

Picobirnavirus Detection in Animals From Amazon Biome

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Abstract

This study aimed to detect picobirnavirus (PBV) in the fecal samples of wild and domestic animals from 2014 to 2016 in the Amazon biome. For detection, Polyacrylamide Gel Electrophoresis (PAGE) and RdRp gene based Polymerase Chain Reaction (PCR) preceded by Reverse Transcription (RT) were used. Subsequently, statistical analyses were performed using the Chi-square G test and nucleotide analyses for the construction of the phylogenetic tree. A total of 258 fecal samples from different animals, including birds (n=41) and mammals (n=217) were used. The PAGE test showed negativity for PBV genome in all samples while in the RdRp gene based RT-PCR test 32 samples showed amplification, corresponding to 12.4% (32/258) positivity. Among the positive samples, mammals, including pigs and cats, both with 28.12% (9/32), registered the highest frequencies, and in birds, the positivity was 4.9% (2/41). In phylogenetic analysis, eight sequences from positive samples grouped in the genogroup 1 of PBV (GI). The statistical test was significant for PBV in relation to the groups of animals studied (birds, mammals, and rodents), and in relation to the cities of origin of the samples, with a value of $p < 0.05$. Nucleotide analysis demonstrated similarity among the feline group, but the absence of a defined structure between the clades. PBVs are highly widespread viruses that can affect the most diverse types of hosts in the Amazon biome.

Introduction

Picobirnaviruses (PBVs) are small viruses approximately 40 nm of diameter, non-enveloped, icosahedral symmetry and have two genomic segments, the larger segment with 2.2-2.7 kbp size and the smaller segment with 1.2-1.9 kbp [1] [2]. The larger segment (segment I) plays a role in the coding of structural proteins, and the smaller (segment II) codes for RNA-dependent viral RNA polymerase (RdRp) and gives PBV its classification [2–5].

The current PBVs classification in Genogroup I and II was based on strains 1-CHN-97 (GI) and 4-GA-91 (GII) from humans, infecting vertebrate animals [6, 7]. The Genogroup III, recently proposed, and unlike the others, affects invertebrate animals [5].

PBVs are associated with gastroenteritis in both humans and animals, and may present as an apparent symptom of diarrhea that may be directly linked to the virus as a single pathogen or other enteric pathogens, attributing to it the characteristic of a secondary pathogen in mixed infections. These viruses further be classified as opportunistic pathogens when detected in immunocompromised patients or being treated with their weakened immune system and who have diarrheal conditions [3, 4].

PBVs are considered emerging, opportunistic and suggestive of zoonotic potential [8]. The first report on PBV appeared from an outbreak of gastroenteritis in Brazil, where these were detected in human stools specimens and in a species of rodent [9]. Since then, PBVs have been described as pathogens that infect a variety of animal species, such as mammals, reptiles, birds, humans, and also in sewage, showing a genetic diversity among circulating strains [11] [11] [12] [13] [14] [15]. Recent studies suggest that PBVs

are bacteriophages of prokaryotes housed in the digestive tract of possible viral hosts of this pathogen [16] but need further investigations before reaching to this conclusion.

Currently, several pathogens, including enteric viruses that affect humans and animals, have been registered as responsible for 60% of zoonotic infections in humans. Zoonoses are responsible for 75% of emerging and reemerging diseases that affect humans and cause at least 20% of losses in animal production [17]. Thus, the investigation of PBV in samples from different hosts has its importance in relation to public health related to the transmission of these interspecies viruses and mainly due to their potential for zoonotic transmission [8]. Therefore, it is imperative to monitor animals (wild as well as domestics) for the presence of different human pathogens and to identify a potential reservoir of infectious diseases since the contact and interaction between humans and animals may highlight possible events of zoonotic transmission and trigger the emergence of emerging and reemerging pathogens.

This study aimed to detect the presence of PBV in fecal specimens of wild and domestic animals collected in deforestation areas of Amazon biome.

Materials And Methods

Ethical aspects

The National Council for Animal Control and Experimentation (CONCEA), System of Authorization and Information in Biodiversity—SISBIO/ICMBIO/Ministry of the Environment approved this research under protocol No. 37174–1, and the Ethics Commission at the Evandro Chagas Institute (CEUA) under protocol number 35/2016.

