Evidence for Occurrence of Human group B rotavirus in Central India Based on Characterization of NSP2 Gene

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Abstract Human group B rotavirus (HuGBR) was first described as the causative agent of severe gastroenteritis, affecting millions of people in China during 1982–1983. In spite of serological evidences for the presence of HuGBR in many countries of the world, the virus has only been detected from China, Bangladesh and some parts of India. The present study describes a HuGBR (designated as MP-1 isolate) which was confirmed in an adult patient suffering from gastroenteritis in 2008 in Madhya Pradesh, central India. The RNA electrophoresis in polyacrylamide gel (RNA-PAGE) and NSP2 gene based RT-PCR assays and later sequencing was used to confirm the isolate. The nucleotide and deduced amino acid sequences of this HuGBR (MP-1) isolate were analyzed and their relationship with corresponding gene of other Indian, Bangladeshi and Chinese HuGBR and animal group B rotaviruses (AnGBR) was determined. The isolate showed a typical RNA banding pattern of 4:2:2:3 in RNA-PAGE which was indicative of group B rotaviruses (GBR). The sequence comparison of MP-1 isolate with NSP2 gene revealed that MP-1 isolate had 98.6 and 97.7% nucleotide sequence homology and 93.8% amino acid similarity with Bang373 and CAL-1 strains, respectively. The nucleotide and amino acid sequence

similarity of MP-1 isolate with one of the Chinese ADRV (WH-1) strain was 92.8 and 92.5%, respectively. While sequence homology with another Chinese strain ADRV (J19) was considerably lower (45.6 and 48.3%, respectively). The percent identity with AnGBRs (porcine and murine) was also lower at nucleotide and amino acid level (66 to 80%). The phylogenetic analysis suggested that MP-1 isolate is closer to Bangladeshi (Bang373) as compared to Indian strain (CAL-1). Our findings indicated that MP-1 isolate might have originated from a common ancestral HuGBR virus but distinct from AnGBR lineage. Occurrence of GBR in other parts of India warrants further epidemiological and molecular studies to develop effective control strategies for GBR infection in adults as well as children.

Keywords Rotavirus · Group B · NSP2 gene · Sequence analysis

Introduction

Rotavirus is one of the main causes of acute viral gastroenteritis in humans and animals worldwide [17]. It belongs to the family *Reoviridae* and characterized by 11 segmented dsRNA genome [12]. It is classified into seven distinct serogroups (A to G), of which all groups (A to G) infect animals; whereas humans are infected mainly by group A, B and C. The group B rotaviruses (GBR) which predominantly infect human adults and a variety of animal species [3, 9, 16] are considered as emerging enteric pathogens. The GBR were first time reported during 1982–83 from a large epidemic of adult human diarrhoea in China and named as adult diarrhoea rotavirus (ADRV) [10]. Though, seroepidemiological studies have demonstrated that GBR infection is widespread in different parts

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of the world [16], still epidemics of human diarrhoea have not been reported outside China, Bangladesh and India.

In India, Krishnan et al. [15] first identified GBR in five sporadic cases of adult diarrhoea in Kolkata during 1997–1998 and named CAL-1. This was the first description of HuGBR cases outside China. This GBR strain was found genetically more close to Chinese ADRV strain [14, 20]. Later, in 2004, a report from Pune, Maharashtra, also described 3 HuGBR strains from adult diarrhoea, the partial nucleotide sequences of NSP2 gene of these strains showed a high nucleotide sequence homology to CAL-1 [13]. Except these reports, information is lacking on the occurrence of GBR from other parts of India. RT-PCR assay was used as a method of choice for the detection of GBR [6]. The present study describes detection and characterization of HuGBR isolated from central India (Madhya Pradesh) based on RNA electrophoresis in polyacrylamide gel (RNA-PAGE), RT-PCR, restriction digestion analysis (RFLP) and NSP2 partial gene sequence analysis.

