

Characterization of human symptomatic rotavirus isolates MP409 and MP480 having 'long' RNA electropherotype and subgroup I specificity, highly related to the P6[1],G8 type bovine rotavirus A5, from Mysore, India

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Summary. In an epidemiological study of symptomatic human rotaviruses in Mysore, India during 1993 and 1994, isolates MP409 and MP480 were isolated from two children suffering from severe, acute dehydrating diarrhea. Both isolates exhibited 'long' RNA pattern and subgroup I specificity suggesting the likelihood of their animal origin. Both isolates did not react with monoclonal antibodies (MAbs) specific for serotypes G1 to G6 as well as G10. To determine the genetic origin of these isolates, complete nucleotide sequences of genes encoding the outer capsid proteins VP4 and VP7, nonstructural proteins NSP1 and NSP3 and viral enterotoxin protein NSP4 from MP409 and partial sequences of genes from MP480 were determined. Comparison of the 5' and 3' terminal sequences of 250 nucleotides revealed complete identity of the gene sequences in both strains suggesting that MP409 and MP480 are two different isolates of a single strain. Comparison of the nucleotide and deduced amino acid sequences of VP4, VP7, NSP1 and NSP3 of MP409 with published sequences of strains belonging to different serotypes revealed that both outer capsid proteins VP4 and VP7 and NSP1 are highly related to the respective proteins from the P6[1], G8 type bovine rotavirus A5 isolated from a calf with diarrhoea in Thailand and that the NSP3 is highly homologous to that of bovine rotaviruses. The NSP4 protein showed greatest sequence identity with NSP4s belonging to the KUN genetic group to which NSP4s from human G2 type strains and bovine rotaviruses belong. MP409 and MP480 likely signify interspecies transmission of P6[1], G8 type strains from cattle to humans and represent the first P6[1] type rotaviruses isolated in humans. These and our previous studies on the asymptomatic neonatal strain I321 are of evolutionary and epidemiological significance in the context of close association of majority of the Indian population with cattle.

Introduction

Rotavirus, a member of the *Reoviridae* family, is the single most important aetiological agent of severe, acute dehydrating gastroenteritis in infants and young children and in the young of many avian and mammalian species [38]. Rotavirus particles consist of triple-layered protein capsids enclosing a genome of eleven segments of double-stranded RNA [23]. The genome primarily encodes six structural and five non-structural proteins [23, 45]. VP4 and VP7 encoded by gene segments 4 and 7, 8 or 9 (depending on the strain), respectively, form the outer capsid, and VP6, the product of gene 6 forms the intermediate capsid. Segment 2 codes for the inner capsid protein VP2 that encloses the core consisting of the genome and the virion proteins VP1 and VP3. The nonstructural proteins NSP1, NSP2, NSP3, NSP4 and NSP5 are encoded by gene segments 5, 8, 7, 10 and 11, respectively [23].

The outer capsid proteins VP7 and VP4 specify two distinct serotype specificities, called the G and P serotypes, respectively. To date at least 14 G serotypes have been identified in humans and other species [38]. Among the 14 G serotypes, G1 to G4 are predominantly found in humans [38]. Serotypes G5, G6, G8 and G10 are usually detected in animals, but have also been detected in humans in recent years [19, 38, 54]. By serological assays, so far 11 P serotypes have been identified [38]. Due to lack of immunological reagents, rotaviruses have also been classified into P genotypes based on amino acid sequence identity. VP4s showing > 89% identity are considered to belong to the same genotype, and to date at least 20 genotypes have been identified. While the P serotype is represented by a number immediately after P, the genotype is indicated by a number in a square bracket [23].

Rotaviruses are also classified into groups and subgroups (SGs) based on the antigenic epitopes present on the intermediate capsid protein VP6. At least 7 groups termed A to G have been recognized. Group A rotaviruses constitute the major pathogens in humans as well as domestic animals [38]. Four subgroups I, II, I+II and non I/II have been identified among group A rotaviruses [28]. Rotaviruses can also be identified as 'long' or 'short' electropherotypes based on the faster or slower electrophoretic mobility of gene segment 11 in polyacrylamide gels, respectively. While human strains with 'short' RNA pattern generally exhibit subgroup I specificity, those with 'long' RNA pattern possess subgroup II VP6. In contrast, 'long' RNA pattern and subgroup I VP6 are commonly associated with animal strains [38]. Isolation of rotaviruses having 'long' RNA electropherotype and subgroup I-specific VP6 strongly suggests animal origin of the strains [28].

