Detection of Reticuloendotheliosis Virus in Brazil

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Abstract

Reticuloendotheliosis retroviruses (REV) are known to cause immunosuppressive and oncogenic disease that affects numerous avian species. REV is present worldwide and recently has been reported in South America with cases of infected commercial flocks in Argentina. We surveyed for the presence of REV in birds from a state in the northern region of Brazil using real-time PCR. We report the first cases of REV in Brazil, detected in Muscovy ducks (*Cairina moschata*), wild turkeys (*Meleagris gallopavo*), and chickens (*Gallus gallus*) at a relatively high prevalence rate (16,8%). Phylogenetic analysis indicated a close relationship of this strain to variants in the United States. This study provides evidence of REV in the Amazon biome and provides a baseline for future surveillance of the virus in the region and throughout Brazil.

Key words: avian retrovirus, Brazil, reticuloendotheliosis virus

Main Text

Reticuloendotheliosis viruses (REV) are a group of immunosuppressive and sometimes oncogenic retroviruses that affect numerous species of birds, including waterbirds (Anseriformes), game birds (Galliformes), and perching birds (Passeriformes) (Nair et al. 2013). REV has previously affected commercial poultry and has been a recurring obstacle in the conservation of endangered species (Luan et al. 2016). Within vaccinated flocks, REV infection can lead to decreased efficacy of vaccinations for avian influenza virus, Newcastle disease virus, Marek's Disease virus (MDV), and turkey herpesvirus due to the reduced humoral response resulting from immunosuppression (Sun et al. 2009). The virus was first identified in the US in 1958, and later in China, Taiwan, Australia, Argentina, and Canada (Singh et al. 2003; MacDonald et al. 2018). REV prevalence varies from 0–50% depending on the region, setting, and avian species (Jiang et al. 2013; Ferro et al. 2017).

Evidence of REV in South America was recently demonstrated in fowlpox-vaccinated commercial poultry flocks in Argentina (Buscaglia, 2013). REV proviral DNA has been detected in the genomes of the attenuated MDV vaccine (MD-2 strain) and the field and vaccine strains of fowlpox viruses (FWPV-REV) (Isfort et al. 1992; Hertig et al. 1997). Vaccine-integrated REV can be either infectious or non-infectious (Hertig et al. 1997; Moore et al. 2000). The Brazilian coast serves as an important stopover site for migratory birds coming from the northern hemisphere. Several species migrate during the austral winter from Argentina and Chile to Central Bolívia and Brazil (Somenzari et al. 2018). In addition, several birds, including passeriformes, migrate from the US, Mexico, and Central America to northern Brazil (Somenzari et al. 2018). However, no prior studies have surveyed for the presence of REV in Brazil. With more than 2,000 species of wild birds present in the country, many of which are endangered,

67 determining the presence of REV and establishing a baseline prevalence rate could be of value
68 for future conservation efforts in the Amazon biome.

69 During 2005–2006, a total of 441 samples were collected (blood and pooled
70 cloacal/orotrachea swabs) near eight different cities in the northeastern region of Pará state. Most
71 of the samples were from Muscovy ducks (*Cairina moschata*, *n*=379), and the remaining
72 samples were from turkeys (*Meleagris gallopavo*, *n*=41), and chickens (*Gallus gallus*, *n*=21).
73 Total cDNA from orotrachea and cloacal swab samples was obtained and previously prepared
74 from an avian influenza virus detection study (Thomazelli et al. 2012). As an internal control, a
75 conventional PCR method for avian mitochondrial DNA was performed using cytochrome *b*
76 primers (Kocher et al. 1989). The presence of REV proviral cDNA was detected using real-time
77 PCR and primers specific to the *gp90* gene (*env*) (Li et al. 2013). Reactions were prepared using
78 2.5mM of each primer, 7.5µL of water, 12.5µL of MasterMix SYBR® Green
79 (LifeTechnologies®, Brazil), and 3µL of purified cDNA. Plasmids containing genes of interest
80 were constructed from commercially-synthesized inserts (GenScript, USA) to serve as positive
81 controls. In addition, partial fragments of LTR-U5, Gag, and envelope from REV-positive
82 samples were amplified and sequenced by Sanger method using previously described primers
83 (Singh et al. 2003; Barbosa et al. 2007; Li et al. 2013). For the envelope region, an additional
84 primer was designed (gp90-7242R 5'–GCCAGTATGCACAGCCCTATCCA–3').

