tube) RT-PCR, and describe the procedure that we used to extract RNA from turkey feces.

**Key words:** pan-coronavirus PCRs; coronavirus detection; coronavirus discovery; avian coronaviruses; galliform birds

### 1. Introduction

In recent years sequence analysis has shown that turkey coronavirus (TCoV) and pheasant coronavirus (PhCoV) have a gene order that is the same as that of the longer-studied infectious bronchitis virus (IBV) of the domestic fowl (chicken): (5'-replicase-S-3-M-5-N-3' (2–5), placing them all in coronavirus group 3 (6). Protein sequence identities are frequently greater than 80% among

these CoV species, except for the spike (S) protein of TCoV, which has only about 30% identity with that of IBV and PhCoV, and only about 17% with the S1 subunit of the S protein (5). Recently, a coronavirus in quail has been shown to have an S1 sequence with about 80% identity with that of TCoV (7). In gene 1, which encodes RNA replication functions, there are sequences with moderately high nucleotide identity among all known coronaviruses, which has led to the design of oligonucleotide combinations for the detection of all known coronaviruses—pancoronavirus primers (see, e.g., (1) and other chapters in this book). Herein we demonstrate the utility of the Stephensen primers for the detection of a coronavirus in parrots (8).

The terminal region of the 3'UTR of group 3 coronaviruses is very highly conserved, including in coronaviruses from pigeon, duck, and goose (9). In our field studies of TCoV, we used a 3' UTR primer pair very successfully to detect TCoV in feces and gut contents [(10) and unpublished observations]. In this chapter we describe the conditions for use of these primers in conjunction with RNA extracted from feces.

## 2. Materials

### 2.1. RNA Extraction from Feces

1. We used the QIAamp DNA stool mini kit (Qiagen) for RNA extraction from feces, which has a special step designed to remove inhibitors that are present in feces (see **Notes 1 and 2**).
2. Absolute ethanol.

### 2.2. One-Step Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

1. We performed a one step-RT-PCR using Ready-to-go RT-PCR beads (Amersham Biosciences), essentially as described by the manufacturer (See **Note 9**).
2. Nuclease-free water.
3. Oligonucleotide primers dissolved in nuclease-free water to a concentration of either 10 or 100 pmol/ $\mu$ l. The composition of the oligonucleotides is shown in **Table 1**.
4. Mineral oil.
5. Thermocycler.

### 2.3. Detection of PCR Products

1. Agarose.
2. Distilled water.
3. Ethidium bromide.
4. 10X Tris borate EDTA buffer (TBE): 1 M Tris, 0.9 M boric acid, 10 mM ethylenediaminetetraacetic acid.

**Table 1**  
**Details of the Oligonucleotide Primers Described in this Chapter (see Note 12)**

Primer name	Sequence	Length	Sense	IBV 5' position <sup>a</sup>	IBV 3' position <sup>a</sup>	Tm (°C)	Region amplified	Avian CoV amplified <sup>b</sup>	Reference
2 Bp	act ca(ag) (at)t(ag) aat (ct) t(aget) aaa ta(ct) gc	23	+	13953	13976	52	gene 1	I, T, Ph, Pa	(1)
4 Bm	tea ca(ct) tt(at) gga ta(ag) tcc ca	20	-	14184	14204	53	gene 1	I, T, Ph, Pa	(1)
UTR41+	aTg tct atc gcc agg gaa aTg tc	23	+	27342	27364	60	3' UTR	I, T, Ph	(2)
UTR11-	gct cta act cta tac tag cct a	22	-	27586	27607	56	3' UTR	I, T, Ph	(2)

<sup>a</sup> Relative to the genome of the Beaudette strain of IBV (Accession number AJ311317).

<sup>b</sup> The two primer combinations were tested using RNA extracted from IBV (I), TCoV, (T), pheasant coronavirus (PhCoV), and parrot coronavirus (PaCoV).

