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RESEARCH

Circulation of picobirnavirus in Neotropical free-ranging mammals

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Abstract

Picobirnavirus (PBV) is a family of non-enveloped double-stranded RNA viruses with bisegmented genomes. Segment 1 encodes the capsid protein and segment 2 encodes RNA-dependent RNA polymerase. They exhibit high genomic heterogeneity and infect a wide range of vertebrate hosts, including humans. The objective of this study was to expand our knowledge of the circulation of PBV in free-living animals from two regions (Brazil and Argentina) of the Atlantic Forest. Fecal samples were analyzed from free-living animals: tapir, brocket deer, peccary, and different species of rodents and marsupials. A total of 133 samples were collected and analyzed by RT-PCR, of which 44 (33.08%) were PBV-positive. Nine amplicons were sequenced, five species from Argentina and four from Brazil, and phylogenetic analysis was performed. The nucleotide and amino acid identities of the PBV strains detected in animals from Argentina and Brazil were between 66.3% and 82.5% and between 55.3% and 74.2%, respectively. The analysed strains presented conserved nucleotide blocks without distinction of the host species. The phylogenetic tree showed that PBV strains from Atlantic Forest animals belonging to genogroup I were grouped into different clusters, without defining groups according to host species (human or animal) or the geographical area of detection. This is the first study on PBV in free-living animals in the Atlantic Forest. Our analysis suggested that PBV strains can infect different animal species, leading to PBV transmission between animals and humans. This reinforces the hypothesis of previous crossover points in the ecology and evolution of heterologous PBV strains.

Keywords Picobirnavirus · Atlantic Forest · Cerrado · Wild ungulates · Rodents

Introduction

The American tropics (the Neotropics) are the most species-rich realm on Earth (Antonelli and Sanmartín 2011), comprising many distinct biomes and habitats, such as seasonally dry forests, arid zones, high-elevation grasslands, young and old mountain systems, and extensive rainforests, such as the Cerrado and the Atlantic Forest (Hoorn et al. 2010). The latter is one of the most representative forests in South America and is considered one of the five hotspots for biodiversity in the world (Myers et al. 2000). It is a set of tropical and subtropical rainforests made up of 15 ecoregions extending from the coast of Brazil, eastern Paraguay and the province of Misiones in northeastern Argentina (Di Bitetti et al. 2003; Oyarzabal et al. 2018). Its climate is humid subtropical with annual rainfall between 1000 and 1200 mm. Still, the Cerrado is also a diverse biome with mosaics of forest, savanna, and wetland habitats and occurs in northeastern Brazil, throughout central, southeastern, and southwestern Brazil, and into eastern Paraguay and Bolivia (Oliveira-Filho and Ratter 1995). In the past decades, both the Cerrado and the Atlantic Forest have suffered great habitat loss due to human-induced activities (Colli et al. 2020; da Silva et al. 2020; Lingner et al. 2020). Human actions, such as

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habitat fragmentation and the introduction of animal species, promote the transmission of infectious diseases to wild animals (Jones et al. 2013; Wilkinson et al. 2018). Monitoring infectious diseases in wildlife populations can provide an early warning of the potential risk of emerging infections in the region (Machalaba et al. 2021). However, owing to the lack of systematic epidemiological surveillance, the magnitude of the circulation of different viruses and the incidence rate of infectious diseases are difficult to define. One of the viruses that could be present in the wild environment belongs to the *Picobirnaviridae* family and genus *Orthopicobirnavirus*. Picobirnavirus (PBV) belongs to a family of small, non-enveloped, double-stranded RNA viruses (Delmas et al. 2019). They have a bisegmented genome structure with segment 1 encodings (2.2–2.7 kb) capsid protein, and segment 2 (1.2–1.9 kb) encoding the RNA-dependent RNA polymerase. The genomic segment 1 was found to encode two open reading frames (ORF1 and ORF2); ORF1 encodes a hydrophilic protein with 224 amino acids of unknown function. ORF2 encodes a 552 amino acid capsid protein. Smaller segment 2 has a single ORF of 534 amino acids and encodes viral RNA-dependent RNA polymerase (RdRp). Based on the genetic variability of genomic segment 2, PBVs are classified into five genogroups with high sequence variability: viruses infecting vertebrates (Genogroups I and II), viruses found in invertebrates (Genogroup III) (Ganesh et al. 2012; Delmas et al. 2019) and virus identified in fungal and prokaryotic host cells (Genogroups IV and V) (Kashnikov et al. 2023). Genogroup I (GI) has a wider distribution and usually a much higher prevalence than Genogroup II (GII), which is reflected in the GenBank database, where over 80% of PBV sequences are GI (Malik et al. 2014). Conversely, Genogroup III (GIII) has only been obtained from invertebrate hosts and differs largely from the PBVs of GI and GII (Delmas et al. 2019). In 2017, an RNA-polymerase gene-based RT-PCR diagnostic assay was developed for the initial detection of viral RNA in stool samples (Malik et al. 2017). The expected size of the PCR amplicon was 275 bp and the sensitivity, specificity, and validation of the assay were accomplished using field samples. Expected PCR amplicons of 275 bp were observed in a high percentage of the samples tested (53.7%). However, the authors propose not only the use of the designed PCR method, but also, in combination with GI and GII specific PCR, for the primary detection of PBV infection in animals and subsequent genogroup confirmation for new and emerging PBVs (Malik et al. 2017).

PBV strains infect different animal species and humans (Ganesh et al. 2014; Reddy et al. 2023; Kumar et al. 2020; Masachessi et al. 2015). The virus has been detected in fecal samples of humans and domestic animals, such as pigs, sheep, cattle, dogs, cats, donkeys, chickens, horses, rats, hamsters, rabbits, and wild animals, such as armadillos, giant anteaters, big cats, orangutans, geese, pelicans, Darwin, emus, and pheasants (Masachessi et al. 2007, 2012, 2015; Chen et al. 2014; Ganesh et al. 2014; Bányai et al. 2017; Navarro et al. 2017; Wilburn et al. 2017; Ghosh et al. 2018; Knox et al. 2018; Yinda et al. 2018; Woo et al. 2019; Kleymann et al. 2020). The detection of PBVs in the feces of numerous diarrheic and healthy humans as well as animals has increased concern in public health aspects regarding the transmission of these viruses and their potential zoonotic transmission (Ganesh et al. 2014; Kumar et al. 2020).

