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Occurrence of avian reovirus and picobirnavirus in wild birds from an environmental protection area in the Brazilian Amazon

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ABSTRACT

Wild birds have great prominence in the transmission of diseases to humans, mainly due to their ease of access to human populations, raising concerns about the potential impact of that proximity on public health. The present study reports ARV and PBV occurrence in wild birds from an environmental protection area in the Amazon biome, in Pará state, Brazil. We obtained 155 fecal specimens from 47 species of wild birds for RNA extraction, ARV and PBV detection utilizing molecular methods, nucleotide sequencing, and phylogenetic analysis. ARV prevalence was 0.6% (1/155), being positive in an individual of *Myrmotherula longipennis*, and PBV prevalence was 1.29% (2/155), affecting two individuals of *Guira guira*. The PBV strains were assigned to genogroup I based on phylogenetic analysis, and they shared a strong phylogenetic link with strains isolated from different geographic locations. The ARV strain was more closely related to strains that had previously circulated in the same region. The presence of ARV and PBV in this habitat suggests that infection cycles of these agents occur naturally in the wild ecosystem, potentially triggering transmission events between bird species and humans. This is the first study on ARV detection in wild birds in Brazil and the first report on the occurrence of PBV in wild *Guira guira*. Additional studies are required to determine the epidemiology, origin, evolution, and emergence of new potentially pathogenic viruses in the Amazon.

KEYWORDS: enteric viruses, epidemiology, RT-PCR, phylogeny, *Myrmotherula longipennis, Guira guira*

Ocorrência de reovírus aviário e picobirnavirus em aves silvestres de uma área de proteção ambiental na Amazônia brasileira

RESUMO

As aves silvestres têm grande destaque na transmissão de doenças ao homem, principalmente pela facilidade de acesso à população humana, levantando preocupações sobre o potencial impacto dessa proximidade no contexto da saúde pública. O presente estudo relata a ocorrência de ARV e PBV em aves silvestres de uma área de proteção ambiental no bioma Amazônico. Obtivemos 155 espécimes fecais de 47 espécies de aves silvestres, que foram submetidos a extração de RNA, detecção de ARV e PBV por técnicas moleculares, sequenciamento nucleotídico e análise filogenética. A prevalência de infecção por ARV foi 0,6% (1/155), sendo positivo em um indivíduo de *Myrmotherula longipennis*, e a prevalência de PBV foi 1,29% (2/155), afetando dois insdivíduos de *Guira guira*. A análise filogenética agrupou as cepas de PBV no genogrupo I, mostrando intensa relação filogenética com cepas isoladas de outras regiões geográficas. A cepa de ARV esteve mais relacionada filogeneticamente a cepas previamente circulantes na mesma região. Este é o primeiro estudo sobre detecção de ARV em aves silvestres no Brasil e o primeiro relato sobre a ocorrência de PBV em *Guira guira*. A circulação de ARV e PBV nesse ambiente demonstra que os ciclos desses agentes estão ocorrendo de forma natural no ecossistema silvestre, o que pode desencadear possíveis eventos de transmissão entre as espécies aviárias e humanos. Estudos adicionais são necessários para determinar a epidemiologia, origem, evolução e surgimento de novos vírus potencialmente patogênicos na Amazônia.

PALAVRAS-CHAVE: virus entéricos, epidemiologia, RT-PCR, filogenia, *Myrmotherula longipennis, Guira guira*

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INTRODUCTION

Wild birds are among the animals with high prominence in the context of disease transmission to humans. The ability to fly propitiates birds ease of access to human populations, increasing the transmission risk of pathogens to humans, and the potential impact on public health (Morais *et al*. 2019).

Viruses are the most important clinical and epidemiological pathogens in birds. Infections that occur in the first weeks of avian life are usually from viral etiology (Luz *et al.* 2014; Santos *et al.* 2015). Rotaviruses (RV), avian reoviruses (ARV), picobirnaviruses (PBV), avian influenza viruses (AIV), astroviruses (AstV), coronaviruses (CoV) and West Nile viruses (WNV) are examples of the most significant viruses for global public health transmitted by birds. This is due to the potential for dispersal of wild birds, especially those that have migratory habits (Bezerra *et al.* 2014; Lu *et al.* 2015; Noh *et al.* 2018; El Taweel *et al.* 2020; Hassan *et al.* 2020; Vidaña *et al.* 2020; Rahman *et al.* 2021).

ARV and PBV are frequently reported in poultry infections, associated with clinical or subclinical diseases, which causes serious economic impacts to the poultry industry (Day and Zsak, 2016; Pankovics *et al.* 2018). ARV are described as important agents of gastroenteric diseases, viral arthritis, and tenosynovitis in birds (Davis *et al.* 2013; Assunção *et al.* 2018). PBV can be detected in excrements with both normal and diarrheic aspect, both from domestic and wild birds, therefore its role as a primary agent of acute gastroenteritis remains unclear (Silva *et al.* 2014; Verma *et al.* 2015).