### 3. Methods

#### 3.1. RNA Extraction from Feces

For RNA extraction from feces we used the QIAamp DNA stool mini kit (Qiagen), which has a step designed to remove inhibitors that are present in feces (*See Notes 1 and 2*). RNA extraction comprises three main steps: 0.2 g of each stool sample is lysed; a reagent in the kit called InhibitEX then absorbs any impurities (by removing PCR inhibitors present in feces—*see Note 3*); finally the RNA is purified on spin columns. The RNA is bound to a silica gel membrane, impurities are washed away, and then pure RNA is eluted from the spin column in nuclease-free water. We followed the manufacturer's instructions:

1. Perform RNA extraction from fresh or frozen material (*see Note 5*).
2. Use a pea-sized amount of feces, which is approximately 0.2 g, fresh or frozen (*see Note 5*).
3. If using fresh, i.e., not frozen, feces, homogenize the feces using a sterile spatula before dispensing into a microfuge tube (*see Note 6*). Feces should be kept on ice until buffer is added.
4. Add 1.4 ml of ASL buffer to each fecal sample. Vortex until thoroughly homogenized (30 sec to 1 min).
5. Heat fecal suspensions in a 70°C water bath for 5 min then vortex each sample for 15 sec.
6. Centrifuge each sample for 1 min to pellet any particulate matter.
7. Pipette 1.2 ml of supernatant into a new microfuge tube and discard the pellet (*see Note 8*).
8. Add one Inhibitex tablet to each sample and vortex for 1 min. Incubate the suspension for 1 min (*see Note 7*). Incubate at room temperature for 1 min before centrifuging the sample to pellet the Inhibitex tablet to which the inhibitors will be bound.
9. Pipette all the supernatant into a new 1.5-ml microfuge tube. Centrifuge the supernatant for 3 min.
10. Add 15 µl Proteinase K (provided in the kit) into a new 1.5-ml microfuge tube. To this add 200 µl of the supernatant and 200 µl AL buffer and mix vigorously. Then incubate for 10 min at 70°C.
11. To this, add 200 µl of 100% ethanol and mix thoroughly. Add the contents of this microfuge tube (steps 9, 10, and 11) to a QIAamp spin tube.
12. Centrifuge for 1 min and place the column into a new collection tube. To the spin column, add 500 µl of AW1 buffer. Centrifuge for 1 min.
13. Place spin column into a new collection tube. To the spin column, add 500 µl of AW2 buffer. Centrifuge for 3 min.
14. Place spin column into a new collection tube. Centrifuge for 1 min. Place spin column into a new microfuge tube. Pipette 200 µl of AE buffer onto the spin column (do not use water). Incubate for 1 to 5 min at room temperature before eluting RNA from the spin column by centrifugation for 1 min.

15. Upon elution, store the RNA on ice for as short a period of time as possible until it can be transferred to  $-20^{\circ}\text{C}$ . It may be stored for several months at this temperature and may be thawed on ice a few times for subsequent RT-PCR.

### **3.2. One-Step Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)**

We used a one-step RT-PCR, i.e., RT and PCR reagents all together in one microfuge tube throughout the procedure, using Ready-to-go RT-PCR beads (Amersham Biosciences) (*see Note 9*). Perform all RT-PCR steps on ice. It is advisable to use filter tips for pipettes when measuring volumes (*see Note 4*). For this one-step RT-PCR reaction:

1. Add nuclease-free water to a 0.5-ml microfuge tube containing beads (comprising, most importantly, lyophilized *Taq* DNA polymerase, dNTPs, murine Maloney leukemia virus reverse transcriptase, and a ribonuclease inhibitor).
2. Incubate microfuge tube containing nuclease-free water and bead on ice for 5 min, ensuring that the beads are resuspended in the water. The amount of water added depends on the volumes of RNA and primers; the final volume, after addition of oligonucleotide primers (*see next step*) should be 50  $\mu\text{l}$ .
3. To this add 2  $\mu\text{l}$  forward primer (1 pmol/ $\mu\text{l}$ ) and 2  $\mu\text{l}$  reverse primer (1 pmol/ $\mu\text{l}$ ) and 1 to 10  $\mu\text{l}$  of RNA (*see Note 10*). Reminder: upon addition of the primers and template, total reaction volume should be 50  $\mu\text{l}$ .
4. Add a 50  $\mu\text{l}$  mineral oil overlay to the microfuge tube while it is still on ice before placing the tubes into PCR thermocycler (*see Note 11*). The RT-PCR is performed in a thermocycler with a heated lid using the following program:  $42^{\circ}\text{C}$  for 30 min and  $95^{\circ}\text{C}$  for 5 min for the reverse transcriptase step. First-strand cDNA generated with these beads is used as a direct template for PCR. The amplification step is denaturation at  $95^{\circ}\text{C}$  for 1 min, primer annealing at the desired temperature for 1 min, and primer extension at  $72^{\circ}\text{C}$  for 3 min. These cycles are repeated 35 times, with a final step of  $72^{\circ}\text{C}$  for 10 min. Use  $50^{\circ}\text{C}$  for the gene 1 oligonucleotide combination 2Bp/4Bm, and  $48^{\circ}\text{C}$  for 3' UTR oligonucleotide combination UTR11-/UTR41+. Visualize PCR products on a 1% agarose gel containing ethidium bromide (1  $\mu\text{g}$ ).

## **4. Notes**

1. We used the Qiagen QIAamp stool extraction kit, which has been employed by others for extraction of RNA for pathogen detection, including the detection of SARS-CoV in human feces (*11*) and by ourselves for the detection of turkey coronavirus from turkey feces (*10*).

2. When RNA extraction or RT-PCR is being carried out in a laboratory on a routine basis it is important to put safeguards in place to prevent the detection of false positives or false negatives, which may occur owing to several sources of cross-contamination. For example, it is advisable to have separate areas for the handling of PCR reagents and areas in which the RT-PCR is set up. If possible each step of the process should be carried out in a separate room, e.g., one area for the storage of reaction components, one area for the reaction to be set up, another area where the reaction tube will be opened after PCR has been carried out, and another for the corresponding sequencing reactions.
3. The surfaces of the bench at which RNA extraction or RT-PCR is carried out should be prepared by cleaning with an RNase inhibitor spray and 70% ethanol solution.
4. Filter tips should be used for all pipettes. If available, separate sets of pipettes should be used for RNA extraction and RT-PCR. The design of the experiment should incorporate positive and negative controls.
5. Freeze feces for storage. For subsequent sampling, simply scrape frozen feces off the surface; there is no need to thaw the feces.
6. We found that sterile ice-cream sticks (wooden birch sticks) were useful for this purpose, as they are cheap and disposable.
7. Feces contain materials that may adversely affect enzyme function or nucleic acid integrity. Although avian feces have a different composition from that of mammals, the QIAamp DNA stool kit of Qiagen was certainly effective when used with turkey and chicken feces.
8. When opening and closing microfuge tubes during RNA extraction, be careful of any aerosol or droplets spraying and contaminating work surfaces or other samples.
9. This is a one-step RT-PCR reaction to reduce the amount of handling required. In our laboratory it has been used to amplify RT-PCR products of between 200 bp and 2.5 kb in length.
10. We found that 2  $\mu$ l of RNA was usually sufficient. However, if a very small amount of virus is suspected within fecal samples, up to 10  $\mu$ l of RNA extracted from feces can be added without deleterious effect.
11. In our experience, better amplification of RNA products is obtained when mineral oil is added to the reaction, regardless of whether or not a thermocycler, with or without, a hot lid is used.
12. Even the conserved, 3'-terminal part of the 3' UTR of group 3 avian coronaviruses exhibits some variation. It is likely that oligonucleotides UTR11- and UTR41+ will require modification to increase their universality for group 3 coronaviruses. For example, we modified our initial versions of these primers [UTR1- and UTR4+ (*I2*)] to the current UTR11- and UTR41+ as more IBV sequences became available. We did this by moving the position of the oligonucleotides very slightly to avoid variation at the extreme 3'- end of the oligonucleotides. The data of Jonassen et al. (*9*) for group 3 coronaviruses in pigeon, mallard duck, and greylag goose suggests that our UTR11- and UTR41+ primers should be

modified slightly to increase the likelihood of them working with coronaviruses in a greater number of species. Modification might well include degeneracy, especially with respect to the 3'-most nucleotide.

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