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Isolation and genotypic characterization of rotavirus

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Two out of 12 fecal samples from children were found to be positive for rotavirus. Both the strains of rotaviruses were successfully isolated on MA 10^4 cell line where characteristic CPE was observed after 3 passages. The presence of virus was confirmed by SDS-PAGE and RT-PCR. Genotypic characterization was carried out by amplification of *VP7* and *VP4* genes followed by 2 separate multiplex PCR for G genotyping and another 2 for P genotyping using genotype specific primers. These isolates were characterized as G1P[6] and G11P[8] by RT-PCR and confirmed by nucleotide sequencing. Epidemiological investigation in the field need to be performed continuously at molecular level to ascertain whether G11P[8] specificity will predominate in human, and also to know the nucleotide exchange between porcine and human strains of rotavirus. Such type of emergence of noval strains may lead to change or update of exhisting anti-rotaviral vaccine component strains. Therefore, proper molecular epidemiological investigation is required not only in humans but also in animals.

Key words*:* Genotype, Isolation, Rotavirus, RT-PCR, SDS-PAGE

Within enteric viruses, rotaviruses have been recognized as the most common cause of severe gastroenteritis worldwide. Out of 7 groups, group A rotaviruses are considered as an important viral diarrhoeal agents in children and young animals. It can be further typed based on the proteins of the outer capsid that elicit neutralizing antibodies- VP7 (G serotypes) and VP4 (P serotypes) or based on sequence comparison as G genotypes or P genotypes. To date, 23 distinct G genotypes and 31 P genotypes have been identified (Mukherjee *et al*. 2010). It has been documented in the previous studies that, there is significant diversity of rotavirus strains along with prevalence of unusual strains in India. The most prevalent strains in India are G1, G2, G3, G4 and G9 and P[8], P[6] and P[4], however some other G types strains such as G6, G8, G10 and G12 have also been identified as cause of diarrhoea (Ramani and Kang 2007). Therefore it is very important to standardize the tissue culture method for isolation of rotavirus as well as PAGE and RT-PCR for detection of rotavirus from fecal samples to use these methods for screening of the various fecal samples collected from humans and animals.

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MATERIALS AND METHODS

In this study, we isolated rotavirus from diarrhoeal stool samples collected from children suffering from acute gastroenteritis. Upon isolation, these strains were confirmed by SDS-PAGE, RT-PCR and characterized as G and P genotypes. We also presented the partial nucleotide sequence of the *VP7* and *VP4* genes and compared them with those of the corresponding genes of human rotavirus strains.

Isolation of rotavirus by cell culture: Isolation of rotavirus from stool samples was carried out as per Babiuk *et al.* (1977) with little modifications. SDS-PAGE and RT-PCR positive fecal samples were diluted to 20% suspension in phosphate-buffered saline (PBS) and centrifuged at 10,000g for 20 min, and the supernatant was filtered through 0.45 μm membrane filter. The samples were then treated with trypsin (20 μg/ml) for 30 min at 37ºC and inoculated in duplicate on washed MA-10^4 cell monolayers. Viruses were allowed to adsorb for 1 h at 37ºC with intermittent shaking and the medium containing unadsorbed virus was decanted and discarded carefully. DMEM maintenance medium (6 ml) was added into each flask and further

Table 1. The genes targeted in PCR with the cyclical conditions

Targeted Gene	PCR Method	Primers	Cyclical conditions		Reference
VP7 gene (Partial)	—	Rota1 (F) Rota 2 (R)	94°C for 5 min 94°C for 1 min 55°C for 1 min 72°C for 2 min 72°C for 7 min	30 cycles	Husain *et al.* (1995)
VP7 gene (Full length)	—	Beg9 (F) End 9 (R)	94°C for 1 min 55°C for 1.30 min 72°C for 1.30 min 72°C for 7 min	30 cycles	Gouvea *et al*. (1990)
VP7 gene	1st Multiplex	9T1-1, 9T1-2, 9T-3P, 9T-4, 9T-9B	94°C for 1 min 42°C for 2 min 72°C for 1 min 72°C for 7 min	30 cycles	Gouvea *et al*. (1994 a, b)
	2nd Multiplex	sBeg9, FT5, DT6, HT8, ET10, BT11	94°C for 1 min 55°C for 2 min 72°C for 1 min 72°C for 7 min	30 cycles	
VP4 (Partial)	—	Con 3 (F) Con2 (R)	94°C for 1 min 48°C for 2 min 72°C for 2 min 72°C for 7 min	30 cycles	Gentsch *et al.* (1992)
VP4 Gene	3rd Multiplex	1T-1, 2T-1, 3T-1, 4T-1	94°C for 1 min 48°C for 2 min 72°C for 2 min 72°C for 7 min	30 cycles	Gouvea *et al*. (1994b)
	4th Multiplex	Con2, pNCDV, pUK, pGott, pOSU, pB233	94°C for 1 min 55°C for 2 min 72°C for 1 min 72°C for 7 min	30 cycles	