In Brazil, there are few studies on the occurrence of ARV and PBV in free-living wild birds, especially in natural environments close to urban centers, where the risk of zoonotic transmission of infectious agents by wild animals to the human populations is higher. Studies on molecular epidemiology are essential to describe the prevalence of infectious agents, characterize variants and estimate the potential impact of wild reservoirs on public health.

This study aimed at describing the occurrence of ARV and PBV in wild bird samples collected in an environmental protection area in the Amazon biome.

MATERIAL AND METHODS

Ethical and legal aspects

This study is in accordance with the ethical principles of animal experiments and was approved by the Animal Ethics Committee of Universidade Federal Rural da Amazônia (protocol # 025/2018 CEUA/UFRA), and by the Biodiversity Information and Authorization System (SISBIO) (license # 63488-1).

Study area

The study area included forest areas close to deforested areas for pasture and/or urbanization in an area in the campus of Universidade Federal Rural da Amazônia (UFRA) (1º27'21"S, 48º26'12"W), within the environmental protection area of the metropolitan region of the city of Belém (*Área de Proteção Ambiental da Região Metropolitana de Belém*, or APA Belém) (Figure 1), in Pará state (Brazil), a protected area of 5,647 ha that harbors a wide variety of wild animals.

Bird capture and clinical specimen collection

From February to October 2019, birds were collected in the months of February and March (rainy season), June and

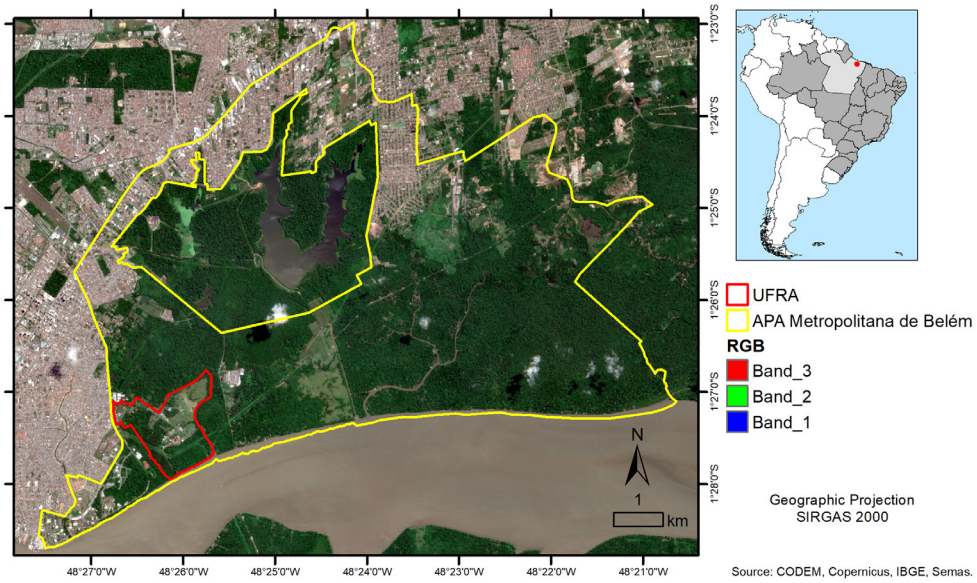

Figure 1. Location of the APA Belém environmental protection area in the outskirts of Belém (Pará state, Brazil) and the campus of Universidade Federal Rural da Amazônia (UFRA) therein, where the wild birds used in this study were captured.

July (dry season), and October (transition season). Seven mist nets were used (Figure 2), fixed to the ground with metal wattle, opened from 5:00 am to 10:00 am, and checked every thirty minutes. Captured birds were weighed and identified to species level, sex (male or female), and life stage (young or adult) (Gwynne *et al.* 2010; Sigrist 2014). The birds were kept individually in cardboard boxes lined with aluminum foil paper. Fecal specimens deposited in the boxes were collected or obtained directly from the bird's cloaca using sterile swabs, kept in cryogenic tubes, and stored at -20ºC until processing. After collecting the fecal samples, the birds were marked with non-toxic ink (Raidex®) to identify in case of recapture and released back into the environment. Due to stress, some birds died during handling, so that the collection of feces was not possible. The birds that died were submitted to necropsy and their intestine was stored for future studies.

RNA extraction and electrophoresis

Suspensions were prepared at 10% by diluting the feces and/or intestinal samples in Tris/HCl/$CaCl^{2+}$ buffer (pH 7.2 0.01M), clarified by centrifugation at 4,000 rpm/10 minutes. The supernatant was submitted to RNA extraction according to the protocol by Boom *et al.* (1990).

The products of RNA extraction were submitted to polyacrylamide gel electrophoresis (PAGE) for ARV and PBV detection by electrophoretic profiles according to Pereira *et al.* (1983).

