



Genetic diversity of bovine *Picobirnavirus*, Brazil

Juliana de Oliveira Navarro¹ · Marcelo Candido¹ · Sabrina Ribeiro de Almeida-Queiroz¹ · Maria da Glória Buzinaro² · Márcia Cristina Livonesi³ · Andrezza Maria Fernandes¹ · Ricardo Luiz Moro de Sousa¹

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Abstract

Picobirnaviruses (PBVs) are emerging and opportunistic viruses with possible zoonotic potential. In this study, we present the detection, molecular characterization, and genotypic differentiation of PBVs from genogroup I in bovine stool samples from different Brazilian regions. A high proportion of PCR-positive samples (23.4%) was detected in a total of 77 analyzed. Nucleotide identity, alignment, and phylogenetic analyses revealed high diversity among the studied sequences. The results obtained indicate, for the first time, the circulation of bovine PBVs belonging to genogroup I in different Brazilian states, with heterogeneous phylogenetic-clustering profiles.

Keywords Bovine *picobirnavirus* · Molecular diagnostics · Bovine viral diseases · Animal RNA virus · Molecular epidemiology

Picobirnavirus (PBV) is the unique genus of the family *Picobirnaviridae*, classified into two different species: *Human picobirnavirus* and *Rabbit picobirnavirus* [1, 2]. It is a non-enveloped double-stranded segmented RNA virus (dsRNA) constituted of an icosahedral symmetry capsid with a diameter ranging from 35 to 40 nm that protects the two segments of the genomic dsRNA [3, 4].

The molecular characterization of the segment two of human and animal strains revealed that PBV are highly variable, and due to these variations, they are divided into two distinct genogroups, genogroup I (GI) and genogroup II (GII) [5–7]. The first detection of PBV was from a child's

stool sample [8]. Subsequently, several studies reported the presence of *Picobirnavirus*-like in different animals: cattle [9–12], monkeys [13], puma, jaguar and cat [14], chickens [15], and other more.

PBV has been reported in different countries [3]. Although epidemiological studies have reported the presence of the virus in fecal samples of adults and children with diarrhea, the etiological relationship of PBV with diarrheal syndrome has not yet been established [16]. Detection of PBV in feces from a wide range of hosts increases public health concern, given its zoonotic threat, and since the natural host or species that serve as the reservoir of the virus remain undefined [3]. In this study, we report the presence, molecular characterization, and genotypic differentiation of PBV in fecal samples from cattle from different Brazilian municipalities.

Fecal samples from 77 diarrheic or asymptomatic animals were collected in the period from May 2013 to October 2015, in five Brazilian states: Sao Paulo (35 samples, 45.5%), Minas Gerais (36 samples, 46.7%), Mato Grosso do Sul (three samples, 3.9%), Goiás (two samples, 2.6%), and Rio Grande do Sul (one sample, 1.3%). Of the 77 analyzed samples, 53 (68.9%) were from calves under 6 months old. Sixty-three animals (81.8%) were dairy cattle, and the majority (56 animals, 72.7%) were female. Fourteen animals (18.2%) were raised in extensive farming conditions and 63 (81.8%) in semi-intensive or intensive farming.

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✉ Marcelo Candido
marcelo.c@usp.br

- ¹ Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo (FZEA/USP), Avenue Duque de Caxias Norte, 225, Jardim Elite, Pirassununga, São Paulo 13635-900, Brazil
- ² Department of Preventive Veterinary Medicine and Animal Reproduction, São Paulo State University (UNESP), Access Route Prof. Paulo Donato Castellani, rural, Jaboticabal, São Paulo 14884-900, Brazil
- ³ Department of Clinical Analysis, Alfenas Federal University (UNIFAL), Street Gabriel Monteiro da Silva, 700, Center, Alfenas, Minas Gerais 37130-000, Brazil

Sample harvesting was performed according to Hoet et al. [17]. RNA was extracted using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription was performed using the ImProm-II™ Reverse Transcription System (Promega, USA) and random primers (Invitrogen, USA), in accordance with the manufacturer's instructions, having as a negative control, nuclease-free water. The synthesized complementary deoxyribonucleic acid (cDNA) was directly used in the PCR reactions.

Highly specific primers were selected to amplify genomic fragments of the RNA-dependent RNA polymerase region of the PBV bovine genotypes (GI and GII) [18]. Partial amplification of bovine β -actin gene was used as an internal control [19].

GoTaq™ Colorless Master Mix (Promega, USA) was used for PCR amplification, following the manufacturer's recommendations. Nuclease-free water was used as a negative control. The amplified products (201 bp) were extracted from an 1.5% agarose gel and directly sequenced on both strands with the same primers used in the PCR, in an automated ABI 3730 DNA Analyzer (Applied Biosystems, USA).