incubated at 37ºC. The daily observations were undertaken for the appearance of cytopathic effect (CPE). The monolayers showing about 90% CPE were harvested by repeated freeze thawing and presence of rotavirus was confirmed by SDS-PAGE and RT-PCR.

Extraction of dsRNA and detection by PAGE and RT-PCR: dsRNA was extracted by phenol: chloroform: isoamyl (25: 24: 1) alcohol method (Sambrook and Russel 2001) from 1 ml of fecal suspension before isolation and 500 µl cultured medium after isolation of rotavirus. The RNA pellet was suspended in 20 µl of RNA stabilizing solution and stored at –20°C for further use. The extracted dsRNA was subjected to SDS-PAGE (7.5% resolving gel and 5.0% stacking gel) as per Laemmli (1970) and Herring *et al*. (1982) with little modifications followed by silver staining (Kumar 2006) to determine the presence of rotavirus. The group A rotavirus was detected by using the 2 primers (Rota1 and Rota 2), which were chosen from the region of gene segment 9 (Husain *et al*. 1995). The details of the genes targeted in PCR with the cyclical conditions are given in Table 1. Briefly, for partial amplifications of gene segment 8 or 9 (*VP7* gene) of all group A rotaviruses, 5 µl portion of each extracted rotavirus dsRNA was used for reverse transcription to synthesize 25 µl cDNA copies from both the strands. The denaturation of dsRNA was carried out in 14 µl reaction mixture, which consists of 2.5 µl of 5× RT buffer (containing 250 mM Tris-HCL, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$ and 50 mM dithiothreitol), 1.5 µl dimethyle sulfoxide, 10 pM each of rota 1 and rota 2 primers, extracted dsRNA and nuclease free water in DEPC treated PCR tube, kept at 95°C for 5 min in thermocycler followed by quick chilling on ice for 2 min. The master mixture for RT was prepared separately @11 µl per reaction which consists of 0.2 mM dithiothreitol (DTT), 0.4 mM deoxynucleoside triphosphates, 20 units of RNAse inhibitor and 20 units of M-MuLV reverse transcriptase. The reaction tubes were incubated for 1 h at 37°C followed by 10 min incubation at 65°C to inactivate the enzyme. The PCR reaction was carried out in total volume of 25 µl, which contained 5 µl of cDNA, 10X PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 15 mM $MgCl_2$), 0.2 mM concentrations of the deoxynucleoside triphosphates, 10 pM each of rota 1 and rota 2 primers and 1.0 U of *Taq* DNA polymerase. After polymerization, the amplification products were analyzed by electrophoresis on 1.5% agarose gel containing 2 µl ethidium bromide (10 mg/ml) in Tris-borate buffer and was visualized with UV transilluminator and photographed by gel documentation system.

G and P typing by RT-PCR: G genotyping was carried out by amplifying full length *VP7* gene as per the procedure described by Gouvea *et al.* (1990) and (Gouvea *et al*. 1994 a, b), with little modifications followed by 2 multiplex PCRs

(Gouvea *et al.* 1994a, Das *et al*. 1994). Briefly, the RT-PCR was carried out in the similar way as described earlier with the exception of primers (Beg9End9 instead of Rota 1 Rota 2). All the PCR products were diluted for 50 times and subsequently used for identification of G genotype by performing 2 separate semi-nested multiplex PCRs. The first multiplex PCR was carried out in a final volume of 25 µl using diluted PCR product, type specific primers [9T1–1 (G1), 9T1–2 (G2), 9T-3P (G3), 9T–4 (G4), and 9T-9B (G9) each at a concentration of 10 pM] in a reaction mixture containing 10X PCR buffer, dNTP and *Taq* DNA polymerase at same concentration as described earlier. Similarly, second multiplex was carried out with the exception of primers [sBeg9, FT5 (G5), DT6 (G6), HT8 (G8), ET10 (G10) and BT11 (G11)].

