Research Article

Avian Coronaviruses in Wild Birds along Brazilian Segment of Atlantic Flyway

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Abstract | Degradation of natural habitats has been described by many previous studies as a major factor in the transmission of infectious diseases, once it leads to changes on migration patterns and dispersal of birds, as resting and feeding points become scarcer resulting in agglomeration of different species. One pathogen of greatest importance that may be dispersed by birds are the coronaviruses (CoVs). Despite its relevance and complexity, studies on the detection and genetic characterization of these viruses are scarce, especially in South American wild birds. Thus, this paper aims to detect and discuss the diversity of these agents in birds at migratory stopping and wintering sites, along Brazilian Segment of the Atlantic Flyway. Therefore, 738 avian samples from different regions of Brazil were subjected to a RT-Nested-PCR protocol, with primers targeting RdRp region. Positive samples were confirmed using Sanger followed by Ion Torrent S5 sequencing platforms and results were submitted to phylogenetic analysis. From total of 8 confirmed samples, 3 clustered to Deltacoronavirus genus and 5 to *Gammacoronavirus*. Two of those samples showed partial sequences of other regions of CoVs confirming their genotyping to *Gammacoronavirus* genus and proximity to important poultry diseases causative agents.

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Introduction

Deforestation and man-made changes in the environment that cause drastic changes in migration patterns. Continued habitat loss and fragmentation at stopping points along major migratory routes creates bottlenecks, resulting in overpopulation and clustering of different species, favoring transmission of infectious diseases (Sehgal, 2010). Additionally, several aspects of long-distance migrations contribute to the acquisition of pathogens by birds, such as stress, a known risk factor for causing immunosuppression (Reed *et al.*, 2003). In Brazil, five major routes, also called flyways are specially used by

Nearctic or Septentrional migratory birds: Atlantic, Northeast, Central, Central Amazon/Pantanal, and Western Amazon (ICMBio, 2019). If, on the one hand, migratory birds can carry pathogens to different areas, on the other, resident hosts, can act as local amplifiers and transmitters of circulating diseases to newly arrived animals (Verhagen, 2014).

One pathogen of greatest importance that may be dispersed by birds are the coronaviruses (CoVs), described within the order of *Nidovirales*, in the suborder *Cornidovirineae*, *Coronaviridae* family and *Orthocoronavirinae* subfamily, currently divided into the genera Alpha, Beta, Delta and Gamma coronavirus. The latter two affect birds (ICTV, 2022). *Coronaviridae* family comprises enveloped and pleomorphic viruses, with the largest known RNA genomes (about 27,500 bp) (Milek and Blicharz-Domańska, 2018). Characterized by a high genetic diversity that can lead to the emergence of new viruses, CoVs have a great ability to adapt new hosts and ecological niches (Chan *et al.*, 2013). SARS, MERS and more recently SARS-CoV-2 viruses the most notable examples, all having animal reservoirs (Lu *et al.*, 2020; Perlman, 2020).

Among the avian CoVs, the most well-known is the Infectious Bronchitis-causing Gamma coronavirus associated with great economical losses in poultry industry, though their impact on wild birds remains unknown (Milek and Blicharz-Domańska, 2018). Initially, this virus infects the respiratory tract, where it is restricted to mucus-producing hair cells. Later, similar concentrations of viruses are found in the lungs and air sacs, and with the loss of cilia of the epithelium, secondary bacterial infections are common (Feng *et al.*, 2012).

Besides the Gamma coronavirus group, a wide variety of Delta coronavirus strains and genotypes have been detected in birds (Woo *et al.*, 2012). Belonging to this group, PorCoV HKU15 has been associated with fatal swine outbreaks revealing the potential for bird to mammal transmission and emergence of CoVs (Lau *et al.*, 2018).

Despite their relevance, studies on the detection and genetic characterization of CoVs are scarce, especially in wild birds from South America. Therefore, here we aim to detect these agents in migratory and resident birds from 5 sites along Atlantic Flyway in Brazilian territory, in order to analyze and discuss viral diversity through phylogenetic analysis of their nucleotide sequences, contributing to the understanding of the epidemiology of CoVs and its impairments on wild birds' health.

Materials and Methods

Sample collection

Cloacal and orotracheal swabs of 738 birds were collected between 2015 and 2019 in stopping and wintering areas along the Brazilian segment of the Atlantic flyway (Supplementary Material 1).

