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Maintenance of picobirnavirus (PBV) infection in an adult orangutan (*Pongo pygmaeus*) and genetic diversity of excreted viral strains during a three-year period

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ABSTRACT

The present work provide data about the maintenance of picobirnavirus (PBV) infection during adulthood in a mammalian host. For this purpose PBV infection was studied in an adult orangutan (*Pongo pygmaeus*) by PAGE/SS, RT-PCR and nucleotide sequencing. PBV infection in the animal was asymptomatic and was characterized by interspaced silent and high/ low active viral excretion periods. The PBV strains excreted by the studied individual were identified as genogroup I and revealed a nucleotide identity among them of 64–81%.

The results obtained allowed to arrive to a deeper understanding of the natural history of PBV infection, which seems to be characterized by new-born, juvenile and adult asymptomatic hosts which persistently excrete closely related strains in their feces. Consequently, picobirnaviruses could be considered frequent inhabitants of the gastrointestinal tract, leaving the question open about the molecular mechanisms governing persistent and asymptomatic coexistence within the host and the potential host suitability to maintain this relationship.

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1. Introduction

Picobirnaviruses (PBVs) are a new group of viruses that belong to the genus Picobirnavirus, family *Picobirnaviridae* (ICTV, 2008). Virions are isometric, non-enveloped, 35 nm in diameter and they present a bi-segmented double-stranded RNA (dsRNA) genome (Duquerroy et al., 2009; Nates et al., 2011; Pereira et al., 1988b). The large genome segment (segment 1) is 2.3–2.6 kb in size and encodes the capsid protein and a polypeptide of unknown function. The small genome segment (segment 2) is 1.5–1.9 kb and encodes the viral RNA-dependent RNA polymerase (RdRp) (Wakuda et al., 2005). Based on the sequences of the small segment, PBVs are classified into genogroup I (prototype strain, 1-CHN-97) and II (prototype strain, 4-GA-91) (Bányai et al., 2003; Rosen et al., 2000). Recently a PBV genogroup III was reported (Smits et al., 2014).

After being reported in the year 1988 (Pereira et al., 1988b), PBVs have been detected in stools samples of several species of animals and from different countries, including rabbits (Gallimore et al., 1993; Ludert et al., 1995), dogs (Costa et al., 2004), cattle (Buzinaro et al., 2003; Malik et al., 2014; Vanpodenbosch and Wellemans, 1990), foals (Ganesh et al., 2011b), pigs (Bányai et al., 2008; Carruyo et al., 2008; Ganesh et al., 2012; Martínez et al., 2010), guinea pigs (Pereira et al., 1989), rats (Pereira et al., 1988a), monkeys (Wang et al., 2007), giant anteaters (Haga et al., 1999), orangutans, armadillos (Masachessi et al., 2007), Pantheraleo, Pantheraonca, Puma concolor, *Oncifelis geoffroyi* (Gillman et al., 2013), snakes (Fregolente et al., 2009), chickens (Tamehiro et al., 2003), geese, pheasants, pelicans (Masachessi et al., 2007) and humans (Gallimore et al., 1995; Ganesh et al., 2010, 2011a; Giordano et al., 1998; Grohmann et al., 1993; Pereira et al., 1988b). PBVs have been detected in feces from animals with or without diarrhea (Bhattacharya et al., 2006, 2007; Gatti et al., 1989). Nowadays, they could be considered as

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opportunistic diarrheagenic agents (Bányai et al., 2003; Gallimore et al., 1995; Ludert et al., 1991; Rosen et al., 2000).

The host-PBV interaction as well as the factors that facilitate higher production of viral progeny in mammals and birds (particularly in porcine and rhea animal models) are now better understood (Martínez et al., 2010; Masachessi et al., 2012). The results demonstrated that PBV infection is characterized by asymptomatic and persistently infected carriers which acquire PBV infection very early in their lives and hold the viral infection at least until the beginning of adulthood.

So far, there are no reports about the maintenance of the PBV infection during adulthood in individuals infected by PBV.

The aim of this study is to provide the first data about the progression of PBV infection during adulthood in a mammalian host, an orangutan (Pongo pygmeus), kept in captivity in a zoo of Córdoba city, Argentina, as well as to reveal the intra-host PBV strain genetic diversity excreted by the animal during a three-year period and to study the relationship of these PBV strains with the strains circulating in other hosts.