Study area

The study area and the collection of biological samples comprised three cities: Santa Bárbara, Peixe-Boi and Viseu, located in the North of state of Pará. In terms of distance, these places are covered by 278.15 km², 450.29 km², and 4.934.54 km², respectively. Thus, the cities are part of a portion of the Brazilian Amazon, where the strong anthropic pressure increases the interaction between humans and animals, and the contact and handling of animals is becoming increasingly greater due to agribusiness, which is the main source of subsistence for families living there.

These areas have already been investigated for other enteric viruses and other pathogens that affect animals and are detailed in a previous study [18].

Sample collection

Sample collection was carried out from October 2014 to April 2016, comprising two annual visits to each city in the study area. The capture of wild animals (bats, birds, and rodents) was done from areas of

forest fragment and urban areas adjacent to fragment areas, where supposedly there would be a greater concentration of these species.

Different traps were used to capture wild animals. For birds and bats, fog nets were set up, at different times, from 4:00 am - 9:00 am, and 6:00 pm - 7:00 am, respectively, and inspected periodically. The other wild animals were captured using Tomahawk (45x16x16cm), Sherman (30x9x8cm), and Pitfall traps. The baits used to attract the animals to the traps were peanut butter, sardines, bacon, and fruit (pineapple, banana, and apple).

The biological material was collected by stimulation of the rectum using urethral probe (Nº 10) for the non-fly wild mammals and rectal swab "Zaragatoa" for wild birds and bats (small animals) through stimulation of the rectal ampulla. In relation to companion animals (dogs, cats, and poultry) and other mammals (pigs, horses, and cattle), sample collection and necessary information about the animals were carried out with authorization and contribution from their respective owners.

All samples were stored in sterile plastic vials and properly packed (-30°C) to maintain the quality of the material before processing in the virology laboratory.

Samples

A total of 258 fecal samples were used in this study. The sample groups were composed of mammals (flying and non-flying) and birds (domestic and wild). The non-flying mammals include bovines (n = 23), canines (n = 34), equines (n = 41), felines (n = 33), rodents (n = 33), and swine (n = 23), and the flying mammals, are the chiropters (n = 30). The birds group includes domestic birds (n = 3) and wild birds (n = 38).

Sample preparation

Fecal suspensions were prepared at 20% (w/v) in Tris/Ca⁺⁺ and clarified by centrifugation at 5000 rpm for 10 minutes at 4°C. Then, supernatants were collected and stored at -20°C for later use in the extraction, detection and characterization of viral genetic material.

The viral genome was extracted from the fecal suspensions according to the method described by Boom et al. [19]. During the extraction process, all contamination control measures were performed, including the use of positive and negative control (ultrapure water). The PAGE test for analysis of the electrophoretic profile was performed on all samples, the protocol followed the method described by Pereira et al. [20].

RT-PCR was used to amplify the genes using the PicoB25 and PicoB43 primers for Genogroup I, PicoB23 and PicoB24 for Genogroup II, which amplify 201 bp and 369 bp products, respectively [6]. Subsequently, the positive samples were subjected to amplification with the primer pairs FP-AAGGTTCGGKCCRATGT and RP-TTATCCCYTTTCATGCA [21] producing ~1200 bp amplicon for the first stage (RT-PCT) and PBV-F580 (5'-TGGGWTGGCGWGGACARGARGG-3') and PBV-R580 (5'-YSCAYTACATCCTCCAC-3') with ~580 bp

amplicon, for the second stage (Nested-PCR) (designed by Dr Yashpal S. Malik, Indian Veterinary Research Institute, India). After purifying the samples, the product was subjected to sequencing reaction following the protocol of the Big Dye Terminator® v.3.1 kit (Applied Biosystems).

Sequence analysis

The sequences obtained were aligned and edited by the Geneious program (version 8.1.9) and compared with sequences available in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) using the BLAST program (version BLAST + 2.8.0-alpha released) [22].

Phylogenetic trees were built using the maximum likelihood method (Maximum Likelihood-ML) by the FastTree program [23]. To determine the best nucleotide replacement model, the GTR (General Time Reversible) program was used. Bootstrap analysis (1,000 replicates) was used to give reliability to phylogenetic groups [24]. The tree was visualized using the Evolview software [25].

The construction of the phylogenetic tree included PBV sequences available from GenBank, belonging to different animal hosts and samples from previous studies from the same collection region, and belonging to PBV-GI.

Statistical analysis

Statistical analysis of the data was performed using the SPSS (Statistical Package for the Social Sciences) software [26]. The G and Chi-square tests were used to identify the frequency of pathogens in the cities, the period of collection, and the categorical variables: group of animals, gender (male and female) and city from the samples. The significance value in the results was $\alpha = 0.05$ (error probability of 5%) [27].

