Diverse gammacoronaviruses detected in wild birds from Madagascar

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Abstract To date, infectious bronchitis virus (IBV) is potentially found in wild birds of different species. This work reports the survey of coronaviruses in wild birds from Madagascar based on the targeting of a conserved genome sequence among different groups of CoVs. Phylogenetic analyses revealed the presence of gammacoronaviruses in different species of Gruiformes, Passeriformes, Ciconiiformes, Anseriformes, and Charadriiformes. Furthermore, some sequences were related to various IBV strains. Aquatic and migratory birds may play an important role in the maintenance and spread of coronaviruses in nature, highlighting their possible contribution in the emergence of new coronavirus diseases in wild and domestic birds.

Keywords Gammacoronavirus · Wild birds · Madagascar

Introduction

Coronaviruses (CoVs) are enveloped viruses within the Coronaviridae family with a positive-sense RNA genome ranging from 27 to 30 kb (Cavanagh 1997). They are divided into four genera: Alpha-, Beta-, Gamma- and Deltacoronavirus.

Alpha- and betacoronaviruses have been isolated from mammals; gammacoronaviruses, being the most representative member infectious bronchitis virus (IBV), have also been detected in the beluga whale (Mihindukulasuriya et al. 2008), the Asian leopard cat (Dong et al. 2007), and in several species of wild birds (Chu et al. 2011; Hughes et al. 2009; Jonassen et al. 2005; Muradrasoli et al. 2010; Woo et al. 2009). Viruses of the new proposed genus Deltacoronavirus were found primarily in mammals, terrestrial and aquatic birds (Chu et al. 2011; Dong et al. 2007; Woo et al. 2009).

In animals, CoVs are associated to respiratory and intestinal infections, as well as hepatic, renal, and neurological disorders (Cavanagh 2005). Human coronaviruses infections were generally related to the common cold, but the emergence and pandemic potential of severe acute respiratory syndrome (SARS) in 2003 and the Middle East respiratory syndrome (MERS) in 2012, both belonging to genus Betacoronavirus, led to the discovery of several CoVs hosted by humans and animals (Hilgenfeld and Peiris 2013). Over the past years, the wildlife has been under epidemiological surveillance worldwide, as they can play an important role as reservoir of emerging viruses that may pose a risk to mankind and threaten wildlife itself. Wild birds are known to be important reservoirs of avian influenza A virus (Brown and Stallknecht 2008; Spackman 2009), as well as other respiratory and enteric viruses like IBV (Cavanagh and Gelb 2008). In poultry, the disease is controlled through the use of vaccines and biosafety measures, including avoidance of contact with...
wild birds. Upon vaccination, IBV generates antigenic variants that can result in incomplete protection (Cavanagh 2005).

Coronaviruses phylogenetically related to IBV were largely detected in several wild bird species in Northern England, as well as novel viruses that are not associated to classical IBV types (Hughes et al. 2009). This was also reported in the Beringian area between Alaska and Siberia (Muradrasoli et al. 2010) and in wild bird surveillance carried out in Hong Kong and Cambodia (Chu et al. 2011). These studies highlight the wide host range, geographic distribution, and diversity of avian CoVs, suggesting a possible impact on animal health and later a possible risk for human health (Muradrasoli et al. 2010).

In the present work, we report the detection and the phylogenetic aspects of coronaviruses detected in wild birds sampled at Alaotra Lake, Madagascar. This lake constitutes an exceptional site for the settlement of both endemic and migratory birds (BirdLife International 2014a). The sanitary and economic impacts of avian coronaviruses in the Madagascar poultry sector are still unknown.

### Material and methods

For this work, we used part of the samples collected in the framework of the GRIPAVI project (http://gripavi.cirad.fr/en/) launched in 2007, and concluded in 2011 by CIRAD in collaboration with the Département de Recherche Zootechnique et Vétérinaire du Centre National de la Recherche Appliquée au Développement Rural (FOFIFA-DRZV) in Madagascar, with the support of the French Ministry of Foreign Affairs. Cloacal swabs collected in 2011 from 357 free-ranging birds at Alaotra Lake on Madagascar highlands were used. The samples were dipped in a transport medium consisting of isotonic phosphate-buffered saline, pH 7.0–7.4, with antimicrobial additives (penicillin 10,000 U/mL, streptomycin 10 mg/mL, amphotericin B 25 μg/mL, and gentamycin 250 μg/mL) supplemented with 20 % glycerol and stored in liquid nitrogen containers. They were shipped in dry ice and kept in the laboratory at −80 °C until processing. All samples were manipulated in a biosafety level 3 laboratory.