The data obtained in the present study would reinforce the knowledge about the natural history of PBV circulation in nature.

2. Materials and methods

2.1. Background

In 2003 we identified PBV in the stool samples of an orangutan kept in captivity in a single cage. The orangutan had no contact with other animals and received food and water individually in a zoo of Córdoba city, Argentina (Masachessi et al., 2007). This finding expanded the knowledge of the host range of PBV. There was no reference about the moment in which the orangutan acquired the PBV infection due to the fact that the animal was adult (approximately 28 years old) at the time of sampling. In addition, we had no information about the animal health status during the period of sampling (infections with any other virus/pathogen), but in general, the animal neither showed any symptoms of diarrhea nor other visible illness.

2.2. Stool sample collection

A follow-up study was carried out during 3 years (from June-2005 to June 2008) with the same orangutan. A total of 117 stool samples were collected (2–5 times a month), immediately after defecation.

The specimens collected were classified as diarrheic or normal on the basis of their consistency. Fecal specimens were stored at −20 °C until analysis.

Samples from the follow-up study were analyzed by PAGE/SS ($n = 117$) and a number of them ($n = 80$) were also assayed by RT-PCR using a genogroup-I-specific primer pair (Pico B25/Pico B43) derived from genomic segment 2 of the 1-CHN-97 prototype strain (Rosen et al., 2000).

2.3. Viral RNA extraction

Nucleic acids were extracted directly from 10% stool suspensions in 0.2 M Tris–HCl, pH 7.2, and clarified by centrifugation at 2000×*g* for 10 min. Nucleic acids were extracted as described by Perry et al. (1972). Briefly, approximately 400 µl of the supernatant were mixed with an equal volume of extraction buffer (10 mM EDTA disodium salt, 500 mM LiCl and 1% SDS) and 800 µl phenol chloroform (1:1). After incubation for 10 min at 56 °C, samples were centrifuged at 16,000×*g* for 30 min, and 800 µl of the

Table 1
Follow-up study of PBV excretion in the adult orangutan (*Pongo pygmaeus*) followed for a period of 3 years.

Orangutan																								
Date	Jun-05				Jul-05				Aug-05					Sep-05		Oct-05				Nov-05				
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
PAGE	–	+	–	+	–	–	+	–	–	+	–	–	–	–	–	+	+	–	–	+	–	–	–	–
RT-PCR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+	+
Orangutan																								
Date	Dec-05		Jan-06			Feb-06				Mar-06					Apr-06					May-06				
Sample	25(1)	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42(2)	43	44	45	46	47	48
PAGE	–	+	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	+	–	–	–	+
RT-PCR	+	+	–	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	–	–	+	–
Orangutan																								
Date	Jun-06				Jul-06			Aug-06					Sep-06					Oct-06				Nov-06		
Sample	49(3)	50	51	52	53	54	55	56	57(4)	58(5)	59	60	61	62	63	64	65	66	67	68	69	70	71	72
PAGE	+	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	+	–	–	–	–	+	+	–
RT-PCR	+	–	–	–	–	+	+	–	+	+	–	–	–	–	–	+	+	+	–	–	–	–	+	+
Orangutan																								
Date	Dec-06			Jan-07		Feb-07		Mar-07		Apr-07			May-07		Jun-07		Jul-07		Aug-07		Sep-07			
Sample	73	74(6)	75	76	77	78	79	80	81	82(7)	83	84	85	86	87	88	89	90	91	92	93	94	95	
PAGE	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	
RT-PCR	+	+	–	+	+	–	+	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	
Orangutan																								
Date	Oct-07		Nov-07			Dec-07		Jan-08		Feb-08		Mar-08			Apr-08				May-08		Jun-08			
Sample	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117		
PAGE	–	+	–	–	–	–	+	–	–	+	–	+	–	+	+	–	–	+	+	–	+	+		
RT-PCR	–	–	–	–	–	–	+	–	–	+	–	+	+	+	+	+	+	+	nd	nd	nd	nd		

