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Phylogenetic and phylogeographic mapping of the avian coronavirus spike protein-encoding gene in wild and synanthropic birds

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ABSTRACT

The evolution and population dynamics of avian coronaviruses (AvCoVs) remain underexplored. In the present study, in-depth phylogenetic and Bayesian phylogeographic studies were conducted to investigate the evolutionary dynamics of AvCoVs detected in wild and synanthropic birds. A total of 500 samples, including tracheal and cloacal swabs collected from 312 wild birds belonging to 42 species, were analysed using molecular assays. A total of 65 samples (13%) from 22 bird species were positive for AvCoV. Molecular evolution analyses revealed that the sequences from samples collected in Brazil did not cluster with any of the AvCoV S1 gene sequences deposited in the GenBank database. Bayesian framework analysis estimated an AvCoV strain from Sweden (1999) as the most recent common ancestor of the AvCoVs detected in this study. Furthermore, the analysis inferred an increase in the AvCoV dynamic demographic population in different wild and synanthropic bird species, suggesting that birds may be potential new hosts responsible for spreading this virus.

1. Introduction

Avian coronaviruses (AvCoVs) are enveloped viruses with pleomorphic spherical forms belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* and grouped into the *Gamma-CoV* genus (Carstens, 2010). The AvCoV genome consists of positive-sense single-stranded RNA with an approximate size of 27 kb (Jackwood et al., 2012). The genome is subdivided into nine open reading frames (ORFs) that encode a set of non-structural proteins known as the transcriptase–replicase complex, which functions as the RNA polymerase (Pol-1a, -1b) and four main structural proteins (spike surface glycoprotein (S), membrane (M), envelope (E), and nucleocapsid (N)) (Fauquet et al., 2005; Mardani et al., 2008; Mo et al., 2013).

Among the AvCoVs, we highlight Avian infectious bronchitis virus (AIBV), the aetiological agent of infectious bronchitis, which is one of the most important viral diseases in countries with intensive poultry industries (Cavanagh and Gelb, 2008; Jones, 2010), as well as AvCoVs (IBV-like) that were recently detected in several bird orders, such as *Galliformes*, *Anseriformes*, *Columbiformes*, *Charadriiformes*, *Passeriformes*, *Pelacaniformes*, *Ciconiiformes* and *Psittaciformes*, which may influence strongly AvCoV epidemiology (Domanska-Blicharz et al., 2014).

Due to its highly mutagenic character, the control of AvCoVs is a constant challenge for the poultry industry. The evolutionary dynamics of AvCoVs are characterised by the continuous emergence of new variants, resulting in multiple serotypes that do not confer cross-protective immunity to each other (Jackwood et al., 2012). AvCoV diversity is mainly driven by three factors: the elevated mutation rate of the RNA-dependent RNA polymerase; the great diversity of birds, which provides many potential target hosts for different AvCoV genotypes; and the facilitation of viral transmission and dissemination across large geographic areas and between different species by bird migration and flock lifestyles (Keesing et al., 2010). These factors have driven the generation of a wide

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variety of AvCoV variants, resulting in persistent reservoirs that can become relevant for human populations when wild animals acquire synanthropic habits and start to live in close proximity to domesticated poultry (Jourdain et al., 2007; Felippe et al., 2010).

Despite the health and economic importance of AvCoVs to the poultry industry, knowledge about the prevalence and distribution of these viruses in wild and synanthropic birds remains limited. The variety of potentially infected bird species and the annual use of intercontinental territory by migratory birds increase the risk of the introduction and spread of new pathogens and new variants of CoV (Jourdain et al., 2007; Somveille et al., 2013), requiring the development of new analytical methods.

Phylogenetic inferences are extremely useful tools for the study of the molecular ecoepidemiology of pathogens and for the elucidation of epidemiologically important characteristics. Phylogenetic inferences enable the molecular characterisation of existing variants and the discovery of new viral variants with potential health repercussions. Furthermore, these techniques permit an analysis of the evolutionary history of new variants (Lemey et al., 2009; Yang and Rannala, 2012).

In addition to providing information on the timeline of evolutionary processes, phylogenetic studies can be used to compare and associate individuals from specific geographical regions with the time of the most recent common ancestor (tMRCA) of each region studied. Tools such as phylogeography provide a useful approach for understanding the origins and distributions of different viral strains to elucidate population evolution dynamics and their dependence on the environment. Phylogeographic techniques have been applied to many viral infections that threaten human health, including dengue, rabies, influenza and HIV (Holmes, 2004; Kühnert et al., 2011), but there is a lack of phylogeographic information regarding CoVs.

