Research Square

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Adenovirus Diversity in Wild Birds of Atol das Rocas Biological Reserve, Brazil

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Research Article

Keywords: Adenovirus, Circovirus, Herpesvirus, Wild birds

Posted Date: June 2nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9794703/v1

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Additional Declarations: No competing interests reported.

Abstract

Brazil hosts one of the world's richest avian diversities, with wild birds acting as reservoirs for various viruses that can suppress immune responses and impact disease dynamics. Surveillance of immunosuppressive viruses in wild birds remains limited, especially in isolated ecosystems. Here we screened 815 nasal, cloacal, and ocular swab samples from wild birds across Brazil, employing PCR and sequencing to detect adenoviruses, herpesviruses, and circoviruses. Sixteen samples from Atol das Rocas Biological Reserve tested positive for adenoviruses, specifically aviadenovirus and siadenovirus, marking their first detection in this South Atlantic atoll. Phylogenetic analyses revealed diverse adenovirus lineages circulating among seabirds. These findings underscore the importance of monitoring viral diversity in wild bird populations to better understand virus evolution and potential impacts on avian health and conservation efforts in Brazil.

INTRODUCTION

Wild birds, due to their biological characteristics, are capable of easily disseminating pathogenic microorganisms. They act as a biological or mechanical carrier and are of great importance in the dispersion of various viruses. This constant concern with understanding the viruses that affect this species is aimed at predicting possible outbreaks of emerging diseases. The susceptibility of this animal species to certain viruses can serve as an early warning signal for potential viral diseases in humans.

Of the 1,919 species of birds recorded in Brazil, 198 (10.3%) are migratory. Of these, 127 (64%) were classified as Migratory and 71 (36%) as Partially Migratory [1]. Greater efforts in epidemiological surveillance are necessary to detect viruses transmitted by birds, especially in areas of high avian diversity and endemism. This is intensified by the correlation between migratory routes and the main chicken meat producing regions in Brazil [2].

Some viruses, such as adenoviruses (AdVs), herpesviruses (HVs), and circoviruses (CVs), act subclinically in birds, primarily affecting the immune system. Information regarding the epidemiology of these viruses in wild birds in South America, essential for their conservation, is scarce [3]. AdVs are common infectious agents in domestic and wild birds worldwide, with most capable of causing inclusion body hepatitis, representing a significant risk by increasing mortality rates [4]. HVs cause a wide variety of diseases with diverse lesions and clinical presentations in birds [5]. CV infections in birds are associated with immunosuppression, which predisposes animals to secondary infections that can lead to mortality [6].

Given the importance of studying immunosuppressive viruses in areas of high preservation and with a large passage of wild animals, this study aimed to determine the presence of Herpesvirus, Adenovirus, and Circovirus in wild birds from distinct areas of Brazil.

MATERIAL AND METHODS

Ethics committees

This study was approved by the Animal Use Ethics Committee (CEUA), Rural Federal University of Pernambuco (UFRPE) and Institute of Biomedical Science of University of Sao Paulo (ICB-USP) approvals numbers 14/2017 and 1115/2020, and by the authorization and biodiversity information system (SISBio), license number: 60753-1 of the Chico Mendes Institute for Biodiversity Conservation (ICMBio).

Sampling and Collection Sites

A total of 815 samples from nasal, cloacal, and ocular swabs were collected from wild birds across different regions of Brazil between 2017 and 2019. The collection sites included [S1: Fig. 1]:

- Atol das Rocas Island, Rio Grande do Norte (300 samples) [S2: Fig. 2]
- Fernando de Noronha Island, Rio Grande do Norte (85 samples)
- Panaquatira Beach, Maranhão (300 samples)
- Ibirapuera Park, São Paulo city, São Paulo (85 samples)
- Parque das Aves, Foz do Iguaçu, Paraná (45 samples)

Sample collection

All samples analyzed in this study were collected from the evaluated birds through the introduction and epithelial scraping of disposable sterile swabs in the animal's trachea (oral swab) and cloaca (cloacal swab) and placed together in the same tube containing VTM [S3: Fig. 3], maintained in liquid nitrogen. Only in Atol das Rocas, were collected eyes swab samples from birds that presented ocular discharge, corneal opacification and corneal ulcer. The ocular swabs were placed in individual tubes, identified as right eye (RE) and left eye (LE) [S4: Fig. 4]. All swabs from Atol das Rocas were collected in Lysis buffer, due to the impossibility of keeping samples refrigerated on the Island and impossibility to replace liquid nitrogen. The analyses were done at the Biosafety Level 3 + Laboratory (BSL3+) of the Biomedical Sciences Institute, University of São Paulo.