Note: (a) PAGE: polyacrylamide gel electrophoresis and silver staining. (b) RT-PCR: reverse transcription and polymerase chain reaction. (1);(2);(3), (4); (5); (6); (7) Amplicons sequenced. nd: not done.

aqueous phase were added to 1 ml of ethanol for RNA precipitation (overnight at −20 °C). The pellets obtained after centrifugation (16,000×g, 30 min) were dried under laminar flow for approximately 1 h. After drying, the pellets were resuspended in 20 μl RNase-free water for RT-PCR assays or in 15 μl of sample buffer (62 mM Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 3% SDS, 0.01% bromophenol blue and 30% glycerol), and loaded onto a 10% polyacrylamide gel as described by Laemmli (1970) for PBV detection by the PAGE/SS technique.

2.4. PAGE assay

Polyacrylamide gel electrophoresis (PAGE) was carried out in 10% of polyacrylamide gel, 10 cm × 10 cm × 1 mm thick, in a

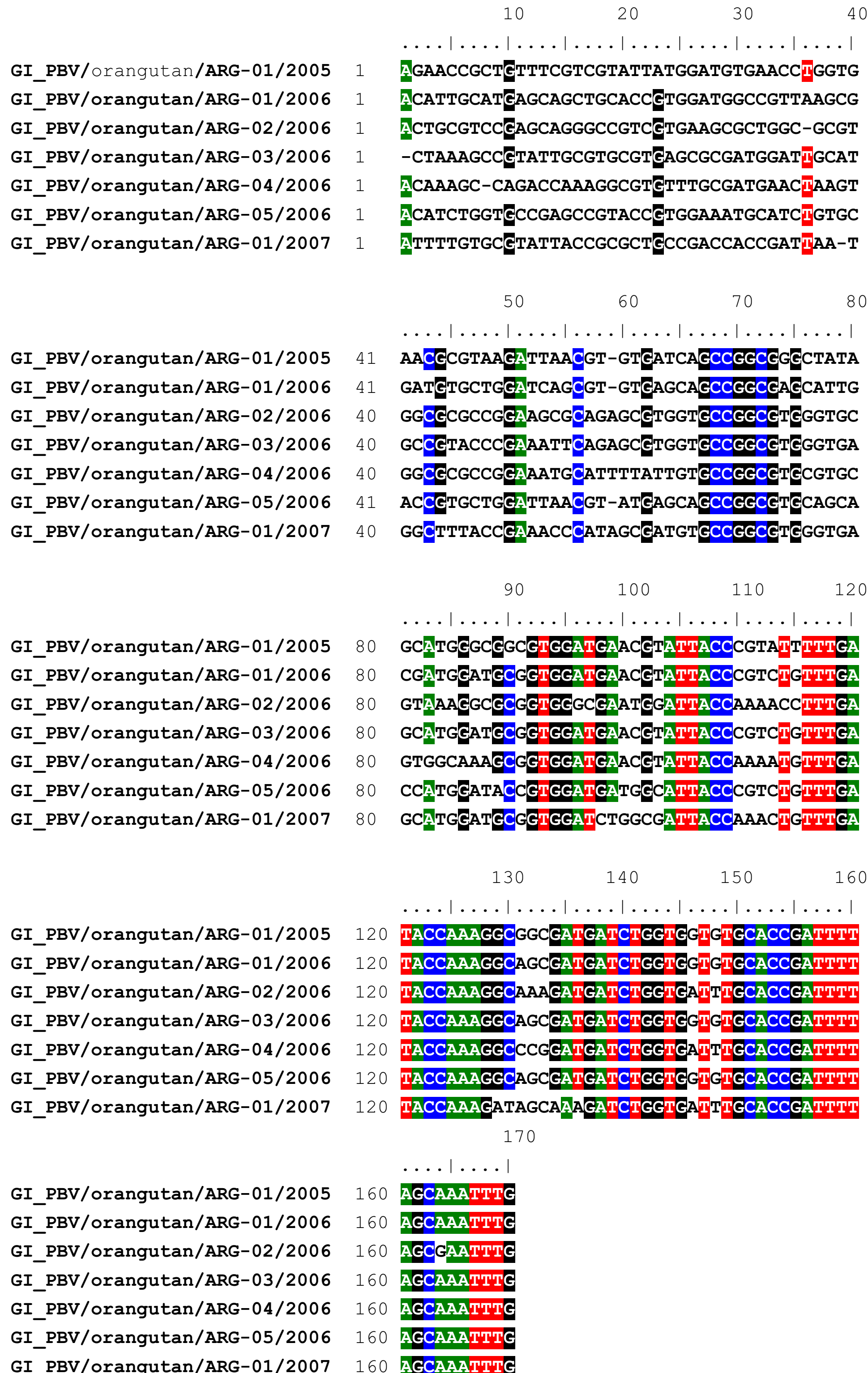

Fig. 1. Alignment of sequences of 170 bp of RT-PCR products from PBV-positive stool samples isolated in Argentina from the orangutan (*Pongo pygmaeus*). The samples were reverse transcribed and PCR amplified using the primers Pico B25 and Pico B43 derived from the 1-CHN-97 strain of PBV.

