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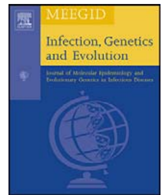

Detection of closely related Picobirnaviruses among diarrhoeic children in Kolkata: Evidence of zoonoses?

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ABSTRACT

The genus, Picobirnavirus (PBV), Spanish '*pico*' = 'small', birna for '*bipartite RNA*' genome, belongs to the family *Picobirnaviridae* under the proposed order *Diplornavirales*. PBV infections have been reported from diarrhoeic animal species and humans as well as from asymptomatic cases. The detection of Picobirnaviruses (PBVs) in diarrhoeic faecal specimens from children aged <5 years, suggestive of zoonotic transmission is being reported. 23 Picobirnavirus positive faecal specimens were detected by polyacrylamide gel electrophoresis (PAGE) and silver staining from a set of 1112 faecal specimens collected from an urban slum community in Kolkata between July and October 2007. The Picobirnaviruses showed either large profile ($n = 22$) or small profile ($n = 1$) for their bisegmented genomic double-stranded RNA (dsRNA). 13/23 positives were amplified by reverse transcription polymerase chain reaction (RT-PCR) as 201 bp amplicon with genogroup I primers [PicoB25(+) and PicoB43(−) specific for RNA dependent RNA polymerase (RdRp) gene fragment encoded by genomic segment 2] and seven amplicons were sequenced [GPBV1–5, 7 and 8]. Sequence analyses showed that four PBV strains [GPBV1–3 and 8] resembled different clones of porcine PBV strains (D4, D6 and C10) reported in 2008 from Hungary and two PBV strains [GPBV4 and 7] resembled human PBV strains (P597, Kolkata and 2-GA-91, USA) with the maximum nucleotide (nt) identity ranging from 78% to 92%. One strain GPBV5 clustered with human PBVs and porcine PBVs that were reported from Hungary, Venezuela and Argentina showing close homology to human-like PBVs. Therefore, the close monitoring of their global spread as well as in-depth molecular characterization is essential for better understanding of emerging PBV strains.

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1. Introduction

The genus, Picobirnavirus (PBV) belongs to the family *Picobirnaviridae* under the proposed order *Diplornavirales*. The virion is non-enveloped, small, spherical, 33–41 nm in diameter and consists of a simple core capsid with distinctive icosahedral pattern (Rosen et al., 2000; Bányai et al., 2003; Duquerroy et al., 2009). The genome is bisegmented, double-stranded RNA (dsRNA) with either large genome profile (2.3–2.6 kbp for the larger and 1.5–1.9 kbp for the smaller segment, respectively) (Rosen et al., 2000) or small genome profile (1.75 and 1.55 kbp for segments 1 and 2, respectively) (Gallimore et al., 1995a,b; Bhattacharya et al., 2006). The RNA segment 1 encodes two open reading frames (ORFs) of 224 and 552 amino acids, and the RNA segment 2 codes for a single ORF of 534 amino acids comprising of amino acid motifs typically encoded by other RNA dependent RNA polymerase (RdRp) genes (Rosen et al., 2000; Martínez et al., 2003; Wakuda et al., 2005). Epidemiological studies carried out in several countries have made it clear that human picobirnavirus strains are highly diverse; they can be grouped into two genogroups represented by the Chinese strain 1-CHN-97 (prototype of genogroup I) and the US strain 4-GA-91 (prototype of genogroup II), based on RT-PCR experiments, which specifically amplify partial fragments of RdRp gene of segment 2, with genogroup specific primers.

Picobirnaviruses were first detected in faecal specimens from humans and rats (*Oryzomys nigripes*) in 1988 (Pereira et al., 1988a,b). Since then, viruses with similar characteristics have

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subsequently been detected in faecal samples from rabbits, chickens, domestic and wild animals in captivity, birds and snakes (Pereira et al., 1988a; Haga et al., 1999; Fregolente et al., 2009) as well as in faecal samples of humans with or without diarrhoea (Gallimore et al., 1995a,b). Studies of PBV infections among children have been conducted in Brazil (Pereira et al., 1988a), Venezuela (Ludert and Liprandi, 1993), Italy (Cascio et al., 1996), Russia (Novikova et al., 2003), and India (Bhattacharya et al., 2006, 2007).

Laboratory diagnosis of PBV infections is mainly based on the detection of two dsRNA genome segments, in silver stained PAGE gels (Herring et al., 1982). PBVs were often detected from faecal specimens of farm mammals (pigs, calves) and birds as virus particles are shed in their faeces (Chasey, 1990; Browning et al., 1991; Gallimore et al., 1993; Ludert et al., 1995; Haga et al., 1999; Buzinaro et al., 2003; Masachessi et al., 2007; Wang et al., 2007).

