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Original Article

Detection and molecular characterization of multiple strains of Picobirnavirus causing mixed infection in a diarrhoeic child: Emergence of prototype Genogroup II-like strain in Kolkata, India

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Abstract: Background: Picobirnaviruses (PBVs) associated with viral gastroenteritis were reported from humans and several animal species to date. PBVs belonging to family *Picobirnaviridae* under proposed order *Diplornavirales* are small, non-enveloped, with bisegmented dsRNA genome. Methods: PBV was detected by polyacrylamide gel electrophoresis (PAGE) and silver staining. Confirmatory RT-PCR using primer pair PicoB25 (+) and PicoB43 (-) for genogroup I PBV and PicoB23(+) and PicoB24(-) for genogroup II PBV, resulted in amplicons of 201bp and 369bp respectively. The amplicons of genogroup I PBV were cloned and sequenced; amplicon of genogroup II PBV was directly sequenced. Further, the phylogenetic relationship and genetic diversity of strains from Kolkata was compared with hitherto reported PBV strains. Results: In PAGE, a faecal specimen showed three sets of PBV with large profile bisegmented genomic RNA with slight variation in migration pattern. Molecular cloning experiments confirmed that PBV/Human/INDIA/GPBV6/2007 had mixed infection comprising four different strains of PBV genogroup I [GPBV6C1P-GPBV6C4P] and one PBV genogroup II strain [GPBV6G2P]. Conclusion: Sequence comparison and phylogenetic analysis of gene segment 2 of GPBV6 clones (C1, C2, C3 and C4) revealed low nucleotide identities (59-63%) and distant genetic relatedness to other human and porcine genogroup I picobirnaviruses. The strain GPBV6G2P represents another PBV genogroup II strain after prototype strain 4-GA-91/USA as genogroup II PBVs have seldom been reported to date, except from Kolkata, India and Netherlands. We are reporting the first incidence of detection of multiple strain (mixed) infection of picobirnavirus [genogroups I and II] from a diarrhoeic child in a slum community of Kolkata, India.

Key words: Genogroup I and II Picobirnavirus, bisegmented dsRNA virus, viral diarrhea, mixed infection

Introduction

The genus Picobirnavirus, a novel double-stranded RNA (dsRNA) virus belongs to the family *Picobirnaviridae* under the proposed order *Diplornavirales*. The taxonomic proposals ratified by International Committee on Taxonomy of Viruses (ICTV) in April 2008 mention: '*Human picobirnavirus'* as the 'type species' and '*Rabbit picobirnavirus'* as a 'designated species' [1]. The virion is non-enveloped, small, spherical, 33-41 nm in diameter, with bisegmented genomic dsRNA as either of two different genome profiles. In PBVs with large genome profile, the segment size ranges from 2.3 to 2.6 kbp and 1.5 to 1.9 kbp for the two segments [2-5]. In PBVs with small genome profile the segment size is approximately 1.75 and 1.55 kbp for the segments 1 and 2, respectively [6-8]. The first complete nucleotide sequence of the two genome segments of PBV isolated from humans was published by Wakuda et al., in 2005. The ge-

nomic segment 1 encodes two open reading frames (ORFs) of 224 and 552 amino acids, respectively. The first ORF codes for a protein of unknown function, whereas the second ORF has been shown to encode the capsid protein (CP). The smaller segment 2 has a single ORF of 534 amino acids encoding the viral RNA dependent RNA polymerase (RdRp). The virion consists of a simple core and capsid with distinctive icosahedral arrangement [9]. It has also been shown that picobirnavirus particles are capable of disrupting biological membranes *in vitro*, indicating that its simple capsid of 120-subunits has evolved animal cell invasion properties. Picobirnaviruses have been detected in faeces of humans and wide range of animal species with diarrhea [10, 11] or without diarrhea [7, 12], and have also been reported as coinfections with other etiological agents of diarrhoea in humans [13-15].

Picobirnaviruses were first detected in the fecal specimens of humans and rats (*Oryzomys nigripes*) in 1988 from Brazil [16-17]. Thereafter, PBVs were detected in faecal specimens of humans from different countries [2, 6, 7, 18, 19]. PBVs in children have been reported from Brazil [16], Venezuela [20], Italy [14], Russia [21], India [4, 5, 8], USA, Australia [22], Argentina [53] and the Netherlands [55]. Early studies in immunocompromised hosts implied that PBVs may be opportunistic pathogens [13, 15, 18, 40, 41]. Asymptomatic PBV was detected in two stool samples obtained at 6 month intervals, from a randomly selected healthy individual [42].

PBVs were reported from feces of a wide variety of farm mammals such as pigs [23-26, 10], calves [27-30], foals [31], lambs [11], rabbits [12, 32, 33], guinea pigs [34], or birds such as chickens [35]. PBVs have also been reported from wild animals and birds kept in captivity [36-38] and also from dogs, rats, and snakes [39].

