Chapter 3

Detection and Discovery of Coronaviruses in Wild Bird Populations

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Abstract

Wild birds are natural hosts of multiple microbial agents, including a wide diversity of coronaviruses. Here we describe a pan-Coronavirus detection RT-PCR method to identify those viruses regardless of the coronavirus genus or nature of the specimen. We also describe a protocol using high-throughput sequencing technologies to obtain their entire genome, which overcomes the inherent difficulties of wild bird coronavirus sequencing, that is, their genetic diversity and the lack of virus isolation methods.

Key words Wild birds, Gammacoronavirus, Deltacoronavirus, Sample collection, Pan-Coronavirus, RT-PCR, Next-generation sequencing, Illumina MiSeq

1 Introduction

Wild birds are natural hosts of multiple microbial agents such as low pathogenic avian influenza viruses or avian avulaviruses but also avian coronaviruses (CoV). Historically, avian CoV mainly referred to CoV from poultry that have been long studied, especially infectious bronchitis virus (IBV). However, the discovery of the first gamma-CoV in wild birds in Norway in 2003 [1], followed by the first delta-CoV in Hong Kong in 2007 [2] changed our perspective on CoV epidemiology in birds. The significant challenges in understanding their ecology include the large numbers of potential host species and the usually short period of viral shedding, combined with the difficulty of catching and sampling representative numbers of individual species. Although some surveillance has started to target CoV in wild birds, more research is necessary to determine which species may serve as reservoirs, carrying and shedding the disease asymptomatically.

In addition, IBV-like strains are occasionally detected in wild birds, and conversely, wild bird gamma- or delta-CoV strains are occasionally identified in domestic poultry [3]. This promiscuity is also raising questions regarding the origin of some genetic
fragments observed in new CoV species such as Turkey Coronavirus and Guinea fowl Coronavirus. The genome of these two poultry viruses is closely related to IBV, except for the S gene, which is of unknown origin [4, 5]. Efforts toward full genome characterization of wild bird CoV are therefore warranted to understand and prevent potential recombination events.

Here we describe a pan-Coronavirus RT-PCR detection method to identify CoV in wild birds, regardless of the expected CoV genus of interest. Given the challenge of sequencing CoV with classical methods due to their genetic variability and the lack of in vitro culture methods, high-throughput sequencing technologies offer faster, cheaper, and less laborious alternatives to obtain complete wild bird CoV genome information. Here we provide a protocol coupling metagenomics approach and high throughput sequencing technology, taking advantage of metagenomics unbiased survey of the genetic material within a sample.

2 Materials

2.1 Specimen Collection (See Note 1)

1. Virus transport medium (VTM; see Note 2).
2. Flocked swabs (see Note 3).
3. 70% alcohol.
4. Screw-cap tubes.
5. Personal protective equipment (see Note 4).

2.2 RNA Extraction and Amplification of the RNA-Dependent RNA Polymerase (RdRp) Gene

1. RNA extraction kit (e.g., QIAamp viral RNA minikit or similar).
2. RNase/DNase-free water.
3. One-step RT-PCR kit (e.g., Qiagen One-Step RT-PCR kit).
4. Taq polymerase 5 U/µl (e.g., Platinum Taq DNA Polymerase).
5. 10 mM dNTPs.
6. 50 mM MgCl₂.
7. RNase inhibitor (e.g., RNaseOUT 40 U/µl).
8. Fwd primer for first round RT-PCR: 5’-GGKTGGGAYTAYCCKAARTG-3’ [6].
9. Rev. primer for first round RT-PCR: 5’-TGYTGTSWRCARAAYTCRTG-3’ [6].
10. Fwd primer for nested PCR: 5’-GGTTGGGACTATCAAGTGTGA-3’ [6].
11. Rev. primer for nested PCR: 5’-CCAACAYTTNGARTCWGCCAT-3’ [7] (see Note 5).
12. Thermocycler.
13. Gel electrophoresis equipment.
14. 50× TAE buffer: 242 g tris base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA (pH 8.0), add double distilled water to reach 1 l.
15. 1.5% agarose gel: 1.5 g of agarose, 100 ml 1× TAE.
16. Molecular weight marker (e.g., 1 kb plus DNA ladder or similar).
17. Gel purification kit (e.g., QIAquick Gel Extraction Kit or similar).
18. PCR product purification kit (e.g., QIAquick PCR Purification Kit or similar).