In the course of study of the genetic variation among Indian rotaviruses, we have previously reported the isolation and characterization of P8[11], G10 rotaviruses from a large number of asymptomatic new-born children in Bangalore [19, 22, 57]. During epidemiological studies of symptomatic human rotaviruses, we have isolated a few unusual strains with 'long' RNA electropherotype and subgroup I specificity suggesting that they are of animal origin ([2] and unpublished data). Since infection by purely heterologous rotaviruses does normally

not result in severe disease due to host range effects, analysis of a few naturally occurring reassortants would be valuable in identifying the putative candidate gene/s involved in host range restriction and pathogenicity.

In this study, two isolates MP409 and MP480 having 'long' RNA pattern and subgroup I specificity, isolated from two children suffering from severe, acute diarrhea in Mysore, a city of tourist attraction in southern India, were subjected to nucleotide sequence analysis of genes encoding VP4, VP7, NSP1, NSP3 and NSP4 which have been implicated in different studies to be associated with important viral properties such as pathogenicity, virulence, host range restriction, plaque morphology phenotype, morphogenesis, viral RNA replication and translation [6, 9, 11, 15, 20, 34, 37, 49, 60].

Materials and methods

Viruses, virus isolation and adaptation to tissue culture

In an epidemiological study during 1993 and 1994 in Mysore, two isolates from among 54 rotavirus-positive samples (representing 3.7%) were observed to possess 'long' RNA pattern and subgroup I specificity. These two stool specimens MP409 and MP480 were collected from two children with severe acute diarrhea admitted to Cheluvamba hospital in Mysore, India. Sample MP409 was collected from a female child aged 2 years and MP480 was from a male child of 2 years and 7 months. Both children were from the same rural neighbourhood near Mysore but belonged to different families separated by a distance of half a kilometer. Residents of this locality rear cattle for their livelihood. The stool samples were processed as described previously [57], and the supernatants were stored at -20°C . MP409 was adapted to culture in vitro in MA104 cells. Tissue culture-grown viruses, Wa (SGII, P1A[8], G1), S2 (SGI, P1B[4], G2), RRV, SA11 (SGI, P5[3], G3), ST3 (SGII, P2A[6], G4), NCDV (SGI, P6[1], G6), WI61 (SGII, P1A[8], G9) and I321 (SGI, P8[11], G10) were used as controls in subgroup and serotype analysis.

Extraction and electrophoresis of rotavirus RNA

Procedures for extraction of viral genomic RNA from clinical samples and from viruses grown in tissue culture, electrophoresis of the RNA in polyacrylamide gels and detection of the dsRNA by staining with silver nitrate were described previously [57].

Subgroup and serotype analysis

Subgrouping ELISA and serotyping ELISA were performed as described earlier [28, 57]. For subgrouping ELISA, hyperimmune anti-RRV antiserum R2, the SGI-specific monoclonal antibody (MAb) 255/60 and SGII-specific MAb 631/9 were used. MAbs specific for serotypes G1–G6 and G10 used in serotyping ELISA were described previously [57]. All the antibodies were kindly provided by Dr. Harry B. Greenberg, Stanford University School of Medicine, Stanford, CA, USA.

Cloning of genes 4, 5, 7, 9 and 10 of MP409 and MP480 by RT-PCR

Double-stranded (ds) RNA from the cell culture-grown MP409 and the clinical isolate MP480 was purified by the method described earlier [19]. Purified dsRNA was reverse transcribed using AMV-reverse transcriptase and amplified by polymerase chain reaction (PCR) using a method previously described [51]. Gene-specific primers were used in cDNA synthesis and PCR amplification. Gene 4-specific 5' and 3' primers were 5'-CTAAGCTTCCCG