Laboratory diagnosis mainly relies upon the detection of bisegmented dsRNA genome by PAGE and silver staining [43]. For RT-PCR experiments, the two sets of primer pairs described by Rosen et al [2] have been widely used worldwide for molecular detection and characterization of PBVs. These RT-PCR primers specifically amplify small fragments within the RNA dependent RNA polymerase (RdRp) gene. They are also capable of differentiating 2 major PBV genogroups. The genogroup I and II of PBVs are represented by the prototype strains 1-CHN-97 (China) and 4-GA-91 (USA), respectively. The two sets of primer pairs yield specific amplicons of 201bp and 369bp representing Genogroup I and Genogroup II PBVs, respectively [2]. With the advent of sequence-independent amplification and high-throughput sequencing, analysis of the etiological agents in human feces on metagenomic aspects, has resulted in detecting divergent novel subtypes or genotypes of viruses including picobirnaviruses [22, 42, 44].

In this study, we report the occurrence of multiple PBV strains detected in the faecal specimen of a diarrhoeic child by PAGE and further characterization by RT-PCR, cloning, and sequencing. Phylogenetic analyses was carried out with four different genogroup I PBV strains which had been selected from cloning experiments and one genogroup II PBV strain that was sequenced directly. The genogroup I PBV strains detected during this study clustered on different branches of human and porcine PBV strains reported from different geographical locations. The genogroup II PBV strain clustered with the prototype strain 4 GA-91 (68% nt identity and 72% aa identity) and this is another rare instance of a PBV genogroup II strain from Kolkata, India that has shown genetic resemblance to the prototype strain outside USA since 1991.

Materials and methods

Fecal specimen

The fecal sample had been collected on 1, October 2007 from a male child aged 43 months with acute watery diarrhea after obtaining a written consent from his parents. An aliquot of the fecal specimen diluted with 1x PBS was thoroughly vortexed and centrifuged at 3000rpm for 15 mins at 4°C, for clarification. The supernatant was taken in a fresh microfuge tube and again centrifuged (7000rpm at 4°C for 15mins); the supernatant was saved in a fresh microfuge tube as virus suspension and stored at 4°C.

Routine microbiological examination

Each fecal sample was routinely screened for different etiological agents of diarrhoea comprising bacterial, viral and parasitic pathogens using a combination of conventional microbi-

ological, biochemical, immunological and molecular assays as described by Nair et al., 2010 [45]. The viral enteric pathogens screened were Rotavirus, Norovirus, Sapovirus, Adenovirus and Astrovirus. The specimens were screened for several bacteria viz. *Vibrio spp*, *Shigella spp*, *Klebsiella spp*, *Escherichia coli*, *Aeromonas spp*, and *Campylobacter spp*). The parasites screened for were *Giardia lamblia*, *Cryptosporidium spp*, and *Entamoeba histolytica*.

Extraction of dsRNA from virus suspension and polyacrylamide gel electrophoresis for detection of picobirnavirus

PBV dsRNA was extracted from virus suspension using phenol-chloroform-isoamyl alcohol mixture for PAGE experiments as previously described[4] and subsequent visualization of dsRNA migration patterns after PAGE and silver staining was performed according to Herring et al. 1982 [43].

RNA extraction for RT-PCR

Extraction of viral RNA was carried out using the commercially available QIAGEN QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per manufacturer's instructions.

RT-PCR for detection of Picobirnavirus

The primer pair (A) PicoB25[+] (5'TGGTGTGGATGTTTC3') and PicoB43[–] (5'A (G,A)TG(C,T)TGGTCGAACTT3') was used to amplify the 201 bp fragment of RdRp gene (genomic segment 2), related to PBV strain 1-CHN-97 (Genogroup I) and (B) PicoB23[+] (5'CGGTATGGATGTTTC3') and PicoB24[–] (5'AAGCGAGCCCATGTA3') was used to amplify the 369bp fragment of RdRp gene (genomic segment 2), of strains related to strain 4-GA-91 (Genogroup II). RT-PCR was carried out following the protocol of Bhattacharya et al. 2006 [4].

Amplicons were checked in 2% agarose gel run in Tris-Boric acid-EDTA buffer, pH8, along with 1 Kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA), stained with ethidium bromide; the gel images were recorded in a BioRad Gel documentation system.

Purification of PCR product and sequencing

The amplicons were purified using the commercially available QIAGEN QIAquick PCR product purification kit (QIAGEN GmbH, Hilden, Germany), according to manufacturer's instructions. Cycle sequencing was performed separately with forward and reverse primer for Genogroup I or II picobirnavirus, respectively, using the BigDye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) and sequenced in an automated sequencer (ABI PRISM 3100).

Cloning and sequencing

The RT-PCR amplicons of genogroup I PBV were cloned in pCR2.1-TOPO vector according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, the steps involved were as follows: An aliquot of fresh PCR product was mixed with molecular biology grade distilled water to 4µl and taken in a microfuge tube. 1µl of salt solution containing 1.2M Sodium chloride and 0.06M Magnesium chloride mixture was added to it followed by 1µl of TOPO vector to make up the final volume to 6µl. The contents of the microfuge tube was gently mixed and incubated for 5 minutes at room temperature. The reaction tube was next kept on ice and 2µl of reaction was used for 1 vial of TOP10 competent cells during transformation. Later, plasmid was isolated using the commercially available Plasmid extraction Miniprep kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. The isolated plasmids were checked by restriction analysis to confirm the presence of insert of appropriate size and correct orientation. The transformants were amplified by PCR and visualized by agarose gel electrophoresis. Finally, the clones were sequenced in both directions.