Results

From September 2014 to March 2016, a total of 258 biological samples were collected, from Santa Bárbara, Peixe-Boi and Viseu cities, distributed among the classes of mammals ($n = 217$) and birds ($n = 41$). The samples were analyzed using PAGE and RT-PCR technique. For PAGE test the result shows negativity for PBV and by RT-PCR the samples were positive only for genogroup I (G-I).

Of the total of samples analyzed, 12.4% (32/258) amplified for segment 2, specifically for G-I. Of the total of samples analyzed, 12.4% (32/258) amplified for segment 2, specifically for G-I. The frequency of PBV in pigs represented 28.13% (9/32) of positivity. Similarly, the occurrence of this pathogen in cats showed the same value, 28.13% (9/32). The lowest frequency was seen in rodents, representing 3.13% (1/32) of the cases as shown in *Figure 1*. There was no PBV positivity in the samples from bats.

In the statistical analysis, G and Chi-square tests were performed to assess the significance of positivity between animals (birds, cattle, canines, horses, felines, bats, rodents and pigs), comparing gender (male and female) and cities of sample origin (Santa Bárbara, Peixe-Boi and Viseu), as shown in *Table 1*. The

data demonstrated that for the variables group and cities, $p < 0.05$ and $p = 0.003$, respectively, indicating positivity for PBV among animal groups, when compared to the origin of the specimens was significant ($\alpha = 0.05$). However, for the gender the values were approximate, therefore, the frequency of PBV for this variable was not significant ($\alpha = 0.918$).

Referring to phylogenetic analysis, among the 32 samples amplified by RT-PCR for PBV, seven samples (21.87%) showed satisfactory results for genomic sequencing. The genotypic characterization identified all the sequences as belonging to the G-I of the PBVs, as illustrated in *Figure 2*.

The presence of PBV was identified in three classes of animals: feline ($n=5$), swine ($n=1$), and canine ($n=1$). Molecular analysis showed a nucleotide identity of 70.0% to 100% identity among the samples in the study. The sequences of felines (*Felis catus*) of codes PB088 and PB097 remained close together in the same clade with a canine (*Canis lupus familiaris*), PB103, and bootstrap value varied from 70% to 89%. It was also observed that the sequences PB106, from a swine (*Sus domesticus*), and PB089, PB091, and PB100, from felines (*Felis catus*), also remained close, representing bootstrap value from 91% to 100%.

Discussion

Currently, several pathogens, including enteric viruses, which affect humans and the most varied species of animals have been registered as responsible for 60% of zoonotic infections in humans. Zoonoses are responsible for 75% of the emerging and reemerging diseases that affect humans and cause at least 20% of losses in animal production [17], where the wild environment has also been responsible for most zoonotic infections [28].

In the present study, PAGE was one of the techniques used to detect PBV, with the aim of visualizing the profile of electrophoretic migration of genomic segments. However, this test is characterized by having a high specificity and low sensitivity. Then, suggesting that the negative results, obtained from fecal samples from animals, have a correlation with low viral excretion and the low sensitive by PAGE technique. However, such samples may show positivity when analyzed by RT-PCR, since this technique has greater sensitivity and specificity [3], in addition to the detection of viral nucleic acid may occur even though fecal samples do not show high viral titers [29].

Despite the low sensitivity of PAGE, several epidemiological studies use this technique in the screening stage of diarrheal infections due the electrophoretic profile of these viruses, which helps to outline the scenario of infections caused by these agents and its reach zone in the world [30] [31] [32] [4].

Clinical aspect of animals and fecal specimens were not considered, since detection may happen even though hosts, in many cases, remain asymptomatic [4, 8]. However, this fact could have influenced on PAGE results, suggesting the quantity of viral particles eliminated in fecal excrement was not sufficient for detection by the test.

Previous studies carried out with fecal samples of broilers (*Gallus gallus*), from northeastern region of the state of Pará, reported positivity for PBV, by PAGE from 15.3% [33] to 30% [34]. The same protocols were used to perform the PAGE technique, although divergent results when compared to this study, believing that the reduced viral load excreted was the interferer to positivity.