DNA Extraction

The extraction of viral genetic material, using 100 µl of the sample, was automated using the “MagMAX ™ -96 Isolation Kit“, according to the manufacturer's instructions, in the “MagMAX ™ Express” device from Applied Biosystems® (Foster City, CA, USA)., obtaining total RNA and DNA.

PCR Followed by Nested PCR Detection

Molecular detection of immunosuppressive viruses was performed using Polymerase Chain Reaction (PCR) and Nested PCR (N-PCR) assays targeting specific viral genes previously published [7, 8, 9]. For the detection of Adenovirus (AdV), a protocol (Pan 41) targeting the DNA Polymerase gene (pol) was employed to ensure broad detection across the four genera of adenoviruses known to infect various

hosts, including birds. The first-round PCR utilized the forward primer FLTR (5’ TIMGNGGIGGIMGNTGYTAYCC 3’) and the reverse primer RTR (5’ GTDGCRAAISHICCRTABARIGMRTT 3’). For the nested amplification, the primers used were RNR (5’ GTITWYGAYATHTGYGGHATGTAYGC 3’) and TS-TERM_708as (5’ CCAICCBCDRTTRTGIARIGTRA 3’). Herpesvirus (HV) detection involved two separate Nested PCR protocols, both of which were based on pan-herpesvirus consensus primers. Protocol 32 targeted the DNA Polymerase gene, using the outer primer pair DFA (5’ GAYTTYGCNAGYYTNTAYCC 3’), ILK (5’ TCCTGGACAAGCAGCARNYSGCNMTNAA 3’) and KG1 (5’ GTCTTGCTCACCAGNTCNACNCCYTT 3), and the inner primer pair TGV (5’ TGTAACTCGGTGTAYGGNTTYACNGGNGT 3’) and IYG (5’ CACAGAGTCCGTRTCNCCRTADAT 3’), yielding an amplicon of approximately 215–315 base pairs (bp). Protocol 33 targeted the Terminase gene, using the outer primer pair TS-TERM_707s (5’ TTGTGGACGAGRYESAYTTYAT 3’) and TS-TERM_707as (5’ ACAGCCACGCCNGTICCIGAIGC 3’), and the inner pair TS-TERM_708s (5’ GCAAGATCATNTTYRTITCITC 3’) and TS-TERM_708as (5’ TGTTGGTCGTRWAIGCIGGRT 3’), which resulted in a product of approximately 419 bp. For Circovirus (CV) detection, primers commonly associated with the Beak and Feather Disease Virus (BFDV) were used. The forward primer BFDVF (5’ CCGAGAAGTATTGCAGTAAAGAGGG 3’) and the reverse primer BFDVR (5’ TCTGGGAACTCTCGCGCGAC 3’) were utilized for the amplification, targeting a specific region of the viral genome, which resulted in a product of approximately 142 bp.

PCR and NESTED were performed using platinum taq kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amplification was performed in the thermocycler GeneAmp PCR Systemm 9700 following the conditions previously described by authors. As control, a well-known positive clinical sample (human AdV and HV, CV) was used, and DNA/RNAse free water was used as the negative control.

Detection of the amplified product by PCR and NESTED was performed by 2% agarose gel electrophoresis (LifeTechonologies corporation) in TAE buffer [0.5x]. AdV positive agarose gel samples were then submitted for sequencing.

Purification and Sequencing

Purification of PCR product was performed with the ExoSap kit (exonuclease I - Amersham Pharmacia Biotech), according to the manufacturer's instructions. After purification of the PCR product, the DNA sequences of the polimerase protein gene were sequenced using the "ABI PRISM Dye™ Terminator Cycle Sequencing Ready Reaction Kit" ("Big Dye v3.1" - Applied Biosystems, Inc., USA), following the manufacturer's instructions. The enzymatic extension was performed in GeneAmp PCR System 9700 thermocycler for 25 cycles of 96 ° C for 10 seconds for denaturation of the template DNA, 50 ° C for 10 seconds for annealing of the primers and 60 ° C for 4 minutes for extension. The obtained product was further purified with the X-terminator kit (Applied Biosystems Inc., USA) according to the manufacturer's instructions. The purified product was subjected to POP7 polymer electrophoresis using ABI model 3130 automatic sequencer (Applied Biosystems, Inc., USA).

