

# Chapter 2

## Identification of a Novel Coronavirus from Guinea Fowl Using Metagenomics

Mariette F. Ducatez and Jean-Luc Guérin

### Abstract

While classical virology techniques such as virus culture, electron microscopy, or classical PCR had been unsuccessful in identifying the causative agent responsible for the fulminating disease of guinea fowl, we identified a novel avian gammacoronavirus associated with the disease using metagenomics. Next-generation sequencing is an unbiased approach that allows the sequencing of virtually all the genetic material present in a given sample.

**Key words** Next-generation sequencing, Illumina, Ultracentrifugation, Nuclease treatment, Random RT-PCR

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### 1 Introduction

The field of pathogen discovery gained a completely new dimension when new genomics tools, next-generation sequencing (NGS), became available. Isolation and identification by electronic microscopy used to be the gold standard techniques to identify a new pathogen. However, some pathogens are difficult to culture, and/or to separate from co-infecting agents. PCR has improved pathogen discovery, but even when pan-species/Genus/family PCRs may be developed (which is not always possible, especially for highly variable RNA viruses), the primers chosen do condition the nucleic acids that will be amplified: it is still a “biased” technique with which only known (or closely related to known) pathogens whose presence was suspected may be detected [1, 2]. NGS allows for (1) massive sequencing of genetic material and (2) unbiased sequencing, to a much lower cost per sequenced base than Sanger sequencing technology. Here we describe an NGS technique that can be used to identify novel pathogens, which we recently used to identify the pathogen responsible for the guinea fowl fulminating disease [3].

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## 2 Materials

### 2.1 Materials for Preparation of Specimens and Concentrating Viral Particles

1. PBS.
2. PBS containing 100 U/ml Penicillin and 0.1 mg/ml streptomycin.
3. 0.45 µm filter.
4. RNase.
5. DNase (10 U/µl).
6. 10× DNase buffer: 200 mM Tris-HCl (pH 8.4), 20 mM MgCl<sub>2</sub>, 500 mM KCl.

### 2.2 Materials for RNA Extraction and Amplification

1. TRIzol reagent or similar.
2. Chloroform.
3. RNA extraction kit, e.g., Nucleospin RNA virus kit (Macherey-Nagel) or similar.
4. Reverse transcription kit, e.g., RevertAid kit (ThermoFisher) or similar.
5. 10 mM dNTPs.
6. RNase inhibitor.
7. 20 µM tagged random hexamer: 454-A: 5' ATG-GTC-GTC-GTA-GGC-TGC-TCN-NNN-NNN-N 3' (*see Note 1*).
8. PCR kit, e.g., Phusion (NEB) or similar.
9. 20 µM PCR primer (tag only): 454-A: 5' ATG-GTC-GTC-GTA-GGC-TGC-TC 3'.
10. Thermocycler.
11. 1 % agarose gel.
12. 1× TBE buffer: 89 mM Tris, 89 mM orthoboric acid, 2 mM EDTA.
13. Sybr® safe (Life technologies) or similar.
14. 1 kb plus DNA ladder.
15. Gel purification kit.
16. PicoGreen® quantitation assay (Life technologies) or similar.

