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Novel human astrovirus strains showing multiple recombinations within highly conserved ORF1b detected from hospitalized acute watery diarrhea cases in Kolkata, India

Madhusudhan Pativada, Rittwika Bhattacharya, Triveni Krishnan *

Division of Virology, National Institute of Cholera and Enteric Diseases (NICED), P-33, C.I.T. Road, Scheme-XM, Beliaghata, Kolkata 700 010, West Bengal, India

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ABSTRACT

Human astroviruses (HAstVs) associated with acute watery diarrhea among hospitalized infants, children and adults as sole or mixed infection, were earlier reported from Kolkata, India. Further, novel recombinations have been detected through sequencing of the highly conserved ORF1b (RdRp) region of seven human astrovirus strains in Kolkata, India. Primers were designed and the ORF1b region was amplified by RT-PCR and sequenced. To examine the evolutionary pressures influencing the evolution of human astroviruses we implemented evolutionary genetics analysis. Maximum recombination break points detected in Kolkata strain IDH1300 were 8 and a single break point location was detected at 1205nt position. Partition-wise phylogenetic analyses of the IDH1300 Kolkata strain did not show close homology to the reference strains. Further phylogenetic analyses of full length ORF1b region of the seven human astrovirus strains showed that they formed a close cluster with each other and displayed a separate lineage in comparison to reference human astrovirus strains worldwide. This study shows the emergence of novel recombinant human astrovirus strains in Kolkata, India, warranting stringent surveillance to monitor the genetic diversity of human astrovirus strains infecting different age groups.

1. Introduction

Human astroviruses (HAstVs) are presently recognized as an important etiological agent of acute gastroenteritis (AGE) among infants, young children, adults and immunocompromised patients worldwide besides rotavirus (Matsui and Greenberg, 1996). Human astrovirus strains have also been reported to infect a wide range of domestic, synantropic and wild animals in terrestrial as well as aquatic environments (De Benedictis et al., 2011). The family *Astroviridae* comprises two genera, *Mamastrovirus* (MAstV), which infects mammalian species and *Avastrovirus* (AAstV), which infects avian species. Recently, the *Astroviridae* family has officially been classified by the International Committee on Taxonomy of Viruses (ICTV) based on the full length amino acid sequences of the ORF2 (capsid) region. The ICTV official classification showed three species of *Avastrovirus* [Avastrovirus 1 (GI.A), Avastrovirus 2 (GI.B) and Avastrovirus 3 (GII.A)] and nineteen species of *Mamastrovirus* [Mamastroviruses 1 (GI.A-Human), Mamastrovirus 2 (GI.B-Feline), Mamastrovirus 3 (GI.C-Porcine), Mamastrovirus 4 (GI.D-California sea lion), Mamastrovirus 5 (GI.E-Canine), Mamastrovirus 6 (GI.F-Human), Mamastrovirus 7 (GI.G-Bottlenose dolphin), Mamastrovirus 8 (GII.A-HMOAstV-A and HAstV-VA2), Mamastrovirus 9 (GII.B-HMOAstVs-B and-C and HAstV_Va1), Mamastrovirus 10 (GII.C-MiAstV), Mamastrovirus 11 (GII.D-California sea lion AstV-1), Mamastrovirus 12 (GII.E-BatAstV) Mamastrovirus 13 (GII.F-OAstV), Mamastroviruses 14 to 19 (GII.G to GII.L-BatAstVs)]. The astrovirus virions are 28–30 nm in diameter, icosahedral, *non*-enveloped with single stranded positive (+) sense genomic RNA of approximately 6.8–7.9 kb. The genome consists of untranslated (UTR) regions at 3′ and 5′ ends, and a poly (A) tail at 3′ end. The viral genome comprises three overlapping open reading frames (ORFs): ORF1a, ORF1b and ORF2. In Astroviruses, the ORF1a encodes the non-structural serine protease (nsP1a) and ORF1b encodes the RNA dependent RNA polymerase (nsP1b) and both are flanked by ribosomal frameshift mechanism event. In astroviruses, the ORF2 region is translated from a sub genomic RNA and encodes capsid protein precursor (Carter and Willcocks, 1996; Lewis et al., 1994). Genome analysis of astroviruses has shown that ORF1b is most conserved and ORF2 is highly divergent due to selective pressure in this region. To date, eight serotypes of human astroviruses identified in humans displayed good correlation with eight known human astrovirus genotypes in the phylogenetic grouping of nucleotide sequence analyses of limited partial ORF2 region (Belliot et al., 1997; Méndez-Toss et al., 2000). Recently, two novel Mamastrovirus 6 species (GI.F and AstV–MLB2) identified in patients displayed close genetic relatedness to urban

* Corresponding author. Tel.: +91 33 2363 3852; fax: +91 33 2363 2398.
E-mail addresses: drtriveni.krishnan@gmail.com, venihics@yahoo.com (T. Krishnan).