While the majority of research focuses on captive wild animals, there have been documented cases of PBV in freely living wild mammals. For instance, studies have reported PBV infections in fur seals along the coast of Brazil (Kluge et al. 2016), roe deer in Slovenia (Kuhar et al. 2017), bats in Cameroon, and gorillas in the Republic of the Congo (Duraisamy et al. 2018).

Due to the lack of information about the activity of PBV in natural environments, such as the Atlantic Forest and Cerrado, it is important to monitor wildlife to identify a PBV potential reservoir and circulation in and between free-ranging mammals. Therefore, our objective was to contribute to the understanding of the natural diversity and host range of PBV by analysing fecal samples from various free-living animal species. Additionally, we aimed to expand knowledge regarding the circulating PBV strains in the biomes of the Atlantic Forest and the Cerrado, as well as their similarities and differences with strains found in different animal species, including those detected in humans.

Materials and methods

Study site

We carried out the research in three high conservation value natural reserves, the Iguazu National Park (INP), in the northern province of Misiones, Argentina (25° 32' to 25° 44' S lat; 54° 09' to 54° 33' W), and two Brazilian natural reserves, the Córrego do Veado Biological Reserve (-18.34379 and -40.12316), Espírito Santo state, and Chapada das Mesas National Park (7° 19′ 0″ S lat; 47° 20′ 6″ W long), Maranhão State. (Fig. 1).

Host species

Iguazú National Park: In the present study, we included the following species: tapir (*Tapirus terrestris*), brocket deer (*Mazama americana*), and peccaries (Tayassuidae). These species are large ungulates with a wide home range and

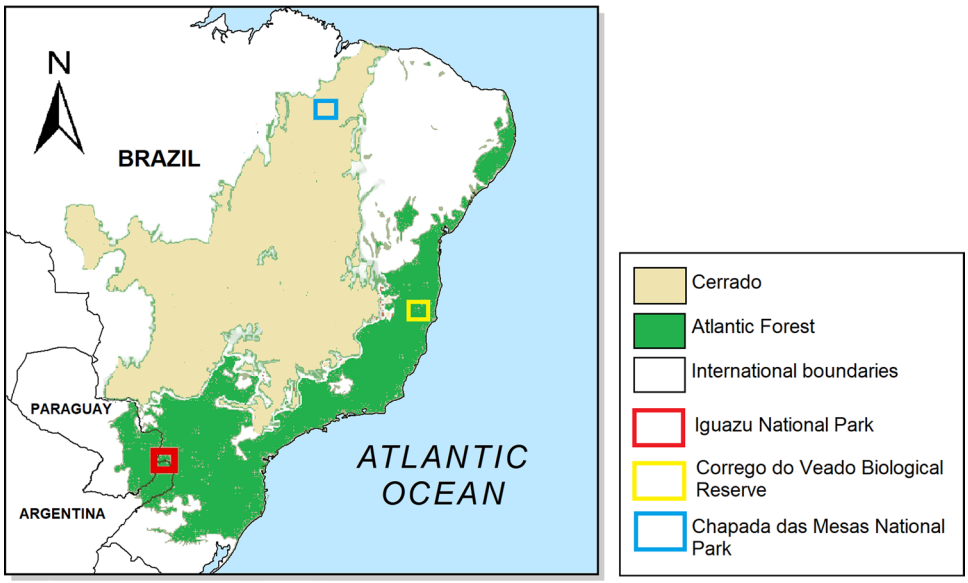

Fig. 1 Map illustrating the study area in the Atlantic Forest and the Cerrado biome where animals were sampled

are distributed throughout the Atlantic Forest. Sampling was done under the authorization from the National Parks Administration of Argentina, permits Dispo 40–2015. Project Number DCM 492.

Brazilian Natural Reserves: We sampled small rodents that included the following species:

Oligoryzomys sp., *Cerradomys subflavus*, *Necromys lasiurus*, *Akodon cursor*, *Thricomys* sp., *Proechimys ssp.*, *Thalpomys sp*., *Akodon montensis*, *Rhipidomys* sp. and marsupials: *Marmosa murina*, *Monodelphis americana*, *Cryptonanus agricolai*, *Thylamys karimii*, *Gracilinanus* ssp.

Sampling was done under the authorization from the Brazilian Environment Ministry, Instituto Chico Mendes de Conservação da Biodiversidade permit number: 46596–1.

Stool collection

Iguazú National Park: The fieldwork was performed in the months from May to July of 2016. We collected samples through walks in trails and roads of the five different sites helped by a trained scat detection dog, a Golden Retriever called "Potolo". The stool of the species (tapir,, peccary, and brocket deer) included in this study had a distinctive morphology, and its identification was possible without having to resort to genetic analyses. To ensure the independence of the sample, sampling sites had a distance greater than 3 km. All samples were geolocalized,stored in nylon bags and refrigerated at 5 °C in coolers containing cooling gels until arrival at the laboratory.

Brazilian Natural Reserves: To sample small mammals we used pitfalls and Sherman's traps, baited with peanut paste, sardines, bananas and oats (Mills et al. 1995; Sikes and Animal Care and Use Committee of the American Society Mammalogists 2016). The sampling was performed under the months of May till September of 2016.

Viral RNA extraction

Stool samples were clarified at 10% with sample buffer, 0.2 M Tris (ULTRA PURE™ TRIS-Invitrogen-Argentina)-HCl (Merck KGaA, Darmstadt, Germany), pH 7.2, and centrifuged at 2000 g for 10 min (i.e. an aliquot of sample buffer (0.5 mL) was added to 5 g stool, mixed by vortex, and centrifuged at 2000 g). The aqueous phase was recovered and stored at -20 °C until analysis. The solid phase was stored at -80 °C for future investigations.