GGCTATAAAATGC/GC/GTTC-3' and 5'-CTAAGCTTCCCGGGTCACATCC/TT-3', respectively. The respective primers for gene 9 were 5'-CTTCCCGGGCTTTAAAAGA/CGA-GAAT-3' and 5'-CTTCCCGGGTCACATCA/GT/AA/CCA-3'. Gene 5-specific 5' and 3' primers respectively were 5'-ACCCCGGGCTTTTTTATGAAAAGTC-3' and 5'-CACCCG-GGTCACATA/TTTTTAT-3'. The sequences of gene 7-specific 5' and 3' terminal primers were described previously [51]. The gene 10 primers corresponded to the 5' and 3' regions of the ORF and the respective sequences are 5'-ATCCCGGGATGGAAAAGTTTCCGACCTC-3' and 5'-CTTAAGCTTCATA/C/GGC/AT/CGCAGTC/TACTTC-3'. Gene 10 from the asymptomatic neonatal strain I321 was also amplified using the same primers. All the primers have sites at the 5' end for restriction enzymes of convenience. Rotavirus gene-specific sequences in the primers are underlined. PCR-amplified DNAs were digested with appropriate restriction enzymes and cloned into either pBluescript KS⁺ or PUC18 vectors that were also digested with the corresponding enzymes.

Nucleotide sequencing and comparative sequence analysis

Nucleotide sequence of the cloned genes was determined by the dideoxynucleotide-mediated chain termination method [53]. At least two independent clones for each gene were used for sequencing. Complete nucleotide sequences of the genes from MP409 and only the 5' and 3' terminal 250 nucleotides of the genes from MP480 were determined using gene-specific and vector-specific primers as well as by sequencing of the subclones generated using internal restriction enzyme sites. The nucleotide and deduced amino acid sequences of genes of MP409 were analyzed and compared with the previously published rotaviral gene sequences in the data bases using GCG sequence analysis software. Phylogenetic analysis was carried out using the neighbour-joining method of Saitou and Nei [52], and Distances Program of the GCG package, Wisconsin. The accession numbers for the VP4, VP7, NSP1 NSP3 and NSP4 genes of strain MP409 and for the NSP4 gene of strain I321 are AF 143408, AF 141918, AF 141916, AF 141917, AF 141919 and AF 165066, respectively.

Results

Electropherotype, subgroup and serotype analysis of MP409 and MP480

Clinical isolates MP409 and MP480 showed identical 'long' RNA electropherotype. Of the two strains, MP409 was adapted to tissue culture and it grew rapidly in MA104 cells. Since the RNA from the clinical isolate MP480 was very limited, only the electrophoretic pattern of the tissue culture-grown MP409 is shown in Fig. 1. Subgroup analysis revealed that the clinical isolates as well as the cell culture-grown MP409 were of subgroup I specificity (data not shown). 'Long' RNA pattern and SGI specificity of these strains suggested that they are of animal origin [57]. In serotyping ELISA, neither MP409 nor MP480 reacted with MAbs specific for G serotypes 1–6 and 10 which further suggested the likelihood of their animal origin (data not shown).

Sequence analysis of VP7 and VP4 genes of MP409

Preliminary sequence analysis of about 250 nucleotides from the 5' and 3' termini of VP7 and VP4 genes of MP409 and MP480 revealed that the corresponding sequences of the two isolates were identical. This observation in conjunction with the observed identical subgroup and electropherotypic properties of the two



Fig. 1. Electrophoretic analysis of the cell culture-adapted MP409 strain. 1 A P[4], G2 type isolate 1040 (short electropherotype) 2 MP409; 3 NCDV (P6[1], G6)

strains indicated that both MP409 and MP480 represent two isolates of a single strain. Hence only MP409 was subjected to further sequence analysis as it was adapted to growth in tissue culture.

The VP7 gene of MP409 was 1062 nucleotides long with a single ORF stretching from nucleotide position 49 to 1026 and encoding a polypeptide of 326 amino acids. Comparative sequence analysis revealed that MP409 VP7 shared greatest sequence identity (94.8%) with that of the G8 bovine strain A5 previously isolated from a calf with diarrhea in Thailand [59]. The percent amino acid sequence identities with VP7 from other G8 strains 69M, B37, NCDV-cody I-801, 678 and HAL1166 ranged between 91.7 and 93.9 and the nucleotide sequence homologies were between 83.2% and 84.6% (Table 1). A comparison of the three neutralization epitope-determining regions A, B and C of MP409 with those of all other serotypes also showed that MP409 shared greater homology in these regions with G8 strains A5, NCDV-cody I-801, 69M, B37 and 678 (Fig. 2).