### 2.3 NGS Sequencing

#### 2.3.1 RNA Extraction

**Prior to NGS Sequencing**

1. RNA extraction kit (e.g., QIAamp RNeasy Mini Kit or similar).
2. TRIzol LS Reagent.
3. Chloroform.
4. Molecular biology-grade ethanol.
5. DNase mix: 5 μl DNase (6 U/μl), 75 μl DNase Digestion Buffer mixed in a nuclease-free tube.
6. Round bottom 2 ml collection tube.
7. Chemical fume hood.
8. Refrigerated microfuge.
9. Vortex.

#### 2.3.2 Quantification and RNA Integrity Check

1. Qubit® 2.0 Fluorometer (Invitrogen).
2. Qubit RNA HS Assay Kit (Invitrogen).
3. Thin-wall, clear 0.5 ml PCR tubes Qubit® assay tubes (Invitrogen) or Axygen PCR-05-C tubes.

#### 2.3.3 cDNA Synthesis and Quantification

1. SuperScript IV First Strand Synthesis Reaction or similar.
2. 3′→5′exo− Klenow DNA polymerase.
3. Thermocycler.
4. PCR plate.
5. Qubit® 2.0 Fluorometer (Invitrogen).
6. Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen).
7. Thin-wall, clear 0.5 ml PCR tubes Qubit® assay tubes (Invitrogen) or Axygen PCR-05-C tubes.

#### 2.3.4 Library Preparation for Sequencing with Illumina MiSeq

1. Nextera® XT Library Preparation Kit (Illumina).
2. Nextera XT Index Kit (Illumina).
3. TruSeq Index Plate Fixture Kit (Illumina).
4. MiSeq Reagent Kit (Illumina).
5. PhiX Control (Illumina).
6. Hard-Shell skirted PCR plate or similar.
7. Microseal adhesive film.
8. 96-well 0.8 ml polypropylene deep-well plate.
9. Agencourt AMPure XP beads.
10. Molecular biology-grade ethanol.
11. 0.1 N NaOH.
12. Ultrapure water.
13. High Sensitivity DNA chip (Agilent).
14. Thermocycler.
15. Magnetic stand.
16. Microplate centrifuge.
17. Agilent Technology 2100 Bioanalyzer.
18. Illumina® MiSeq System.

3 Methods

3.1 Specimen Collection [8, 9]

1. Swabs taken from the cloaca, oropharynx, and trachea or fresh dropping samples are suitable for CoV detection (see Note 1).
2. Wear appropriate personal protective equipment when handling birds and samples (see Note 4).
3. Unwrap a swab, according to the size of the bird, from the stem end of the packaging without touching the swab tip.
4. Tracheal swab: open the bird’s mouth and gently pull the tongue forward to expose the trachea at the rear end of the tongue.
5. Wait until the bird breathes and the cartilage protecting the trachea is open.
6. Insert the swab into the trachea and gently touch the sides and back of the trachea.
7. Oropharyngeal swab: open the bird’s mouth and gently roll the swab tip around the inside of the bird’s mouth and behind the tongue.
8. Cloacal swab: insert the entire tip of the swab into the cloaca and swab with two to four circular motions while applying gentle pressure against the mucosal surfaces.
9. Fresh droppings: insert the entire tip of the swab into the fresh droppings, swab with two to four circular motions, taking care to not touch the underlying surface (see Note 6).
10. Place the swab tip into a screw cap vial containing VTM, ensuring the swab tip is fully immersed into VTM.
11. Cut or snap the stem of the swab so that the swab remains in the vial and the cap can be screwed on tightly.
12. Wipe the tube with 70% alcohol.
13. Label the tube with appropriate information.
14. Record sample tube number on data sheet.
15. Immediately store all samples on ice (preferably dry ice), transferring to a $-70 \, ^\circ C$ freezer as soon as possible (see Note 7).