Sequence analysis

All sequences were read using FinchTV (v.1.4.0) and sequence data obtained was compared with other reference sequences in the DNA databases, using BLAST [46]. Amino acid prediction was carried out using DNASIS (Version 2.1). ClustalW (Version 1.83) was used for multiple alignments of all the sequences [47]. LAlign program (Version 2.0) was used for the global alignment of consensus with reference sequences [48].

MEGA (Version 4.0) [49] was used for constructing phylogenetic tree. The bootstrapped phy-

logenetic tree (bootstrap of 1000 replicates) was constructed using Neighbor-Joining method [50], following Juke-Cantor's parameter. The phylogenetic tree for genogroup I PBV strains was constructed with 4-GA-91, the prototype strain of Genogroup II, defined as the outgroup strain. The phylogenetic tree for genogroup II PBV strains was constructed with 1-CHN-97, the prototype strain of Genogroup I, defined as the outgroup strain.

Nucleotide sequence accession numbers

The sequence data of four 201bp amplicons [from clones of genogroup I] and one 369bp amplicon (nucleotide sequence fragment covering partial RdRp gene of genomic segment 2 of genogroup II PBV strain) analysed during this study from Kolkata, India were submitted to the DNA Data Bank of Japan (DDBJ; http://www.nig.ac.jp/) under the following accession numbers: GPBV6C1P: AB526253, GPBV6C2P: AB526254; GPBV6C3P: AB526255; GPBV6C4P: AB526256 [representative of 4 types of Genogroup I clones] and GPBV6G2P: AB526257[Genogroup II amplicon].

Ethics approval

The study was approved by ethics committee of National Institute of Cholera and Enteric Diseases, Kolkata, India.

Results

Picobirnaviruses as mixed infection was detected from a 43 month old male child who had severe diarrhoea and was passing loose stool more than 6 times a day, without fever or vomiting. In PAGE, the presence of three larger and three smaller, well separated segments, representing three sets of PBV genome with 'large' profile migration pattern were detected (**Figure 1**). The sample was also positive for Norovirus [NVGII].

Using the two pairs of genogroup specific primers [PicoB25(+) and PicoB43(–) for Genogroup I PBV and PicoB23(+) and PicoB24(–) for genogroup II PBV] reverse transcription-polymerase chain reaction (RT-PCR) indicated that both genogroups of PBV were present in the sample. The amplicons of 201bp for genogroup I and 369bp for genogroup II respectively were observed (**Figure 2**). The genogroup

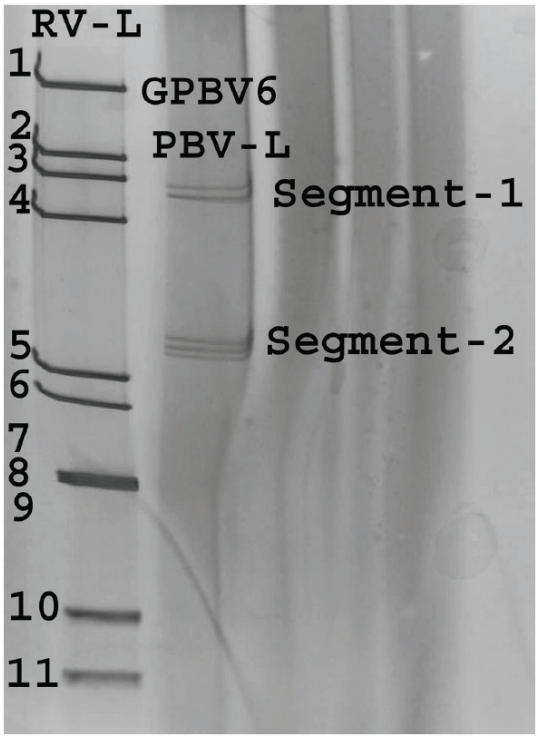

Figure 1. Mixed infection of human picobirnavirus in a diarrhoeic child with slight variation in migration pattern of three sets of PBV large profile, bisegmented, genomic double-stranded RNA (Lane 2), alongside long electropherotype pattern of eleven segmented, genomic dsRNA profile of human Group A rotavirus (Lane 1).

nature of strains was further confirmed by cloning into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), and sequencing 20 clones in both directions. The sequence analysis revealed that the clones of PBV/Human/IND-GPBV6/2007 consist of multiple PBV strains that exhibited absolute identities within themselves. A representative clone from the four types was designated as C1P, C2P, C3P and C4P for sequence submission. Based on the proposed nomenclature for Picobirnavirus [49] the PBV positive sample is described in this study as: PBV/ Human/ IND/ GPBV6/ 2007. The four representative genogroup I clones of PBV of above strain are denoted as: GI strain PBV/ Human/ IND/ GPBV6C1P/ 2007; GI strain PBV/ Human/ IND/ GPBV6C2P/ 2007; GI strain PBV/ Human/ IND/ GPBV6C3P/ 2007; GI strain PBV/Human/ IND/ GPBV6C4P/ 2007. The phylogenetic tree (**Figure 3**) also indicated that four sets of genogroup I PBV strains were present,

