

# 1 **Detection of Reticuloendotheliosis Virus in Brazil**

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30 **Abstract**

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

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41 Key words: avian retrovirus, Brazil, reticuloendotheliosis virus

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44 **Main Text**

45 Reticuloendotheliosis viruses (REV) are a group of immunosuppressive and sometimes  
46 oncogenic retroviruses that affect numerous species of birds, including waterbirds  
47 (Anseriformes), game birds (Galliformes), and perching birds (Passeriformes) (Nair et al. 2013).  
48 REV has previously affected commercial poultry and has been a recurring obstacle in the  
49 conservation of endangered species (Luan et al. 2016). Within vaccinated flocks, REV infection  
50 can lead to decreased efficacy of vaccinations for avian influenza virus, Newcastle disease virus,  
51 Marek's Disease virus (MDV), and turkey herpesvirus due to the reduced humoral response  
52 resulting from immunosuppression (Sun et al. 2009). The virus was first identified in the US in  
53 1958, and later in China, Taiwan, Australia, Argentina, and Canada (Singh et al. 2003;  
54 MacDonald et al. 2018). REV prevalence varies from 0–50% depending on the region, setting,  
55 and avian species (Jiang et al. 2013; Ferro et al. 2017).

56 Evidence of REV in South America was recently demonstrated in fowlpox-vaccinated  
57 commercial poultry flocks in Argentina (Buscaglia, 2013). REV proviral DNA has been detected  
58 in the genomes of the attenuated MDV vaccine (MD-2 strain) and the field and vaccine strains of  
59 fowlpox viruses (FWPV-REV) (Isfort et al. 1992; Hertig et al. 1997). Vaccine-integrated REV  
60 can be either infectious or non-infectious (Hertig et al. 1997; Moore et al. 2000). The Brazilian  
61 coast serves as an important stopover site for migratory birds coming from the northern  
62 hemisphere. Several species migrate during the austral winter from Argentina and Chile to  
63 Central Bolívia and Brazil (Somenzari et al. 2018). In addition, several birds, including  
64 passeriformes, migrate from the US, Mexico, and Central America to northern Brazil (Somenzari  
65 et al. 2018). However, no prior studies have surveyed for the presence of REV in Brazil. With  
66 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, 7.5 $\mu$ L of water, 12.5 $\mu$ L of MasterMix SYBR® Green  
79 (LifeTechnologies®, Brazil), and 3 $\mu$ L 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 Marabitaná, 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

90 MG953804–MG953809. Consensus sequences of partial LTR/gag (808 bp), gag (1168 bp), and  
91 envelope (~600 bp) were aligned and compared to reference strains and other sequences  
92 retrieved from GenBank representing worldwide REV ( $n=16$ ). Phylogenetic trees were  
93 reconstructed under maximum likelihood method in PhyML software using K80 as the best  
94 nucleotide substitution model for all fragments as determined by jModeltest (Guindon et al.  
95 2010; Durriba et al. 2012). Only sequences built with high quality reads (phred >30) were used  
96 for phylogenetic reconstructions. Genetic distance ( $p$ -distance) between the main REV strains  
97 and one representative Brazilian REV sample was estimated for LTR/gag, gag, and envelope  
98 partial fragments. To establish the phylogenetic relationships among Brazilian and worldwide  
99 REV, phylogenetic trees were reconstructed using LTR/gag and envelope fragments representing  
100 Brazilian and worldwide REV genomic sequences ( $n=16$ ) (Fig 1). Brazilian REV clustered  
101 together with sequences sampled in the US, such as APC-566. Genetic distance at the nucleotide  
102 level ( $p$ -distance) between the main REV strains and one representative Brazilian REV also  
103 agreed that Brazilian REV is more closely related to the US samples representative of subtype 3,  
104 such as MD-2 and APC-566, rather than to spleen necrosis virus (SNV) strain or China isolates  
105 (Fig. 1 and Table 1).

106 Pará state is located in the northern coastal region of Brazil and has a tropical rainforest  
107 climate. Due to its geographic location and ecosystem, Pará is a stopover site for shorebirds  
108 coming from the northern hemisphere during the migration season. In addition, several species of  
109 migratory birds that have Argentina and the US as their final destination pass through Brazil  
110 during their flight. The similarity between Brazilian REV and the US viruses suggests that the  
111 US could be the source of REV detected in Brazil. However, there is no sequence data available  
112 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.

