Chapter 2

Discovery of Novel Coronaviruses in Rodents

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Abstract

The recent emergence of SARS, SARS-CoV2 and MERS and the discovery of novel coronaviruses in animals and birds suggest that the Coronavirus family is far more diverse than previously thought. In the last decade, several new coronaviruses have been discovered in rodents around the globe, suggesting that they are the natural reservoirs of the virus. In this chapter we describe the process of screening rodent tissue for novel coronaviruses with PCR, a method that is easily adaptable for screening a range of animals.

Key words Discovery, Rodents, PCR, Coronavirus detection, Alphacoronavirus, Betacoronavirus

1 Introduction

Rodents are known to be an important source of emerging viral infections [1]. Rodentia include approximately 2200 species such as voles, mice, and rats and is the single largest mammalian order comprising ~40% of all mammals [2, 3].

Historically, virus discovery relied on relatively inefficient in vitro or in vivo virus isolation methods. These led to the identification of two rodent coronaviruses (CoVs); rat sialodacryoadenitis coronavirus (SADV) [4] and murine hepatitis virus (MHV) [5], both of which are from the same viral species within the Betacoronavirus genus. However, the advent of degenerate primer PCR and unbiased viral metagenomics has significantly enhanced our ability to detect novel viruses, and their use has led to the discovery of numerous alpha- and betacoronaviruses in a range of rodent species, including field voles, bank voles, rats, and mice in East Asia and Europe [6–11]. These findings are paving the way toward a better understanding of the longer-term evolution and origins of these important viral species [12].
In this chapter we describe a degenerate primer PCR method that we have used [10] to detect coronaviruses in a variety of tissues obtained from rodent species sampled postmortem.

2 Materials

2.1 Sample Acquisition
1. Ethics approval.
2. Live traps and snap traps of different sizes to collect wild rodents (voles, rats).
3. −80 °C freezers.
4. RNA preservative such as RNAlater or DNA/RNA shield.
5. Class I biosafety cabinet.
6. Disposable scalpels.

2.2 Total RNA Extraction from Mammalian Tissue
1. Class I biosafety cabinet.
2. Plastic tweezers.
3. Petri dishes.
4. Homogenizer.
5. Rodent tissue sample (preferably liver) in a preservative such as RNAlater stored at −80 °C (see Note 1).
6. Total RNA extraction kit from mammalian tissues such as GenElute™ Mammalian Total RNA Miniprep Kit (see Note 2).
7. Thermocycler.
8. NanoDrop® spectrophotometer or similar.

2.3 cDNA Synthesis with Random Hexamers
1. Random hexamers, such as RNA to cDNA EcoDry (see Note 3).
2. PCR-grade water.
3. Thermocycler.

2.4 PCR
1. Taq polymerase and buffer, such as HotStarTaq DNA Polymerase.
2. Thermocycler.
3. PCR-grade water.
4. 10 mM deoxynucleotide mix.
5. Primers (see Table 1; see Notes 4 and 5).

2.5 Agarose Gel Electrophoresis
1. Gel electrophoresis tank.
2. 2% agarose gel with 0.5 µl/ml ethidium bromide (from a 10 mg/ml stock).
3. 6× loading buffer: 10 mM Tris–HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM
EDTA (or equivalent commercial ones such as Thermo Scientific 6× DNA Loading Dye).

4. Tris–acetate–EDTA (TAE): 40 mM Tris–acetate and 1 mM EDTA (pH 8.3) buffer containing 0.5 μl/ml ethidium bromide (from a 10 mg/ml stock) (see Note 6).

5. UV transilluminator.

### 3 Methods

#### 3.1 Sample Acquisition

1. Acquire ethical approval from relevant committees to trap, euthanize, and handle wild rodents.

2. Wild rodents can either be live trapped and euthanized later, or snap-killed by the trap. In either case, the carcasses should be stored as soon as possible at −80 °C.

3. In a Class I biosafety cabinet, the carcasses should be dissected using disposable scalpels and collect the organs of interest (in this case liver and intestine).