Birds were captured by field team of the Laboratory Clinical and Molecular Virology, University of São Paulo (USP), São Paulo, Brazil in collaboration with Chico Mendes Biodiversity Institute (ICMBio) and Wildlife Department of São Paulo Municipality (DEPAVE) using mist nets and released after sample collection, according to licence permit for collection of biological material from ICMBio (SISBIO n. 65230-1) and Ethics Committee on Animal Experimentation of the Institute of Biomedical Sciences, USP (n. 105/09/CEUA). Oral and cloacal swabs were placed together in cryotubes containing 500 µL of Viral Transport Medium (VTM) and immediately stored in liquid nitrogen.

Sample purification, nucleic acid extraction and reverse transcription

Samples were filtered in 0.45 µm membrane and a reaction containing 233 µL of the flowthrough, 30 µL 10x DNase Buffer, 25 µL Turbo™ DNase (Thermofisher Scientific, Waltham, Massachusetts, USA) (2 U / µL) and 12 µL RNase Cocktail Enzyme™ mix (Ambion) was prepared resulting in 300 µL final volume, incubated at 37°C for 2 hours. This purified material was submitted to total nucleic acid extraction was performed using MagMax™ 96 Total Nucleic Acid Isolation Kit (Thermofisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions and stored at −80°C Ultra freezer until processed.

High-Capacity cDNA Reverse Transcription Kit (Thermofisher Scientific, Waltham, Massachusetts, USA) was used for reverse transcription reaction as described by the insert. This reaction was then placed in the thermal cycler at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and cooled to 4°C

until removal.

Coronavirus detection

Detection of coronaviruses was achieved through Nested-PCR protocol described by Chu *et al.* (2011) using the PCR primers 5'-GGKTGGGAY-TAYCCKAARTG-3' and 5'-TGYTGTSWR-CARAAYTCRTG-3'; and Nested-PCR primers 5'-GGTTGGGGACTATCCTAAGTGTGA-3' and 5'-CCATCATCATCAGATAGAATCTCAT-3' that target replicase gene, resulting in a 440 bp amplicon of RdRp coding region.

Sanger sequencing

The 440 bp Nested-PCR products were purified using ExoSap-IT® Kit (Thermofisher Scientific, Waltham, Massachusetts, USA), according to manufacturer's instructions. Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). A second purification was conducted using the BigDye X Terminator® Purification kit (Applied Biosystems, Foster City, California, USA and products were resolved in ABI PRISM 3130XL DNA automated sequencer (Applied Biosystems, Foster City, California, USA).

To confirm which sequences actually belonged to *Coronavirus* family, they were submitted to GenBank Blast-n on the National Center for Biotechnology Information (NCBI) page (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using as parameters the higher nucleotide query coverage and percent identity.

Second DNA strand synthesis

For the synthesis of the second DNA strand, 0.5 µL of 3'-5' exo- DNA Polymerase I, Large (Klenow) Fragment (Thermofisher Scientific, Waltham, Massachusetts, USA) was added to each microtube containing cDNA of samples confirmed through Sanger method on the previous step. Reaction was then incubated at 37°C for 60 minutes and 75°C for 10 minutes.

Ion S5™ system

DNA quantification was performed using the Qubit™ 2.0 fluorimeter (Invitrogen). Samples showing concentration higher than minimum requirement for NGS platform Ion S5™ System were selected. DNA Ion Xpress™ Fragment Library Kit (Thermofisher Scientific, Waltham, Massachusetts, USA) was chosen for the fragmentation of double-stranded DNA, followed by purification using AMPure XP kit (Beckman Coulter, Brea, California, USA).

To perform PCR emulsion, Ion Chef™ System equipment was used with Ion 540™ kit. The device was prepared according to the manufacturer's instructions. Once ready, the chip was transferred to the Ion S5™ System for the sequencing per se.

Data analysis

The raw data was retrieved in FastQ format sequencer and submitted to FastQC v. 0.11.8 software (Patel and Jain, 2012) in which adapter sequences were trimmed, initial quality analysis was performed as well as reads coverage.

For reads assembly, strains which showed highest identity to the coronavirus RdRp partial sequence previously obtained that had complete genomes available at GenBank (http://www.ncbi.nlm.nih.gov) and used as reference.

Phylogenetic trees were generated through neighbor-joining distance algorithm and substitution models were provided by Bayesian Information Criterion (BIC) with 500 bootstrap replicates using MEGA X software (Kumar *et al.*, 2018).