Materials and Methods

Stool Samples

During the routine epidemiological survey for animal and human group A rotaviruses in Madhya Pradesh (2007–08), one sample (MP-1) was collected from a 28 years old woman suffering from gastroenteritis. She was mildly dehydrated and having stool frequency three to four times a day.

Extraction of Viral RNA for PAGE and RT-PCR

For extraction of viral nucleic acid, 10% faecal suspension (v/v) was prepared in phosphate buffer saline (PBS; pH 7.2) and centrifuged at 10,000 rpm for 30 min. From supernatant, 1 ml was used for RNA extraction using GIT lysis method [4]. The isopropanol precipitated RNA was pelleted and washed with equal volume of 70% pre-chilled ethanol by centrifugation at 12,000 rpm for 5 min at 4°C. The air dried pellet was dissolved in 2× RNA lysis buffer for viral RNA genomic electrophoresis and for RT-PCR assay in nuclease free water (NFW) separately. The viral RNA was quantified using Nanodrop Spectrophotometer (Thermo Scientific, USA).

Viral RNA Electrophoresis

The RNA electrophoresis in polyacrylamide gel was carried out for the initial detection of rotavirus dsRNA genome segments. The electropherotyping of viral RNA was carried out in 10% polyacrylamide gel (PAGE) by loading 500 ng

viral RNA per well. Samples were electrophoresed at 100 V using Tris–Glycine buffer. The gel was stained with silver nitrate as described by Herring et al. [8] and photographed.

Oligo-Nucleotide Primers

The virus detection was confirmed by RT-PCR assay based on NSP2 gene with GBR specific primers as described by Gouvea et al. [6]. Primers B1 (nt 18–40 bp, CTATTCA GTGTGT CGTGAGAGG) and B3 (nt 430–451 bp, CGAAGCGGGCTAGCTTGTCTGC) were used to amplify the 434 bp product of NSP2 gene, whereas B1 and B4 (nt 485–506 bp, CGTGGCTTTGG AAAATTCTTG) were used to amplify the 489 bp product.

RT-PCR Assay

RT-PCR assay was standardized to amplify GBR partial NSP2 gene sequence. Single tube BioRT one step RT-PCR Kit (Taurus Scientific, USA) was used for the cDNA synthesis and later PCR. Initially viral RNA was heated at 65°C for 5 min in Thermocycler (GeneAmp®, PCR System 9700, Applied Biosystems, USA). The master mix and template were mixed together with B1 and B3 primers for 434 bp product and B1 and B4 primers for 489 bp product and following RT-PCR conditions were used: 30 min at 42°C for reverse transcription followed by 5 min denaturation at 94°C and 30 cycles of 94°C for 1 min (denaturation), 42°C for 2 min (annealing), 72°C for 1 min (extension) with final extension step of 72°C for 7 min. The RT-PCR products were visualized under UV Transilluminator and documented.

Sequencing

The sequencing of the RT-PCR amplified product was carried out using ABI Prism Big Dye Terminator Cycle Sequencing kit with an ABI Prism 3100 automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystem, USA) using forward primer (B1).

The nucleotide sequence determined in this study has been deposited in the NCBI GenBank sequence database with accession number EU436849.

Sequence Analysis

Sequence analysis of the sample was carried out using multiple alignment program MEGA4 and DNASTAR (CLUSTAL W). The evolutionary lineage history was inferred using the Neighbor-Joining method with the bootstrap consensus tree inferred from 1000 replicates being taken to represent the evolutionary history.

PCR–RFLP

Further characterization of MP-1 isolate of HuGBR was carried out by direct digestion of PCR products of partial length NSP2 gene using *Hinf* I (MBI Fermentas, USA) restriction enzyme. The restriction enzyme was selected from the restriction map created using the sequence available in GenBank and restriction mapper version 3 software. The RT-PCR and PCR–RFLP products were analyzed by electrophoresis on 2% agarose gel in running buffer containing ethidium bromide and visualized under UV Transilluminator and photographed.