Viral RNA was extracted from 140 µL of the sample using the commercial QIAamp Viral RNA kit (Qiagen Inc., Hilden, Germany). The manufacturer's protocol was followed, and the purified viral RNA was eluted in a 30 µL elution buffer.

Amplification by RT-PCR

The extracted RNA was reverse-transcribed into cDNA using 0.5 µl of random hexamer primers (10 µM) and 0.5 µl of MML-V (200U/µl) reverse transcriptase (Invitrogen™ CA, USA) Briefly, in the first RT step, 3 µL of the extracted RNA was denatured at 97 °C for 5 min in a thermocycler, followed by 5 min in an ice bath. Then, cDNA was obtained by adding 0.5 µL of 10 mM hexamer primers, 1.25 µL of 10 µM dNTP, 1.25 µL of 5X buffer, 0.6 µL of DMSO, 0.5 µL of reverse transcriptase enzyme, 2.9 µL of RNase/DNase-free H_2O and 3 µL of denatured RNA making up a final volume of 10 µL. The reaction mixture

was first incubated at 22 °C for 10 min, followed by incubation at 37 °C for 50 min, and then at 75 °C for 15 min. cDNA products were used as templates for the amplification of PCR gene 2. Our previous results on the use of PicoB23/PicoB24 in different animal species (data not shown) confirmed the limited information on the occurrence of GII in animals (Masachessi et al. 2012, 2015). Considering that GII is uncommon, the PICOB25/PICO B43 primer pair that amplified GI was used (Rosen et al. 2020). Briefly, a volume of 6.25 µL of resulting cDNA was added to 18.75 µL of the PCR mixture yielding a total of 25 µL of a reaction mixture consisting of 10 × reaction buffer (Taq DNA polymerase, Invitrogen) diluted to a final concentration of 1 x, 2.5 mM of $MgCl_2$, 26 pmol of primer B25, 80 pmol of B43, 10uM of dNTP and 1.5 U of Taq DNA polymerase (Thermo Fisher Scientific Inc, USA). Conditions for PCR amplifications were as follows: 2 min at 94 °C, followed by 40 three-step cycles of 1 min at 94 °C, 2 min at 49 °C, and 3 min at 74 °C and by a final single incubation at 74 °C for 7 min. To avoid and control cross-contamination between samples, aerosol-resistant tips were used. Furthermore, several negative controls and a positive PBV sample were included for each extraction. Amplification products were examined by 10% polyacrylamide gel electrophoresis (Laemmli 1970), followed by silver staining (Herring et al. 1982) to achieve high resolution of the products obtained. The amplified genome products were submitted for bi-directional sequencing with the primer pair PicoB25/PicoB43 to Macrogen Laboratory Services (Seoul, Korea).

Sequence analysis

We performed sequence similarity analyses between the obtained PBV sequences and reference sequences from GenBank using BLASTn software, version 2.0. We edited and assembled consensus contig nucleotide sequences using BioEdit v. 7.0.9 and MEGA X (Kumar et al. 2018).

Phylogenetic study

A phylogenetic tree was constructed with all PBV sequences obtained in this study and sequences published in the GenBank database using the MEGA X (Kumar et al. 2018) and the IQTree free web server (http://iqtree.cibiv.univie.ac.at/). The best Composite Maximum Likelihood (ML) model was selected using MEGA X and verified by IQTree free web server. The tree was supported by bootstrapping with 10,000 replicates.

Results

Iguazú National Park

We collected 63 fecal samples from peccaries (n = 6), tapir (n = 27), and brocket deer (n = 30). Of the total samples collected, 36 (57.1%) resulted positive for PBV. Table 1 depicts the rate of PBV detection for each animal species. Neither positive nor negative samples presented signs of diarrhea, we did not observe any loose or liquid stools or with blood

Table 1 Total samples tested for each animal species and positive samples for Picobirnavirus

Animal species	N	PBV +	%	CI (%)	Country	Study site
Tapirus terrestris	27	10	37.03	18.82–55.25	Argentina	INP
Mazama sp.	30	21	70	53.60–86.40	Argentina	INP
Tayassuidae	6	5	83.33	53.51–100	Argentina	INP
Akodon montensis	12	2	16.67	0–37.75	Brazil	ES
Necromys lasiurus	5	2	40	0–82.95	Brazil	MA
Cerradomys subflavus	6	1	16.66	0–86.68	Brazil	ES
Proechimys ssp.	16	2	13.33	0–30.54	Brazil	MA
Thalpomys sp.	4	1	25	0–67.44	Brazil	MA
Akodon cursor	12	0	0	0	Brazil	ES
Oligoryzomys sp.	2	0	0	0	Brazil	ES
Thricomys sp.	2	0	0	0	Brazil	MA
Rhipidomys sp.	2	0	0	0	Brazil	MA
Marmosa murina	1	0	0	0	Brazil	ES
Gracilinanus spp.	3	0	0	0	Brazil	ES
Cryptonanus agricolai	1	0	0	0	Brazil	ES
Monodelphis americana	1	0	0	0	Brazil	ES
Thylamys karimi	3	0	0	0	Brazil	MA
Total	133	44	-	-	-	-

traits. In four of the five sites tested in Iguazú National Park, we found positive samples.

All samples positive for PBV by RT-PCR produced amplicons with the expected size of 201 bp. We selected ten total RT-PCR products based on the quality and concentration of the genomic bands for PBV partial-length RdRp segment sequencing. It was possible to obtain five of the ten sequences, generating a 201 bp amplicon, four obtained from brocket deer, and one obtained from peccary. As expected, all sequences corresponded to PBV-GI. The global nucleotide identity of the amplicons detected among all brocket deer collected from different sampling sites in the Iguazú National Park was between 72.7% and 94% (201 bp), with the highest nucleotide identity between PBV/brocket/ARG-01/2017 and PBV/brocket/ARG-02 (identity: 94%). On the other hand, the lowest nucleotide identity was detected between PBV/brocket/ARG-03 and all others (identity between 72.2 and 75.2%; PBV/brocket/ARG-03vs PBV/brocket/ARG-01/2017, 72.7%; PBV/brocket/ARG-03 vs PBV/brocket/ARG-02, 75.2; PBV/brocket/ARG-03 vs PBV/brocket/ARG-04, 75.2%) (Fig. 2). Similar genomic identity was obtained when comparing PBV strains from peccary versus PBV strains from brocket deer, being the highest nucleotide identity between PBV/peccary/ARG-01 and PBV/brocket/ARG-04/ (identity:83.6%) and as observed in the brocket deer, the lowest nucleotide identity was detected between PBV/peccary/ARG-01 and PBV/brocket/ARG-03 (identity 72.2%).