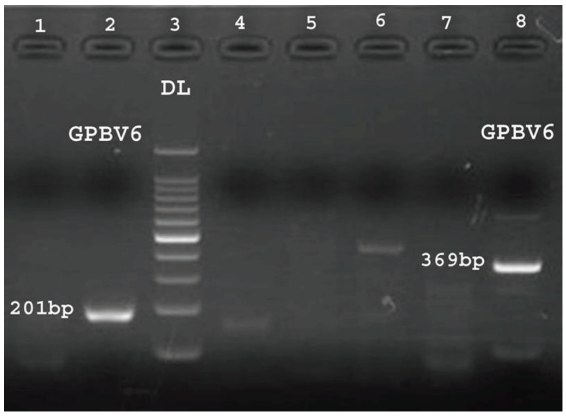

Figure 2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) showing amplicon of 201bp with genogroup I specific primer pair [PicoB25(+) and PicoB43(–)] in Lane 2, and amplicon of 369bp with genogroup II specific primer pair [PicoB23(+) and PicoB24(–)] in lane 8 with 1 Kb plus DNA marker (Invitrogen, Carlsbad, CA, USA) (Lane 3).

represented by a clone from each set thereby confirming that PBV genogroup I clones [C1P – C4P] were distinct as they clustered on separate branches showing close homology to other human or porcine PBV strains.

The comparison of partial length deduced amino acid sequences of gene segment 2 (stretch of 56 amino acids) among the picobirnavirus strains detected in Kolkata and related human/porcine strains is given in **Table 1**. The deduced stretch of 56 amino acids (nucleotide sequence of 201bp amplicons) from Hu/GPBV6C1P-Hu/GPBV6C4P and other PBVs showed that 19 amino acids were conserved, whereas distinct amino acid changes were observed in other positions. The Hu/GPBV6C1P showed close homology with clones of porcine PBV strain C10/AM706366 and strain E2/AM706392, which were earlier reported to resemble human-like PBVs (Hu/2-HUN-01/AJ504795) from Hungary.

The strain Hu/GPBV6C1P* showed only 2 amino acid changes from porcine strain C10/AM706366' in amino acid position 46 S* to P' i.e., from 'polar' to 'non-polar and aa47 G* to D i.e., from non-polar to acidic with negatively charged group respectively.

Similarly, there were variations in the polarity (either 'polar' to 'non-polar' or *vice versa*); other variations were observed in the nature of charge for amino acids viz. from 'a basic positively charged group' to 'an acidic negatively charged group' or *vice versa*). Thus molecular analysis of the PBV strains detected during the present study showed that they were genetically divergent and rapidly evolving. Similarly, in some instances, though an amino acid change occurred, its 'charge' or the 'polarity' remained within the same class.

Similarly, there were variations in the polarity

Table-1. Comparison of partial length deduced amino acid sequence of gene segment 2 (stretch of 56 amino acids) among genogroup 1 picobirnavirus strains detected in Kolkata and related human/porcine strains. The conserved proline residues (aa13 and aa25) and other amino acids are underlined and shown in bold face.

Amino acid	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 # 21 # # # 25 # 27 # # # 31 # # # 35 # 37 # # # 41 # # # 45 # 47 # # 50 51 52 53 54 55 56
Hu/GPBV6-C1P	F A V H I E E L R Y Y Q P A I E A A Q H F H L V P A W V S M E S V D D R I T R M F D T K G S G D L V I C T D F S
Po/C10/AM70636	. . . . . P D . . . . .
Hu/2-HUH-01	Y . . . . . S V . . . L . . K V . S . . . . . R . . . A . . . . . A . H . . . V . . . . .
Hu/2-GA-91(USA	. . . . . Q . . . L . . . L . . . S C . K . . . . . A . . R . . . Q L . . . . . K D . D I . . . . .
Po/C10/AM70636	. . . . L C . . Q V . . . M . . S C . R . . . . . A . . Q . . . H . . . . . V D . . . . .
Hu/GPBV6-C2P	. S . . . R . . Q V . . . L . . S C . K . D . . . . . R . . . K . . . S . . K D . V . . . . .
Po/D6/AM706382	. . . . . C . . Q V . . . L . . . C . K . . . I . . . . . Q . V . K . . . . . T D . V . . . . .
Po/D4/AM706367	. G . . . H . . Q V . . . L . . C . . K . . . . . I . . D E . . Q . . . . . K D . V . . . . .
Hu/415-ARG-95	. . . . . R . . Q V . . . L . L T F . R L G . . . . . A . . R . . . K . . . . . P H . V . V . . . . .
Hu/Hy005102	. . . . . R . . Q V . . . L . L T F . R L G . . . . . A . . R . . . K . . . . . P R . V . V . . . . .
Hu/GPBV6-C4P	Y . . . . R . . Q F . . . . . L . K R E L . . . . . G . D . . . V . . . . L . . . . . A K S E . I V . . . . .
Po/PBV1-Por	Y . . . . R . . Q F . . . . . L . . K H L . . . . . G . D A . . V . . . . L . . . . A K S . . I V . . . . .
Hu/3-HUH-01	Y . . . . Q . . Q V . . . M . . . F . Y H . . . . . G . D A . . V E . . . L . . . . K P S . . E F . . . . .
Hu/1-CHH-97	. G . . V K . . Q F . . . . . I . . K R W I T . . . I G . . A . . Q . . . K L . . . . A K S . . . . .
Hu/1-HUH-01	. G . . . R . . Q V . . . . . L . . R S . . . . . G . . A . . K A V . K L . . . . . K H . . . . . E
Hu/GPBV6-C3P	. G I . V L . . Q L . . . L . . . L . A H S D . . . . . G . D A . . L A . . K L . . . . D . K . . . . .
Hu/1-GA-91	. G I . V L . . Q L . . . L . . . L . A H S D . . . . . G . D A . . L A . . K L . . . . D . K . . . . .
Hu/1-ARG-97	. G I . V L . . Q L . . . L . . . L . A H S D . . . . . D A . . L A . . K L . . . . D . K . . . . .
Hu/615-ARG-97	. G I . V L . . Q L . H . L . A . L . A H S D . . . H . . . V A . . W A . . A L . . . . E T K . S I L . . . . .
	: . : * : * * * : * * : * * . * * : * : * : * . * * * * .
	F A V H I E E L R Y Y Q P' A I E A A Q H F H L V P' A W V S M E S V D D R I T R M F D T K G S G D L V I C T D F S