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brown rat astroviruses identified in Hong Kong (Chu et al., 2010). Further, three novel Mamastrovirus 8 and 9 species (GII.A and GII.B) were detected in human stool samples from Nepal, Pakistan and Nigeria (Kapoor et al., 2009). Several molecular epidemiological studies indicated that human astrovirus genotype 1 (MAstV_G1) strains are most predominant, human astrovirus genotypes 2–4 (MAstV_G2–MAstV_G4) are also often reported (Jeong et al., 2011). In recent years, evidence of recombination within the classic HAstV strains has been detected in astroviruses infecting humans. A recombinant wild type human astrovirus strain reported from a Kenyan child with acute gastroenteritis showed close genetic relatedness to HAstV_6/7 within ORF1a, ORF1b and clustered with HAstV_3 whereas ORF2 resembled HAstV_2 (Wolfaardt et al., 2011). Ahmed et al. (2011) reported novel recombination events in human astrovirus strains from Egyptian children with close genetic relatedness to VA2 strain (ORF1b) and MLB1 strain (ORF2). Further, a recombination event between porcine astrovirus (PAstV) and human astrovirus genotype 3 (HAstV_G3) was reported from piglets and children from various regions of Colombia (Ulloa and Gutiérrez, 2010). They identified a high predominance of porcine astrovirus strains (PAstVs) in pigs that was closely related to human astrovirus strains. This study describes the interesting changes and genetic diversity within highly conserved complete ORF1b region from seven human astrovirus positives in the course of genetic characterization of human astrovirus strains causing acute gastroenteritis (AGE) among infants, children and adults in Kolkata, India.

2. Materials and methods

2.1. Sample collection

Seven out of 60 positive samples (single or mixed infection) for humanastrovirus by RT-PCR collected from Nov' 2007 to Oct' 2009, from hospitalized infants, children and adults admitted to Infectious Diseases & Beliaghata General Hospital, Kolkata, India with severe acute gastroenteritis (AGE), were included in this study.

The fecal samples were collected after getting approval from the institutional ethics committee of National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India for the study.

2.2. Virus suspension preparation

Each fecal sample was collected aseptically and directly transferred into a clean sterile collection container; the specimen was suspended [30% w/v] in 1× Phosphate buffered saline (PBS, pH 7.4) and thoroughly vortexed before slow centrifugation at 1400*g* for 20 min; the supernatant was next transferred into a fresh microfuge tube and centrifuged at 30,700*g* for 20 min. The pellet was discarded and the supernatant [virus suspension] was immediately transferred into a fresh microfuge tube and stored at 4 °C for RNA extraction.

2.3. RNA extraction and RT-PCR

The viral RNA was extracted with QIAmp Viral RNA extraction kit (QIAGEN, GmbH, Hilden, Germany) according to manufacturers' guidelines. At first, astrovirus-positive cases were confirmed by RT-PCR (Matsui et al., 1998) with the primer pair viz. forward primer PRECAP [+]: 5′-GGACTGCAAAGCAGCTTCGTG-3′ and reverse primer 82b [−]: 5′-GTGAGCCACCAGCCATCCCT-3′ that targeted to amplify and generate 719 bp amplicon of all known HAstV genotypes. Further, the cDNA synthesis step was performed for full length highly conserved ORF1b region of the seven Kolkata strains of human astrovirus under the following conditions: 25 °C for 10 min, 42 °C for 60 min and denaturation step was carried out at 72 °C for 10 min. The cDNA of full length ORF1b region was amplified and sequenced with designed primers by PCR method. The PCR conditions and size of amplicons of human astrovirus strains are shown in Table 1. The amplicon fragments covering the complete ORF1b for these human astrovirus strains were loaded in separate wells on 2% agarose gel and stained with 0.5 μg/ml ethidium bromide, visualized and documented in GelDoc unit (BioRad, Japan).

2.4. Nucleotide sequencing and phylogenetic analyses

PCR amplicons were purified and sequenced using ABI prism 3100 Genetic Analyzer (PE, Applied Biosystems, Foster City, California, USA) and Big Dye® Terminator v3.1 cycle-sequencing kit (Applied Biosystems, USA) according to manufacturer's protocol. Fig. 1 provides a schematic representation of nucleotide sequencing, phylogenetic and recombination analyses of Kolkata human astrovirus strains. The nucleotide sequence data was manually read by Finch TV Version 1.4.0 (Geospiza). The complete consensus sequence of conserved ORF1b of seven astrovirus strains was obtained for comparison with all available astrovirus reference strain sequences in the GenBank database (http://www.ncbi.nih.gov/blast). The deduced amino acid sequence was next obtained from nucleotide sequence with the help of DNASIS software program. The LAlign program (version 2.0) (Huang and Miller, 1991) was performed for global alignment of consensus with reference sequences.

The pair-wise nucleotide and amino acid sequence alignment was performed by the ClustalW program (Thompson et al., 1994). The phylogenetic analysis was carried out with MEGA 5.05 version software (Tamura et al., 2011). Phylogenetic distances were measured by the Jukes–Cantor model and phylogenetic trees were statistically supported by bootstrap test value of 1000 replicates.

2.5. Recombination analyses

In this method, the clustalW aligned files were first uploaded to Datamonkey for detection and identification of any potential recombination event and best nucleotide substitution bias model for the Kolkata strains. In Datamonkey, GARD was initially performed with an automatic model selection tool which showed that

Table 1
Amplification conditions of primers used to characterize the highly conserved ORF1b region of human astrovirus strains in this study.

Primer pair	Amplicon size (bp)	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Prot10 [+]: 5′-AAGGGCCCAGAAGACCAA-3′ R1 [−]: 5′-CCTCTAAGCACGCCCCTAT-3′	645	94 °C–4 min	94 °C–60 s	56 °C–60 s	72 °C–50 s	72 °C–7 min
Rd1 [+]: ATAGGGCGTGCTTAGAG-3′ R2 [−]: 5′-AAATAAGCAAACTCAAAAGCC-3′	419	94 °C–3 min	94 °C–45 s	51 °C–45 s	72 °C–45 s	72 °C–7 min
Rd2 [+]: 5′-GGCTTTTGAGTTTGCTTATTT-3′ R3 [−]: CTAGCCATCACACTTCTTTG-3′	508	94 °C–5 min	94 °C–50 s	52 °C–50 s	72 °C–50 s	72 °C–7 min