Brazilian Natural Reserves

A total of 61 fecal samples were collected from rodent species and nine fecal samples from marsupial species. Overall, eight rodents samples (12.2%) were positive for PBV, and no positive samples were detected in the marsupials. In Table 1 it is depicted the rate of PBV detection in each one of the animal species studied. Neither positive nor negative samples presented signs of diarrhea.

All eight PBV-positive samples showed strong signals in the RT-PCR assay and were further sequenced for the partial-length RdRp gene. Only four fecal samples had reliable nucleotide sequences to test, giving a total of six PBV sequences.; identifying a co-detection with two PBV strains in rodent 1 and two strains in rodent 4 (Named sequences: rodent 1: PBV_rodent/BR-1/2015; rodent 2, in co-detection with PBV_rodent/BR-2a/2015 and PBV_rodent/ BR-2b/2015, rodent 3: PBV_rodent/BR-3/2015 and rodent 4 in co-detection with PBV_rodent/BR-4a/2015 and PBV_rodent/BR-4b/2015). All sequences corresponded to PBV-GI and were used for analyses and construction of the phylogenetic tree.

The global nucleotide identity of the six amplicons was between 68.8% and 80.6%, being the highest nucleotide identity between PBV_rodent/BR-1/2015 and PBV_rodent/BR-4b/2015 (identity: 80.6%), and the lowest nucleotide identity was between PBV_rodent/BR-3/2015 and PBV_rodent/BR-4b/2015 (identity: 68.8%) (Fig. 3). The nucleotide identity between the strains detected in the same animal was 82.6% for the strains: PBV_rodent/BR-2a/2015 and PBV_rodent/BR-2b/2015 and 80.1% between the strains: PBV_rodent/BR -4a/2015 and PBV_rodent/BR-4b /2015.

The sequences obtained have been submitted to the GenBank under the following accession numbers: ON309129, ON309130, ON309131, ON309132, ON309133, ON309134, ON309135, ON309136, ON309137, ON309138, ON309139.

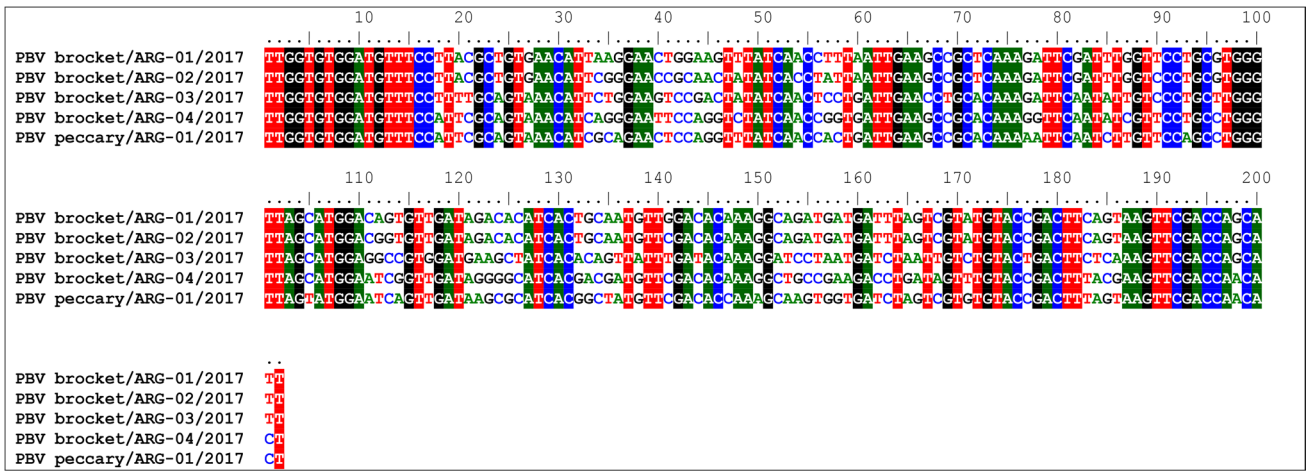

Fig. 2 Alignment of nucleotide sequences corresponding to a region of genomic segment 2 of PBV strains isolated from brocket and peccary from the Iguazú National Park and amplified with the pair of primers Pico B25 / PicoB43. The colored blocks indicate an identity between 80 and 100%