### 3.2 RNA Extraction and Amplification of the RdRp Gene

1. Prior to the extraction, vortex the swab or fecal material 20% w/v in VTM for 1 min.
2. Centrifuge for 5 min at 2000 rpm ($400 \times g$).
3. Extract RNA from 140 μl of clarified supernatant on a silicate column with a RNA extraction kit, such as QIAamp viral RNA minikit according to the manufacturer’s instructions, including all necessary positive and negative controls.
4. RNA samples can be stored up to 48 h at $+4 \, ^\circ C$ or immediately at $-80 \, ^\circ C$ for later use.
5. Perform the RT-PCR by mixing 13.9 μl of RNase/DNase-free water, 5 μl of $5 \times$ enzyme buffer, 0.5 μl MgCl$_2$, 0.5 μl dNTPs, 1 μM of each primer (final concentration), 0.1 μl RNase inhibitor, 1 μl enzyme, and 2 μl RNA.
6. Perform the RT-PCR using the following cycle conditions: 50 °C for 30 min, 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min.
7. Analyze the PCR products on a 1.5% agarose gel, let samples migrate for 45 min at 130 V. The expected size of the fragment is 602 bp (see Note 8).
8. Perform the nested PCR reaction by mixing 16.9 μl of RNase/DNase-free water, 2.5 μl of $10 \times$ enzyme buffer, 1 μl MgCl$_2$, 0.5 μl dNTPs, 0.7 μM of each primer (final concentration), 0.2 μl enzyme, and 2.5 μl of first round PCR products (see Note 9).
9. Amplify the PCR products using the following cycle conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min.
10. Analyze the PCR products on a 1.5% agarose gel, let samples migrate for 45 min at 130 V. The expected size of the fragment is 555 bp (see Note 10).
11. The samples with a band at the expected size can be directly purified using a PCR product purification kit if specific, or perform a gel extraction using a commercial kit if more than one band are visible.
12. Sequence purified PCR fragments in both directions using your preferred Sanger sequencing platform to confirm positivity.

3.3 NGS Sequencing
(See Note 11)

3.3.1 RNA Extraction
Prior to NGS Sequencing

This protocol making use of QIAamp RNaseasy Mini Kit is suitable for the purification of RNA from 250 μl sample volumes. Sample material can be liquid samples (serum/plasma, cell culture supernatant, EDTA-blood diluted 1:2 in PBS, supernatant from swabs) or samples homogenized and lysed (tissue and stool homogenates).

1. Thaw the samples on ice and keep them on ice during the entire procedure to prevent degradation.
2. Mix each sample by pulse-vortexing for 20 s.
3. Working in a chemical hood, pipet 750 μl TRIzol LS Reagent into a 2 ml tube, add 250 μl sample and thoroughly shake manually for 15 s.
4. Briefly centrifuge for 5 s at 8000 rpm and incubate 5 min at room temperature.
5. Add 200 μl chloroform.
6. Thoroughly shake manually for 15 s and incubate for 10 min at room temperature. Centrifuge for 10 min at 13,000 rpm (16,000 × g) in a precooled refrigerated microfuge set at 4 °C.
7. Carefully transfer the upper aqueous phase without disturbing the DNA containing interphase into a new sterile 2 ml tube.
8. Add 600 μl 75% ethanol, mix by pulse-vortexing and briefly centrifuge for 5 s at 8000 rpm (6000 × g).
9. Apply 600 μl of the sample from step 8 to the column, and centrifuge at 10,000 rpm (9600 × g) for 20 s.
10. Discard the collection tube and place the column into a clean 2 ml collection tube, apply residual sample from step 8 to the column and centrifuge at 10,000 rpm for 20 s.
11. Discard the collection tube and place the column into a clean 2 ml collection tube, add 350 μl buffer RW1 to the column and centrifuge at 10,000 rpm for 20 s.
12. Discard the collection tube and place the column into a clean 2 ml collection tube, apply 80 μl DNase mix solution to the center of the membrane without touching the membrane.
13. Incubate for 15 min at room temperature, add 400 μl buffer RW1 to the column and centrifuge at 16,000 rpm (>21,000 × g) for 30 s.
14. Discard the collection tube and place the column into a clean 2 ml collection tube, add 500 μl buffer RPE and centrifuge at 16,000 rpm for 30 s.
15. Discard the collection tube and place the column into a clean 2 ml collection tube, add 500 μl buffer RPE and centrifuge at 13,000 rpm for 1 min.