P genotyping was carried out by amplifying partial length *VP4* gene as per Gentsch *et al*. (1992) with little modifications followed by 2 multiplex PCRs (Gouvea *et al*. 1994b). Briefly, the RT-PCR was carried out in the similar way as described earlier with the exception of primers (Con 3 Con 2 instead of Rota1Rota 2). Diluted PCR products were used for identification of P genotype by performing 2 separate semi-nested multiplex PCRs. The third multiplex PCR was carried out in a final volume of 25 µl using diluted PCR product, type specific primers (1T-1 [P8], 2T-1 [P4], 3T-1 [P6], 4T-1 [P9] each at a concentration of 10 pM) in a reaction mixture (similar to first and second multiplex PCR) and cyclical conditions were similar to that used for amplification of partial length *VP4* gene using Con 3 Con 2 primers. Fourth multiplex PCR was carried out in a similar way as described for third with the exception of primers [Con 2, pNCDV [P1], pUK [P5], PGott [P6], POSU [P7] and pB223 [P11]] and cyclical conditions (similar to second multiplex).

Sequencing and sequence analysis: The sequencing of *VP7* (G genotype) and *VP4* genes (P genotype) was carried out commercially. Sequences were assembled using Pregap4 and Gap4 programs within the molecular biology software STADEN package (Staden *et al*. 1998). Multiple sequence alignment of partial *VP7* and *VP4* genes of these isolates were carried out with already reported sequences available in GenBank, NCBI using Clustal W or DNA star programme and checked mannualy. The pair-wise nucleotide sequence homology was determined with previously reported isolates and per cent identity matrix was determined by Clustal W or DNA star programme. Based on these results, phylogenetic tree and root distance matrix of tree was constructed using PhyloDraw programme or DNA star programme. Partial nucleotide sequence of the *VP7* and *VP4* gene of 2 rotaviral isolates were deposited in GenBank under the accession no. GQ 412143, GQ 412146, GQ 412147 and GQ 412148.

RESULTS AND DISCUSSION

The main aim of this study was to standardize the methods for isolation of rotavirus, extraction of dsRNA, detection of rotavirus by SDS-PAGE and RT-PCR and, genotypic characterization of rotaviral isolates by RT-PCR for analysis of field fecal samples from animals as well as humans. The viruses were successfully isolated on MA 10^4 cell line from SDS-PAGE and RT-PCR positive fecal samples of children. The characteristic cytopathic effects (CPE) were observed after 3 passages (Fig. 1a, 1b and 1c). CPE includes increased cytoplasmic granularity, rounding of cells, clumping with flagging and cytoplasmic extensions. Most of the cells were detached from surface by 48–72 h of post infection. It appeared to be very difficult to isolate rotavirus from fecal samples in 1980s. However, Wyatt *et al.* (1978) reported successful adaptation of HRV in cell culture using virus after passaging 11 times serially in gnotobiotic piglets. Our results showed that HRV propagated in MA-10^4 cells were adapted well enough to stationary culture to produce complete CPE 72 h after infection. Isolation of rotavirus from fecal samples was successfully carried out by Ward *et al*. (2005) and many other researchers using same MA-10^4 cell line.

The dsRNA of rotavirus from stool samples were extracted before and after the isolation of rotavirus in tissue culture flask. This dsRNA was detected by SDS-PAGE yielded a typical 11 banded pattern of rotavirus due to its segmented genome. Both the isolates showed long electrophoretic mobility pattern. In the majority of earlier studies long pattern was found common (Kaur *et al*. 2008). Early detection of rotavirus from stool samples by highly sensitive and specific method is of prime importance. RT-PCR is more sensitive than SDS-PAGE and can detect as less as 10^4 rotavirus particles per ml. In the present study, the standardized RT-PCR gave an expected product of the size 304 bp on amplification of *VP7* gene. These 2 samples were shown to be positive by SDS-PAGE as well as by RT-