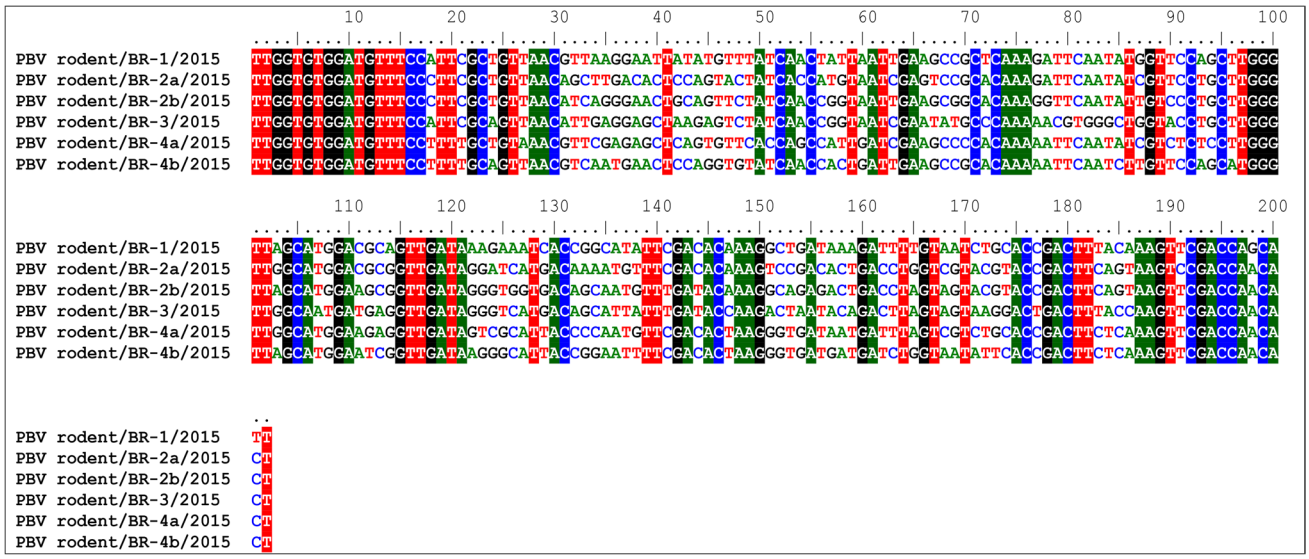

Fig. 3 Alignment of nucleotide sequences corresponding to a region of genomic segment 2 of PBV strains isolated from rodent and amplified with the pair of primers Pico B25 / PicoB43. The colored blocks indicate an identity between 80 and 100%

Genomic analysis of strain detected in animal species from Argentina and Brazil

The PBV strains detected in animals from Iguazú National Park versus those from Brazilian Natural Reserves, have a nucleotide identity ranging from 66.3% to 82.5% (Fig. 4). The predicted amino acid sequences of all PBV amplicons showed the motif 1, which is common among the RNA polymerases (RdRps) of dsRNA viruses and some single-stranded RNA viruses (Fig. 5). The amino acid identity of the strains was within a range from 64.1% to 85.0% (Fig. 5).

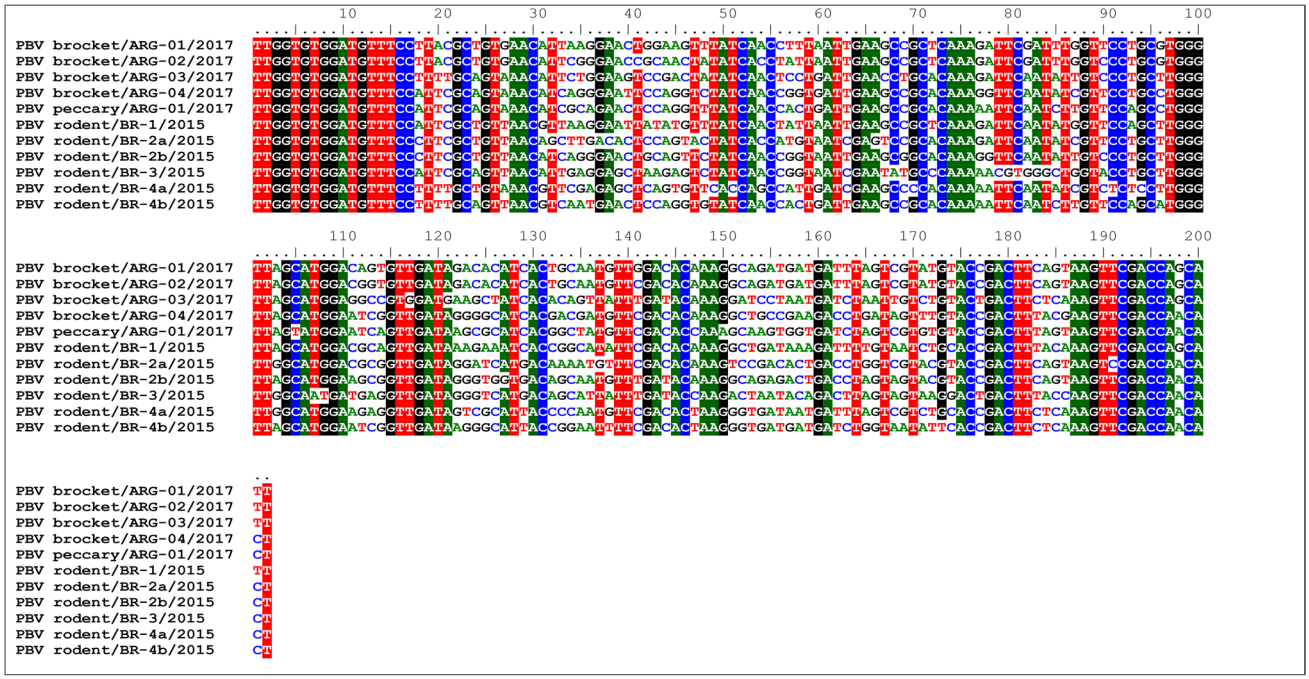

Fig. 4 Region of genomic segment 2 of PBV strains isolated from rodent, peccary and brocket from Brazilian Natural Reserves and Iguazú National Park and amplified with the pair of primers Pico B25 / PicoB43. The colored blocks indicate an identity between 80 and 100%

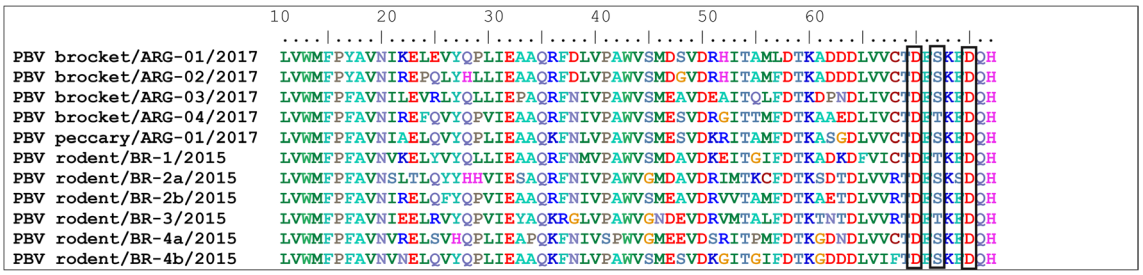

Fig. 5 Predictive amino acid sequence containing the characteristic specific motif 1 of RNA polymerase (D-S–D) identified in sequences of genomic segment 2 of PBV strains excreted by peccaries, brocket and rodent from the Atlantic Forest

Phylogenetic analysis

The phylogenetic tree obtained showed that the strains belonging to GI were grouped into different clusters, without defining representative groupings according to host species or geographic area of strain detection (Fig. 6).