PBV are reported in some different hosts, 3.63% in cats from Portugal [35], 14.28% in horses [8], 11.15% in pigs [36], and 0.73% in cattle in India [37]. In Brazil, the positivity in swine is concentrated from 12.45% [38] to 43.24% [39], 8.30% in cattle [40], 0.86 % [39] to 1.84% [41] in canines, and 3.4% [32] to 49.4% [33] in broilers. There have no positivity in rodents of the *didelphideos* family and in wild birds. Therefore, the present study corroborates the previously reported data, considering that the population with the highest frequency of PBVs (28.13%) was in swine.

The cities of samples came from in this study showed high rates of deforestation during 2014 to 2016, due the use of the land for agricultural and livestock production, where families residing in these areas use these activities as a source of subsistence [42]. It becomes evident that the increase in the area of fragmentation, the alteration of the habitat and ecological niches of animals caused by anthropic pressure, and consequently the closer contact between humans and wild animals, contribute to the occurrence of infectious diseases, spread of emerging pathogens and new hosts [43–45]. This fact may have contributed to the fact that the percentage of positivity among the studied species occurred due to the wide interaction between the species, facilitated by the change in the environment where they live.

The dissemination of PBV among the most varied animal hosts in the present study could be confirmed by phylogenetic analysis. The virus presence in canines, felines, cattle, poultry, swine, and in humans, shows the lack of host specificity, corroborating what has already been demonstrated in studies with swine [46, 47].

Phylogenetic analysis reported the virus circulated in several species of animals, regardless of time and geographic area of detection, as previously reported, suggesting a potential of spreading that pathogen presents [10].

Rodents, birds and bats are among the wild animals in this study. Considering a fecal-oral infection route, and that the diet of rodents (*Marmosa sp*, family *Didelphidae*) is composed of small vertebrates (baby birds, chickens), fruits, decomposing organic matter (carrion) even invertebrates [48], suggesting that the feeding habits of these animals may have contributed to their infection by ingesting nutrients exposed in the contaminated environment.

Chiropterans, except *Desmodus rotundus* species (blood-sucking habit), the other species: *Carollia perspicillata* and *Phyllostomus hastatus* are frugivorous [49]. Characteristics such as diet, ability to fly, seasonal migration, behavioral patterns, affinity to live in colonies, and other, including this group as a great zoonotic potential host [50]. However, there was no positivity for this order (*Chiroptera*) in this study

Birds, and chiropterans, are of great importance for public health and are considered as potential reservoirs of pathogens that cause zoonotic diseases and due to their ability to fly, they can act in the dispersion of viruses and thus establish new foci of emerging diseases or reemerging along their paths [28, 51].

Referring to pets (canines and felines) and livestock animals (cattle, horses, pigs and birds) due they share the same space and environment, circulate in forest and home area, and interact with each other and with humans, the chances of viral transmission are high, especially in cases of animals infected in a phase of viral excretion. Therefore, the strong anthropic pressure in Amazon has been an important factor of spread zoonotic diseases, infecting new hosts, and expanding pathogens with wide-reaching potential through the interaction between species, including enteric pathogens such as PBV and RV [18].

In conclusion, in this study the incidence of PBV GG-I occurred in all groups of animals, except in bats. This study is a pioneer in characterizing PBV, G-I, in felines (*Felis catus*) in Brazil. These data suggest the possible interspecies transmission of PBV among the animals included in this study, considering that some animals were grouped with different taxon and some samples formed clusters with strains similar to strains isolated in porcine detected mainly in China.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The National Council for Animal Control and Experimentation (CONCEA), System of Authorization and Information in Biodiversity—SISBIO/ICMBIO/Ministry of the Environment approved this research under protocol No. 37174–1, and the Ethics Commission at the Evandro Chagas Institute (CEUA) under protocol number 35/2016.

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Tables

Table 1 - Data referring to statistical analysis of the frequency of PBV in fecal samples of animals, according to the group, gender and origin city, in the period from October-2014 to April-2016.

CATEGORIES	PICOBIRNAVIRUS				Total		p
	Positive		Negative				
	n	%	n	%	n	%	
GROUP							0.000 [#]
Avian	2	4.9	39	95.1	41	100.0	
Bovine	3	13.0	20	87.0	23	100.0	
Canine	5	14.7	29	85.3	34	100.0	
Equine	3	7.3	38	92.7	41	100.0	
Feline*	9	27.3	24	72.7	33	100.0	
Bats	0	0.0	30	100.0	30	100.0	
Rodent*	1	3.0	32	97.0	33	100.0	
Swine*	9	39.1	14	60.9	23	100.0	
GENDER							0.918 [¥]
Female	14	14.0	86	86.0	100	100.0	
Male	15	13,5	96	86,5	111	100,0	
CITY							0,003 [¥]
Peixe-Boi*	8	27,6	21	72,4	29	100,0	
Santa Bárbara	10	19,6	41	80,4	51	100,0	
Viseu*	14	7,9	164	92,1	178	100,0	
Total	32	12,4	226	87,6	258	100,0	

p: Probability of the statistic test. [#]G Test. [¥]Chi-square Test. *Categories that will contribute for statistical significance.