16. Discard the collection tube and place the column into a clean 2 ml collection tube, centrifuge for 2 min at 13,000 rpm (to let the column dry).

17. Discard the collection tube and place the column into a clean 1.5 ml tube, add 50 μl RNase-free water to the center of the membrane, incubate 1 min at room temperature and centrifuge for 1 min at 10,000 rpm.

18. RNA can be used immediately or stored at ≤70 °C.

3.3.2 Quantification and RNA Integrity Check

1. Store the dye and the buffer at room temperature. Store the DNA, RNA, and protein standards at 4 °C. Ensure that all assay reagents are at room temperature before starting.

2. Set up two Assay Tubes for the standards and one tube for each user sample.

3. Prepare the Qubit™ Working Solution by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepare 200 μl of Working Solution for each standard and sample.

4. Prepare the Assay Tubes according to Table 1.

5. Vortex all tubes for 2–3 s.

6. Incubate the tubes for 2 min at room temperature.

7. Insert the tubes in the Qubit® 2.0 Fluorometer and take readings.

3.3.3 cDNA Synthesis and Quantification

1. Prepare a mastermix 1 and 2 for the first strand synthesis using the SuperScript IV First Strand Synthesis Reaction according to Table 2.

2. Add 5 μl of mastermix 1 to all necessary wells, then add 8 μl of samples.

3. Perform the primer annealing step by placing the plate on a thermocycler at 65 °C for 5 min, then place the plate directly on ice for 1 min.

4. Add 7 μl of mastermix 2 to all necessary wells.

5. Perform the reverse transcription by placing the plate on a thermocycler at 23 °C for 10 min, followed by 50 °C for 10 min, then 80 °C for 10 min. At the end, place the plate directly on ice for 1 min.

6. Add 0.5 μl 3′→5′exo− Klenow DNA polymerase for synthesis of dsDNA.

7. Perform the dsDNA synthesis by placing the plate in a thermocycler at 37 °C for 60 min, 75 °C for 10 min, then hold at 4 °C.
8. Store cDNA at −20 °C or continue directly with the library preparation.

9. Quantify the cDNA on a Qubit® 2.0 Fluorometer, similarly to RNA quantification, using the Qubit dsDNA HS Assay Kit.

10. Use 2 μl of each DNA sample with 198 μl of the Qubit working solution (prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer).

11. Target a 260/230 ratio of 2.0–2.2 values.

### 3.3.4 Illumina MiSeq Library Preparation—DNA Tagmentation

Fragment DNA and then tag DNA with adapter sequences in a single step as follows (see Note 12):

1. Record information about your samples before beginning library preparation.

2. Thaw the reagents on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly before use. Add the following volumes in the order listed to each well of a new PCR plate:

| Table 1 |
|---|
| **Volume of specific reagents to prepare standards and samples to be quantified on the Qubit® 2.0 Fluorometer** |

| Reagent | Volume to add to prepare Standard Assay Tubes | Volume to add to prepare Sample Assay Tubes |
|---|---|---|
| Working solution (from step 3) | 190 μl | 180–199 μl |
| Standard (from kit) | 10 μl | – |
| Sample | – | 1–20 μl |
| Total volume | 200 μl | 200 μl |