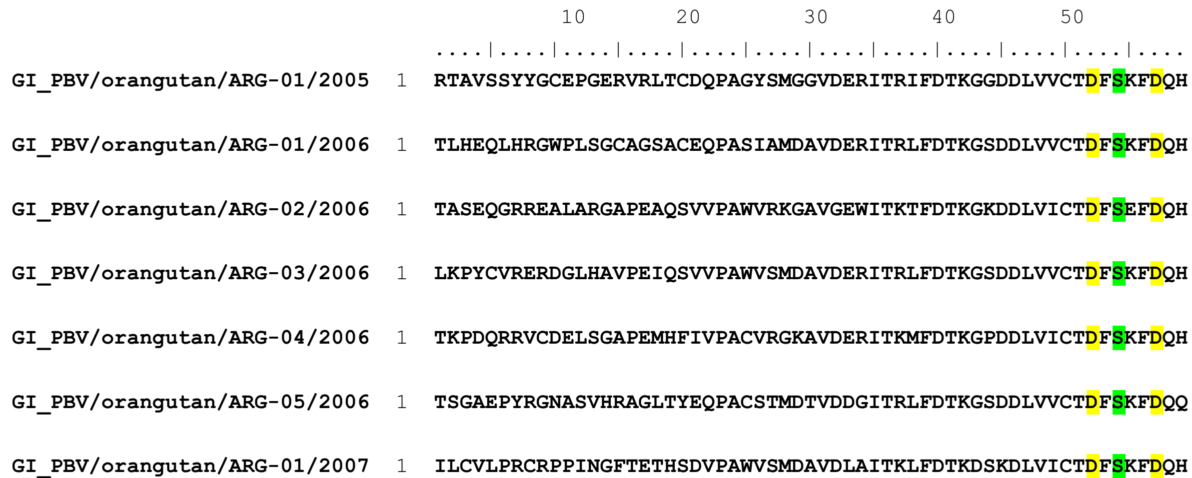

Fig. 2. Alignment of deduced amino acid sequences of orangutan (*Pongo pygmaeus*) PBV strains. Amino acid sequences D-S-D: conserved residues typical of motif 1 of RNA-dependent RNA polymerases of RNA viruses.

vertical device (Bio-Rad, Hercules, CA) at 60 mA in constant current for 3 h. PBV genomic segments were visualized by silver staining (SS) (Herring et al., 1982).

2.5. Amplification by RT-PCR

The RNA was denatured by heating at 97 °C for 5 min. Complementary DNA (cDNA) was obtained by reverse transcription according to Iturriza-Gomara et al. (1999) with minor modifications (Masachessi et al., 2012). PCR was carried out with primer Pico-B25/Pico-B43, according to Rosen et al. (2000). To avoid and control cross-contamination between samples, aerosol resistant tips were used. Furthermore, several negative controls and positive PBV samples were included for each extraction. Amplification products were examined by PAGE/SS. The amplicons obtained were submitted for bi-directional sequencing with the primer pair PicoB25/ PicoB43 to Macrogen Laboratory Services (Seoul, Korea).