Figures

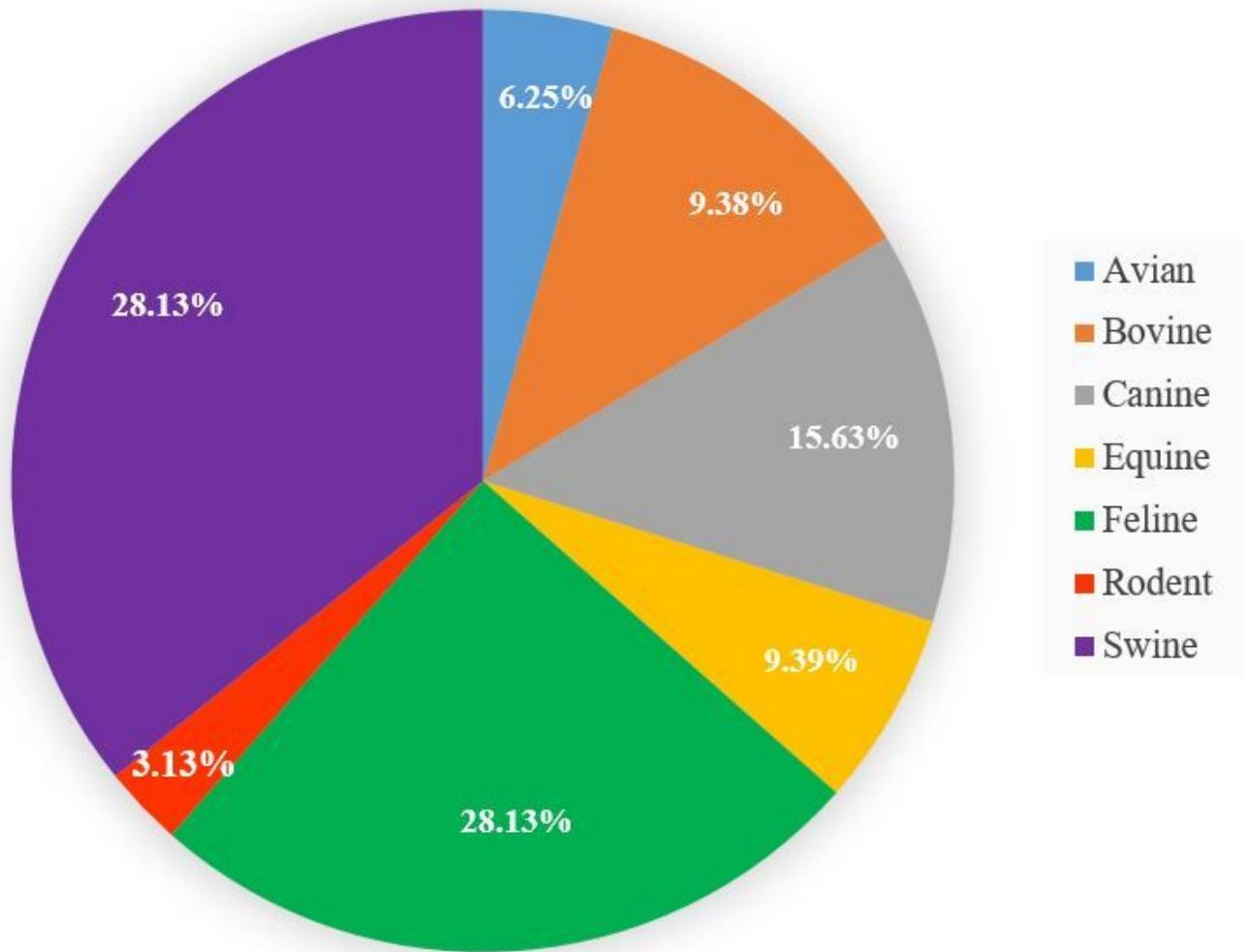


Figure 1

Frequency of positivity of PBV in 32 fecal animal samples from Santa Bárbara, Peixe-Boi, and Viseu in the period from October-2014 to April-2016.