| Table 2 |
|---|
| **Composition of mastermixes for cDNA synthesis** |

| Mastermix | Reagent | Volume for 1 sample |
|---|---|---|
| Mastermix 1 | DEPC-treated water | 3 μl |
| | Random hexamer [50 ng/μl] | 1 μl |
| | 10 mM dNTPs | 1 μl |
| | RNA (up to 5 μg) | 8 μl |
| | Total volume | 13 μl |
| Mastermix 2 | 5×SSIV buffer | 4 |
| | DTT [0.1 M] | 1 |
| | RNaseOUT [40 U/μl] | 1 |
| | SuperScript IV [200 U/μl] | 1 |
| | Total volume | 7 μl |
5 μl Tagment DNA Buffer (TD), 2.5 μl DNA (0.2 ng/μl per sample), 2.5 μl Amplicon Tagment Mix (ATM) to each well. Pipet to mix.

3. Centrifuge at 280 × g at 20 °C for 1 min.

4. Run the tagmentation program on a thermocycler with pre-heated lid (55 °C for 5 min, hold at 10 °C). When the sample reaches 10 °C, immediately proceed to the next step because the transposome is still active.

5. Add 5 μl Neutralize Tagment Buffer (NT) to each well. Pipette to mix (at room temperature).

6. Centrifuge at 280 × g at 20 °C for 1 min.

7. Incubate at room temperature for 5 min.

This step amplifies the tagmented DNA using a limited-cycle PCR program.

1. Setup of TruSeq Index Plate Fixture according to the provided kit protocol and arrange the tubes and tips provided with the kit.

2. Add the following reagents and mix by pipetting after adding each reagent: 7.5 μl Nextera PCR Master Mix (NPM), 2.5 μl Index 2 primers (S5XX), 2.5 μl Index 1 primers (N7XX).

3. Centrifuge at 280 × g at 20 °C for 1 min.

4. Place the plate on a thermocycler and run the following program: 72 °C for 3 min, 95 °C for 30 s, 12 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s followed by a final step at 72 °C for 5 min, and hold at 10 °C.

5. Seal the plate and store at 2–8 °C for up to 2 days. Alternatively, leave on the thermocycler overnight.

This step uses AMPure XP beads to purify the library DNA and remove short library fragments.

1. Centrifuge at 280 × g at 20 °C for 1 min.

2. Transfer 50 μl PCR product from each well of the PCR plate to corresponding wells of a deep-well plate.

3. Add 15 μl AMPure XP beads to each well.

4. Shake at 1800 rpm for 2 min then incubate at room temperature for 5 min.

5. Place on a magnetic stand and wait until the liquid is clear (approx. 2 min).

6. Remove and discard all supernatant from each well.
7. Wash two times as follows: add 200 μl fresh 80% ethanol to each well, incubate on the magnetic stand for 30 s, remove and discard all supernatant from each well.

8. Using a 20 μl pipette, remove residual 80% ethanol from each well. Then let dry on the magnetic stand for 15 min before removing the plate from the stand.

9. Add 52.5 μl Resuspension Buffer (RSB) to each well, and shake at 1800 rpm for 2 min.

10. Incubate at room temperature for 5 min.

11. Place on a magnetic stand and wait until the liquid is clear (approx. 2 min).

12. Transfer 25 μl supernatant from the midi plate to a new PCR plate.

13. Seal the plate and store at −25 °C to −15 °C for up to 7 days.

3.3.7 Illumina MiSeq Library Preparation—Library Check and Pooling

1. Run 1 μl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip. A typical library shows a broad size distribution (Fig. 1).

2. Pooling libraries combines equal volumes of normalized libraries in a single tube. After pooling, dilute and heat-denature the library pool before loading libraries for the sequencing run.

3. Centrifuge at 280 × g at 20 °C for 1 min.

4. Transfer 5 μl of each library from the new 1.5 ml tube. Invert to mix.

![Fig. 1 Typical fragment size distribution of a library](image)
5. Dilute pooled libraries to the loading concentration for your sequencing system. For instructions, see the denaturing and dilution library guide for your system.