RT-PCR for ARV detection

RT-PCR was performed targeting the ARV S2 gene to amplify a partial fragment of 625 bp of the S2 gene, using forward primer PAF (5' - ACT TCT TYT CTA CGC CTT TCG - 3') and reverse PAR (5' - ATY AAW DCW CGC ATC TGC TG - 3') (Zhang *et al.* 2006). To complementary DNA strain (cDNA), 4 µL of extracted dsRNA and 2 µL of pair of primers (20 mM) were used. The reaction followed an incubation of 5 minutes at 97ºC for denaturation, followed by a heat shock of 5 minutes at 0ºC.

Reverse transcription was completed to a final volume of 25 µL. The mix was obtained by adding 19 µL of RT mixture including 11 µL of DNAse/RNAse free H_2O (Hyclone™), 1 µL of dNTPs (20mM, Promega®), 5 µL of buffer (5x, Promega®), 1.5 µL of $MgCl_2$ (25 mM, Promega®) and 0.5 µL of RT (4U, Promega®), followed by incubation at 42ºC for 60 minutes. After reverse transcription, PCR was performed, adding to the cDNA 25 µL of the PCR mixture containing 15.25 µL of H2O free DNAse and RNAse (Hyclone™), 3 µL of dNTPs (20mM, Promega®), 5 µL of buffer (5x, Promega®), 1.5 µl of $MgCl_2$ (25mM, Promega®) and 0.25 µl of Taq DNA Polymerase (5U, Promega®). Zhang *et al.* (2006) described the cycling conditions used.

The amplicons obtained by PCR were performed using agarose gel electrophoresis at a concentration of 1.5% in Tris/Borate/EDTA (TBE) buffer, and gel stained with SYBR® Safe DNA Gel Stain (Invitrogen®). GEL DOC 1000 image processor (Bio-Rad Laboratories, Inc., Hercules, CA) performed photo documentation.

RT-PCR for PBV detection

RT-PCR was performed targeting the PBV RdRp gene. To amplify 201bp genogroup I fragment, PicoB25 forward primers (5'-GCN TGG GTT AGC ATG GA-3') and PicoB43

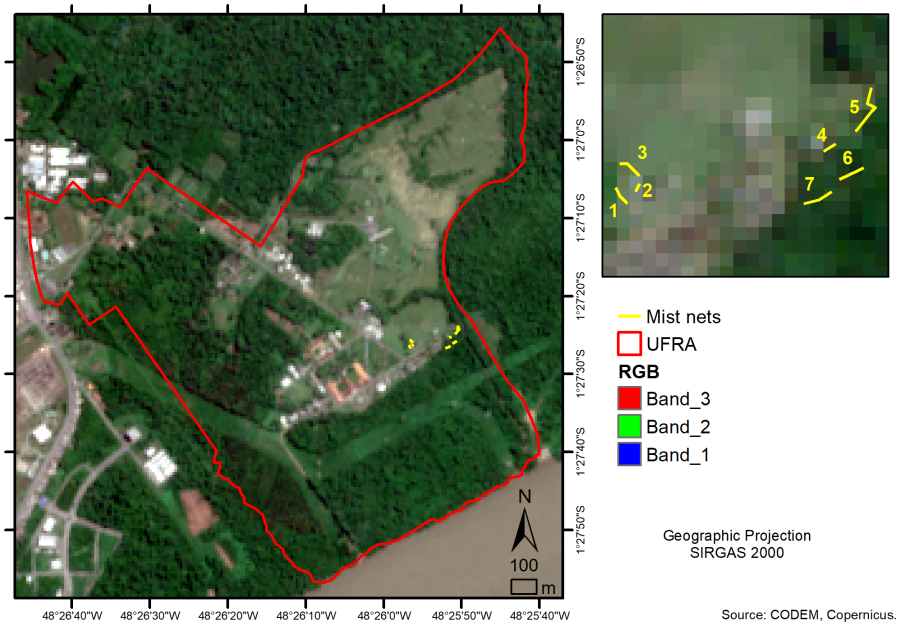

Figure 2. Location of the mist nets used to capture the wild birds used in this study in the campus of Universidade Federal Rural da Amazônia (UFRA) in Belém (Pará state, Brazil).

reverse (5'-A(GA)T G(CT)T GGT CGA ACT T-3')) were used (Rosen *et al.* 2000). For genogroup II, PicoB23 forward primers (5'-CGG TAT GGA TGT TTC-3') and PicoB24 reverse (5'-AAG CGA GCC CAT GTA-3') were used to amplify fragments of 369bp (Rosen *et al.* 2000). To obtain the cDNA, 4 µL of extracted dsRNA and 1 µL of primer pair (20mM) were used, followed by 5 minutes of incubation at 97ºC for dsRNA denaturation, and 5 minutes of heat shock at 0ºC.