Viral RNA was extracted from samples by a high-throughput-automated workstation Biomek FX® (Beckman Coulter) using the Nucleospin RNA virus kit, according to manufacturer’s instructions (Macherey Nagel). The viral RNA was resuspended in nuclease-free water and stored at −80 °C. Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers were used in 20 μL reactions to generate cDNAs from 10 μL of the extracted RNA suspension, according to the manufacturer’s instructions.

The obtained cDNAs were submitted to a pancoronavirus nested PCR (nPCR) with Top Taq DNA polymerase (Qiagen), according to manufacturer’s instructions. A segment of the RNA-dependent RNA polymerase (RdRp) was targeted with the following primers: CoV-F1 (5′-GGKTGGGAYTAYC CKAARTG-3′), CoV-R1 (5′-TGTYGTSWRCARAAAYTCR TG-3′), CoV-F2 (5′-GGTTGGGACTATCTCTAAGTGTGA-3′), and CoV-R2 (5′-CCATCATCAGATAGATCATCATA-3′) (Chu et al. 2011). Briefly, the nPCR was performed as follows: the first reaction was performed in a 25-μL volume containing 20 ng of cDNA with primers (CoV-F1 and CoV-R1). The cycling conditions were 5 min at 95 °C, 40 cycles of 45 s at 94 °C, 40 s at 94 °C, 45 s at 72 °C and a final incubation at 72 °C for 7 min. For the second (nested) reaction, we used 2 μL of the first reaction under the same amplification conditions of the first PCR, but using primers CoV-F2 and CoV-R2 in the assay.

Standard precautions were taken to avoid PCR contamination; blank controls without template were included in every set of five RT-PCR assays. PCR products were electrophoresed in 1.5 % agarose gels and the products visualized on UV light. The products with the expected size (400 to 440 bp) were excised and purified using QiaQuick gel extraction Kit (Qiagen) following manufacturer’s instructions.

The positive samples were sequenced (Cogenics) with primers CoV-F2 and CoV-R2, using Big dye Terminator v3.1 (Applied Biosystems) cycle sequencing kit according to the manufacturer’s instructions.

The BLAST was used to detect homologous regions in sequence databases. Sequences were aligned by ClustalW and MEGA version 6 (Tamura et al. 2013). Phylogenetic analysis was performed based on a fragment containing approximately 350 bp of the RdRp CoV gene using the neighbor-joining method with bootstrap support (1000 replicates) in MEGA version 6 (Tamura et al. 2013).

### Results and discussion

Samples originating from 17 bird species were tested for CoVs polymerase RdRp gene by RT-PCR, and 28 of the 357 cloacal samples were positive (7.8 %). Positives were found in 11 species (Table 1). All sequences were submitted as GenBank:KM093872 to GenBank:KM093897; GenBank:KM093899 and GenBank:KM093901. Phylogenetic tree reconstructions enabled us to classify all the coronaviruses identified in this study as gammacoronaviruses (Fig. 1).

These *gammacoronaviruses* were detected in a wide broad of bird orders, such as the following: Gruiformes, Passeriformes, Ciconiiformes, Anseriformes, and Charadriiformes. Nucleotide distances between CoVs sequences in this study were 0.0–23.5 %. Sequences formed five different subgroups within two main clusters, revealing the diversity among avian CoVs strains from the same geographic
The number of samples analyzed in this study was limited to predict the real prevalence of gammacoronavirus in Madagascar, but our results emphasize the circulation of genetically diverse coronaviruses in apparently healthy populations of wild birds from different species. Additional genetic data on coronavirus surveillance in a wide number of individuals and species, as well as the complete genomic characterization of these wild bird coronaviruses are required to better understand their evolution, the relationship between these viruses and wild bird population, and the risk of transmission to poultry.
Fig. 1 Phylogenetic relationship between the nucleotide sequences corresponding to a portion of 350 bp of the *Coronavirus* gene (RNA-dependent RNA polymerase gene) amplified from wild birds at Alaotra Lake. *Coronavirus* detected in wild bird species in this study are denoted by black diamonds and their corresponding GenBank accession numbers. Sequences from other coronaviruses were included for comparative purposes.
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