6. Store unused pooled libraries at \(-25^\circ\text{C}\) to \(-15^\circ\text{C}\) for up to 7 days.

### 3.3.8 Sequencing of Pooled Libraries with the MiSeq Instrument

1. Mix 2.5 μl of diluted NaOH (0.1 N NaOH, max. 7 days old) and 7.5 μl of a library pool for 5 min at room temperature.

2. Add 940 μl of hybridization buffer to the denatured library and 50 μl PhiX Control.

3. Load 600 μl of the library mix onto the reagent cartridge.

4. Run the MiSeq system.

5. Wash the instrument with incorporation buffer PR2.

6. Inspect cluster density for the data output quality.

### 4 Notes

1. Before planning studies in wild birds necessitating capture and sampling, all the necessary permits from local and national wildlife authorities in the country should be obtained. Handling endangered species may require additional permits. Wild birds may be captured using several methods, including nets, live traps, and spotlighting. Once wild birds are captured, handling by authorized personnel should be done according to the recommendations of the local wildlife authorities. Captured birds are kept in a well-ventilated and quiet environment to prevent them from overheating and to minimize the stress.

2. A variety of viral transport media are commercially available. However, viral transport media can also be prepared as following: Hanks balanced salt solution supplemented with 10% glycerol, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 μg/ml gentamicin, and 50 U/ml nystatin.

3. If you are planning to sample different species of birds, consider purchasing different sizes of swabs for large and small bird species to avoid injuries.

4. When handling wild birds, the presence of an infectious agent should always be assumed. It is therefore critical to take standard precautions to prevent exposure and the spread of pathogens. Visiting several sampling sites must be avoided unless microbial decontamination of equipment and clothing has been carried out.
5. Several protocols for the detection of coronaviruses in (wild) birds have been published (reviewed in [10]). The primers presented here offer the advantage of a pan-Coronavirus detection, not only amplifying gammacoronaviruses but the four genera of coronaviruses. The use of such pan-Coronavirus assays has led to the characterization, among other, of a plethora of gamma- and deltacoronaviruses in wild birds. The existence of betacoronaviruses in wild birds [11] is still debated. No alpha- or betacoronaviruses were detected in our cohorts using those primer sets [3] while they were successfully used to detect and characterize alpha- and betacoronavirus strains in bats [7].

6. Alternatively, fresh droppings can be collected using a spoon and later resuspended 20% w/v in VTM in the laboratory.

7. Samples should be kept in an undisrupted cold chain. Fast freezing and rapid handling of the samples for the laboratory investigations are critical for the conservation of nucleic acid in the samples. If using dry ice for sample shipment, enough dry ice should be foreseen to ensure that some is remaining until the samples arrive at the laboratory. This requires a minimum of 1 kg dry ice for every kg of samples.

8. Samples with a band at the expected size (602 bp) can be sequenced directly, without the need for a nested PCR. Refraining from performing a nested reaction on the first round PCR product that is already quite concentrated prevents cross contamination during the subsequent steps.

9. Dilution (e.g., 1:5 in RNase/DNase-free water) of the first round PCR products prior to the nested PCR is often useful to remove leftover reagents, diminishing carry-over into the nested reaction, which then often gives better results.

10. Due to the degenerate nature of the primer sequences to allow for a pan-Coronavirus detection, nonspecificity is expected. Therefore, confirmation of positivity by Sanger sequencing is often useful and advisable. In addition, the genetic information generated is useful to infer the coronavirus genus and perform phylogenetic analyses.

11. The risk for cross contamination is high during next generation sequencing steps due to clonal amplification. To minimize the risk for carryover of the amplified library DNA into original sample material or sequencing ready libraries, use separate areas for sample processing/library construction and run preparation and never mix materials from the sequencing and library preparation area.

12. To avoid contamination, do not use the same barcode as in the sequencing run before.
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