The reverse transcription was completed by adding 20 µL of the RT mixture containing 12.25 µL of DNAse/RNAse free H_2O (Hyclone™), 1 µL of dNTPs (20mM, Promega®), 5 µL of buffer (5x, Promega®), 1.5 µL of $MgCl_2$ (25mM, Promega®) and 0.25 µL of RT (4U, Promega®), followed by incubation at 42ºC for 60 minutes. For the PCR test, 25 µL of the PCR mixture were added to the cDNA containing 15.25 µL of DNAse/RNAse free H_2O (Hyclone™), 3 µL of dNTPs (20mM, Promega®), 5 µL of buffer (5x, Promega®), 1.5 µL of $MgCl_2$ (25mM, Promega®) and 0.25 µL of Taq DNA Polymerase (5U, Promega®). The cycling conditions used were described by Silva *et al.* (2014).

The amplicons obtained by PCR were performed using agarose gel electrophoresis at a concentration of 1.5% in Tris/Borate/EDTA (TBE) buffer, and gel stained with SYBR® Safe DNA Gel Stain (Invitrogen®). GEL DOC 1000 image processor (Bio-Rad Laboratories, Inc., Hercules, CA) performed photo documentation.

Nested-PCR for PBV genogroup I

Samples that presented amplicons of 201bp by previous RT-PCR for PBV GI were submitted to a new RT-PCR followed by a nested-PCR to amplify a larger region of the RdRp gene. The forward primers PBV 1.2F (5'-AAG GTC GGK CCR ATGT-3') and reverse PBV 1.2R (5'-TTA TCC CYT TTC ATG CA-3') were used to amplify a fragment of 1229bp (Malik *et al.* 2018). In nested-PCR, Malik-2-FP forward primer (5'-TGG GWT GGC GWG GAC ARG ARGG-3') and Malik-2-RP reverse (5'-YSC AYT ACA TCC TCC AC-3') were used, which amplified a fragment of 580bp of RdRp gene (Malik *et al.* 2018). The cycling conditions used were described by Malik *et al.* (2018).

Nucleotide sequencing

Amplicons were purified using ExoSAP-IT™ kit (Applied Biosystems™) according to the manufacturer's recommendations. After purification, products were subjected to nucleotide sequencing using RT-PCR/nested-PCR primers, and Big Dye Terminator® v.3.1 kit (Applied Biosystems™) according to the manufacturer's recommendations. The final reaction was submitted through ABI PRISM 3130 Automated Genetic Sequencer (Applied Biosystems™).

Phylogenetic analysis

The sequences were edited and aligned with programs BioEdit v.7.2 and MEGA v.10.0.537 (Tamura *et al.* 2013), respectively, and compared with other sequences deposited in GenBank (www.ncbi.nlm.nhi.gov) through the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were constructed with MEGA v.10.0.537 program using the neighbor-joining method and the Kimura two-parameter model (Kimura 1980). A bootstrap of 2000 replicates was used for phylogenetic grouping. Nucleotide similarity was calculated with Geneious v.10.0.7 (Kearse *et al.* 2012).

Accession numbers

The nucleotide sequence accession numbers are available at www.ncbi.com/nucleotide under the codes: OM287555, OM287556, and OM287557.

RESULTS

A total of 155 clinical specimens from 47 different species of wild free-living birds were collected (Table 1) from February to October 2019, including 144 fecal specimens and 11 cloacal specimens.

The PAGE test was negative for all the samples tested, with no electrophoretic profile consistent with ARV or PBV. There was a prevalence of 0.6% (1/155) for the ARV S2 gene through RT-PCR. The positive sample belonged to a long-winged-antwren (*Myrmotherula longipennis*). The prevalence for PBV GI was 1.29% (2/155), both samples belonging to guira-cuckoo (*Guira guira*). There were no positive samples for PBV GII.

The partial sequences of the PBV RdRp gene of the two strains obtained from our samples were compared with other prototype PBV sequences isolated in Brazil and other countries and deposited in GenBank. Phylogenetic analysis grouped the two strains from this study into PBV GI, however, the sequences were heterogeneously related, grouping divergently (Figure 3). One strain, GI/PBV/Guira-cuckoo/BRA/UFRA-115/2019, grouped with a sequence from a duck in Australia in 2018, with a bootstrap of 94%. The group was phylogenetically related to other strains isolated from toucan in Brazil and chicken in Brazil and South Korea (bootstrap of 72%). The other strain, GI/PBV/Guira-cuckoo/BRA/UFRA-114/2019, grouped with a strain isolated from swine in the USA, with a bootstrap of 85%. This cluster was phylogenetically related to other PBV isolated from primates.

