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Molecular Evidence of Multiple Viral Infections in an Infant Hospitalized with Acute Gastroenteritis in India

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Acute diarrhea caused by an array of infectious agents is the most important disease of childhood with high mortality worldwide. Multiple enteric infections are serious threat with fatal outcome compared to infection by one causative agent. Initial identification of those multiple infections is essential for formulating the better treatment recipes and preventive measures in long term. Therefore we describe here such infection in an infant with acute gastroenteritis from Haldwani city, Uttarakhand, India. The molecular investigations were carried out for the common enteric infection viz. rotavirus, astrovirus, calicivirus and picobirnavirus by RT-PCR, cloning and sequencing followed by phylogenetic analysis. The investigation detected the presence of group A (RVA) and B (RVB) rotaviruses with picobirnavirus (PBV) simultaneously. Further, RVA was genotyped as P[6] and PBV as of genogroup I. Phylogenetic analysis clustered RVA, RVB and PBV with American bovine group A isolate, human Myanmar isolate and Chinese porcine isolate, respectively suggestive of respective species origin. This report is the first to describe a concomitant infection of PBV [genogroup I] with RVA and RVB. The investigation emphasizes that the presence of mixed infections should always be kept in mind in enteric infections with great demand of a reliable test that detects the presence of RVA, RVB and PBV simultaneously.

Key words: Viral Gastroenteritis, Concurrent Infection, RVA, RVB, PBV, Molecular Characterization.

Diarrhoea, the second-most-common cause of neonatal death worldwide kills 2,195 children every day, more than AIDS, malaria, and measles, if combined together[1]. It is caused by an array of microorganisms including viruses, bacteria, and parasites contributing to the disease burden either individually or in congruent way[2]. Among viral causes, rotaviruses (RVs) is the foremost cause of deaths in children up to 5 years of age[3] and have been detected predominantly in several animal species[4]. However, recently picobirnaviruses (PBVs) have been described to be associated with human diarrhoeal cases and other animal species from India as well as other parts of the world[5-7]. Together, concomitant infections pose more threat with fatal outcome than infections with sole pathogen[8,9]. We describe here a case of mixed viral infection seen in an infant travailing with acute gastroenteritis from Haldwani city, Uttarakhand, India.

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MATERIALS AND METHODS

Sample collection and processing

In August 2011, approximately 5 ml of fresh diarrhoeic stool sample from one month old male child hospitalized due to severe dehydration and diarrhoea was collected aseptically. The stool sample was processed as described in previous studies[7, 9, 10-12].

Molecular detection of enteric viruses

The stool sample was screened for different etiological agents of diarrhoea i.e. group A (RVA) and B (RVB) rotaviruses, astrovirus, calicivirus and picobirnavirus (PBVs) using a combination of conventional and molecular detection assays at Govt. Medical College, Haldwani and Enteric Virus Laboratory, Division of Virology, IVRI, Mukteswar Campus, Nainital, Uttarakhand, India. The viral extraction, silver staining and visualization of genome segments in electrophoresis were performed as described earlier[7, 9, 10]. RT-PCR was carried out following either the protocols described for detection of targeted viruses, as for astrovirus[13], calicivirus[14] and in-house optimized protocol for RV and PBV. The primers as listed in Table 1 were custom synthesized from Integrated DNA Technologies, Inc. (IDT), India.

Genotyping of enteric rotavirus and picobirnavirus

For genotyping of group A rotavirus (RVA), multiplex nested PCR was performed as per the method and primers described earlier[15-18] while for Picobirnavirus, the method described by Rosen et al.[19] were followed. The amplicons were visualized in ethidium bromide stained 1.5% agarose gel and documented using Transilluminator-UV®300 (UVP Inc., Upland, USA).

Sequence and phylogenetic analysis

The RT-PCR amplicons were cloned and sequenced by ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). The VP6 gene sequences of rotaviruses (RVA and RVB) and RdRp gene of Picobirnavirus along with same gene sequences of different species from India and across the world were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned by ClustalW and dendrograms were constructed with MEGA5 by neighbour-joining statistical method with 2000 bootstrap replicates[20].

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study for RVA and RVB have been submitted in the GenBank database under accession numbers; JX187432 and JX187433, but accession number could not be assigned to PBV being short sequence of less than 200 bp.

RESULTS AND DISCUSSION

The RVA associated infections remains a common enteric problem affecting young children and infants in developing countries[4], whereas RVB is more commonly known to be associated with the adult diarrhoea[21]. The association of RVB with

Table 1. List of primers used during the study for molecular detection of viral infections

Virus	Gene targeted	Sense primer (5'-3')	Antisense primer (5'-3')	Amplicon size (bp)	References
RVA	VP6 gene	TTTGATCAC TAAYTATTC ACC	GGTCACATCCT CTCACTA	227	This study
RVB	VP6 gene	ATCTACTGA TGATTAYGA TGA	TATTACCYGAC CAYCTRCCAA	343	This study
Astrovirus	ORF1b	ACTGCCTRT CWCGGACTG	TGTGACACCYT GTTTCCT	328	Brown et al.[13]
Calicivirus	Polymerase gene	ATACCACTA TGATGCAGATTA	CATCATCACCA TAGAAAGAG	326	Vinje et al.[14]
Picobirnavirus	RdRp gene	YGGTRTNCA TGGTATGGG	YSCAYTACATC CTCCAC	179	This study