Discussion

Picobirnavirues (PBVs) have been detected in a wide variety of host species in various geographical areas worldwide (Masachessi et al. 2018; Duarte Júnior et al. 2021; Huamán et al. 2021; Nazaktabar 2021; Ramesh et al. 2021; Ullah et al. 2022). In South America, the occurrence of PBV has been documented in both wild and captive animals, spanning various geographic regions, such as the central region of Argentina (Masachessi et al. 2007, 2012, 2015), the Amazon region (Duarte Júnior et al. 2021), and Uruguay (Gillman et al. 2012). This works allowed to expand susceptible species for PBV infection, the viral excretion pattern in infected animals, and the potential association with PBV diarrheic illness (Masachessi et al. 2007, 2012, 2015, Martínez et al. 2010, Giordano et al. 2011). However, this is the first study carried out on free-living animals in the Atlantic Forest and Cerrado biome, regions that belong to an area shared between Brazil and Argentina. Our research reports for the first time the circulation of PBV in tapirs (*Tapirus terrestris*), peccaries (Tayassuidae), brocket deer (*Mazama americana*) and five rodent species (*Akodon montensis*, *Necromys lasiurus*, *Cerradomys subflavus*, *Proechimys* sp, *Thalpomys* sp). While it is not possible to definitively establish whether these species serve as natural reservoirs for PBV, the findings may provide valuable insights in this regard. Further investigations focusing on the epidemiology of this virus in wildlife, expanding sample sizes, and meticulously identifying each infected animal, may help to determine this.. The role of PBV as an etiologic agent of diarrhea remains highly controversial. In this sense, it is well known than in more than one opportunity, the infection is present without gastrointestinal symptoms. As mentioned, in Misiones, it was not possible to follow up on the animals under study, since the animals were free-living and only fecal matter was observed. None of the fecal samples showed signs of diarrhea; the fecal matter was not liquid or bloody, nor did it show any other signs compatible with diarrhea. Similarly, the animals sampled in Brazil, belonging to the Atlantic Forest or Cerrado, did not show signs of diarrhea. Whether PBV is a direct cause of diarrhea or a commensal virus is still under debate. Studies that have reported diarrhea in PBV-positive samples have been done in animals that were in stressful situations such as captivity or confinement, and in humans it has been reported in immunosuppressed adults and children (Ganesh et al. 2011; Atasoy et al. 2022; Karayel-Hacioglu et al. 2022; Smoľak et al. 2022; Yang et al. 2022;). Although we do not exclude that PBV might have influenced the manifestation of diarrhea, it is more likely that this symptomatology is also caused by other stressors. In this regard, our results would indicate that in populations of free-living animals under non-stress conditions, the presence of PBV would not be related to clinical manifestations related to diarrhea. However, it is important to continue further studies on the natural history of PBV infection and the virus-host relationship to continue collecting evidence of the association between PBV and a defined pathology.

Since 2000, PBV detection techniques have evolved, allowing the detection of PBV in a more sensitive and specific way. From the use of the PAGE S/S technique to visualise the genomic bands of PBV, to the design of molecular techniques such as RT-PCR designed from a portion of the genomic fragment 2 (Rosen et al. 2000; Malik et al. 2018) or based on the complete genomic fragment (Wakuda et al. 2005) of the RdRp, and the use of more complex techniques such as metagenomics, for the characterization of PBV diversity in an animal sample (Atasoy et al. 2022; Du et al. 2022). Molecular-based tests, such as RT-PCR, have gained importance for the detection of genome cloning and sequencing. The primers developed by Rosen et al. (2000) allowed the characterization of PBV strains as GI and GII. These primers are widely used worldwide, and it was shown that most of