The homology of the PBV sequences obtained in this study represented 59.5% of nucleotide identity, showing a high genetic diversity. Compared with the prototype PBV sequences, the values ranged from 55.0 to 81.4%. The GI/PBV/Guira-cuckoo/BRA/UFRA-115/2019 strain showed higher nucleotide similarity (81.4%) with the Australian duck sequence (MH453875.1), and two sequences isolated from chicken in Brazil (KC865823.1) (76.6%) and South Korea

Table 1. Overview of the wild birds (N = 155) sampled for prevalence of avian reovirus (ARV) and picobirnavirus (PBV) in an environmental protection area in Belém (Pará state, Brazil). N = sample size; ARV = ARV prevalence; PBV = PBV prevalence.

Species	N	ARV (%)	PBV (%)
Order Accipitriformes			
Family Accipitridae			
Rupornis magnirostris (Gmelin, 1788)	1	0	0
Order Apodiformes			
Family Trochilidae			
Glaucis hirsutus (Gmelin, 1788)	1	0	0
Order Caprimulgiformes			
Family Caprimulgidae			
Nyctidromus albicollis (Gmelin, 1789)	3	0	0
Order Columbiformes			
Family Columbidae			
Columba livia (Gmelin, 1789)	1	0	0
Columbina minuta (Linnaeus, 1766)	1	0	0
Columbina passerina (Linnaeus, 1758)	1	0	0
Leptotila rufaxilla (Richard & Bernard, 1792)	6	0	0
Order Coraciformes			
Family Alcedinidae			
Chloroceryle anea (Pallas, 1764)	2	0	0
Chloroceryle inda (Linnaeus, 1766)	1	0	0
Order Cuculiformes			
Family Cuculidae			
Coccycua minuta (Vieillot, 1817)	1	0	0
Crotophaga ani (Linnaeus, 1758)	1	0	0
Guira guira (Gmelin, 1788)	6	0	33.3
Order Passeriformes			
Family Dendrocolaptidae			
Dendroplex picus (Gmelin, 1788)	6	0	0
Family Furnaridae			
Synallaxis gujanensis (Gmelin, 1789)	9	0	0
Family Icteridae			
Molothrus bonariensis (Gmelin, 1789)	2	0	0
Family Parulidae			
Myiothlypis mesoleuca (Sclater, 1866)	1	0	0
Family Passerellidae			
Arremon taciturnus (Cabanis & Heine, 1850)	6	0	0
Family Passeridae			
Passer domesticus (Linnaeus, 1758)	7	0	0
Family Pipridae			
Pipra fasciicauda (Hellmayr, 1906)	4	0	0
Family Rhynchocyclidae			
Tolmomyias flaviventris (Wied, 1831)	2	0	0
Family Thamnophilidae			
Myrmotherula longipennis (Pelzeln, 1868)	1	100	0
Sclateria naevia (Gmelin, 1788)	2	0	0
Taraba major (Vieillot, 1816)	3	0	0
Family Thraupidae			
Coereba flaveola (Linnaeus, 1758)	1	0	0
Eucometis penicillata (Spix, 1825)	2	0	0
Ramphocelus carbo (Pallas, 1764)	27	0	0
Saltator maximus (Statius Muller, 1776)	3	0	0
Tachyphonus rufus (Boddaert, 1783)	2	0	0
Tangara episcopus (Linnaeus, 1766)	4	0	0
Tangara palmarum (Wied, 1821)	1	0	0
Family Tityridae			
Pachyramphus rufus (Boddaert, 1783)	2	0	0
Family Troglodytidae			
Cantorchilus leucotis (Lafresnaye, 1845)	3	0	0
Pheugopedius coraya (Gmelin, 1789)	1	0	0
Troglodytes musculus (Naumann, 1823)	2	0	0
Family Turdidae			
Turdus fumigatus (Lichtenstein, 1823)	5	0	0
Turdus leucomelas (Vieillot, 1818)	11	0	0
Family Tyrannidae			
Atilla cinnamomeus (Gmelin, 1789)	2	0	0
Cnemotriccus fuscatus (Wied, 1831)	1	0	0
Elaenia flavogaster (Thunberg, 1822)	1	0	0
Legatus leucophaius (Vieillot, 1818)	2	0	0
Myiarchus ferox (Gmelin, 1789)	1	0	0
Myiopagis flavivertex (Sclater, 1887)	1	0	0
Myiozetetes similis (Spix, 1825)	2	0	0
Pitangus sulphuratus (Linnaeus, 1766)	4	0	0
Family Vireonidae			
Hylophilus pectoralis (Sclater, 1866)	1	0	0
Family Xenopidae			
Xenops minutus (Sparrman, 1788)	5	0	0
Order Psittaciformes			
Family Psittacidae			
Brotogeris versicolurus (Statius Muller, 1776)	3	0	0

(KM254161.1) (75.6%). The GI/PBV/Guira-cuckoo/BRA/UFRA-114/2019 strain showed higher nucleotide similarity (63.4, 61.9, and 60.7%) with sequences obtained from two chicken in Brazil (KC865823.1, KC865829.1), and a green monkey on Saint Kitts and Nevis (KY053143.1), respectively.