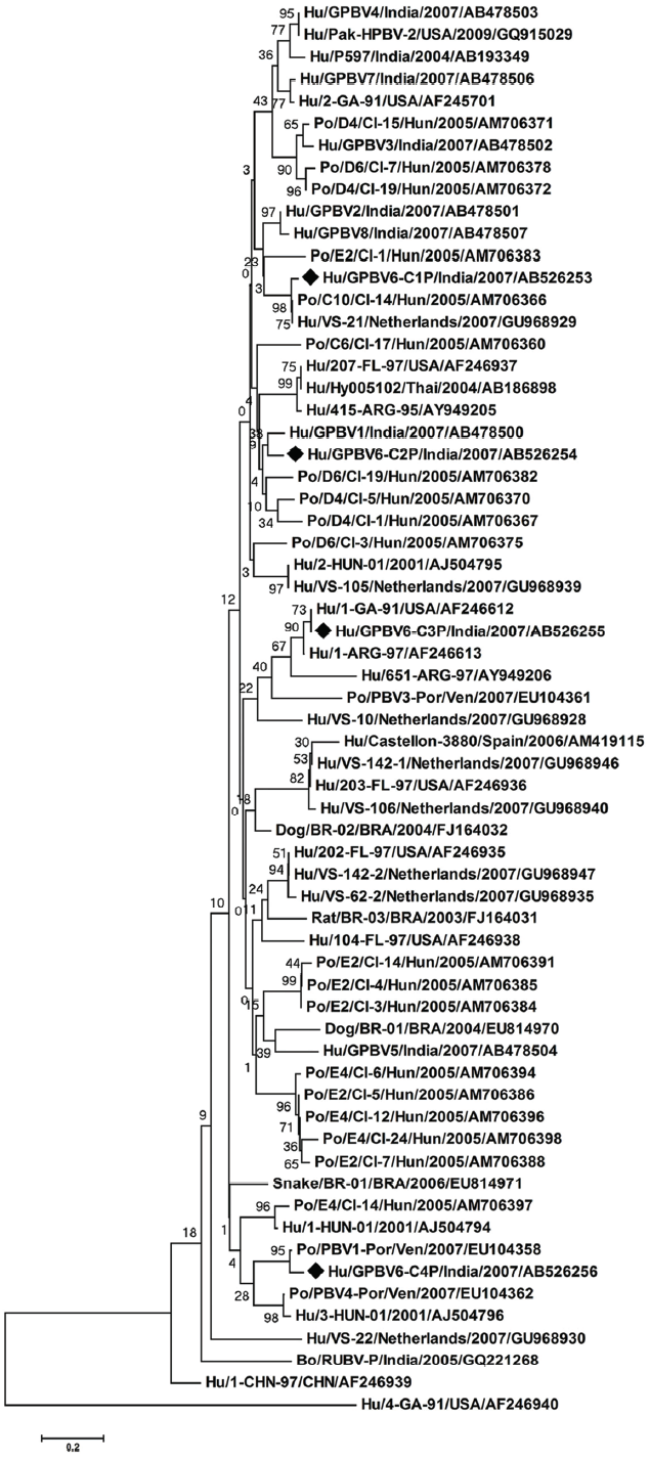

Figure3. Phylogenetic tree showing representative clones of four sets of PBV genogroup I (GPBV6C1P, GPBV6C2P, GPBV6C3P, GPBV6C4P) with cognate stretch of hitherto reported human, porcine, bovine, dog, rat and snake genogroup I PBV strains, based on partial amino acid sequence [56 amino acids (aa)] of genomic segment 2. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software (Version 4.1). Phylogenetic distances were measured by the Kimura two-parameter model, and the tree was statistically supported by bootstrapping with 1000 replicates. The genogroup I clones of PBV strain GPBV6 are indicated with a ♦ symbol. The tree was rooted with cognate stretch of gene segment 2 of genogroup II prototype strain 4-GA-91 defined as the outgroup strain. Bar indicates 0.2 substitutions per nucleotide. Abbreviations: Hu, Human; Po, Porcine; Bo, Bovine; IND, India; USA, United States of America; HUN, Hungary; THAI, Thailand; ARG, Argentina; BRA, Brazil; VEN, Venezuela.

viz. either polar to non-polar or *vice versa*; variations were observed in the nature of charge for amino acids viz. from a basic positively charged group to an acidic negatively charged group or *vice versa*. Thus molecular analysis of the PBV strains detected during the present study showed that they were genetically divergent and rapidly evolving. Similarly, in some instances, though an amino acid change occurred, its charge or the polarity remained within the same class.

The strain Hu/GPBV6C1P* showed 12 amino acid changes from Hu/2-HUN-01 in amino acid (aa) position 1 F* to Y' ie non-polar to polar; aa9 R* to S' ie from a basic positively charged group to polar; aa10 Y* to V' ie from polar to non-polar; aa14 A* to L'; aa17 A* to K' ie from non-polar to a basic positively charged group; aa18 A* to V'; aa20 N* to S'; aa35 D* to R' ie from an acidic negatively charged group to a basic positively charged group; aa39 R* to A' ie from a basic positively charged group to non-polar group; aa45 G* to A'; aa47 G* to N' ie from non-polar to polar group and aa51 I* to V'.