85 Of the 441 samples tested and amplified, REV PCR products were detected in 74 samples
86 (16,7%). Infected individuals included 65 Muscovy ducks, 6 wild turkeys, and 2 chickens. REV
87 positive samples came from Marabitana, Vila Maracajá, and Marajó. Sequencing reads were
88 inspected for quality and consensus sequences were built using CLC genomic workbench v5
89 (https://www.qiagenbioinformatics.com/) and submitted to GenBank, accession numbers

MG953804–MG953809. Consensus sequences of partial LTR/gag (808 bp), gag (1168 bp), and envelope (~600 bp) were aligned and compared to reference strains and other sequences retrieved from GenBank representing worldwide REV (*n*=16). Phylogenetic trees were reconstructed under maximum likelihood method in PhyML software using K80 as the best nucleotide substitution model for all fragments as determined by jModeltest (Guindon et al. 2010; Darriba et al. 2012). Only sequences built with high quality reads (phred >30) were used for phylogenetic reconstructions. Genetic distance (*p*-distance) between the main REV strains and one representative Brazilian REV sample was estimated for LTR/gag, gag, and envelope partial fragments. To establish the phylogenetic relationships among Brazilian and worldwide REV, phylogenetic trees were reconstructed using LTR/gag and envelope fragments representing Brazilian and worldwide REV genomic sequences (*n*=16) (Fig 1). Brazilian REV clustered together with sequences sampled in the US, such as APC-566. Genetic distance at the nucleotide level (*p*-distance) between the main REV strains and one representative Brazilian REV also agreed that Brazilian REV is more closely related to the US samples representative of subtype 3, such as MD-2 and APC-566, rather than to spleen necrosis virus (SNV) strain or China isolates (Fig. 1 and Table 1).

Pará state is located in the northern coastal region of Brazil and has a tropical rainforest climate. Due to its geographic location and ecosystem, Pará is a stopover site for shorebirds coming from the northern hemisphere during the migration season. In addition, several species of migratory birds that have Argentina and the US as their final destination pass through Brazil during their flight. The similarity between Brazilian REV and the US viruses suggests that the US could be the source of REV detected in Brazil. However, there is no sequence data available for viruses found in Argentina. As such, the source of Brazilian REV cannot be definitively

113 determined. Representative strains of REV include the defective REV-T, the non-defective REV-
114 A, SNV, duck infectious anemia, and chicken syncytial virus (CSV), and the MD-2 and FWPV
115 vaccine-integrated strains. As there is relatively little genetic variation among REV strains, it is
116 difficult to determine the origin and classification of an isolate (Bohls et al. 2006). In our
117 analysis, Brazilian REV differs only 0.0–0.5% from both FWPV and MD-2-integrated REVs and
118 non-integrated APC-566 and CSV. We inspected for point mutations, which have been
119 characterized as specific for FWPV-REV (Tadese et al. 2008). According to the genetic pattern
120 observed, the Brazilian REV samples are all non-FWPV-integrated strains. However, not all
121 FWPV-REV envelope sequences available on GenBank (KY498002, AF246698, and
122 JX217830) contain this signature, indicating that some genetic variability among integrated
123 strains exists and determining the integration status based on few point mutations is inaccurate
124 (Tadese et al. 2008). Therefore, it remains undetermined if Brazilian REV is present as infectious
125 particles or is instead integrated within a large DNA virus genome.

126 Reticuloendotheliosis viruses can infect a number of species, including captive and wild
127 perching birds, game birds, and waterbirds. Although we detected REV in Muscovy ducks, wild
128 turkeys, and chickens, we could only amplify and sequence the virus from ducks. It is possible
129 that the virus was present in a low viral load in other species, and we could not generate large
130 amplicons. Muscovy ducks are native to Mexico, Central, and South America, but populations of
131 Muscovy ducks reside in the US, mainly in Florida and southern Texas. These birds are
132 essentially non-migratory or irregular migrants without any established migration patterns, only
133 migrating short distances to avoid dry weather and fluctuating water conditions. Chickens and
134 turkeys, frequently associated with REV infections, are also non-migratory birds. Therefore, we
135 consider it is unlikely that the positive birds found in Brazil were infected elsewhere.