Results and Discussion

A total of 738 samples were analyzed by the screening protocol (Chu *et al.*, 2011) resulting in 8 Nested-PCR positives confirmed by Sanger sequencing method (Table 1), from the species *Anser cygnoides* (from Ibirapuera Park, São Paulo State), *Calidris alba*, *Rynchops niger* and *Calidris fuscicollis* (from Lagoa do Peixe National Park, Rio Grande do Sul State) and another *Calidris fuscicollis* as well as an *Anas bahame*nsis (from Jurubatiba National Park, Rio de Janeiro State) (Table 2).

All samples came were collected at sites at the Atlantic Route, and their relations can be seen at Figure 1.

The phylogenetic tree of the RdRp gene originated from 440 bp partial nucleotide sequences obtained through Sanger sequencing method is shown in Figure 2.

When compared to reference genomes two samples,

DEP16 and PNRJ46, have shown partial sequences matching to known coronavirus on the regions of N gene and Untranslated Terminal Region 3' (3' UTR), these relations are depicted in Table 3.

The sequences generated by the DEP16 and PNRJ46 samples were aligned to coronavirus N-region sequences available from GenBank. The resulting phylogenetic tree is shown in Figure 3.

Sequenced region in sample PNRJ46 also includes Untranslated Terminal Region 3' (3'UTR). For this analysis Gammacoronavirus sequences of this region available on GenBank were selected. The phylogenetic tree referring to the 3'UTR region alignment is shown in Figure 4.

Table 1: *Distribution of avian samples according to collection site and number of Coronavirus positives at screening Nested-PCR protocol (Chu et al., 2011) confirmed through nucleotide sequencing using Sanger method.*

Collection Site	Number of samples	Sanger method positives
Canelas Island (Pará State)	24.1% (178/738)	0% (0)
Ilha Comprida (São Paulo State)	24.7% (182/738)	0% (0)
Ibirapuera Park (São Paulo State)	11.0% (81/738)	37.5% (3/8)
Jurubatiba National Park (Rio de Janeiro State)	14.2% (105/738)	25.0% (2/8)
Lagoa do Peixe National Park (Rio Grande do Sul State)	26.0% (192/738)	37.5% (3/8)
Total	100% (738)	100% (8)

Table 2: *Coronavirus positive samples sequenced by the Sanger method in the present study, according to collection site, host species and sampling year.*

Sample number	Sample identification	Accession number	collection site	Host species	Sampling year
1	DPV 5	RdRp gene (KU321643)	Ibirapuera Park (São Paulo State)	Anser cygnoides	2015
2	DPV 10	RdRp gene (KU321644)	Ibirapuera Park (São Paulo State)	Anser cygnoides	2015
3	DPV 16	RdRp gene (KU321645) N gene (MN689139)	Ibirapuera Park (São Paulo State)	Anser cygnoides	2015
4	PNLP 100	RdRp gene (KU321640)	Lagoa do Peixe National Park (Rio Grande do Sul State)	Calidris alba	2009
5	PNLP 115	RdRp gene (KU321641)	Lagoa do Peixe National Park (Rio Grande do Sul State)	Rynchops niger	2009
6	PNLP 159	RdRp gene (KU321640)	Lagoa do Peixe National Park (Rio Grande do Sul State)	Calidris fuscicollis	2009
7	PNRJ 46	RdRp gene (MN623247) N gene (MN689140) 3' UTR (MN622961)	Jurubatiba National Park (Rio de Janeiro State)	Anas bahamensis	2019
8	PNRJ 49	RdRp gene (MN623248)	Jurubatiba National Park (Rio de Janeiro State)	Calidris fuscicollis	2019

Table 3: *Relation of samples identifications to GenBank accession number of selected avian coronavirus reference sequence and their positions.*

Sample identification	Reference genome	Contigs	Region
DEP16	MK359255 LC364345 LC364342	26232pb to 26639pb - -	Gene N
PNRJ46	MK359255 LC364345 LC364342	26173pb to 27408pb - -	N Gene and Terminal Untranslated Region (3' UTR)

Figure 1: *Map showing major migratory routes in the Americas. Reproduction sites are represented with stars, wintering areas are depicted as circles and sample collection sites are represented as triangles. Names depicted with asterisks refer to sites that presented positive samples.*