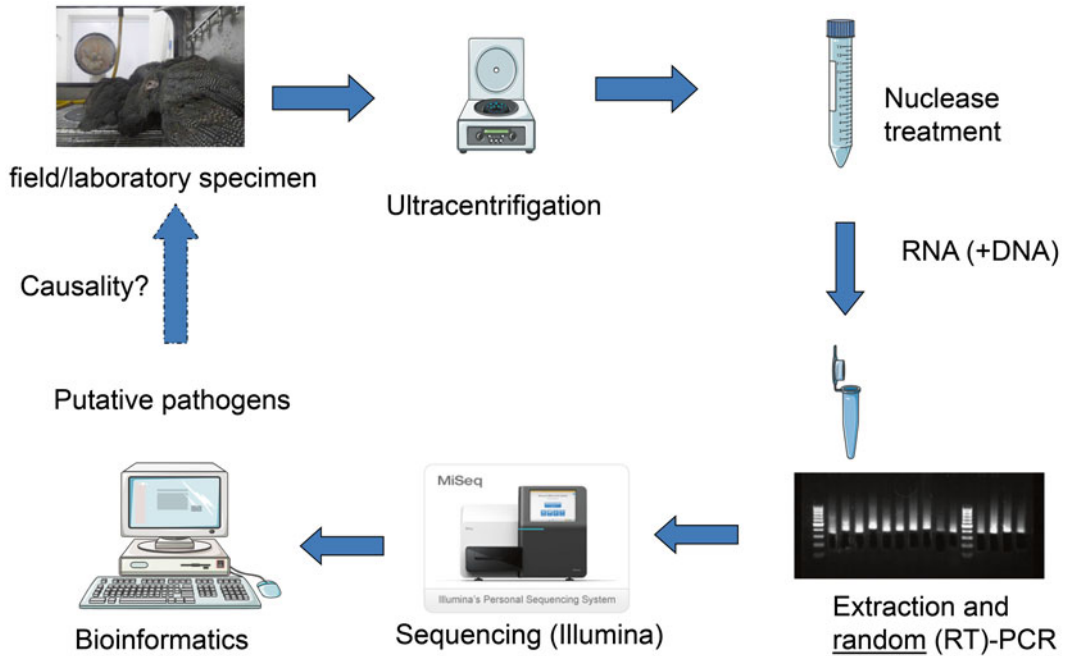
### 2.3 Next-Generation Sequencing

1. Miseq reagent kit (Illumina).
2. Miseq (Illumina).

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## 3 Methods

Figure 1 summarizes the methodology used for the identification of a novel guinea fowl gammacoronavirus.



**Fig. 1** High-throughput unbiased sequencing of intestinal contents of guinea fowls. The scheme represents the main steps of the novel guinea fowl gammacoronavirus detection by NGS

### 3.1 Sample Preparation

1. Pool intestinal contents of experimentally infected guinea fowl poults and resuspend in 500  $\mu\text{l}$  PBS with penicillin and streptomycin. Vortex.
2. Filter (0.45  $\mu\text{m}$  filter) the solution to eliminate eukaryotic- and bacterial-cell-sized particles.
3. Centrifuge the digested content at 10,000 $\times g$  for 30 min twice to clarify the solution and collect the supernatant in a new tube.

### 3.2 Concentration of Viral Particles

1. Pellet the concentrated material by ultracentrifugation at 100,000 $\times g$  for 2 h.
2. Treat with RNase and DNase to remove non-particle-protected nucleic acids: make a mix of 500  $\mu\text{l}$  of sample, 10  $\mu\text{l}$  DNase (100 U), 12  $\mu\text{l}$  RNase (20  $\mu\text{g}/\mu\text{l}$ ), 60  $\mu\text{l}$  10 $\times$  DNase buffer, 16  $\mu\text{l}$  PBS. Incubate the mix for 20 min at 37  $^{\circ}\text{C}$  and then 10 min at 75  $^{\circ}\text{C}$  to stop the reaction.

### 3.3 RNA Extraction and Amplification (See Note 2)

1. Add 750  $\mu\text{l}$  TRIzol to 250  $\mu\text{l}$  sample from Subheading 3.2, step 2 and incubate 5 min at room temperature.
2. Add 200  $\mu\text{l}$  chloroform, vortex vigorously, and incubate 10 min at room temperature.
3. Centrifuge for 15 min at 11,000 $\times g$  at 4  $^{\circ}\text{C}$ .