Here, we detected REV in Brazil for the first time and presented the first DNA sequences of REV provirus from South America. REV found in Brazil is similar to other common circulating strains, including those found in the US. Additionally, considering that REV was present in samples from three of the five regions collected from Pará from 2005–2006 (located 200–500 km apart from each other), it is very likely that the virus has spread to other states in Brazil. We hypothesize that the virus could be carried by migratory birds which stopover in the northern part of the country on their way to and from North America. These results suggest the need for additional studies to further determine the prevalence and genetic variability of REV in Brazilian wild and captive birds and also to perform risk assessment studies dedicated to free-range and captive commercial avian species.

All procedures involving wild and captive birds were approved by the Animal Ethics Committee from the Instituto de Medicina Tropical de São Paulo under protocol 000283A, and licensed by the Ministério do Meio Ambiente-MMA at the Instituto Chico Mendes de Conservacão da Biodiversidade (ICMBio/SISBIO) under protocols 34605-7 45527-2, 54616-1, and 201/2006 CGFAU. This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Project #2015/05958-3). G. Caleiro holds a CAPES scholarship.

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Table 1

Genetic Identity at nucleotide level between Brazilian REV and reference strains.

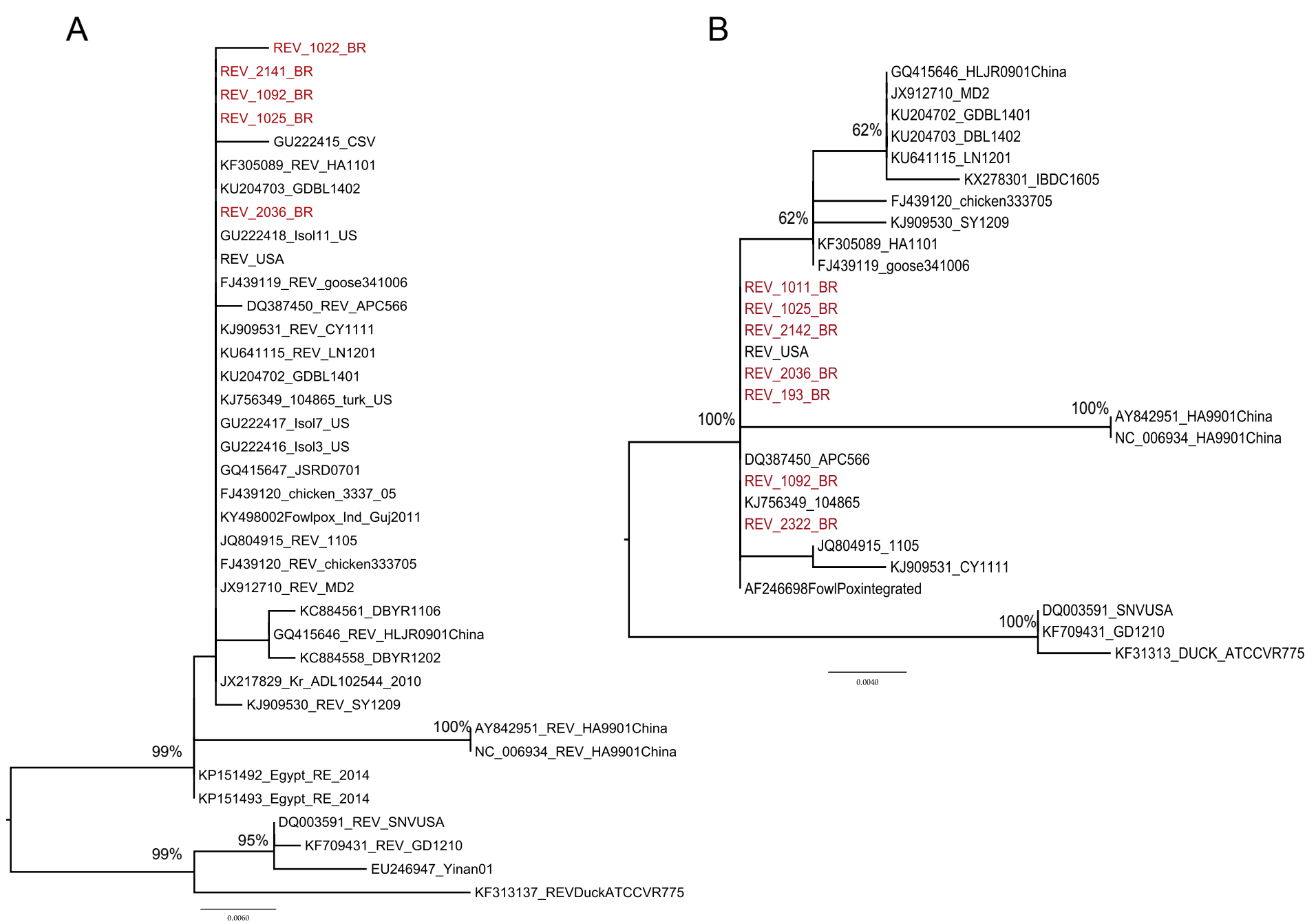
		REV_2036_PA_Br		
Reference	GenBank ID	LTR/gag	Gag	Env
APC-566	DQ387450	99.8%	100%	99.8%
SNV	DQ003591	96%	97.5%	96.4%
MD-2	JX912710	99.4%	100%	100%
FPV	AF246698	99.6%	100%	100%
CSV	DQ237905	NA[a]	NA	99.5%
HA9901 (China)	AY842951	96%	98%	97.6%