Published information on the prevalence of the virus has varied from a frequency of 2.14% in Venezuela (Ludert and Liprandi, 1993), to a frequency as low as 0.45% in Brazil (Pereira et al., 1988a), or 0.43% of PBV detected in Italy (Cascio et al., 1996) and 0.09% in Argentina (Giordano et al., 2008). Earlier, the data about PBV association with gastroenteritis in children was not conclusive and the potential role of pathogenic PBV was unclear (Ludert and Liprandi, 1993; Cascio et al., 1996). Likewise, prospective evaluation of diarrhoea among HIV-infected persons was conducted (Grohmann et al., 1993; Giordano et al., 1998; González et al., 1998), but the role of PBV as a causative agent of gastroenteritis in the immunocompromised population was not proven. Recently, the presence of PBV genome in two faecal samples has been reported, that were obtained at six month intervals, from a randomly selected healthy individual (Zhang et al., 2006). This constitutes new evidence that PBV can also be isolated from asymptomatic individuals. Similarly in Venezuela and Argentina (Carruyo et al., 2008), PBVs were frequently excreted by piglets without any sign of disease. PBV strains obtained from pigs in Hungary, Venezuela and Argentina have been recently reported to be genetically related to the human genogroup I PBVs (Bányai et al., 2008; Carruyo et al., 2008).

Since no attempts to culture PBV *in vitro* have been made to date and no animal model of infection and disease exists, laboratory diagnosis relies upon the detection of dsRNA bisegmented genome by PAGE and silver staining. The RT-PCR detection assay with two pairs of primers targeted to genomic segment 2 of 1-CHN-97 (GGI) and 4-GA-91 (GGII) PBV strains isolated in China and USA, respectively, greatly improved the detection and molecular characterization of PBV worldwide (Rosen et al., 2000). To date, PBV related to the Chinese strain is the predominant virus detected in faecal specimens and the primers from the 1-CHN-97 strain have the broadest reactivity.

Molecular epidemiological data presented in different reports (Rosen et al., 2000; Martínez et al., 2003; Bhattacharya et al., 2006), showed a limited efficacy of these sets of primers to detect PBV circulating in USA, Argentina, and India. On the other hand, the use of PAGE technique is limited to few research laboratories, but it allows rapid detection of circulating PBVs. As a consequence of the limited diagnostic methodology available, PBV is only detected in research laboratories with specific interest in this agent or eventually during rotavirus surveillance by PAGE (Bhattacharya et al., 2006, 2007; Giordano et al., 2008).

Therefore, the potential PBV involved in diarrhoeal illness was an interesting issue to be defined since PBV was the only detectable enteric pathogen in faecal specimens of watery diarrhea in many of the children in Kolkata during the year 1999–2003 (Bhattacharya et al., 2007). The present study has shown an important association of human PBVs closely related to porcine PBVs as sporadic, emerging etiological agents of diarrhea among the children, besides other PBV strains with considerable genetic diversity, circulating in Kolkata.

2. Materials and methods

2.1. Geographical location

The study was carried out in an urban slum in the city of Kolkata (Ward No. 66 of Kolkata Municipal Corporation), with an estimated population density of 24,718 persons/km^2. It was estimated that about 32.5% of total Kolkata population live in slum areas (Census of India, 2001). The study population comprised of 60,000 people with 4000 children under 5 years of age who were recruited for this study.

2.2. Faecal specimens

Faecal specimens ($n = 1112$) of children aged below 5 years were collected from July to October 2007 after obtaining a written consent in the consent form, from parents/guardians. Routine screening of viral agents such as Rotavirus and Picobirnavirus was carried out by PAGE and each case that was positive for PBV was referred back to the Bacteriology and Parasitology laboratory to check for any co-infection with bacterial and/or parasitic agents, respectively.

2.3. Extraction of double-stranded RNA from virus suspension and polyacrylamide gel electrophoresis for detection of picobirnavirus and rotavirus

Preparation of virus suspension and dsRNA extraction using phenol–chloroform–isoamyl alcohol mixture for PAGE experiments was performed as previously described (Bhattacharya et al., 2007) and subsequent visualization of dsRNA migration patterns after PAGE and silver staining was done according to Herring et al. (1982).

2.4. RNA extraction for RT-PCR

Extraction of viral RNA was carried out using the commercially available QIAGEN QIAamp® Viral RNA mini kit (Qiagen Sciences, MD, USA) as per manufacturer's instructions.

2.5. RT-PCR for detection of Picobirnavirus

The two different sets of primer pairs as described (Rosen et al., 2000; Bányai et al., 2003) were used: (A) PicoB25^{+} (5′TGGTGTGGATGTTTC3′) with PicoB43^{−} (5′A(G,A)TG(C,T)-A(G,A)TG(C,T)TGGTCGAACTT3′) to amplify the 201 bp fragment of the RdRp gene (genomic segment 2), related to picobirnavirus strain 1-CHN-97 (Genogroup I) and (B) PicoB23^{+} (5′CGGT-ATGGATGTTTC3′) with PicoB24^{−} (5′AAGCGAGCCCATGTA3′) to amplify the 369 bp fragment of the 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. (2007).

2.6. Purification of PCR product and sequencing

The PCR products were purified using the commercially available QIAGEN QIAquick PCR product purification kit (Qiagen Sciences, MD, USA), according to manufacturer's instructions. An aliquot of each purified product was quantitated in a GeneQuant *pro* UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, UK). Cycle sequencing was performed separately with forward or reverse primer for Genogroup I and II picobirnavirus, respectively, using the BigDye Terminator v3.1 Cycle Sequencing Reaction Kit

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