4. Collect the top aqueous phase.
5. Extract RNA from 150  $\mu\text{l}$  of the collected aqueous phase on a silicate column with a RNA extraction kit.
6. Perform a reverse transcription reaction using random primers by mixing 7.5  $\mu\text{l}$  RNA, 5  $\mu\text{l}$  tagged random hexamer.
7. Incubate at 65  $^{\circ}\text{C}$  for 5 min then keep on ice.
8. Add 4  $\mu\text{l}$  5 $\times$  reaction buffer, 0.5  $\mu\text{l}$  RNase inhibitor, 2  $\mu\text{l}$  dNTP, and 1  $\mu\text{l}$  RevertAid reverse transcriptase.
9. Incubate at 25  $^{\circ}\text{C}$  for 10 min, 42  $^{\circ}\text{C}$  for 60 min, and 70  $^{\circ}\text{C}$  for 10 min.
10. Reactions can now be stored at  $-20^{\circ}\text{C}$  or used immediately for PCR.
11. Perform a random PCR by mixing 10  $\mu\text{l}$  5 $\times$  Phusion HF reaction buffer, 1  $\mu\text{l}$  dNTP, 0.5  $\mu\text{l}$  tag only primer, 5  $\mu\text{l}$  cDNA, 0.5  $\mu\text{l}$  Phusion polymerase, and 33  $\mu\text{l}$  water.
12. Perform PCR using the following cycle: 98  $^{\circ}\text{C}$  30 s followed by 40 cycles of 98  $^{\circ}\text{C}$  10 s, 65  $^{\circ}\text{C}$  20 s, and 72  $^{\circ}\text{C}$  30 s followed by a final incubate of 72  $^{\circ}\text{C}$  10 min.
13. Analyze the PCR products on a 1 % agarose gel, migrate for 1 h at 60 V.
14. Excise the 300 bp bands and perform a gel purification with a commercial kit.
15. Quality assessment of the prepared library: quantify the DNA generated by a fluorescence-based method (PicoGreen<sup>®</sup> quantitation assay) and aim at 1  $\mu\text{g}$  DNA as input; check the DNA quality and aim for a 260:280 ratio  $>1.8$ .

### **3.4 Cluster Generation Using the Miseq Reagent Kit (Illumina)**

1. Hybridize sample to flow cell.
2. Amplify sample (bridge amplification).
3. Linearize fragments.
4. Block fragments.
5. Hybridize sequencing primer.

### **3.5 Sequencing on the Miseq (Illumina)**

1. The library DNA fragment act as a template, from which a complementary strand is synthesized.
2. ddNTPs are added one by one (one cycle = one ddNTP added, a picture taken and defluoration of the ddNTP to be able to add a new ddNTP the next cycle) by a DNA polymerase. The addition of ddNTP is digitally recorded as sequence data cycle after cycle.

### **3.6 Data Analysis**

1. Preprocess the data to remove adapter sequences and demultiplexing using splitbc (several samples can be multiplexed and run together on the MiSeq Illumina sequencer to reduce cost).

2. Preprocess the data to remove low-quality reads and compiling paired sequences using `illumina-paired-end`.
3. Map the data to a reference genome or *de novo* align the sequence reads (alignment with `bwa` [4], consensus computed with the `SAMtools` [5] software package. Display the results with the `IGV` [6] browser).
4. Analyze the compiled sequence with the `GAAS` software (<http://gaas.sourceforge.net/>) with an expected value of  $10^{-3}$ .

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## 4 Notes

1. Victoria et al. [7] described a panel of tagged primers (named with alphabet letter), and while we selected “454-A” for the present study, any of the tagged primers described in [7] could be used instead (454-B, 454-C, etc).
2. To identify pathogens with DNA genomes, a DNA extraction would be performed, followed by a Klenow step before the random PCR:
  - (a) DNA extraction: High Pure template preparation kit (Roche) can be used, following the manufacturer’s instructions.
  - (b) Klenow step on DNA: mix 2.5  $\mu$ l tagged random primer, 3  $\mu$ l 10 $\times$  buffer, 1  $\mu$ l 0.5 mM dNTP, 10  $\mu$ l DNA, 1  $\mu$ l 0.5 U/ $\mu$ l DNA polI and 12.5  $\mu$ l water. Incubate at room temperature for 1 h.
  - (c) Proceed from Subheading 3.3, step 10.

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