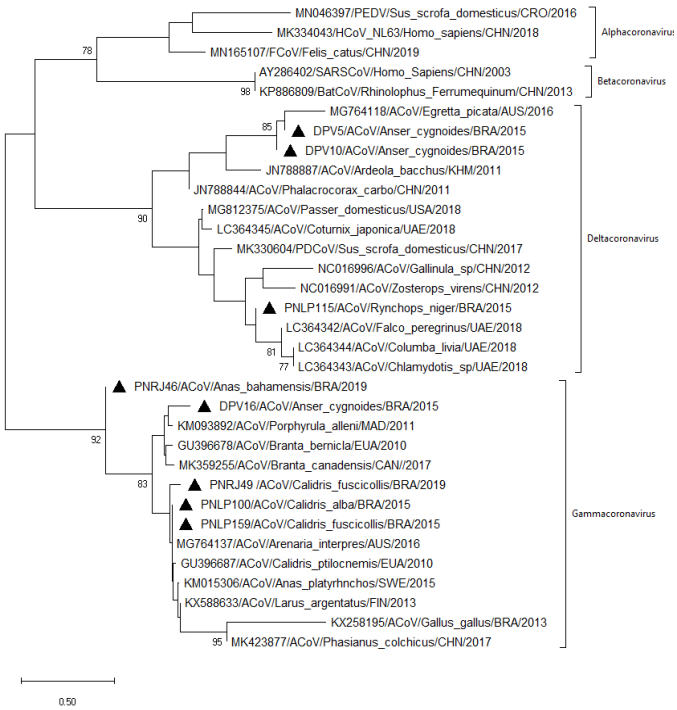

Figure 2: *Nucleotide neighbor-joining distance tree (Tamura 3-parameter substitution model) for the partial RdRp (440 bp) showing the known groups. Strains detected in the present study are preceded by black triangles. The numbers at each node are bootstrap values greater than 70 from 500 replicates. The bar represents the number of substitutions per site.*

This study has shown 1.1% (8/738) of CoV positives, confirmed by nucleotide sequencing. Similar results were found in South Korea (Kim and Oem, 2014) and Hong Kong (Woo et al., 2012) using the same PCR primers and also sampling a variety of species. However, frequency of occurrence may vary significantly in wild birds, according to a myriad of variables like the age of the birds sampled, bird order/species, and their behavior (Milek and Blicharz-Domańska, 2018). There are few studies on CoVs prevalence in wild birds from Brazil, therefore, precluding comparisons with data from these sites.

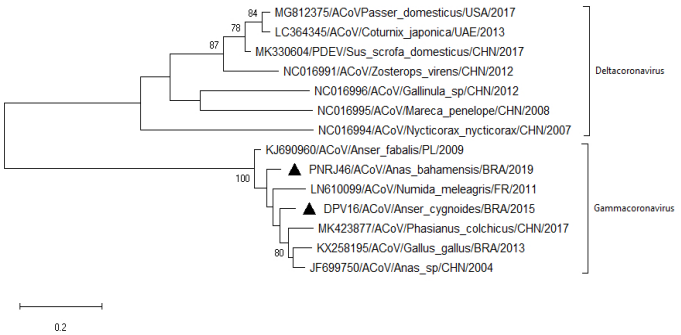

Figure 3: *Phylogenetic tree showing sequences of the coding region of Gamma coronaviruses and Delta coronavirus N protein. Nucleotide neighbor-joining distance tree (Tamura 3-parameter substitution model) for partial N gene sequence (407 bp). Strains detected in the present study are preceded by black triangles. The numbers at each node are bootstrap values greater than 70 from 500 replicates. The bar represents the number of substitutions per site.*

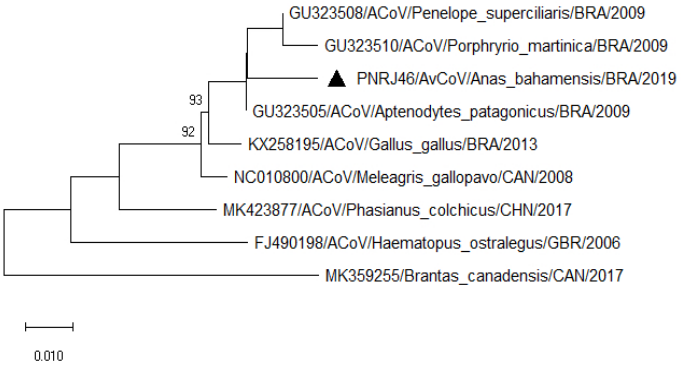

Figure 4: *Phylogenetic tree showing sequences of the coding region of Gamma coronaviruses close related to chicken AvCoVs. Nucleotide neighbor-joining distance tree (Tamura 3-parameter substitution model) for the partial 3' UTR (397 bp). Strains detected in the present study are preceded by black triangles. The numbers at each node are bootstrap values greater than 70 from 500 replicates. The bar represents the number of substitutions per site.*

Degradation of natural habitats has been described by many previous studies as a major factor on predisposition of avian species to viral diseases (Sehgal, 2010; Wille *et al.*, 2017; Barros *et al.*, 2019). Urbanization leads to changes on migration patterns and dispersal of birds, once resting and feeding points become scarcer and smaller (Sehgal, 2010). This process results in agglomeration of different species,

including migratory, resident, synanthropic and domestic animals, exposing these populations to novel pathogens (Reed *et al*., 2003).