The strain Hu/GPBV6C4P* showed 19 amino acid changes from the genogroup I prototype strain Hu/1-CHN-97 in amino acid position 1 Y* to F' ie from polar to non-polar; aa2 A* to G'; aa5 I* to V'; aa6 R* to K'; aa17 L* to I'; aa19 K* to Q' ie from a basic positively charged group to polar, aa20 R* to K', aa21 E* to R' ie from an acidic nega-

Table 2. Comparison of the percentage nucleotide identity (percentage of amino acid identity in parentheses) between clones of genogroup I PBVs detected in Kolkata and some of the hitherto reported Human and Porcine PBV strains

	Hu/GPBV6-C2p	Hu/GPBV6-C3p	Hu/GPBV6-C4p	Hu/1-CHN-97	Hu/1-GA-91 (USA)	Hu/1-ARG-97	Hu/615-ARG-97	Hu/3-HUN-01	Po/PBV1-Por	Hu/2-HUN-01	Po/C10/AM706366	Po/E2/AM706392	Po/D6/AM706382
Hu/GPBV6-C1p	67 (73)	61 (63)	65 (68)	63 (63)	61 (63)	62 (64)	59 (52)	66 (66)	64 (68)	66 (79)	89 (96)	68 (77)	73 (77)
Hu/GPBV6-C2p		65 (61)	69 (63)	66 (63)	66 (61)	68 (63)	59 (52)	66 (59)	67 (64)	66 (73)	67 (75)	65 (70)	80 (84)
Hu/GPBV6-C3p			63 (59)	64 (66)	96 (--)	96 (98)	85 (79)	65 (66)	62 (64)	65 (59)	62 (61)	63 (59)	69 (61)
Hu/GPBV6-C4p				70 (66)	64 (59)	61 (57)	59 (50)	67 (73)	89 (93)	67 (68)	65 (68)	63 (60)	65 (59)
Hu/1-CHN-97					64 (66)	64 (64)	58 (52)	63 (59)	74 (71)	63 (57)	64 (63)	58 (55)	65 (63)
Hu/1-GA-91 (USA)						97 (98)	86 (79)	66 (66)	64 (64)	66 (59)	64 (61)	62 (59)	71 (61)
Hu/1-ARG-97							88 (80)	67 (64)	64 (63)	67 (61)	63 (63)	62 (60)	71 (63)
Hu/615-ARG-97								67 (55)	60 (55)	67 (52)	58 (52)	59 (49)	63 (54)
Hu/3-HUN-01									65 (79)	-- (66)	67 (68)	70 (62)	69 (63)
Po/PBV1-Por										65 (68)	63 (68)	57 (60)	60 (61)
Hu/2-HUN-01											67 (77)	70 (79)	69 (73)
Po/C10/AM706366												68 (81)	73 (79)
Po/E2/AM706392													70 (76)

tively charged group to a basic positively charged group, aa22 L* to W' ie from non-polar to polar group, aa23 L* to I', aa24 V* to T' ie from non-polar to polar group, aa28 V* to I', aa31 D* to E', aa32 S* to A' ie from polar to non-polar group, aa35 V* to Q' ie from non-polar to polar group, aa39 R* to K', aa48 E* to D', aa50 I* to V', and aa51 V* to I'.