The VP4 gene was 2362 nucleotides long with an open reading frame extending from nucleotide position 9 to 2340 and that encoded a protein of 776 amino acids in length. Comparison of MP409 gene 4 sequence with the published gene 4 sequences revealed that gene 4 of MP409 is highly related to the P6[1] type VP4 gene of the bovine strain A5 [59] with nucleotide and amino acid sequence identities of 95.3% and 96.1%, respectively (Table 1). It also shared 92.0% and 91.0% amino acid sequence identities with the P6[1] type VP4 of the simian SA11 4f and bovine NCDV strains, respectively (Table 1). Of note, the carboxy-terminal half of the VP5* region [23] of the VP4 of SA11 4f and NCDV exhibited more divergence than that of A5 from MP409 (Fig. 3). Whereas the VP5* (aa position 250–776) region of MP409 VP4 exhibited percent amino acid identities of 97.0, 90.7 and 91.9 with that of strains A5, NCDV and SA11 4f, the corresponding amino acid percent identities of the P serotype determining VP8* region (position 1–250) [23] were 94.4, 91.5 and 92.3. The cysteine residues at positions 203, 216, 318 and 380 are conserved in MP409 as well as in A5, NCDV and SA11 4f.

Thus, comparison of nucleic acid and deduced amino acid sequences of VP7 and VP4 of MP409 with those of rotaviruses belonging to other serotypes clearly

Table 1. Percent nucleotide and amino acid identities of MP409 gene sequences with those of strains belonging to the 14G serotypes

Strain	Species origin	Serotype/genotype	VP4		VP7		NSP1		NSP3		NSP4	
			Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA
Wa	Human	P1A[8], G1	70.6	71.4	72.8	75.4	70.4	57.8	80.8	83.4	78.5	81.7
RV5	Human	P1B[4], G2	70.3	70.7	NA	NA	NA	NA	NA	NA	88.2	90.9
KUN	Human	P1B[4], G2	NA	NA	NA	NA	NA	NA	NA	NA	89.3	94.3
S2	Human	P1B[4], G2	NA	NA	NA	NA	NA	NA	76.4	77.7	88.2	93.7
1076	Human	P2A[6], G2	70.1	71.9	ND	ND	NA	NA	NA	NA	91.2	94.3
E210	Human	P1B[4], G2	NA	NA	NA	NA	NA	NA	NA	NA	88.6	93.7
DS1	Human	P1B[4], G2	NA	NA	ND	ND	70.5	55.4	NA	NA	NA	NA
SA11	Vervet Monkey	P5B[2], G3	75.8	83.2	74.5	82.5	57.1	35.9	74.5	74.4	84.6	90.0
RRV	Rhesus Monkey	P5[3], G3	76.4	84.7	NA	NA	56.3	36.4	78.3	79.9	78.8	81.7
AU1	Human	P3[9], G3	68.9	70.6	76.8	83.1	72.4	71.9	NA	NA	77.8	82.3
ST3	Human	P2A[6], G4	70.0	72.6	74.6	73.9	70.0	56.0	81.9	85.0	77.5	80.0
Gottfried	Porcine	P2A[6], G4	70.1	73.5	72.8	73.9	70.9	56.6	81.4	83.4	NA	NA
OSU	Porcine	P9[7], G5	75.1	82.0	75.4	80.7	70.7	58.2	81.1	83.8	77.5	81.7
Hg17	Bovine	P?[?], G6	NA	NA	NA	NA	NA	NA	93.5	90.8	NA	NA
UK	Bovine	P7[5], G6	70.9	75.8	74.7	80.7	74.8	80.1	81.7	85.5	85.1	91.4
NCDV	Bovine	P6[1], G6	NA	91.0	75.2	80.4	NA	NA	90.2	91.7	85.3	90.9
TY-1	Equine	P?[?], G7	NA	NA	66.9	58.9	NA	NA	NA	NA	NA	NA
MP409	Human	P?[1], G8	–	–	–	–	–	–	–	–	–	–
A5	Bovine	P6[1], G8	95.3	96.1	89.6	94.8	88.0	91.2	NA	NA	NA	NA
SA11	Vervet Monkey	P6[1], G3	80.6	92.0	NA	NA	NA	NA	NA	NA	NA	NA
4f	Monkey											
NCDV-cody	Bovine	P6[1], G8	NA	NA	83.2	93.3	NA	NA	NA	NA	NA	NA
678	Bovine	P7[5], G8	NA	NA	84.6	93.9	NA	NA	NA	NA	NA	NA
69M	Human	P4[10], G8	74.6	82.7	84.6	93.8	70.3	55.6	75.1	78.7	NA	NA
B37	Human	P4[10], G8	NA	NA	84.1	91.7	NA	NA	NA	NA	NA	NA
HAL	Human	P11[4], G8	68.8	71.4	83.9	93.8	NA	NA	NA	NA	NA	NA
1166												
WI61	Human	P1A[8], G9	NA	NA	75.7	81.0	NA	NA	80.0	83.4	NA	NA
B223	Bovine	P8[11], G10	61.2	59.2	74.4	77.9	NA	NA	NA	NA	NA	NA
I321	Human	P8[11], G10	61.1	58.7	74.3	77.0	71.2	56.4	81.3	84.4	83.6	89.7
YM	Porcine	P9[7], G11	75.2	82.7	75.4	82.8	72.5	57.1	NA	NA	76.8	80.6
L26	Human	P1B[4], G12	70.1	71.1	72.7	77.3	71.5	55.0	NA	NA	NA	NA
L338	Equine	P12[18], G13	77.4	83.6	73.5	75.5	58.0	36.6	NA	NA	NA	NA
FI23	Equine	P?[12], G14	74.2	79.6	74.5	79.1	58.2	36.5	NA	NA	NA	NA