136           Here, we detected REV in Brazil for the first time and presented the first DNA sequences  
137 of REV provirus from South America. REV found in Brazil is similar to other common  
138 circulating strains, including those found in the US. Additionally, considering that REV was  
139 present in samples from three of the five regions collected from Pará from 2005–2006 (located  
140 200–500 km apart from each other), it is very likely that the virus has spread to other states in  
141 Brazil. We hypothesize that the virus could be carried by migratory birds which stopover in the  
142 northern part of the country on their way to and from North America. These results suggest the  
143 need for additional studies to further determine the prevalence and genetic variability of REV in  
144 Brazilian wild and captive birds and also to perform risk assessment studies dedicated to free-  
145 range and captive commercial avian species.

146           All procedures involving wild and captive birds were approved by the Animal Ethics  
147 Committee from the Instituto de Medicina Tropical de São Paulo under protocol 000283A, and  
148 licensed by the Ministério do Meio Ambiente-MMA at the Instituto Chico Mendes de  
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212 Table 1

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

Reference	GenBank ID	REV_2036_PA_Br		
		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 <sup>a</sup>	NA	99.5%
HA9901 (China)	AY842951	96%	98%	97.6%

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215 Distance matrix (*p*-distance)

216 <sup>a</sup>NA – sequence not available

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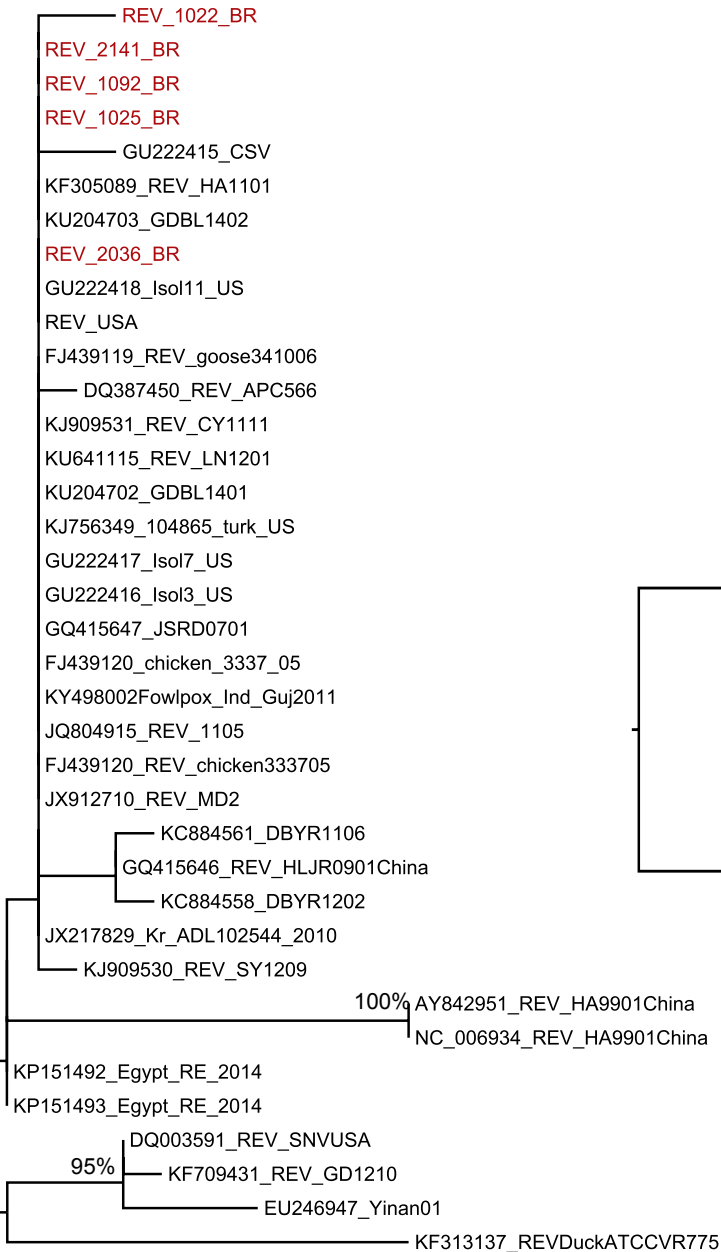
219 Figure 1

220 Maximum likelihood Phylogenetic reconstruction showing the relationships between REV from

221 Brazil and other isolates. Brazilian sequences (in red) are from Muscovy ducks (*Cairina*

222 *moschata*). The trees were made with partial sequences from REV env (A) and LTR/gag (B)

223 available at GenBank.

**A****B**