Two African Geese (*Anser cygnoides*) (DPV5 and DPV10) from Ibirapuera Park were detected with coronaviruses (Table 1). Placed in the center of the city of São Paulo, in São Paulo State, this is most visited park in South America (over 14 million visitors every year). Around 120 avian species may be found, including ducks, swans, mallards, and other wild birds, some of which use the place as stopping points during migration (Nunes and Amaral, 2010). Sequences retrieved from these samples clustered with Deltacoronavirus genus (Figure 1) and could be related to a clade that includes CoVs from waterfowl species such as Common Heron (*Egretta picata*) (Accession Number: MG764118) and Chinese Heron (*Ardeola bacchus*) (Accession Number: JN788847).

Lagoa do Peixe National Park in Rio Grande do Sul receives both austral and boreal migratory birds, what makes it a key site to understand the epidemiology of avian diseases. It is also important for the ecology of shorebirds, once it is the only wintering area in Brazil (ICMbio, 2019). Samples collected in this site presented a CoV associated with the genus Deltacoronavirus could be isolated in a Skimmer (*Rynchops niger*) sample (Sample Identification PNLP115). The clade to which this sample belongs is formed by CoVs of different wild bird species. Skimmers (*Rynchops niger*) breed in central Brazil and migrate to the coast during winter, traveling long distances each year, which may contribute to the spread of CoVs.

Restinga de Jurubatiba National Park is in the north of Rio de Janeiro State and contains beaches and costal lagoons used as shelter for many species, including birds and other animals. From there, a sample belonging to a White-cheeked pintail (*Anas bahamensis*) (PNRJ 46) was detected with a CoV related to the one isolated from an African Goose (*Anser cygnoides*) captured in Ibirapuera Park. When analyzed together, these sequences form a clade within the genus Gammacoronavirus, along with other waterfowl CoVs such as the Black-faced Goose (*Branta bernicla*) (Accession Number: GU396678), the Canadian Goose (*Branta canadensis*) (Accession Number: MK359255) and one species of Moorhen (*Porphyrulla alleni*) (Accession Number: KM093892). Samples PNRJ49 and PNLP159 belong to the species *Calidris fuscicollis* (White-rumped Sandpiper) from Restinga of Jurubatiba National Park and Lagoa do Peixe National Park while sample PNLP100 was collected from a *Calidris alba* (White Sandpiper), also from this second location. These two species belong to the order of *Charadriiforms*, breed in the northern US and southern Canada migrating to South America during the winter (Araújo *et al*., 2018). CoVs found in these samples belong to Gammacoronavirus genus and were grouped into a clade containing CoVs from other birds of the same order as the Ruddy Turnstone (*Arenaria interpres*) (Accession Number: MG764137) and Rock Snadpiper (*Calidris ptilocnemis*) (Accession Number: GU396687).

Because of the low concentration of RNA available in the samples, we had success in only partial sequences of DPV 16 and PNRJ46. Both sequences from region N grouped together in the same clade, confirming that they belonged to the Gammacoronavirus genus. The sequence of the 3' UTR region generated by the PNRJ46 sample confirmed close correlation with waterfowl CoVs, including a species of waterfowl (*Porphyrio martinica*) and King Penguin (*Aptenodytes patagonicus*). This region is highly variable between the genera of CoVs, only allowing the alignment of CoVs similar to those of *Gallus gallus* (formerly Infectious Bronchitis virus) which allows the inclusion of this sample in this group.

The proximity of wild birds both in urban and poultry producing areas especially in South and Southeast regions of Brazil, where the most important sites are found should be considered in the improvement of poultry health programs.

Conclusions and Recommendations

This study demonstrates that wild birds have been carrying coronaviruses between different migratory sites along the Brazilian segment of Atlantic Route, demonstrating the importance of monitoring. These CoVs belong to the Delta and Gamma coronavirus genus and could be found especially in the South and Southeast regions of the country. Although they do not represent a direct risk to human health, these viruses can also cause disease in birds and, in the case of Delta coronaviruses, possibly in other animals therefore, their presence along migratory routes should be constantly monitored.

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Author's Contribution

All the authors contributed equally and approved the manuscript for submission.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.hv/2022/9.38.45

Conflict of interest

The authors have declared no conflict of interest.

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