The partial sequence of the ARV S2 gene was compared with other ARV prototype sequences from domestic birds (chicken, turkey, duck, and goose) and wild birds (pheasant, wild duck, brown-eared bulbul, and crow) isolated in Brazil and other countries. The REO/Long-winged-antwren/BRA/UFRA-118/2019 strain obtained in this study was phylogenetically related to strains previously reported in chicken in Brazil (KY783741.1, KY783739.1), with a bootstrap of 85% (Figure 4).

Regarding nucleotide identity, the S2 gene sequence showed 51.9% to 86.4% similarity when compared with prototype ARV sequences. The highest similarity was observed with sequences obtained from chicken in Brazil (KY783741.1, KY783743.1, KY783742.1), with values of 86.4, 86.0, and 85.8%, respectively. The lowest similarity was obtained for a sequence acquired from brown-eared bulbul (AB914767.1) isolated in Japan (51.9%) (Figure 4).

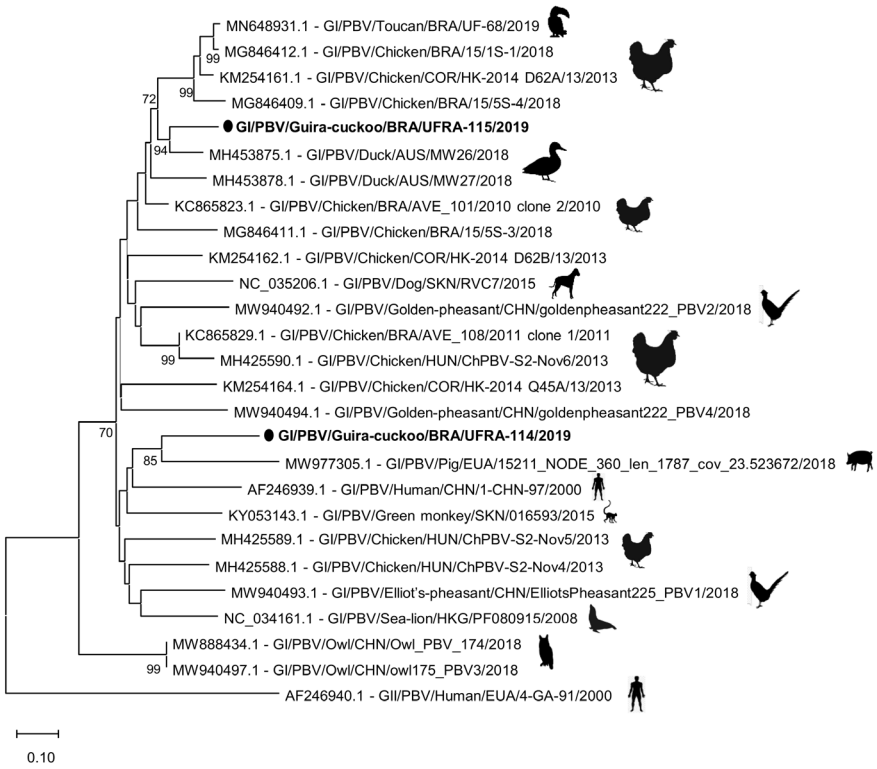

Figure 3. Phylogenetic tree based on partial sequence alignment of the PBV RdRp gene. The sequences of this study are represented in bold. The silhouettes represent zoological families. The numbers next to the nodes indicate bootstrap values >70%. The scale bar is proportional to the phylogenetic distance. The prototype strain GII/PBV/Human/USA/4-GA-91/2000 (AF246940.1) was used as an external group to better understand the phylogenetic relationships between the strains. The phylogenetic tree was constructed using the neighbor-joining method and the Kimura two-parameter model, with bootstrap of 2000 replicas to give consistency to the phylogenetic groups.