bootstrap

● ≤69

● 70~89

● 90~100

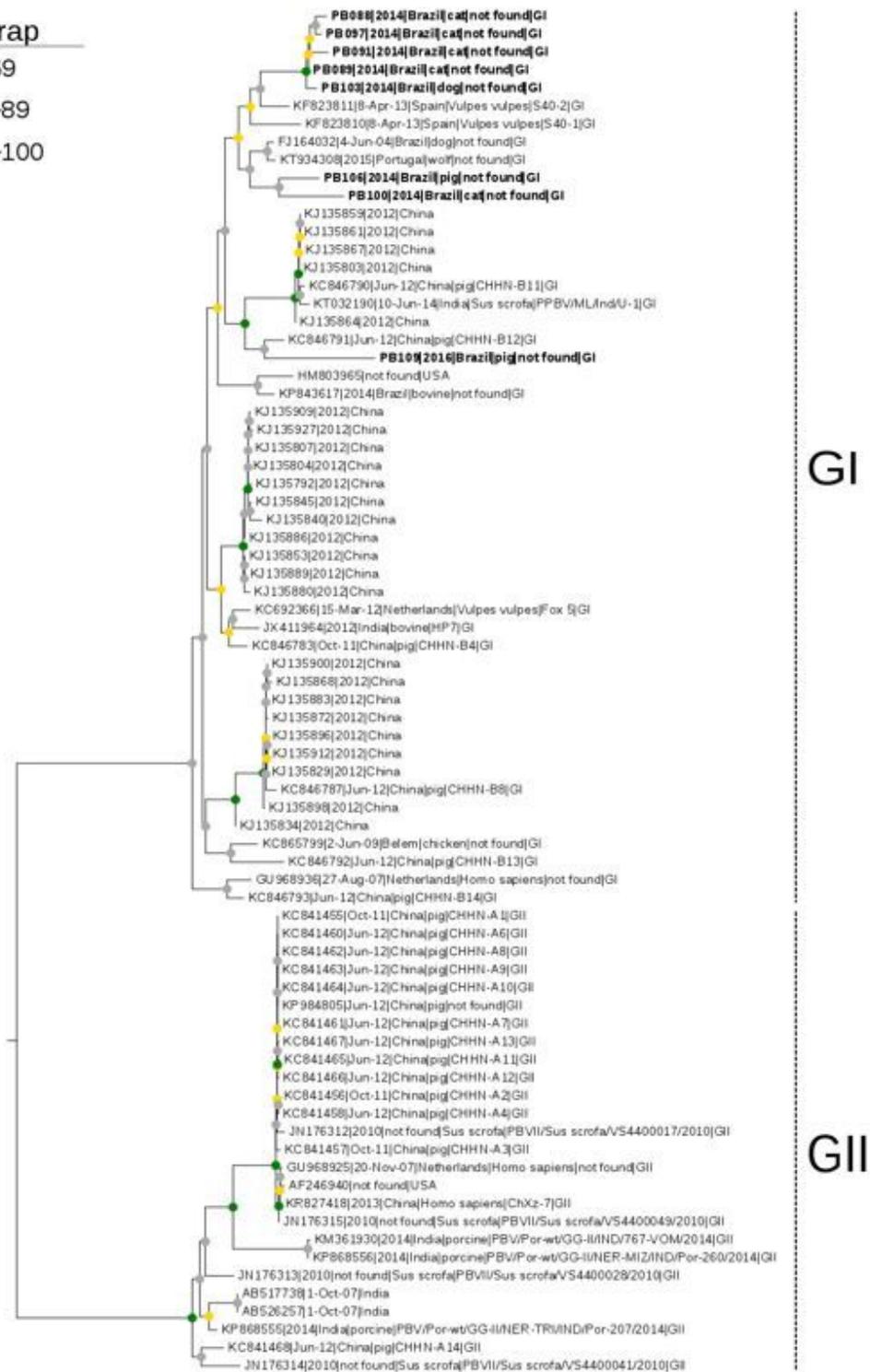


Figure 2

Phylogenetic analysis based on the sequence alignment of segment 2 of the gene that encode for the RdRp protein of PBV. The phylogenetic tree was constructed using the maximum likelihood method (Maximum Likelihood-ML) by the FastTree program. The current study sequences are indicated in bold. The bootstrap values are indicated next to the nodes in three different color circles; GI – PBV Genogroup I, and GII – PBV Genogroup II.