2.6. Sequence analysis

A consensus sequence from each RT-PCR product from the orangutan PBV strains was generated using the Bio Edit Sequence Alignment Editor Program version 5.0.9 (Copyright _ 1997–2001 Tom Hall, North Carolina State University, Department of Microbiology). The data was compared with reference sequences in the GenBank database using the BLAST program. Phylogenetic analysis was performed using the MEGA4 program (Tamura et al., 2007) with the Neighbor-Joining method and Kimura-2 parameter. The bootstrap analysis (1000 replicates) was used to evaluate the robustness of the phylogenetic groupings.

3. Results

All fecal samples collected during the study grouped into the category of normal consistence (not diarrheic). The following characteristics of the PBV infection pattern were detected in the adult orangutan during a period of 3 years (from Jun-05 to Jun-08). The results are depicted in Table 1. PBV was identified intermittently through the PAGE/SS technique during the whole period of the study, detecting PBV-PAGE [+] samples between periods with PBV-PAGE [−] samples. Also, 80 samples were tested through RT-PCR, which were previously analyzed through PAGE/SS. Overall, 45 of the 80 tested samples produced amplicons of the expected size (201 bp). From the total number of positive samples through RT-PCR ($n = 45$), 19 were also detected as PBV-PAGE positive and 26 showed discordant results (PBV-RT-PCR [+] and PBV-PAGE [−]), which suggest that the animal excreted a viral load so low that could not be detected through PAGE/SS. Among the 35 PBV-RT-PCR negative samples, 31 (88,5%) were also PBV-PAGE negative, suggesting periods without excretion of PBV, and 4 samples were PBV-PAGE positive. In general, the follow-up study showed a continuous pattern of PBV excretion, characterized by periods in which the virus can be detected by both PAGE/SS and RT-PCR techniques, only by RT-PCR or by none of them, suggesting silent periods of viral excretion. During the period of the study, all samples that were positive through PAGE/SS showed a similar electrophoretic pattern (approximately 2.9 and 2.6 Kbp for segment 1 and 1.9 and 1.7 Kbp for segment 2, respectively (data is not shown).

Seven of the 45 products obtained through RT-PCR were sequenced. The strains were named GI_PBV/orangutan/ARG-01/2005, GI_PBV/orangutan/ARG-01/2006, GI_PBV/orangutan/ARG-02/2006, GI_PBV/orangutan/ARG-03/2006, GI_PBV/orangutan/ARG-04/2006, GI_PBV/orangutan/ARG-05/2006 and GI_PBV/orangutan/ARG-01/2007, according to the nomenclature for PBV proposed by Fregolente et al. (2009). The amplicons without the pair of primers sequence (170 bp) showed an identity in their nucleotide sequence between 64% and 81% (Fig. 1). The alignment of sequences showed an identity that ranged between 80% and 100% in the following short nucleotide regions: 67–75; 89–99; 102–109; 116–130 and 134–163. In contrast, a significant divergence (between 50% and 70%) was identified between the nucleotides 9 and 66; 76 and 88; 110 and 113; 131 and 133. All amplicons showed in their amino-acid sequence the D-S-D motif (motif 1), which is common in the RNA-dependent RNA polymerases (RdRps) of RNA viruses with double-stranded RNA genome (dsRNA) and in some viruses with single-stranded RNA genome (Fig. 2).

Overall, the sequences obtained from the PBV strains excreted by the orangutan showed a divergence between 16% and 60% with human (from Hungary, Florida-USA, Argentina, Atlanta-USA, India and China), porcine (from Hungary), rat (from Brazil), dog (from Brazil), rhea (from Argentina) and foal (from India) genogroup I PBV sequences. (data not shown), with the following GenBank accession numbers: AJ504795.1; AJ504796.1; AJ504794.1; AF246937.1; AF246938.1; AF246936.1; AY949206.1; AY949205.1; AF246613.1; AF246612.1; AF245701.1; AB517733.1; AF246939.1; AM706361.1; AM706359.1; AM706362.1; AM706376.1; AM706379.1; AM706386.1; AM706387.1; AM706394.1; AM706371.1; AM706368.1; AM706378.1; FJ164031.1; EU814972.1; FJ164032.1; EU814970.1; AB598401.1.