The sequence similarity analyses between the obtained PBV sequences and reference sequences from GenBank were performed using the BLAST software, version 2.0 [20]. Nucleotide sequences were edited and assembled into consensus contigs using BioEdit v. 7.0.9 [21]. Phylogenetic reconstruction was generated by the neighbor-joining method, Kimura 2-parameter model, with bootstrap nodal support for 1000 pseudoreplicates in MEGA software, version 5.0 [22]. MatGAT software, version 2.0 [23], was used for nucleotide and amino acid sequence identity calculations.

From 77 bovine fecal samples tested, 18 (23.4%) were positive for PBV belonging to GI through RT-PCR amplification of the expected size fragment (201-bp). No amplicons were obtained using the GII-specific primers.

From the positive samples, 11 originated from calves ≤ 6 months old (61.1%) and seven were from young animals between 6 months and 2 years old (38.9%). Regarding gender, the majority were from females (77.8%). Thirteen animals (72.2%) did not show diarrhea, while five (27.8%) presented it. All but one of the samples were from dairy cattle (5.5%).

Positive samples were obtained from herds located in the following Brazilian states: 13 samples (72.3%) from São Paulo, four samples (22.2%) from Minas Gerais, and one sample (5.5%) from Goiás. Seven samples were sequenced and identified as PBV-related sequences. Sequences were deposited in GenBank under accession numbers KX964659 to KX964665. Five of these sequences were from samples from three different cities of the São Paulo state (Lins, Pedregulho and Jaboticabal), one from Minas Gerais state (Carmo do Cajuru), and one

from Goiás state (Serranópolis). The average distance between these cities ranges from 141 to 783 km [24].

Figure 1 shows the phylogram obtained by phylogenetic reconstruction. Samples of the different municipalities presented a heterogeneous clustering profile in the phylogenetic tree, grouping with human, ovine, bovine, horse, dromedary, and pig samples.

The identity percentage of samples from different municipalities sequenced in this study shared 31.7–95.5% nt (30.3–94.3% aa). When compared with PBV reference samples retrieved from GenBank, they shared 32.2–76.7% nt (27.3–76.5% aa) identity (Table 1).

Nucleotide differences in aligned 90-bp fragments among sequences of the different Brazilian municipalities are observed (Fig. 2).

Our results demonstrated the circulation of bovine PBV genogroup I in three Brazilian states (São Paulo, Minas Gerais, and Goiás). In the states of Minas Gerais and Goiás, this work shows the first detection of PBV in bovine herds.

The presence of PBV in cattle with clinical signs of diarrhea accounted for 27.8% of the total specimens analyzed, while 72.2% showed no such signs; in terms of age, 61.1% of the positive animals in the RT-PCR for PBV were calves, reinforcing the results previously reported [11, 12].

The nucleotide identity of sequenced samples in this study had an average of 52.7% when compared to each other (Table 1), and the samples that showed greater identity were KX964662-Bovine/BRA/Lins/283 with KX964660-Bovine/BRA/Serranópolis/285-08 (95.5%); compared to the PBV reference samples, those that showed greater identity were KX964662-Bovine/BRA/Lins/283 with JX411964-Bovine/India/HP/2012 (76.7%) and KX964660-Bovine/BRA/Serranópolis/285-08 with JX179289-Human/PB/85 (75.2%). These results corroborate the results of Malik et al. [6], which identified a bovine PBV with sequence identity of 78.7% with human PBV (3-HUN-01/AJ504796).

On the other hand, when samples KX964665-Bovine/BRA/Jaboticabal/77-08 and JX411964-Bovine/India/HP/2012 were compared, the identity obtained was 32.2%, showing a high genetic divergence of the prototype, as described previously [10]. The nucleotide alignment of a 90-bp fragment of the samples detected in this study from the different municipalities supports and adds to previous findings linking high heterogeneity between PBV samples (Fig. 2) [3, 4, 6, 10].