established that MP409 is highly related to the P6[1], G8 type bovine rotavirus A5 and that both outer capsid proteins are likely to be of bovine origin. MP409 represents the first human isolate having the combination of serotype G8 and genotype P1 (P serotype 6) specificities. These results confirm our serological data that MP409 did not belong to any of the common human serotypes.

Sequence analysis of the NSP1 and NSP3 genes

Comparison of the 5' and 3' terminal sequences (250 nucleotides) of NSP1 and NSP3 genes from MP409 and MP480 revealed complete sequence identity between the two isolates suggesting identical origin of the genes in both strains. The gene 5 of MP409 was 1588 nucleotides in length and encoded a protein of 491 amino acids from nucleotide position 34 to 1506. Comparative analysis of the nucleotide and deduced amino acid sequences with the published gene 5 sequences revealed that MP409 gene 5 was highly related to that of the mutant isolate A5-10 of the bovine strain A5 [58] with 88.0% and 91.2% nucleotide and amino acid sequence identities (Table 1). Gene 5 of MP409 contained an additional adenine at position 29 in the 5' UTR which was 33 nucleotides long instead of 32 nucleotides observed in many strains. While gene 5 from A5-10 encoded a truncated non-functional polypeptide of 40 amino acids in length due to the presence of a TGA termination codon at amino acid position 41, the NSP1 gene of MP409 contained a TGG condon coding for tryptophan at this position and encodes a complete NSP1 protein. Of significance, NSP1 from the human G8 strain 69M was far less related to the gene 5 of MP409 and exhibited only 70.3% and 55.6% homology at nucleotide and amino acid levels, respectively (Table 1).

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Table 1 (*footnote*). The accession numbers for gene sequences analyzed in this table and elsewhere in the manuscript are: **VP4**: Wa,L34161; RV5,M32559; 1076,F28839; SA11,X14204; SA11 4f, X57319; RRV,M18736; AU1,D10970; ST3,L33895; Gottfried,M33516; OSU,X13-190; I321,L07657; UK,M22306; NCDV,C31159; A5,D13395; 69M,M60600; B223,M92-986; YM,M63231; L26,M58292; L338,D13399; FI23,D16342; HAL1166,L20875; **VP7**: Wa, K02033; DS1,E27620; SA11,V01190; AU1,D86271; ST3,X13603; Gottfried,X06759; OSU, X04613; UK,X00896; NCDV,M12394; Ty-1,L01098; A5,D01054; NCDV-cody,U14-999; B37,J04334; HAL1166,L20882; 678,L20883; B223,X57852; I321,L07658; YM, M231-94; L26,M58290; L338,D13549; FI23,M61876; **NSP1**: Wa,L18943; DS1,L18945; SA11, X14914; RRV,Z32535; AU1,D45244; ST3,U11492; Gottfried, U08431; OSU,D38153; UK, L12248; A5,D38147; 69M,D38151; I321,U08418; YM,D38154; L26,D38150; L338,D38-158; FI23, D38156; **NSP3**: Wa,X81434; S2,X81428; SA11,X00355; SA114F,M87502; RRV, X81426; ST3,X81436; Gottfried,X81430; OSU,X81431; Hg17,X81427; UK,K02170; NC-DV,X81429; 69M,X81425; WI61,X81437; I321,X81433; **NSP4**: Wa,K02032; RV5,U59103; KUN,D88829; S2,U59104; 1076,U59105; E210,U59107; SA11,K01138; RRV,L41247; AU1,D89873; ST3,U59110; OSU,D88831; UK,K03384; Hg17,Q82047; NCDV,X06806; I321, Q82048; YM,X69485; 69M,Q82051; FRV64,P89063; RV4,Q82033; M37,Q82034. The sequences of VP7 gene of strains 69M and WI61 were from the reference Green KM, Hoshino Y, Ikegami N (1989) 168, 429–433. NA Not available; ND not determined; Nt nucleotide; AA amino acid