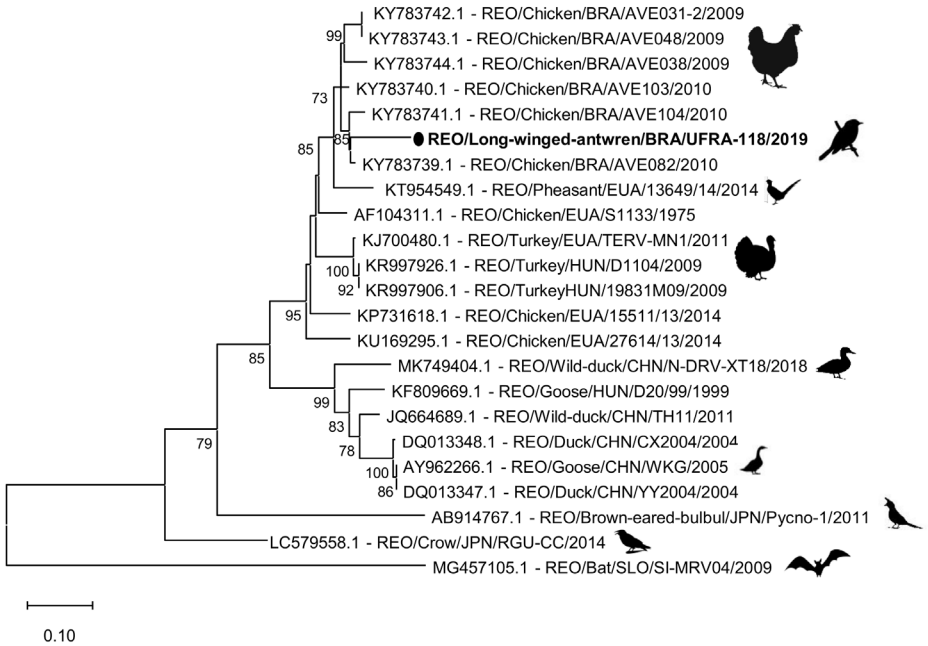

Figure 4. Phylogenetic tree based on the alignment of partial sequences of the ARV S2 gene. The sequence of the present study is represented in bold. The silhouettes represent zoological families. The numbers next to the nodes indicate boostrap values >70%. The scale bar is proportional to phylogenetic distance. The strain REO/Bat/SLO/SI-MRV04/2009 (MG457105.1) represents a prototype of mammalian reoviruses (MRV) and was used as an outgroup to better understand the phylogenetic relationships between the strains. The phylogenetic tree was constructed using the neighbor-joining method and Kimura two-parameter model, with bootstrap of 2000 replicas to give consistency to the phylogenetic groups.

DISCUSSION

ARV and PBV are included in a group of important enteric viruses. They have been widely reported to infect poultry and wild birds, consequently producing impacts on the economy and wildlife (Silva *et al.* 2014; Lu *et al.* 2015; Verma *et al.* 2015; Assunção *et al.* 2018; Wang *et al.* 2019; Duarte-Júnior *et al.* 2021). The characteristic of the ARV and PBV segmented genome is an important factor that contributed to the rapid dispersion, evolution, and adaptation of these viruses in different hosts (Ganesh *et al.* 2014). Wild birds are known to be important reservoirs of several infectious agents (Ogasawara *et al.* 2015).

In the present study, there were no electrophoretic profiles of ARV and PBV in PAGE migration, corroborating previous studies involving wild bird specimens from northern Brazil (Chagas 2018; Guerreiro *et al.* 2018; Duarte-Júnior *et al.* 2021). However, previous studies in Brazil and other countries have already characterized electrophoretic profiles of PBV and ARV in chicken (*Gallus gallus domesticus*, Linnaeus 1758), domestic ducks (*Cairina moschata,* Linnaeus 1758) and wild birds (*Hypsipetes amaurotis,* Temminck 1830) (Yun *et al.* 2013; Silva *et al.* 2014; Ogasawara *et al.* 2015).

The prevalence of PBV by RT-PCR in our samples was 1.29%. Studies carried out in Brazil that investigated PBV in wild birds reported prevalence ranging from 0 to 4.5 %, using the same technique and target gene (Chagas 2018; Guerreiro *et al.* 2018; Duarte-Júnior *et al.* 2021). Higher prevalence of PBV was detected in poultry, with values of 11.8-49.4% in broilers (*G. gallus domesticus,* Linnaeus 1758), and 51.7% in turkey (*Meleagris gallopavo,* Linnaeus 1758) (Silva *et al.* 2014; Verma *et al.* 2015; Ribeiro *et al.* 2019).

The high prevalence of PBV in broilers, especially in poultry farming, suggests this pathogen may be associated to confined systems, facilitating viral dispersion through direct among birds (Silva 2012). As our study dealt with different free-ranging bird species, the probability of virus spread among individuals is reduced, even though species live in flocks.

The GI genogroup was detected in 1.29% of our specimens, corroborating previous studies that detected GI in wedge-billed-woodcreeper (*Glyphorynchus spirurus*, Vieillot 1819) from a deforested area in Pará (Chagas 2018), toucan (*Rhamphastus* sp.) with diarrhea symptoms in Pará (Duarte-Júnior *et al*. 2021), and Australian shelduck (*Tadorna tadornoides,* Jardine & Selby 1828) in Australia (Wille *et al.* 2018).

The present study is the first to report the occurrence of ARV in wild free-ranging birds in Brazil. The low prevalence of ARV in our study (0.6%) is in disagreement with previous studies, that reported a prevalence of 33.3% (5/15) for poultry in Egypt (Al-Ebshahy *et al.* 2019) and 30.2% (58/192) in wild birds in Poland (Styś-Fijoł *et al.* 2017). In Brazil, two previous surveys that assessed the ARV S2 gene in wild birds fecal found no positive results (Chagas 2018; Guerreiro *et al.* 2018), but a prevalence of 32.9% (28/85) was detected in fecal specimens of farm poultry from the region of Belém (Silva 2012).