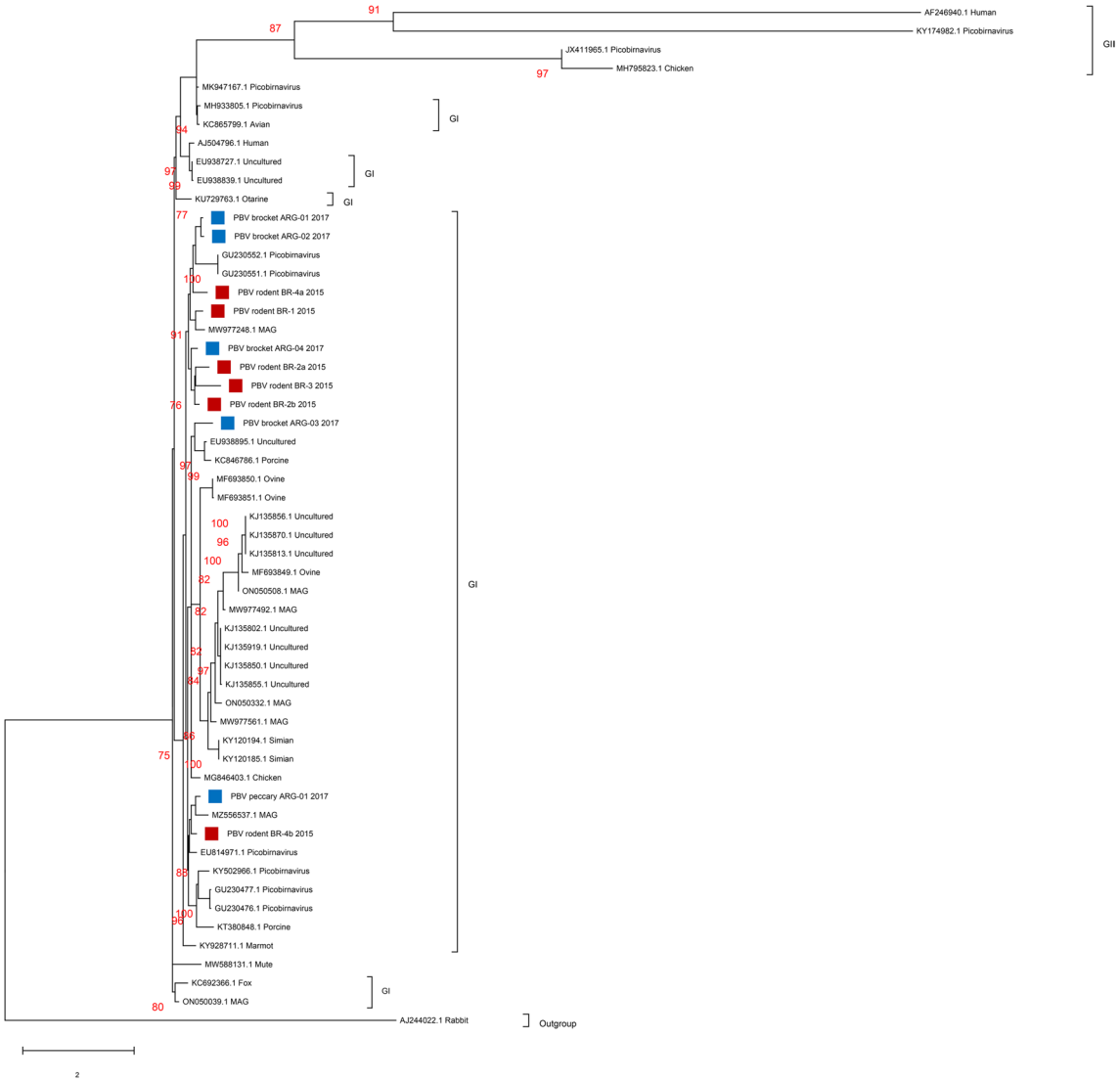

Fig. 6 Phylogenetic analysis based on 201 bp sequences of genomic segment 2 of PBV from different animal species, including in the analysis strains of PBV circulating in brocket deer and peccary from the Iguazú National Park, Argentina (indicated in blue) and in rodents from the reserves of Brazil (indicated in red). The tree was built using the MEGA X program with the neighbour-joining method using the Nucleotide model: Maximum Composite Likelihood. The brackets show the branches that group GI and GII sequences. The AJ244022 sequence belonging to genogroup II was used as outgroup

the identified strains belonged to GI and that these strains were found in a wider variety of animal species compared to GII (Masachessi et al. 2012, 2015; Teng et al. 2021; Atasoy et al. 2022; Karayel-Hacioglu et al. 2022). Therefore, with the aim of studying the circulation and diversity of PBV GI strains, our study focused on the use of the GI primer pair (Rosen et al. 2000). RT-PCR allowed the identification of PBV GI, with an inter-rodent species nucleotide identity between 66.3% and 82.5%. This nucleotide diversity was evidenced in the distribution of PBV strains in the phylogenetic tree, showing rodent PBV sequences widely distributed within the tree, without a defined clade. This result agrees with those previously published by Du et al. (2022) who used viral metagenomic sequencing for virome detection and characterization in 32 rodent species in Xinjiang, northwestern China. The authors found nine viral genotype sequences that corresponded to the PBVs identified in the intestines and liver of seven viscera groups. In agreement with our results, the phylogenetic analysis performed by the authors showed that PBV sequences were distributed throughout the tree that was constructed using the segment 2 RdRp gene with high diversity, sharing 68.42–82.67% nucleotide identities with other strains of PBV from GI. In line with our results, Ghosh et al. (2018) reported high detection rates (54%) of PBVs in the feces and intestinal contents of free roaming, apparently healthy rats on the Caribbean Island of St. Kitts.

In the complete PBV segment-2 of the studied strains, the author found a high PBV genetic diversity. It is important to highlight that regardless of the molecular technique used to identify PBV strains in different animal species (a molecular technique that amplifies a small genomic segment or molecular techniques that allow the characterization of the complete PBV genome), the results indicate that there is a wide diversity of PBV strains with large nucleotide divergence.

It is important to note that during the present study and by using RT-PCR, we observed in feces collected from two rodents, a PBV coinfection with strains with approximately 80% nucleotide identity. The blast analyses performed on the sequences detected in rodent 2, showed that the sequence named as PBV_rodent/BR-2a/2015 aligned with PBV GI strain isolated from snakes in Brazil and the other one, PBV_rodent/BR-2b/2015, aligned with Uncultured picobirnavirus clone Florida_Keys_Raw_Sewage (Fregolente et al. 2009; Symonds et al. 2019). The blast performed on the sequences detected in rodent 4, showed that the sequence named as PBV_rodent/BR -4a/2015, aligned with PBV GI strains isolated from Indian mongooses and the other one, PBV_rodent/BR-4b /2015, aligned with GI avian picobirnavirus detected in Brazilian broiler chickens (Ribeiro Silva et al. 2014; Kleymann et al. 2020).This finding was already observed by our working group in a captive orangutan (Masachessi et al. 2015), who conducted a longitudinal study over a 3-year period. In the work cited above, analysis of the PBV sequences excreted by the orangutan showed a nucleotide identity between 64 and 81%. The variability in the orangutan was explained by the fact that the host was under a long-term infection (at least three years), and as a result, the host may harbour a highly heterogeneous viral population. Furthermore, using metagenomics, the presence of multiple PBVs has been reported in studies exploring viromes in the stool (Chong et al. 2019; Mahar et al. 2020) and plasma (Li et al. 2015) obtained from mammals. In the present study, a longitudinal study was not carried out in rodents, but data were provided on the heterogeneity of the genomic segment 2 of intra-individual and intra-species PBV strains in infected rodents and support the idea that PBVs would form a group of viruses that exist in nature as viral quasispecies (Fregolente et al. 2009).