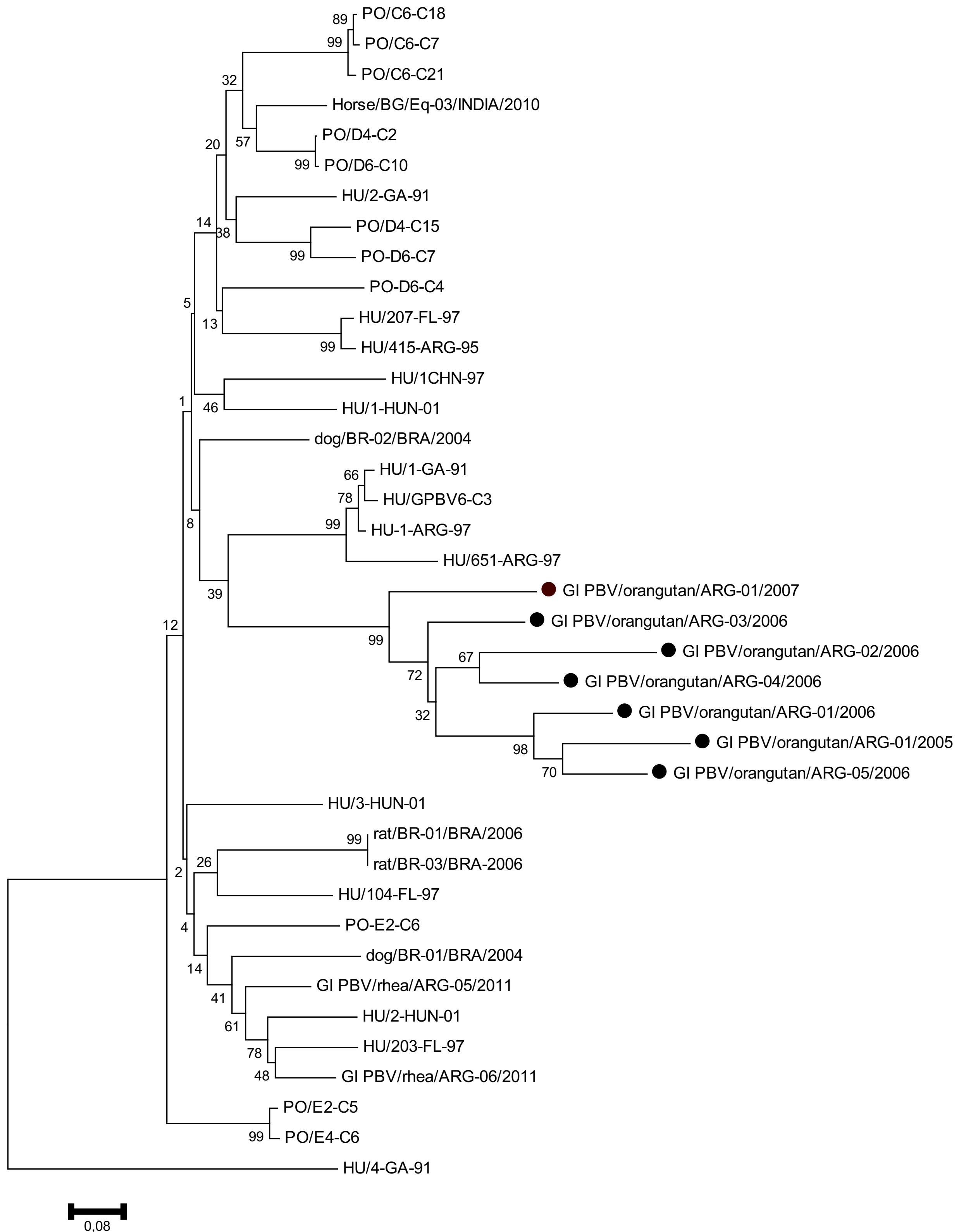

Fig. 3. Phylogenetic analysis of PBV strains based on 170 nucleotides of the genomic segment 2 sequences. The sequences for this study are indicated by ●. Strain 4- GA-91 (genogroup II) was used as an out-group. The tree was inferred by the MEGA4 program using the Neighbor-Joining method with the evolutionary distances computed by the Kimura-2 parameter method. Bootstrap values above 70% are indicated. Bars are in units of substitution per nucleotide.