Distance matrix (*p*-distance)

[a]NA – sequence not available

Figure 1

Maximum likelihood Phylogenetic reconstruction showing the relationships between REV from Brazil and other isolates. Brazilian sequences (in red) are from Muscovy ducks (*Cairina moschata*). The trees were made with partial sequences from REV env (A) and LTR/gag (B) available at GenBank.


A
REV_1022_BR
REV_2141_BR
REV_1092_BR
REV_1025_BR
GU222415_CSV
KF305089_REV_HA1101
KU204703_GDBL1402
REV_2036_BR
GU222418_Isol11_US
REV_USA
FJ439119_REV_goose341006
DQ387450_REV_APC566
KJ909531_REV_CY1111
KU641115_REV_LN1201
KU204702_GDBL1401
KJ756349_104865_turk_US
GU222417_Isol7_US
GU222416_Isol3_US
GQ415647_JSRD0701
FJ439120_chicken_3337_05
KY498002Fowlpox_Ind_Guj2011
JQ804915_REV_1105
FJ439120_REV_chicken333705
JX912710_REV_MD2
KC884561_DBYR1106
GQ415646_REV_HLJR0901China
KC884558_DBYR1202
JX217829_Kr_ADL102544_2010
KJ909530_REV_SY1209
100%
AY842951_REV_HA9901China
NC_006934_REV_HA9901China
99%
KP151492_Egypt_RE_2014
KP151493_Egypt_RE_2014
DQ003591_REV_SNVUSA
95%
KF709431_REV_GD1210
99%
EU246947_Yinan01
KF313137_REVDuckATCCVR775
0.0060
B
GQ415646_HLJR0901China
JX912710_MD2
KU204702_GDBL1401
62%
KU204703_DBL1402
KU641115_LN1201
KX278301_IBDC1605
FJ439120_chicken333705
62%
KJ909530_SY1209
KF305089_HA1101
FJ439119_goose341006
REV_1011_BR
REV_1025_BR
REV_2142_BR
REV_USA
REV_2036_BR
REV_193_BR
100%
100%
AY842951_HA9901China
NC_006934_HA9901China
DQ387450_APC566
REV_1092_BR
KJ756349_104865
REV_2322_BR
JQ804915_1105
KJ909531_CY1111
AF246698FowlPoxintegrated
100%
DQ003591_SNVUSA
KF709431_GD1210
KF31313_DUCK_ATCCVR775
0.0040