diarrhoea in children has been reported earlier from Kolkata[22] and Bangladesh[23]. Epidemiologically, most of the PBVs belong to GI, which infect a wide variety of human and animal species. PBVs GI have been detected in human[24], bovines[7] and pigs[6] from Indian subcontinent indicating the predominance of this genotype. However, its co-existence has never been reported with either rotavirus group A or B. During the investigation, laboratory examination revealed association of some viral cause of the severe gastroenteritis affecting the child. Multiple genomic segments migration patterns in RNA-PAGE analysis with more than fifteen bands also supported existence of multiple viruses in this patient. To determine the causative agents, we performed RT-PCR for the most common causes of gastroenteritis i.e. RVA, RVB, astrovirus, calicivirus and PBV. The amplicons generated by PCR were of 227 bp, 343 bp and 179 bp indicative of presence of RVA, RVB and PBV, respectively. The sample was negative for calicivirus and astrovirus. Further genotyping for RVA revealed P[6] genotype while VP7 gene was untypable. The PBV was typed as genogroup I with the amplicon size of 201 bp.

Phylogenetic analysis of RVA based on the partial VP6 gene sequence clustered the human isolate of this study with group A bovine isolate from USA (GenBank Acc. No. AF411322) with 100% sequence similarity at both nucleotide and amino acid levels (Fig.1A). Similarly RVB strain was matching with human Myanmar strain (GenBank Acc. No. FJ811827) (99.1% and 98.2% at nucleotide and amino acid level, respectively) and distantly related to India bovine strains (Fig.1B). The PBV isolate clustered with porcine isolate of China (88.2% and 94.1% at nucleotide and amino acid levels, respectively) (Fig.1C). Sequence similarity analysis showed that mixed infection of these three viruses (RVA, RVB and PBV) detected in an infant might have origin from bovine, human and porcine species, respectively.

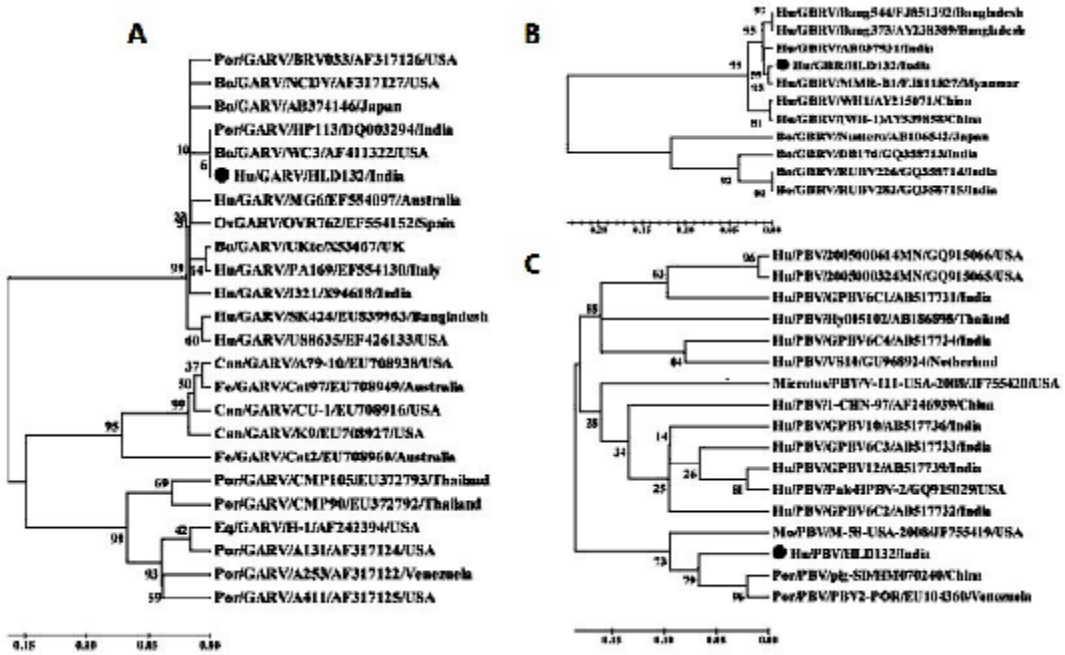

Fig. 1(A). Genetic relatedness of the VP6 genes of human RVA of this study indicated as (´) with other VP6 gene sequences of different host species retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). **1(B).** VP6 genes sequences of human RVB of this study indicated as (´). **1(C).** RdRp gene sequences of human Picobirnaviruses of this study indicated as (´). Phylogenetic tree was constructed based on neighbor-joining (NJ) method implemented in MEGA5 (http://megasoftware.net/). The bootstrap values (2000 replicates) are indicated at the nodes of the branches. Host species depicted are human (Hu); bovine (Bov); porcine (Por); canine (Can); equine (Eq); feline (Fe); ovine (Ovi); mouse (Mo). For each strain, the following data are given: species/virus name/strain name/accession number/country origin

The severity of acute gastroenteritis due to co-infections of RVs with PBVs could be fatal if not handled timely. Still more clinical evidence is required to demonstrate complex interactions between these different viruses belonging to different families. As this is a preliminary work reporting first time the detection of multiple (triple) infections of PBV of genogroup I specificity and RVA and RVB in the diarrhoeic patient, further studies are warranted to confirm the pathogenicity of these viruses which has not been established especially for PBVs.

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