Strain	G-Type	Region A (87–101)	Region B (143–152)	Region C (208–221)
MP409	8	A E A E T E I A D S S W K D T	K Y N A N S E L D R	L T S D T T T F E E V A T A
CodyI-801	8	V - - - - - D - - - - -	- - - - - M	- - T - - A - - - - - R
A5	8	T - - - - - - - - - - -	- - - - - A - - - M	- - - T - - - - - - - - -
69M	8	V - - - - - - - - - - -	- - - - - M	- - T - - - - - - - - -
B37	8	V - - - - - - - - - - -	- - - - - M	- - T - - - - - - - - -
678	8	V - - - - - - - - - - -	- - - - - M	- - T - - - - - - - - -
Wa	1	- - - S - Q - N - G D - - -	S - - D Q S L - - - M	Q - T N V D S - - M I - E N
DS1	2	- - - K N - - S - D E - E N -	R - D N T - - - - A	K - S - V N - - - I - - T S
SA11	3	T - - A - - N - N S - - -	- - - D - - - Q - - M	- - S - A T - - - - - - -
ST3	4	S - - P - Q - S - T E - - -	R F V S G E - - - I	Q - T N - A - - - - - D S
OSU	5	N - - A - - - - T K - T E -	- - - D G - - Q - - M	S - T - I N S - - T - - N A
NCDV-Lincoln	6	V - - S N - - - - T E - - -	- - - D S T Q - - - M	- I T N P D - - - T - - - M
TY-1	7	K - - A - Q M N - A E - - -	S - - D Q - E T - - M	K - T - V S - - - - L T - E
WI61	9	- - - S - Q - G - T E - - -	- - - D S T L - - - M	T - T N - A - - - - - A S
B223	10	T - - R - - N - N E - T S -	R - - S T L K - - M	G - T N - G - - - - - R
YM	11	H - - A - Q - - - D K - - -	- - - D G - - Q - - M	- - T - P G - - - - - S -
L26	12	N S V T - - - T - P D - T H -	Q - Q S T L K - - M	T - T - V A - - - - - N -
L338	13	N - V V S - L N - D - - - N -	- - - S T E L Q - - I	- - T - - E - - - - - L
FI23	14	T - - A - Q - D - - - - - -	- - - D E A I Q - - I	- - T N V E - - - - - S

Fig. 2. Comparison of the deduced amino acid sequences of the VP7 gene of MP409 strain in the variable regions VR5(A), VR7(B) and VR8(C) with those of known G8 and other G serotype rotaviruses. For TY-1, the numbering for the corresponding epitope regions is 91–104, 146–155 and 211–224. The accession numbers for the gene sequences compared in the figure are given in the footnote to Table 1.

The NSP3 gene of MP409 was 1078 nucleotides long with an ORF encoding a protein of 314 amino acids. While the NSP3 proteins of the majority of group A rotaviruses are 313 amino acids in length (except those of SA11 4F and SA11 encoding a protein of 315 amino acids length due to insertion of two amino acids at the carboxyl terminus), the NSP3 of MP409 was longer by one amino acid due to insertion of a TCG codon encoding serine after amino acid position 233. Comparative sequence analysis revealed greatest sequence relatedness of MP409 NSP3 with that of bovine rotaviruses Hg17 [51] and NCDV exhibiting 93.5% and 90.2% sequence identities at nucleotide level and 90.8% and 91.7% identities at amino acid level, respectively. The percent nucleotide and amino acid sequence homologies with other strains were less than 81.9 and 85, respectively (Table 1). As the NSP3 gene sequence of strain A5 is not available for comparison, based on the high degree of homology of MP409 NSP3 with that of bovine strains Hg17 and NCDV, it is likely that the NSP3 gene in MP409 is also of bovine origin.