Phylogenetic Analysis

For the phylogenetic analysis the multiple sequence alignment (MSA) was performed by Jalview using the Clustal-X [10]. The evolutionary model was determined using the tool MEGA [11], in which HKY + G model [12] was proposed. The phylolgenetic tree was builded by the seaview using the maximum likelihood method performed by PhyML [13]. Branch support consistencies were evaluated using the nonparametric bootstrap test [14] with 1000 replicates and the approximate likelihood ratio test (aLRT) [15]. The tree was edited using FigTree software v1.4.4. For the fragments analyzed, we compared our sequences with sequences representative of all subtypes found in GeneBank, to obtain the subtyping of our viruses, as well as the degree of Yesilarity between the sequences.

RESULTS

Seven hundred and fifty birds belonging to different species and zoological orders and families (Passeriformes, Laridae, Fregatidae, Scolopacidae, Anatidae, Tinamidae and Sulidae), were included in this study. Samples were collected opportunistically in different regions (North, Northeast, South and Southeast) of Brazil, between 2016 and 2019 [S1].

During sample collection, were observed that 55 birds presented clinical ocular symptoms, such as ocular discharge, corneal opacification and corneal ulcers in Atol das Rocas [S4]. All these birds belongs the species *Onichoprium fuscattus* were chicks or juveniles.

PCR technique was performed in 815 samples screened for the presence of adenovirus, herpesvirus and circovirus DNA. Of these, 28 samples produced an amplicon of expected size and sixteen were confirmed by sequencing. The species and clinical signs of positive samples are shown in “Table 1” [S5: Table 1]. All samples were negative to herpesvirus and circovirus.

Table 1

List of positive samples. List of birds with positive samples of sequenced for adenovirus, collected in Atol das Rocas, RN with and without clinical symptoms.

ID	Island	Number of GENEBANK	Ring number	Species	Commom names	Clinical signs RE	Clinical signs LE
AR011	Ilha do Farol	OL436124	H106402	*Anous minutus*	Black noddy	No	No
AR012	Ilha do Farol	OL436125	H106403	*Anous minutus*	Black noddy	No	No
AR013	Ilha do Farol	OL436126	H106404	*Anous minutus*	Black noddy	No	No
AR052	Ilha do Farol	OL436127	U25000	*Anous stolidus*	Brown noddy	No	No
AR059	Ilha do Farol	OL436128	U25000	*Sula leucogaster*	Brown booby	No	No
AR074	Ilha do Farol	OL436129	J85541	*Onychoprion fuscatus*	Sooty tern	No	No
AR079	Ilha do Farol	OL436130	No	*Anous minutus*	Black noddy	No	No
AR080	Ilha do Farol	OL436131	H106419	*Anous minutus*	Black noddy	No	No
AR089	Ilha do Cemitério	OL436132	J85529	*Onychoprion fuscatus*	Sooty tern	No	No
AR134	Ilha do Farol	OL436133	V46523	*Sula dactylatra*	Masked booby	No	No
Legend:							
ID: Gene Bank identification							
AR – Atol das Rocas							
RE – Right eye alteration							
LE– Left eye alteration							

ID	Island	Number of GENEBANK	Ring number	Species	Commom names	Clinical signs RE	Clinical signs LE
AR154	Ilha do Farol	OL436134	No	*Sula Sula*	Red boobies - footed	No	No
AR263	Ilha do Farol	OL436135	No	*Onychoprion fuscatus*	Sooty tern	No	Yes
AR269	Ilha do Farol	OL436136	No	*Onychoprion fuscatus*	Sooty tern	Yes	No
AR271	Ilha do Farol	OL436137	No	*Onychoprion fuscatus*	Sooty tern	No	Yes
AR275	Ilha do Farol	OL436138	No	*Anous stolidus*	Brown noddy	No	No
Legend:							
ID: Gene Bank identification							
AR – Atol das Rocas							
RE – Right eye alteration							
LE– Left eye alteration							

Phylogeny showed that nine samples were genetically closest to Aviadenovirus (AR12, AR17, AR74, AR134, AR154, AR263, AR269, AR271 and AR275), and seven closest to siadenovirus (AR11, AR13, AR52, AR59, AR79, AR80 and AR89) [S6: Fig. 5]. All sequences are available in GenBank under accession numbers OL436124 to OL436138, except to AR17 for having a sequence smaller than 200bp.

DISCUSSION

This survey represents the first molecular and epidemiological study of aviadenovirus and siadenovirus in seabirds in “Atol das Rocas Biological Reserve” at Rio Grande do Norte State, Brazil. We found a very large diversity of Adenovirus in seabirds of the archipelago, a very isolated place on the Brazilian coast, but with numerous migratory birds from different regions of the planet.