The Hu/GPBV6C2P showed close homology with human strain [Hu/GPBV1-India] and porcine-like PBVs reported by Ganesh et al. 2010.[5] It was also closely related to other clones of porcine PBV strain D6/AM706382, D6/AM706379 and D4/AM706367. It is noteworthy that most porcine strains have amino acid Serine (S) in amino acid position 32, whereas Alanine (A) is seen at the same position among most human/or human like porcine PBVs. Likewise, in amino acid position 40, Methionine (M) is seen among porcine PBV strains unlike amino acid Leucine (L) found in human/or human like porcine PBVs.

The Hu/GPBV6C3P showed close homology with human strain Hu/1-GA-91 (USA), Hu/1-ARG-97 and Hu/615-ARG-97 from Argentina. The Hu/GPBV6C4P showed close homology with porcine strain PBV1-Por/EU104358 from Venezuela and also human strains 3-HUN-01/AJ504796 from Hungary, 104-FL-97/AF246938 and 203-FL-97/AF246936 from Florida, USA.

The comparison of the percentage nucleotide identity (percentage of amino acid identity in parentheses) between different strains of genogroup I PBVs detected in Kolkata during this study and some of the hitherto reported human and porcine PBV strains is given in **Table**

Table 3. Comparison of LAlign scores for nucleotide (nt) sequences of hitherto detected PBV genogroup II strains

	R227/ AB214978	V957/ AB334530	Pak-HPBV-1/ GQ915028	VS142-3/ GU968925	4-GA-91/ AF246940
V380/AB212175 nt:223-290	nt:129-193 (65%)				
V380/AB212175 nt:3-20			nt:259-276 (89%)		
V595/AB212174 nt:203-393		nt:1-191 (99%)			
GPBV6G2P/AB526257 nt:44-76		nt:81-113 (67%)			
GPBV6G2P/AB526257 nt:83-120			nt:351-388 (76%)		
GPBV6G2P/AB526257 nt:188-272			nt:345-428 (62%)		
GPBV6G2P/AB526257 nt:1-329				nt:1-332 (69%)	
GPBV6G2P/AB526257 nt:1-336					nt:698-1036 (68%)
4-GA-91/AF246940 nt:698-1036				nt:1-339 (97%)	
4-GA-91/AF246940 nt:511-727			nt:79-301 (60%)		
4-GA-91/AF246940 nt:1567-1644			nt:172-245 (60%)		

2. The strains Hu/GPBV6C3P showed 96% nucleotide identity and 98% amino acid identity to the human strain 1-ARG-97 reported earlier from Argentina, whereas it showed only 64% nucleotide as well as amino acid identity with the genogroup I PBV prototype human strain 1-CHN-97. The strain GPBV6C4P showed 70% nucleotide identity and 66% amino acid identity with the human strain 1-GA-91 (USA), but 89% nucleotide and 93% amino acid identity with porcine strain PBV1-Por from Hungary. However all the genogroup I clones of PBV strain GPBV6 showed only 63–70% nucleotide identity and 63–66% amino acid identity for the genogroup I prototype strain 1-CHN-97.

Based on the proposed nomenclature for Picobirnavirus (Fregolente and Gatti, 2009b) the genogroup II PBV strain is denoted as GII strain PBV/Human/IND/GPBV6G2P/2007. The genogroup II PBV strain detected from the GPBV6 sample (Hu/GPBV6G2P/2007/IND) showed 68% nucleotide identity with the genogroup II prototype strain 4-GA-91 and 67% nucleotide identity only with one of the earlier reported strain from Kolkata V957_03_IND. The genetic diversity observed among genogroup II PBV strains detected to date is shown in **Table 3**. The phylogenetic tree (**Figure 4**) indicates that GPBV6G2P clusters closer to the prototype strain 4 GA-91 unlike the PBV genogroup II strains reported earlier from Kolkata which were genetically more distant from the prototype strain. The PBV strain R227_03_IND and V380_00_IND had 65% nt identity and formed a separate cluster whereas the strains V595_01_IND and V957_03_IND had 99% nt identity and formed another cluster. It is noteworthy that genogroup II PBV strain GPBV6G2P is an another PBV strain that has emerged outside USA, after nearly two decades in addition to the prototype strain of genogroup II PBV, 4-GA-91.

Discussion

The molecular epidemiological data reported from USA, Argentina and India has clearly showed that the two sets of primers which are currently being used worldwide for partial mo-

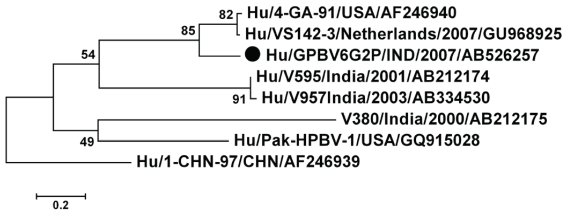

Figure 4. Phylogenetic tree showing PBV genogroup II (GPBV6G2P) and other hitherto reported genogroup II strains from Kolkata, India with the prototype strain 4 -GA-91 from USA based on nucleotide sequence of 369bp amplicon of segment 2 using primer pair PicoB23 and PicoB24 respectively. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software (Version 4.1). Phylogenetic distances were measured by the Kimura two-parameter model, and the tree was statistically supported by bootstrapping with 1000 replicates. The genogroup II PBV strain (GPBV6G2P) is denoted with a ● symbol. The tree was rooted with cognate stretch of gene segment 2 of the PBV genogroup I prototype strain 1-CHN-97 defined as the outgroup strain. Bar indicates 0.2 substitutions per nucleotide. Abbreviations: Hu, Human; IND, India; USA, United States of America; CHN, China.