This pilot study applied a phylogeographic approach to the evolution of the AvCoV spike protein gene to study the spatial arrangements of different genetic lineages within species and between closely related species, to analyse the principles and processes that determine the geographic distribution of lineages, and to establish a resource for improved molecular characterisation of available and as-yet unidentified AvCoV strains.

2. Material and methods

2.1. Ethical statement

The capture and management of wild and synanthropic animals for the collection of biological samples was performed in accordance with current Authorization and Information System of Biodiversity (SISBIO) recommendations, Brazil, protocol no. 34751-1/12.

2.2. Samples and population study

A total of 500 tracheal and/or cloacal swabs were collected from 42 different bird species belonging to 13 orders (Supplementary Table S1). The biological samples were collected in the cities of Campinas and São Paulo in the State of São Paulo (Southeastern Brazil) and the city of Florianópolis (Southern Brazil) in the State of Santa Catarina between 2006 and 2013. The swabs were placed in 1.5-mL microtubes and identified with the name of the bird species and a specific code. The samples were immediately transported in coolers with ice to the Laboratory of Animal Virology (LVA) at the Institute of Biology at UNICAMP and stored at −80 °C until further processing.

2.3. Route map of migratory and non-migratory birds

The maps showing the birds and their specific routes were derived from the BirdLife International (Cambridge, United Kingdom) and NatureServe (Arlington, United States of America) databases. These maps consist of Geographic Information System (GIS) shapefiles that show the geographic extent and distribution of all bird species investigated in the study (Fig. 1).

2.4. RNA extraction and detection of AvCoVs by qRT-PCR

A total of 500 μL of Minimum Essential Medium (MEM) (Nutricell, Brazil) was added to the microtube containing the swab with the biological sample. The remaining procedure was performed according to the QIAamp® Viral RNA Mini Kit (QIAGEN, CA, USA) manufacturer's instructions.

Each extracted RNA sample was subjected to Real Time RT-qPCR using previously published primers for the 5′-Untranslated Region (UTR) of AvCoV (Callison et al., 2006) with the addition of a double Quencher. Amplification of the 143-bp fragment was performed in a 7500 Real Time PCR Cycler (Applied Biosystems™, CA, USA) using the following primers and probes (IDT, Iowa, USA): 5′ UTR forward primer, 5′-GCT TTT GAG CCT AGC GTT-3′; 5′ UTR reverse primer, 5′-GCC ATG TTG TCA CTG TCT ATT-3′; 5′ UTR probe, 5′-/5HEX/CA CCA CCA G/ZEN/A ACC TGT CAC CTC/3IABkFQ/-3′.

2.5. cDNA synthesis and sequencing

The spike glycoprotein gene region was amplified from qRT-PCR-positive samples. Briefly, cDNA was synthesised using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, CA, USA). Next, a nested RT-PCR assay was performed using the following primers in the first step to amplify a 572-bp fragment: forward primer S7 5′-TACTACTACCAGAGTGC(C/T)TT-3′ and reverse primer S6 5′-ACATC(T/A)TGTGCGGTGCCATT-3′. The following primers were used in the second step to amplify a 530-bp fragment: forward primer S9 (5′-ATGGTTGGCATTT(A/G)CA(C/T)GG-3′) and reverse primer S5 (5′-GTGCCATTGACAAAATAAGC-3′) (Bochkov et al., 2007). This assay was performed in an Eppendorf MasterCycler®Pro S (NY, USA).

The amplified PCR products were purified using the MiniElute PCR Purification kit (QIAGEN, CA, USA) and quantified using an Epoch spectrophotometer (Biotek, Vermont, USA). Purified PCR products were sequenced by the Central Laboratory of High Performance Technologies in Life Sciences (LaCTAD/UNICAMP, São Paulo, Brazil). Sequencing reactions were performed using the Big Dye® Cycle Sequencing Terminator 3.1 kit (Applied Biosystems™, CA, USA) following the instructions provided by the manufacturer. The 3730XL DNA Analyser (Applied Biosystems™, CA, USA) platform was used for sequencing.

2.6. Sequence data collection and phylogenetic analyses

All bioinformatics and phylogeny experiments were performed using the Linux Cluster from the High Performance Computer Center, University of Florida, USA (UF HPC). For the bioinformatics and phylogenetic analyses, all available AvCoV spike 1 gene (S1) sequences from different animals were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) (Supplementary Table S2) and filtered using the following criteria: known sampling year; unknown sampling year; and known sampling year and isolation source (Fig. 2). Spike 2 gene regions (S2), clones, patents, and any recombinant sequences were excluded. Additionally, different CoV sequences were downloaded to represent CoVs from the genera *Alpha*, *Beta*, *Gamma*, and *Delta*. The