Results and Discussion

Group B rotaviruses predominantly infect adults and have emerged as important enteric pathogens during past few years [15]. However, Barman et al. [2] detected GBR in children during a course of surveillance conducted in Kolkata [2] emphasizing shift in age preference of GBR infection from adult to children. Study showed 37 (18.5%) sporadic cases of HuGBR in children below 3 years of age, of which 15 (7.5%) showed mixed infection with group A rotaviruses. The authors suggested inclusion of both group A and B rotaviruses in future vaccination strategy to control rotavirus diarrhoea. High prevalence of GBR has also been reported in humans from Australia, USA, England, and China [18]. It is predicted that exposure to GBR might be frequent, however it remains undetected due to difficulty in growing the virus or degradation of the virus particles in stool [22]. Hence, there is paucity of information on GBR epidemics [1, 14, 21]. GBR is identified by RNA electrophoresis in polyacrylamide gel and RT-PCR [2, 5, 6].

In India, sporadic cases of HuGBR have been noticed [13, 15] and similar observations were recorded initially from China, before the epidemic of GBR occurred, affecting more than 1 million people during 1982–83 [23]. In India, HuGBR was reported from Kolkata (CAL strain) and Pune (NIV-HuGBR) and the isolates have been genetically characterized [13, 15]. Approximately 300 km from Kolkata, a HuGBR isolate (Bang373) was reported from Mymensingh, Bangladesh by Sanekata et al. [19].

During the present study, the viral RNA extracted from an adult gastroenteritis case displayed typical RNA banding pattern of 4:2:2:3 indicating the presence of GBR (Fig. 1a, b). The reference GBR RNA banding pattern is shown in Fig. 1c, which was used initially to match the migration pattern of present HuGBR from MP. The MP-1 isolate showed long electropherotype migration pattern with segment 3 and 4 co-migrating, segments 5 and 6 migrating very close and genome segments 7, 8, 9, 10 and 11 migrating distinctly (Fig. 1a, b). The migration pattern

Fig. 1 Comparative RNA banding profiles of Group B rotavirus (GBR) with HuGAR and AnGAR. **a** Migration pattern of HuGBR (4:2:2:3) with long electropherotype AnGAR (4:2:3:2). **b** Migration pattern of HuGBR with short electropherotype human group A rotavirus. **c** RNA banding pattern of reference GBR (4:2:2:3)

of GBR compared with group A rotaviruses obtained from humans (short electropherotype) and animals (long electropherotype) showed a pattern of 4:2:3:2 with clear difference in migration of various genome segments. The migration of GBR genome segments 5 and 6 were faster in comparison to those of group A rotaviruses, while migration of segment 7 was slower than that of group A rotavirus. The movement of genome segments 7, 8, 9, 10 and 11 was in a ladder format, which is similar to a reference GBR RNA banding pattern (Fig. 1c). The RNA-PAGE results indicated that the MP-1 isolate is GBR.

NSP2 gene recommended by earlier workers for detection of GBR [2, 13, 15, 20] was also targeted with a set of previously described primers [6] for detection and confirmation of the present HuGBR strain from MP. These primers were found highly specific for NSP2 gene of MP-1 isolate of HuGBR, as these did not amplify the heterologous group A rotavirus. The expected partial NSP2 gene products of 434 bp and 489 bp were obtained (Fig. 2a). The restriction enzyme (RE) analysis has been used for the characterization of rotaviruses [7]. In the present study, RE analysis of partial NSP2 gene products using *Hinf*I restriction enzyme indicated the presence of two sites as evidenced by presence of three fragments of 250, 180 and 70 bp size (Fig. 2b). Similar RE pattern was obtained *in silico* with other HuGBR strains using *Hinf*I restriction enzyme (Bang 373, CAL-1 and ADRV (WH-1)).