4. The organs should be placed in a tube containing a preservative such as RNAlater and stored immediately at −80 °C until they are processed for RNA extraction.

#### 3.2 RNA Extraction from Mammalian Tissues

1. All the extractions from mammalian tissues should be performed inside a Class I biosafety cabinet to reduce any safety risk while handling the tissues and to avoid contamination.

2. Remove the tissue samples from the freezer, and once thawed remove from the preservative and place the sample in a petri dish (see Note 7).

3. Using a pair of tweezers, tease a section of tissue measuring approximately 1 mm³ in volume from the tissue sample. Put the remaining tissue back in the tube with the preservative and store it at −80 °C.

4. Proceed with the extraction following the steps of the extraction kit protocol.

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### Table 1

| Primer name | Sequence 5'–3' | References |
|-------------|----------------|------------|
| GAPDH_F     | CCATCTTCCAGGAGCGAGA | [10]       |
| GAPDH_R     | GCCTGCTTCACCACCTTCT  |           |
| CoV_F       | GGTTGGGACTATCCTAAGTGTGA | [13]       |
| CoV_R       | CCATCATCAGATAGAATCATCATA |           |
5. Measure the quality and the concentration of the extracted RNA using a NanoDrop® or similar spectrophotometer. Use the elution buffer of the extraction kit as a blank to calibrate the spectrophotometer. A pure RNA sample should have $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios between 1.80 and 2.2 (see Note 8). One $A_{260}$ unit equals 40 μg/ml single stranded RNA (ssRNA).

3.3 cDNA Synthesis with Random Hexamers

1. Add up to 5 μg of the extracted RNA from Subheading 3.2 above to a tube of EcoDry™ cDNA lyophilized premix (random hexamers). If necessary, add molecular biology-grade (RNase/DNase-free) water to yield a total volume of 20 μl.

2. Incubate the reaction at 42 °C for 60 min and then stop the reaction by heating at 70 °C for 10 min.

3.4 PCR Amplification of the GAPDH “Housekeeping Gene” to Assess cDNA Quality

1. For each of the cDNA samples, make up a 12.5 μl PCR reaction containing 1× PCR Buffer, 2.5 units HotStarTaq Polymerase, 0.2 mM of each dNTP, 0.4 μM Forward Primer, 0.4 μM Reverse Primer, molecular biology-grade water, and cDNA template (see Notes 9 and 10). Also include negative control reactions containing water as template.

2. Transfer the reaction tubes to a thermal cycler and perform the amplification using the following cycling parameters: 1 cycle of 15 min at 95 °C; 55 cycles of 20 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C; and a final extension cycle of 60 s at 72 °C.

3. Add 1 μl of 6× gel loading buffer to 5 μl of the PCR reaction and load into the wells of a prepared 2% agarose gel in 1× TAE-EtBr. Load a suitable DNA ladder (e.g., GeneRuler DNA Ladder mix) to allow size estimation of any PCR products.

4. Resolve the DNA products by electrophoresis in 1× TAE-EtBr at 90 V for 40 min.

5. The expected product size for successful amplification of the GAPDH gene is 571 bp (see Note 11).

3.5 PCR Screening for Coronaviruses

1. Samples of cDNA that yield a GAPDH PCR product can then be screened for the presence of coronavirus using a degenerate primer PCR.

2. Similar to Subheading 3.4, for each GAPDH positive cDNA sample, make up a 12.5 μl Coronavirus-specific PCR reaction containing 1× PCR Buffer, 2.5 units HotStarTaq Polymerase, 0.2 mM of each dNTP, 0.4 μM Forward Primer, 0.4 μM Reverse Primer, molecular biology-grade water, and cDNA template (see Notes 10 and 12).

3. Transfer the reaction tubes to a thermal cycler and perform the amplification using the following cycling parameters: 1 cycle of 15 min at 95 °C; 55 cycles of 20 s at 95 °C, 20 s at 48 °C, 30 s at 72 °C; and a final extension cycle of 10 min at 72 °C.
4. Analyse the PCR products by electrophoresis through a 2% agarose gel in 1×TAE-EtBr at 90 V for 40 min. Coronavirus-specific PCR products should appear as a band of approximately 440 bp.