Analyzing the obtained phylogram (Fig. 1), there is a distinct clustering profile, as reported in previous studies, particularly between human and porcine samples [4], indicating a dynamic interspecies transmission (cross-species transmission). The data of this study highlight the genetic relationship profile of these viruses, adding new information related to the heterogeneous molecular profile among

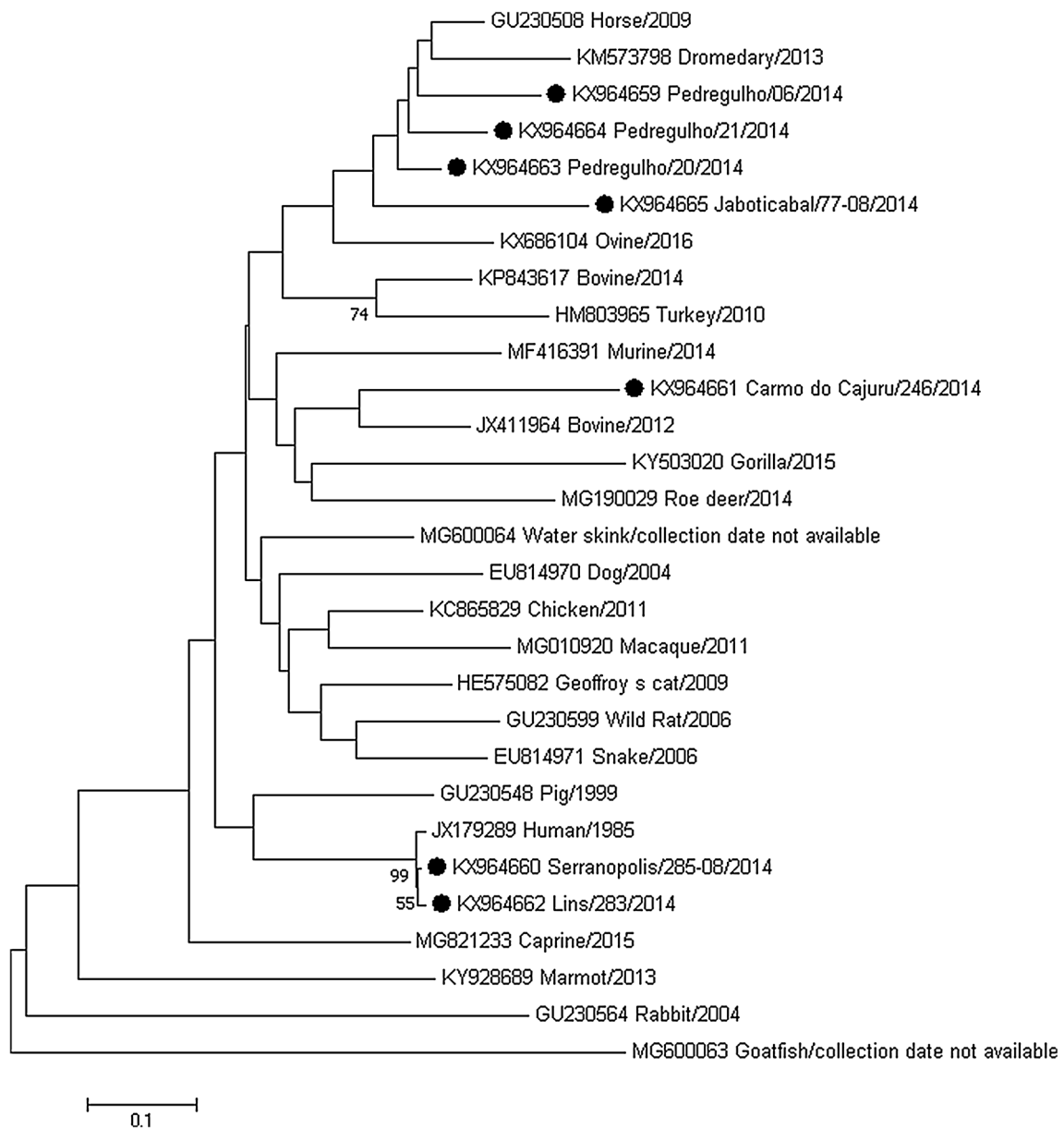


Fig. 1 Phylogram representing phylogenetic reconstruction using a 201-bp sequence of PBV RdRp gene, genogroup I. Bootstrap values higher than 50% for 1000 pseudoreplicates are shown at the nodes. Brazilian sequences obtained in the present study are labeled with a

filled circle. GenBank accession numbers and sample collection date are shown in the tree. The scale bar represents the phylogenetic distance between sequences

PBV strains infecting hosts of the same species, corroborating previous studies [3].

Additionally, only KX964661-Bovine/BRA/Carmo do Cajuru/246 sample demonstrated expected profile clustering, being grouped with another bovine *picobirnavirus* (GenBank: JX411964), originally from India, in a separate clade. In this context, the molecular characterization of partial RdRp gene sequences from bovine PBV revealed a high degree of genetic diversity within genogroup I,

circulating in the south central region of the Brazil and unreported until now.