Despite the distance and little human presence, the study of these seabirds is important in order to understand the spread of the viruses, since Jones et al. 2008 [16] states that most emerging diseases are zoonotic. The epidemiological surveillance focusing on native fauna is a way to identify possible threats to humans and non-human animals that are hidden in reservoirs or that are still unknown. It is

estimated that there are approximately 1.67 million unknown viruses from the main zoonotic viral families in mammal and bird hosts [17].

From 815 samples analyzed, 28 (3.4%) were positive to adenovirus and 16 were genotyped by sequencing. Considering the number of birds that percentage gets even higher (3.7% of birds infected). Most of the positive birds had no apparent symptoms at the time of collection, in addition most of the birds with affected eyes were negative for adenovirus, demonstrating that the virus is probably not the cause of the eye problem.

On phylogenic tree we can observe several clusters, showing the great variability of adenovirus in “Atol das Rocas Biological Reserve”. Adenovirus-like sequences close to Aviadenovirus and Siadenovirus genera were detected in birds at Brazilian Cerrado fauna by Duarte et al. 2019 [18]. Also Park et al. 2011 [19] founded a new Siadenovirus from a South Polar skua (*Catharacta maccormicki*) in Antarctica. It is interesting once the skua species are seabirds.

The samples AR11, AR52, AR79, AR13, AR80 and AR89 are close to Siadenovirus. All these birds were without clinical symptoms at the time of sample collection. Duarte et al. 2019 [18] carried out an eco-epidemiology analysis with birds from the Brazilian Cerrado using a primer based on the hexon protein amino acid sequence with approximately 900 pairs and detected only aviadenovirus. They also used a primer for the conserved polymerase region, however, they did not detect siadenovirus.

Of the five species of seabirds studied in “Atol das Rocas Biological Reserve”, two species (*F. magnificens* and *S. sula*) come from the Fernando de Noronha Archipelago, Brazil and the other three species (*A. stolidus, A. minutus* and *O. fuscatus*) are apparently resident [20, 21, 22]. However, there is a variation throughout the year in the number of individuals of these three species and there are no studies that indicate where these seabirds may be migrating [23, 24, 25].

The limitations of this study include the failure to undertake the sampling of birds in the closest regions at the same time, which makes it difficult to derive any solid ecological picture of what might have been occurring. Another limitation is related to the virus isolation. This methodology could lead to enhance the possibility for us get the full genome of the isolates, but unfortunately the condition of the Atoll does not permit to kept this sample for virus isolation methodology.

This study contributes with the knowledge of the circulation of Adenovirus in seabirds from “Atol das Rocas Biological Reserve” and the adenoviruses variety that may be interesting to future studies. Even though the seabirds in this place apparently do not migrate for long distances, there is a group of shorebirds that visit the island throughout the year [25]. These birds are known for out large migrations and for being able to be carriers and transmitters of different types of viruses and have great potential to infect seabirds in inhospitable environments such as Atoll of Rocks [26, 27].

The aim of this study was to assess the presence of immunosuppressive viruses in the Atol das Rocas Biological Reserve and to characterize any identified viruses. We successfully detected aviadenovirus

and siadenovirus within the avian community, identifying multiple subtypes. Our phylogenetic analysis revealed a high level of genome-wide Yesilarity among the detected aviadenoviruses and siadenoviruses, indicating their close relation to other adenoviruses despite the isolation of the reserve. Further research is needed to clarify the presence of viruses from this adenovirus lineage in birds that are either migrate or residents of this unique atoll in the South Atlantic. Our findings hold significance for the surveillance of emergent and re-emergent viruses in Brazil and contribute to valuable insights in the study of fowl adenovirus in South America. The findings presented here helps for understanding how viral infections in seabirds can affect the health of animal populations, as well as for identifying the genera of adenovirus present in this region.

To conclude, the study successfully identified the presence of adenoviruses in wild bird populations in Atol das Rocas Biological Reserve, one of the most isolated places in the country, highlighting the importance of epidemiological surveillance in native fauna. The results suggest the great genetic variability of fowl adenovirus including the possible presence of new species. This study provides new insights into the diversity, evolution, host range, and distribution of avian adenoviruses in South America.

Declarations

ACKNOWLEDGEMENTS

We thank Dr. Dean D. Erdman from CDC Atlanta for all English suggestions. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (Capes) – Finance Code 001. This study was made possible through the financial support of MCTI- CNPq (403761/2020-4). and FAPESP (2020/06409-1) and was supported by Ministério do Meio Ambiente, Ministério da Ciência, Tecnologia e Inovação.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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