lecular characterization of PBV genomes has limited efficacy in detecting PBV genogroups which are positive by PAGE [2, 4, 5, 18]. This suggests that human PBVs present wide genetic diversity and are evolving rapidly. However, PAGE detects the PBVs readily as it is not dependent on their genomic sequences. PBVs are known to cause chronic diarrhoea with prolonged shedding of the virus in humans [13] and various animals [36, 37] besides frequent infections among piglets [52]. Moreover, the presence of genogroup I PBVs in humans and different animals, rodents and reptiles, suggests that any specific genogroup is not restricted to specific host [39, 51]. This shows that the published epidemiological data on PBV is not the complete profile of virus prevalence or incidence; instead it reflects the data of only a few research laboratories with specific interest in this agent or PBVs detected during surveillance for rotavirus by PAGE [53].

Genogroup I PBVs detected and sequenced from pigs in Hungary [26] and Venezuela and Argentina [52], were observed to be closely related to human genogroup I PBVs. It is also known that porcine PBV strains are genetically diverse, and are related to human strains; they cause frequent infections among young pigs with or without diarrhea or any other sign of illness. The PBVs detected from children in Kolkata, India were reported to be closely related to porcine PBV strains [5]. These results strongly suggest that PBV strains may circulate between humans and pigs.

A research team while working on the protection afforded by the colostrum feeding in calves against rotavirus infection at the Compton Laboratory, U.K. observed that 16 out of 108 faecal extracts from 5 calves were positive for PBVs. [28] Some of the faecal samples showed the presence of mixed infections, as two larger and two smaller, well separated segments in PAGE experiments.

In another study, it has been reported[26] that in swine, genetic diversity was also observed among PBV strains identified in mixed infections. Single point mutations and deleterious mutations within highly related strains suggested that PBVs exist as quasispecies in the swine alimentary tract.

As reported from Hungary[26], Venezuela and Argentina[52] the clones they had isolated showed a number of PBV strains with complete sequence identities originating from different animals that suggested effective, easy animal to animal transmission of the virus.

The highly heterogenous nature of human PBVs is explained due to their segmented genomes [19], and the chances of segment reassortment either *in vivo* and or *in vitro* may lead to emergence of virulent progeny [54]. Therefore, from the results of our study, it is evident that the mixed infection of several genogroup I PBVs along with a genogroup II PBV could result from PBVs found in a habitat shared by humans and different animal species.

Similarly, another study by Carruyo et al. in 2008[52] documented that PBV positive samples from pigs exhibited single electrophoretic pattern in polyacrylamide gels, but contained multiple genogroup I PBVs when sequenced the amplified RdRp gene fragment. It was presumed in the report that the degenerate primers were able to recognize several strains with identical electrophoretic pattern present in the sample and the presence of different strains in the sample occurred with viral loads below the detection

limit of the PAGE technique.

In this study, we report the occurrence of multiple PBV strains in humans as mixed infection that were initially detected by PAGE and further characterized by RT-PCR, cloning and sequencing to determine their phylogenetic relationship. Partial molecular characterization and sequence analyses of human PBV strains[4, 5, 8] from Kolkata, had shown that distinct sequence heterogeneity exists among human PBVs belonging to both the genogroups (GGI and GGII) as well as occurrence of closely related PBV strains, respectively, thereby implicating the importance of stringent surveillance for newly emerging variants of PBVs. Recently, Ghosh et al. 2009 [30] published their study on bovine genogroup I PBV isolated from a diarrhoeic calf in Kolkata that showed the strain was totally unique and distinct from PBVs reported so far either from humans or other hosts.

To the best of our knowledge, this is the first report of detection of PBV multiple (mixed) strain infection with both genogroup I and II PBV strains from a diarrhoeic child in Kolkata, India. Phylogenetic analyses showed that genogroup I PBV strains cloned and sequenced during this study clustered on different branches of human and porcine PBV strains reported from different geographical locations whereas the genogroup II PBV strain clustered with the prototype strain 4 GA-91 (68% nt identity and 72% aa identity). It is important to note that this is another instance that a PBV genogroup II strain has emerged from Kolkata, India showing genetic resemblance to the prototype strain, outside USA since 1991 and recently from the Netherlands [55]. Stringent surveillance and monitoring of PBVs as sporadic, emerging agents is essential to learn more about these genetically diverse and rapidly evolving viruses, shed by humans or domestic animals, living in close proximity to one another in developing countries, for better understanding of evolutionary pattern of PBV strains that circulate in different geographical locations.

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