This genetic heterogeneity can potentially explain the absence of positive samples from genogroup II, investigated in this study. It is plausible that the primers used for detecting genogroup II strains are unsuitable due to the genetic variation of this genogroup although Malik et al. [6] detected the first bovine PBV belonging to GII, using the same primer sequence. Therefore, we should not exclude the possibility

Table 1 Identity of nucleotide and amino acid partial sequences (%) of bovine PBV RdRp gene, genogroup I

Sample	Nucleotide sequence identity (%)					Amino acid sequence identity (%)				
	20 Pedre	77-08 Jaboti	283 Lins	246 Carmo	285-08 Serra	20 Pedre	77-08 Jaboti	283 Lins	246 Carmo	285-08 Serra
GU230564 Rabbit	43.1	43.1	42.4	43.1	41.9	37.3	37.3	37.9	39.4	37.9
KM573798 Dromedary	62.4	52.5	48.0	46.5	49.0	64.2	53.7	52.2	50.7	52.2
HE575082 Geoffroy’s cat	56.4	50.0	55.9	48.5	58.9	47.5	45.0	53.2	48.4	57.4
EU814970 Dog	52.0	49.0	54.5	47.0	53.5	50.7	49.3	57.6	54.5	57.6
EU814971 Snake	45.5	37.6	68.8	64.9	68.8	41.8	39.4	61.4	56.1	61.4
GU230508 Horse	68.3	56.4	51.5	53.0	51.0	68.7	56.7	53.7	50.7	53.7
GU230599 Wild Rat	55.9	44.6	54.0	50.0	53.0	56.7	50.7	55.2	49.3	55.2
HM803965 Turkey	56.4	49.0	47.5	54.2	50.0	57.6	48.5	50.7	56.7	51.5
KC865829 Chicken	59.4	49.5	53.5	49.8	56.4	56.1	51.5	59.7	52.2	60.6
GU230548 Pig	56.7	49.5	56.4	49.5	58.6	53.7	52.2	59.7	52.2	59.7
JX179289 Human	56.9	50.5	72.8	44.6	75.2	61.5	55.4	73.1	44.8	75.8
JX411964 Bovine	41.1	32.2	76.7	75.2	73.8	37.3	27.3	76.5	71.7	73.6
KP843617 Bovine	61.9	53.5	49.0	54.0	53.2	63.6	53.0	53.7	55.2	54.5
KX964663 Bovine—20/Pedre	100.0	78.7	36.1	38.6	40.4	100.0	70.4	38.8	38.8	39.4
KX964665 Bovine—77-08/Jaboti		100.0	31.7	33.2	36.0		100.0	34.8	30.3	35.4
KX964662 Bovine—283/Lins			100.0	68.3	95.5			100.0	60.4	94.3
KX964661 Bovine—246/Carmo				100.0	68.8				100.0	58.5
KX964660 Bovine—285-08/Serra					100.0					100.0

Samples that were detected and discussed in this study are in bold

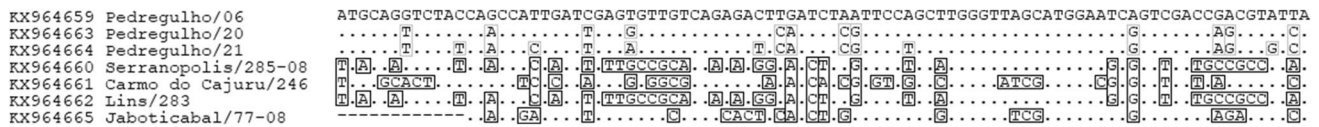


Fig. 2 Alignment of 90-bp nucleotide sequences of bovine PBV RdRp gene, genogroup I, from samples of different cities of the Brazil, using the sample KX964659 of the Pedregulho Brazilian municipi-

ality as reference. Nucleotide substitutions between samples from the same municipality are circled in gray, between different municipalities are circled in black

that, at least in the areas investigated in this study, strains of PBV cattle belonging to the GII are not circulating. Further studies are required to clarify this.

In conclusion, this is the first report of the detection of bovine PBV from genogroup I in the states of Minas Gerais and Goiás in Brazil, and describes the first report of genotypic differentiation of this genogroup infecting cattle in the country. Our results contribute to a better understanding of PBV epidemiology and the subclinical manifestations or unapparent gastroenteritis of PBV infections in Brazilian cattle.

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Author contributions JON, MC, SRAQ, MGB, MCL, and RLMS conceived and designed the experiments. JON and MC performed the

experiments. JON, MC, SRAQ, and RLMS analyzed the data. JON, MC, and RLMS wrote the manuscript. AMF participated in revising the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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