In studies that reported a high prevalence of ARV infection, specimens were from birds with clinical signs of infection, such as arthritis, tenosynovitis, and enteric syndromes, in addition to more severe cases, when the central nervous system is compromised or the bird was dead (Styś-Fijoł *et al.* 2017). In an analysis of tendons, synovial tissue, and viscera of 311 poultry with clinical simptoms of infection in the USA, specimens were positive for ARV (Lu *et al.* 2015). In our study, we did not observe any external sign of disease while handling birds, but we did not measure clinical parameters nor analyzed internal organs, therefore we could not formally evaluate the health condition of the captured birds.

PBV strains isolated in different geographic regions and from different hosts have been shown to be phylogenetically closer than strains isolated in the same region (Silva *et al.* 2014; Verma *et al.* 2015; Malik *et al.* 2018; Wille *et al.* 2018). The similarity of our PBV strains with those of disjunct geographical regions and/or other species corroborates the capacity of these organisms for rapid spread, evolution, and adaptation, which may be related to anthropic dispersion of ancestor sharing strains (Ganesh *et al.* 2014; Ribeiro *et al.* 2019).

The two PBV strains in this study were isolated from two individuals of the same species (*Guira guira*) captured on the same day and location, but showed high genetic diversity, which agrees with the heterogeneous nature of PBV described in the literature (Silva *et al.* 2014; Verma *et al.* 2015). Several factors are considered relevant to explain the high genetic diversity of PBV, such as short fragments analyzed, genetic variability, multiple interspecies transmissions, and genetic rearrangement events between segments of different PBV strains (Ganesh *et al.* 2014; Silva *et al.* 2014; Ribeiro *et al.* 2019).

PBV GI has a worldwide distribution pattern and has been reported to infect a variety of hosts such as mammals, birds, reptiles, and even fish (Kumar *et al.* 2020). The phylogenetic grouping of PBV GI isolated from different hosts indicates these viruses are not species-specific, i.e., they can be transmited from one host to another (Verma *et al.* 2015; Chagas 2018; Malik *et al.* 2018). The great genetic similarity observed between PBV strains isolated from different hosts raises increasing concerns about the zoonotic potential of this virus in the context of public health (Ganesh *et al.* 2014; Kumar *et al.* 2020).

Among 15 ARV S2 strains isolated from farm poultry in the surroundings of Belém, 13 were more phylogenetically related to each other, while two were more closely related to

ARV prototypes from poultry around the world (Silva 2012). All 15 strains showed a nucleotide homology of 90.1-100% among them, and 90.9-94.4% with the reference prototypes from other production birds around the world, further demonstrating that the degree of nucleotide similarity that among strains of ARV is independent of geographic proximity.

Contrary to this notion, however, the ARV strain isolated in the present study was more related to ARV strains that have been circulating for some years in poultry in the same region than to strains from other countries. This result suggests that this ARV strain has adapted to the local environment in the Belém region over the years, and that transmission occurs between domestic and wild birds in the region. Commercial poultry farms in the Belém region are normally located in rural environments close to forest fragments, which can facilitate the transmission of pathogens between poultry and wild fauna.

The results of this study further expand the occurrence of ARV and PBV in wild birds, and confirm the occurrence of these viruses in wild birds in the Amazon region. The documented hosts, *Guira guira* and *Myrmotherula longipennis,* are not long distance migrants, but their proximity to a large urban center and several poultry farms in the Belém region poses a risk as they may act as possible dispersion agents for these viruses (Pacheco *et al*. 2021). The presence of these viruses in wild birds inhabiting an environmental protection area with limited human activity suggests ARV and PBV circulation in natural environments. Yet APA Belém is also partially interspersed with several low-income neighborhoods with poor basic sanitation infrastructure, creating conditions for possible avian to human transmission.

CONCLUSIONS

This study recorded, for the first time, the occurrence of ARV in any wild bird species in Brazil, and the occurrence of PBV in *Guira guira*. The study further confirms the circulation of these viruses in wildlife in close proximity of a large urban center in the Amazon region, prompting additional studies to determine the epidemiology of infectious agents in wild birds in the region, especially concerning segmented genome viruses, for which the processes of transmission, evolution, and adaptation to new environments and hosts occur faster. The molecular characterization and phylogenetic analysis support the notion that the ARV strain detected has been circulating among poultry and wild birds and adapting locally, while the PBV strains were more closely related to geographically disjunct strains, suggesting long distance dispersal through human activities.

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DATA AVAILABILITY

The nucleotide sequences obtained in this study were deposited in GenBank under accession numbers OM287555, OM287556, and OM287557, and can be accessed at https://www.ncbi.nlm.nih.gov/nucleotide/.