In the Iguazú National Park, PBV was detected in brocket deer, peccaries, and tapirs at infection frequencies of 70%, 83.3%, and 37.3%, respectively. As observed in the Brazilian reserves, no signs of diarrhea were found in the samples collected. PBV detection in deer species has been reported by different authors with the aim of identifying a potential reservoir of infectious diseases in wildlife. In these studies, PBV was detected not only in feces (Kuhar and Jamnikar-Ciglenecki 2019), but also in serum samples, nasal swabs (Ribeiro Silva et al. 2014; Mahar et al. 2020), and tracheal samples from deer (Ribeiro Silva et al. 2014) in different countries such as Slovenia, Iceland, Russia, and Australia. Using metagenomic analyses, Huamán et al. (2021) and Sánchez-Romano et al. (2021) found PBV in fecal, blood, and respiratory tract samples from apparently healthy deer, raising questions about the tropism and pathogenicity of PBV. Kuhar et al. (2017) reported a rate of 60% positive for PBV GI in feces collected from roe deer using a new primer pair targeting the segment 2 RdRp gene region that amplifies a 326 bp product.

Overall, independent of the technique used for the detection of PBV in rodents and deer (metagenomics or endpoint RT-PCR), the results obtained by our working group matched those published by different authors all over the world, pointing out the high genetic diversity of PBV GI, without subtypes related to the host species or geographic location, highlighting the need to expand our knowledge on picobirnavirus tropism.

It is well known that large mammals play a key role in modulating plant diversity, primary productivity and existing plant biomass in neotropical forests. Species such as tapir (*Tapirus terrestris*) and peccary (Tayassuidae) help balance areas with different levels of productivity within the Atlantic Forest and spatially structure plant communities (Villar et al. 2022). However, little is known about the epidemiology of viruses that infect tapirs and peccaries in the Atlantic Forest. Therefore, the monitoring of potentially pathogenic viruses that could affect the dynamics of these populations and/or the identification of these animal species as potential reservoirs of viruses that spill over humans and animals is of special interest. Although it was not possible to obtain PBV genomic sequences in tapir samples, the high detection of PBV in samples from these animals without signs of diarrhea evidences the high intraspecies circulation of PBV. To our knowledge, this is the first study to show the incorporation of tapirs into the silent natural history of PBV circulation and infection in nature. In peccaries, PBV was detected in 83.3% of the analyzed samples. Although samples of peccaries were few of the three studied species, the high rate of infection could be linked to their gregarious behaviour, allowing rapid virus transmission in the herd.

Only one sample could be sequenced, and in the phylogenetic tree, it clustered with PBV sequences excreted by avian and fox species, which reinforces the issue that PBV breaks the animal species barrier for infection. In general, when we analyzed all the PBV strains found in the Atlantic Forest and Cerrado regions, the results reflected a highly heterogeneous circulation of PBVs in their nucleotide composition, while keeping all conserved short nucleotide blocks without distinction of the excretory species of the virus and in the absence of distinct regions of the host species. This agrees with recent studies on PBV circulation in pigs at St. Kitts, a small island belonging to the Caribbean Lesser Antilles (Joycelyn et al. 2020). As samples from an island, one would

expect PBV strains to be more homogeneous and similar to each other because, owing to their island status, there would be little contact with other animal species from other regions. However, Joycelyn et al. (2020) reported high heterogeneity in the analyzed samples, both with strains from other animal species and from other geographic regions (Kuhar and Jamnikar-Ciglenecki 2019). On the other hand, studies on sheep and goats in a region at the foot of the Himalayan Mountains in India reached the same conclusions (Malik et al. 2018). Similar to the example of the islands, due to the inhospitable location, it could be assumed that in such a mountainous region exchange with other animal species and other geographical regions is not frequent, even so, the authors reported a high heterogeneity of the strains analyzed. Similarly, the regions studied in the present work are protected natural regions, and there is no contact between the animals that inhabit one region and another.

This is the first report of PBV detected in free-ranging mammal species in South America, inhabiting the Atlantic Forest and Cerrado regions. Global analysis of the results suggests that common strains of PBV could infect different animal species, and this could lead to events of PBV transmission between animals. Considering the PBV data in reference to the genetic relationship between PBV strains excreted by different animal species, without a geographical barrier, the hypothesis of the existence of previous crossing points in the ecology and evolution of heterologous PBV strains is reinforced, as suggested by Ganesh et al. (2012) and Perez et al. (2023).

Finally, our results are an example of the importance of continuing to prioritize long-term work with extensive field work. Collaborative work such as ours that integrates field research with laboratory studies is essential to understand viral threats and prepare for potential outbreaks. By studying the dynamics of virus circulation in natural hosts and ecosystems, we can assess the high risk and prioritize attention to potential viruses that may interact with humans. We believe that field research should continue to be encouraged as it provides valuable data for risk assessment and response strategies, ensuring that funding and coordination with laboratories is secured. Recognizing these efforts is essential for effective virus surveillance and public health protection (Runstadler et al. 2023).

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Authors' contributions E.V, G.M and A.D conceived the study, contributed to the conceptual development and study design. F.V conducted the study and designed the sampling from Brazil, contributed to the conceptual development and study design. E.V. performed the field work and faecal samples collection from Argentina. R.P and I.M performed the field work and faecal samples collection from Brazil. E.V performed the laboratory analyses with the guidance of G.M. E.V and G.M. wrote the first draft of the manuscript, and all authors contributed substantially to further development of the manuscript.

Data availability The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information files.

Sequence accession numbers and link.
ON309129. https://www.ncbi.nlm.nih.gov/nuccore/ON309129
ON309130. https://www.ncbi.nlm.nih.gov/nuccore/ON309130
ON309131. https://www.ncbi.nlm.nih.gov/nuccore/ON309131
ON309132. https://www.ncbi.nlm.nih.gov/nuccore/ON309132
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Declarations

Animal ethics The authors declare that the capture, handling and manipulation of the animals were done under the rules stipulated in the research permits (permit number: 46596–1). All animals were released in the same capture area and this study did not require the euthanasia of the animals to obtain the sample. Samples from tapirs, brocket deer and peccaries were collected in the field without involving the capture, handling and manipulation of any animal.

Competing interests The authors declare no competing financial interests.

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