Fig. 2 a RT-PCR of Human group B rotavirus strain (MP-1). *Lanes-1* 50 bp DNA ladder, *2* PCR product with B1–B3 primers (434 bp) and *3* PCR product with B1–B4 primers (489 bp), **b** PCR–RFLP of NSP2 gene of MP-1 strain. *Lanes-1* Uncut PCR product, *2* 50 bp DNA ladder, *3* Hinf I digested products

Table 1 Percent identities of nucleotides (upper right) and deduced amino acids from ORF portions (lower left) between NSP2 gene of MP-1 strain and those of other group B rotaviruses of human and animal origin

Strain (Origin)	MP-1	Bang-373	CAL-1	ADRV(WH-1)	ADRV(J19)	PRVBRA-16	IDIR
MP-1 (Human)	–	98.6	97.7	92.8	45.6	66.4	75.2
Bang373 (Human)	93.8	–	–99.2	94.5	46.6	66.4	76.1
CAL-1 (Human)	93.8	100	–	93.8	46.6	65.8	76.3
ADRV (WH-1) (Human)	92.5	98.7	98.7	–	47.2	67.8	76.6
ADRV (J19) (Human)	48.3	50.3	50.3	51.0	–	43.7	46.6
PRV BRA-16 (Porcine)	74.4	74.4	74.4	74.4	48.3	–	62.2
IDIR (Murine)	80.8	86.7	86.7	86.6	50.3	74.4	–

Sequencing results obtained with forward BI primer

GenBank accession numbers MP-1 (EU436849), CAL-1 (AY238383), Bang373 (AY238393), ADRV (WH-1) (AY 539861), ADRV (J19) (DQ113903), IDIR (DU03558) and PRV BRA-16 (EF577259)

The sequences of MP-1 isolate of GBR obtained after unidirectional partial sequencing of NSP2 gene with forward B1 primer were compared with 4 human and 2 animal GBRs. Percent identities of nucleotides (upper right) and deduced amino acids from ORF portions (lower left) between NSP2 gene of MP-1 isolate and those of other GBRs of human and animal origin are shown in Table 1. Analysis of the sequences of GBR strains showed that nucleotide sequence identity of MP-1 isolate of HuGBR was very high with Bang373 and CAL-1 (98.6 and 97.7%, respectively) and amino acid identity more than 93% with Bang373 and CAL-1 strains. The nucleotide and amino acid sequence identities with Chinese ADRV (WH-1) strain isolated in 2002 from Wuhan [24] was 92.8 and 92.5%, respectively, while identities with another Chinese ADRV strain (J19) [11] was considerably lower (45.6 and 48.3%, respectively). The sequences

among Indian (MP-1 and CAL-1) and Bangladeshi (Bang 373) strains were more conserved, while point mutations were observed at several nucleotide positions (63, 66, 72, 76, 87, 144, 151 and 155) in ADRV (WH-1) strain, with change in amino acid at 26 (amino acid changed from V to I) and at 51 (amino acid changed from S to A) (Fig. 3a). Yang et al. [24] proposed that Bangladeshi and Indian strains have not evolved from Chinese 1982 strain and these might have been prevalent in these countries before 1982.

The percent identity of MP-1 HuGBR strain with AnGBRs (PRV BRA 16 - porcine origin and IDIR-murine origin) was also lower at nucleotide and amino acid levels (66–80%). This observation supported the previous findings which indicated that AnGBRs may be different genotype than the genotype which infects humans [24]. Despite the low nucleotide sequence identity of HuGBR MP-1 isolate

Phylogenetic analysis of the sequence data indicated that MP-1 isolate is more close to Bangladeshi strain (Bang373), whereas Indian (CAL-1) was closer to ADRV (WH-1) strain. Phylogenetic tree clustered MP-1 isolate with Bangladeshi and Indian strains, and away from Chinese ADRV strains (WH-1 and J19) and AnGBR (Fig. 4). The findings of present study indicated that MP-1 isolate might have a common origin from where other Indian strains of HuGBR have emerged but distinct from AnGBR. This is the first report of detection of HuGBR from central India. Looking into the shift in the age preference of GBR from adults to children and spread to other parts of country, more extensive epidemiological and molecular investigations are required to establish the prevalence of

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