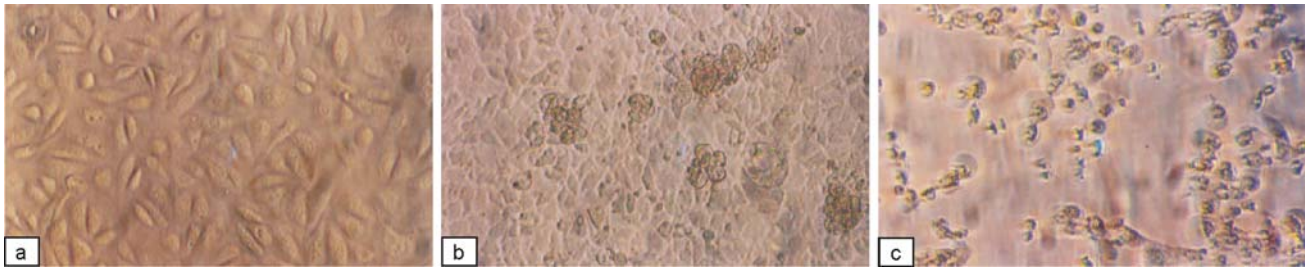

Fig. 1a. Normal MA104 cell line. 1b. Initial stage of infection where rounding and clumping of cells. 1c. Later stage of infection with extensive rounding and destruction of cells.

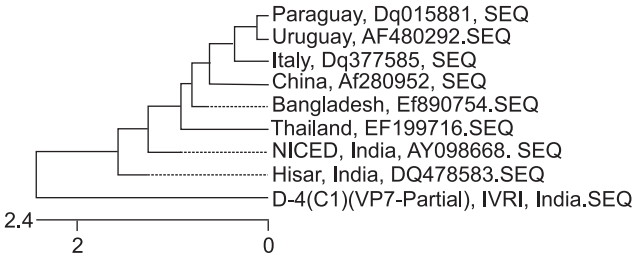

Fig. 3. Phylogenetic tree of the D-C1 (G1 genotype), IVRI, Izatnagar isolate (Access. No. GQ412147).

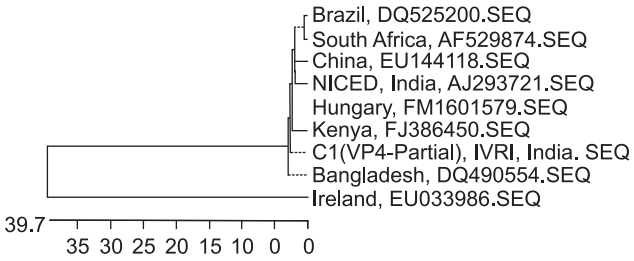

Fig. 4. Phylogenetic tree of the D-C1 (P[6] genotype), IVRI, Izatnagar isolate(Access. No. GQ412148).

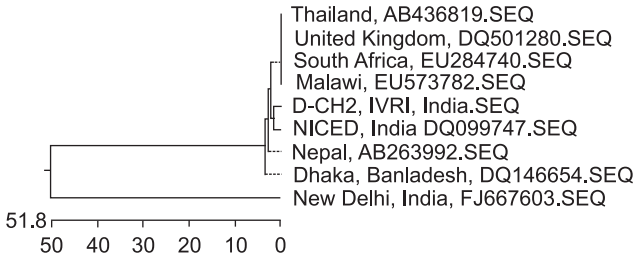

Fig. 5. Phylogenetic tree of the D-C2 (G11 genotype), IVRI, Izatnagar isolate(Access. No. GQ412146).

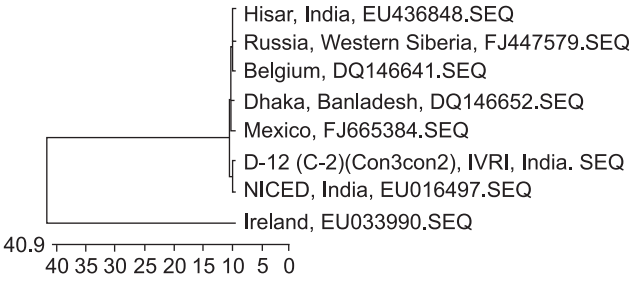

Fig. 6. Phylogenetic tree of the D-C2 (P[8] genotype), IVRI, Izatnagar isolate(Access. No. GQ412143).