5. Any PCR positive samples should be sent for Sanger sequencing using the sense and/or antisense PCR primers (see Note 13).

### 3.6 Agarose Gel Preparation and Electrophoresis

1. Seal the casting tray and add the combs of interest.
2. To prepare a 2% agarose gel add 2 g of agarose in 100 ml of 1×TAE buffer (see Note 6).
3. Place the flask containing the mixture into a microwave oven until it starts boiling. Take the flask out, stir it gently while holding it and place it back to the oven. Repeat the procedure until the mixture is transparent and there is no visible agarose.
4. Cool the flask briefly under the tap with cold water until it is cold enough to place it on your hand.
5. Add 5 μl of EtBr (from a 10 mg/ml stock) and start stirring gently.
6. Pour the mixture into the casting tray steadily and remove any bubbles with a pipette tip.
7. Let it rest for 20–30 min until it becomes solid.
8. Load the gel as described in Subheading 3.4, step 3 and run it at 90 V for 40 min.
9. Visualize the gel on the UV transilluminator.
10. Always be careful when handling EtBr and dispose of as per local regulations. There should be a dedicated space or room for preparing and running agarose EtBr gels.

### 4 Notes

1. For best quality RNA and virus retrieval, the rodent carcasses should be immediately frozen after their death. As soon as they are dissected, each tissue should be placed in a tube containing a preservative such as RNAlater (to preserve the RNA and prevent degradation) and stored immediately at −80 °C. The tissues and the RNA should always be placed on ice when handled. Avoid repeated freeze–thaw cycles as they contribute to the rapid degradation of the nucleic acid.

2. There are several methods for RNA extraction, such as phenol–chloroform and TRIzol. However, it is much easier to use a commercial extraction kit. We routinely use GenElute™
Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) which gives us good RNA recovery.

3. For the purpose of coronavirus discovery, cDNA synthesis with either random hexamers or a specific primer is acceptable. Random hexamers provide the flexibility of performing several PCRs directed at different targets such as different viruses or housekeeping genes for quality control. Primer-specific cDNA, on the other hand, is less flexible but is reportedly more sensitive.

4. A PCR targeting the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene (or any other housekeeping gene) is essential to assess the quality of the cDNA.

5. There are several published primer-sets for coronavirus screening [13–17]. However, in this chapter we describe the use of Woo et al. primer-set [13]. Although it was designed to detect the Human HKU-1 CoV, which is a betacoronavirus, it has been shown to detect alphacoronaviruses [10].

6. It is easier to buy a commercially available 10× TAE buffer and dilute to a final concentration of 1×.

7. RNA is very sensitive and degrades at room temperature; therefore, all the RNA work should be done on ice to keep it stable.

8. The purity of the RNA sample will impact on downstream applications such as cDNA synthesis. Therefore, it is essential to aim for an RNA sample free from contaminants.

9. All the PCR preparation should be performed in a dedicated pre-PCR room where there are no traces of amplicons or plasmids. The amplification and all the post-PCR handling should be done in separate rooms as far away from the pre-PCR room as possible. Thus, the risk of contamination is minimized.

10. The maximum amount of cDNA used in a PCR should not be more than 1/10th of the total PCR volume. If the user wants to add more cDNA template, then the reaction volume should be increased accordingly.

11. In this chapter we describe the conventional PCR method. Quantification of the template is possible with quantitative real-time PCR by doing serial dilutions and using a housekeeping gene as a reference.

12. Sometimes virus titers in a sample can be very low and might not be detected using low levels of template. Larger-volume PCRs, utilizing increased template can be beneficial in these
cases. This approach can also be applied to rescreen potential hits that have yielded low amounts of product (as indicated by the presence of a faint band of the expected size following agarose gel electrophoresis) in the initial screening PCR.

13. Nowadays, Sanger sequencing is commercially available and very affordable. The only requirement usually is to dilute the PCR products (usually 1 in 10 if the gel band is clear and bright) and to provide your primer of interest at a certain concentration.

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