Subsequently, to determine the genetic relationships between the orangutan genogroup I picobirnavirus strains with previously reported genogroup I viruses detected in human, porcine, foal, canine, rat and rhea feces, a phylogenetic tree was constructed on the basis of a 170-nt fragment of the RdRp gene (Fig. 3). The results showed that the strains of PBV excreted by the orangutan grouped themselves in the same branch with the highest bootstrap value, but in general the strain of PBV belonging to genogroup I

revealed no representative groupings, such as host species or geographic areas of detection (Numbers of access are mentioned above).

4. Discussion

In order to investigate the maintenance of PBV infection during adulthood in hosts infected by PBV, we carried out a longitudinally study for a period of 3 years in an adult orangutan. The animal was kept in captivity in a zoological Park situated in Cordoba, Argentina. The stool samples of the animal were analyzed both by PAGE/SS and RT-PCR, since the simultaneous use of these techniques improves the interpretation of the results. That is to say, a positive PBV sample by the PAGE/SS technique reveals a high viral load in the feces whereas the RT-PCR assay provides greater sensitivity than the PAGE/SS technique for the detection of picobirnavirus, providing information about periods of low excretion levels of PBV (Nates et al., 2011). Under these experimental conditions, PBV infection in the orangutan was characterized by interspaced periods of high and low active viral excretion and by silent viral periods, and the animal never showed clinical signs of diarrhea or other symptoms. These results correlated with those reported by our group of work (Martínez et al., 2010; Masachessi et al., 2012) which showed the virus-host interaction as a persistent and asymptomatic infection model. These previous studies were carried out since the animals were primarily infected by the virus, event that occurred very early in their lives. The present work is the first to characterize the pattern of infection in a host during adulthood and provides the first evidence that the adult infected host could actively excrete the virus, contributing to the circulation of the virus in nature.

The mechanisms by which persistent infections are maintained involve both modulation of the virus and cellular gene expression and/or modification of the host immune response. Evident virus reactivation is mainly facilitated by several and different factors such as infection with other viruses (as in HIV), nerve trauma (e.g., herpes facialis following surgery of the trigeminal ganglion), physiologic and physical changes (e.g., fever, menstruation, and sunlight), and immunosuppression (as in CMV disease). Specifically for PBV infections, Giordano et al. (1999), Grohmann et al. (1993) and Martínez et al. (2010), reported the possibility that the virus has to generate highly productive replication cycles is related to the physiological state of the host (the higher virus frequency detected among immunosuppressed humans compared with the general population; and the highest frequency of virus excretion detected at the final stage of gestation and during lactation time in a pig). In the present study, no specific factors could be linked to higher or lower loads of PBV excretion by the orangutan.

The analysis of sequences of PBV excreted for the orangutan during the whole period of study (3 years) shows a nucleotide identity (64% and 81%) lower than those reported by Martínez et al. (2010) and Masachessi et al. (2012) for a pig and rhea. This could be explained by the fact that the host is under a long-term infection (much more than 2 years before initiating the longitudinal study), and as a result, the host presents a highly heterogeneous viral population.

In the phylogenetic study, we observed a cluster with closer evolutionary relationships only by grouping viral strains excreted by the same individual (orangutan). The overall analysis of the distribution of strains in the dendrogram indicates that PBV strains excreted by different animal species do not have defined clusters but rather the genogroup I can co-circulate in nature without characteristic specificity of host species or geographic area of detection. In spite of this, PBV strains circulating intra-host and intra-species are more closely related than PBV strains excreted by different animal species. More studies of other hosts are necessary to establish a deeper understanding of the strain diversity spread of these viruses.

The natural history of PBV infection is beginning to be understood, based on the results obtained in the follow-up studies curried out in pigs, rheas and orangutan, and it seems to be characterized by new-born, juvenile and adult asymptomatic biological containers which persistently excrete closely related strains in their faeces. This could suggest a long period of evolution in the virus-host relationship and define the natural history of infection and maintenance of PBV in nature.

Overall, PBVs could be considered frequent inhabitants of the gastrointestinal tract, leaving the question open for discussion about the molecular mechanisms governing persistent and asymptomatic coexistence with the host and the potential host suitability to maintain this relationship.

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