PCR, before and after isolation of rotavirus, indicative of confirmation of adaptation of rotavirus to the MA-10^4 cell line. The positive results are also indicative of proper dsRNA extraction from stool samples and primers specific for gene segment 9 of rotavirus A, which is thought to be conserved in all group A rotaviruses of animals and human (Green *et al*. 1988).

The samples which were detected positive by SDS-PAGE and RT-PCR were subjected to G and P genotyping by amplifying full length *VP7* and partial length *VP4* gene using specific primers, which produced an expected PCR product of 1062 and 876 bp, respectively. Thus, the results obtained with RT-PCR suggested that, the primers complimentary to 5' end of segment 4 and 9 are suitable for development of RT-PCR assay for detection of rotavirus A from field samples. After dilution, these PCR products were subjected to 2 semi-nested multiplex PCR using type specific primers showing the amplified product of size 158 (G1), 244 (G2), 466 (G3), 403 (G4) and 110 bp (G9) in the first multiplex PCR, and 780 (G5), 500 (G6), 274 (G8), 715 (G10) and 337 bp (G11) in the second multiplex PCR. In this second round for identification of G genotypes, cDNA fragments with 158 bp (in first multiplex) for samples C1 (G1 genotype) and 337 bp (in second multiplex) for samples C2 (G11 genotype) were produced. Similarly, in the second round of PCR for identification of P genotypes, cDNA fragments with 267 bp for samples C1 (P[6] genotype) and 345 bp (in third multiplex) for sample C2 (P[8] genotype) were produced. Thus, the combination of G and P genotypes gives the specificity of G1P[6] and G11P[8] strains. A very faint band of P[8] genotype of 345 bp was observed in agarose gel, which could not be photographed by gel documentation system due to less intensity of the product. G1 strain accounted for 36.5% of episodes of rotavirus diarrhoea in the community and 46.8% in the hospital, showing that, it still continues to be the dominant strain (Banerjee *et al*. 2006). The G11P[8] was also reported by Matthijnssens *et al*. (2010).

Sequence alignment and phylogenetic analysis of G1 genotype of C1 sample revealed close association with isolates from India and other countries (>92% homology). Phylogenetic tree showed close relationship with difference of only 1–2 nucleotide per hundred bases (Fig. 3). However, multiple sequence alignment and phylogenetic analysis of P[6] genotype of same sample showed close clustering with isolates from various countries (>94% similarity) except Ireland isolate (only 22% similarity) (Fig. 4). Sequencing results of another sample (C2) showed interesting findings. After subjecting to second multiplex, the PCR product of this C2 sample showed the band for G11 genotype in agarose gel at 337 bp. However, sequence alignment and phylogenetic analysis of this G11 genotype revealed its close association with G12 genotypes of world isolates (Nepal, Bangladesh and NICED, India) (Fig. 5). Moreover, it was distantly related to G12 genotype isolated from New Delhi, India and G11 genotypes from world isolates. Timenetsky *et al*. (1997) also isolated G11 genotype from human sample, which was identified exclusively as swine pathogen. We have used primer specific for G11 genotype as per Gouvea *et al.* (1994) and obtained the expected PCR product of 337 bp after performing the second multiplex PCR. Therefore, sequence of this genotype was submitted to GenBank, NCBI with the same name as G11 genotype (Access. No. GQ412146). In contrast, the P[8] genotype of the same isolate was closely related to many world isolates with >95% identity except for the strain from Ireland

(EU033990) where the per cent similarity was only 23.7% (Fig. 6). However, the isolate was closely related to Bangladesh isolate (Access. No. DQ146652) and previously reported Indian isolate from NICED (Access. No. EU016497) and Hisar (EU436848), all are clustered in one linage.

Therefore, identification of G11 genotype from human patient raise the question that, whether the strain was of swine origin? The strain G11P[8] might be indicative of human-porcine reassortment i.e. interspecies transmission of one or many segments or part of nucleotide sequence. To our knowledge, this is the first report of G11P[8] strain of rotavirus detected from human case in India. Similar G11P[8] rotaviral strain was reported by Matthijnssens *et al.* (2010) from human stool samples. However, G11 genotype from human of Bangladesh was reported by Rahman *et al.* (2005) but with P[25] genotype specificity.

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