Strain	P Serotype/ Genotype		100	200	300	400
MP409	P?[1],G8		MASLIYRQLLTNSYTVELSD	EQEIGSTKTONVTINPGPEAQT	SYAPVNWGGETNDSTVVEPVL	DGFPYQPTTFNPPVDYWMLLAPT
A5	P6[1],G8					DAGVVVEGTTNNTN
NCDV	P6[1],G6					
SALL 4f	P6[1],G3					
HALL1166	P11[4],G8					
69M	P4[10],G8					
UK	P7[5],G6					
I321	P8[11],G10					
MP409	P?[1],G8		1			
A5	P6[1],G8					
NCDV	P6[1],G6					
SALL 4f	P6[1],G3					
HALL1166	P11[4],G8					
69M	P4[10],G8					
UK	P7[5],G6					
I321	P8[11],G10					
MP409	P?[1],G8		101			
A5	P6[1],G8					
NCDV	P6[1],G6					
SALL 4f	P6[1],G3					
HALL1166	P11[4],G8					
69M	P4[10],G8					
UK	P7[5],G6					
I321	P8[11],G10					
MP409	P?[1],G8					
A5	P6[1],G8					
NCDV	P6[1],G6					
SALL 4f	P6[1],G3					
HALL1166	P11[4],G8					
69M	P4[10],G8					
UK	P7[5],G6					
I321	P8[11],G10					
MP409	P?[1],G8					
A5	P6[1],G8					
NCDV	P6[1],G6					
SALL 4f	P6[1],G3					
HALL1166	P11[4],G8					
69M	P4[10],G8					
UK	P7[5],G6					
I321	P8[11],G10					

Fig. 3 (continued)

Sequence analysis of the NSP4 gene

To understand the species origin of NSP4 in MP409 and MP480 and its possible contribution to the virulent phenotype of these isolates, the complete nucleotide sequences of the protein coding region of the NSP4 gene of the isolate MP409 and the asymptomatic neonatal strain I321 as well as the 5' and 3' terminal sequences (250 nucleotides) corresponding to the protein coding region of the isolate MP480 were determined. The 5' and 3' terminal sequences of the NSP4 gene ORF between MP409 and MP480 were identical. The NSP4 gene of MP409 encoded a protein of 175 amino acids similar to that of other group A rotaviruses. It is of interest to note that the highly conserved residues phenylalanine, tyrosine and arginine at amino acid positions 33, 70 and 101, respectively, were substituted by leucine, cysteine and glutamic acid, respectively, in the MP409 NSP4. Comparative analysis revealed that MP409 NSP4 shared the highest degree of sequence identity with the NSP4 proteins of G2 serotype human rotaviruses (RV5, KUN, S2 and E210), bovine rotaviruses (UK and NCDV) as well as the human asymptomatic strains 1076 (serotype G2) and I321 (serotype G10) belonging to the KUN genetic allele (Fig. 4, Table 1, phylogram of NSP4 not shown) [18, 31, 32, 40]. The amino acid and nucleotide sequence identities of the NSP4 of these strains with that of MP409 ranged from 90.9 – 94.3% and 85.1 – 91.2%, respectively (Table 1). While the NSP4 of Simian rotavirus SA11 showed 90.0% amino acid identity with that of MP409, sequences from other strains representing the Wa, AU1 and EW NSP4 alleles exhibited less than 82.3% identities (Table 1, Fig. 4) [32]. Within the KUN genetic group, MP409 NSP4 exhibited slightly more relatedness with that of human G2 strains with striking similarity of the region between amino acids 135 and 140. As the NSP4 sequence of strain A5 is not available, a comparison could not be carried out. Because of the high degree of relatedness between the NSP4 proteins of G2 human strains and bovine strains, a definite conclusion on the origin of the gene in MP409 and MP480 could not be drawn.

Discussion

In recent years, G6, G8 and G10 serotype rotaviruses that are the principal cause of diarrhoea in cattle [27, 29, 55, 56, 59] are also being detected in humans in significant numbers [1, 7, 17, 19, 24, 26, 30, 57]. Of significance are the recent studies reported from Brazil and Malawi. In the Brazilian study, 16% of the rotaviruses isolated from children suffering from diarrhoea were found to belong to G10 serotype. Also, G8 serotype strains accounted for 4% of rotavirus diarrhoea in that study [54]. In an epidemiological study in Malawi, a striking 42% of rotavirus isolates from children with diarrhoea were of P[6], G8 type and another 9% were of P[4], G8 specificity [17]. Thus G8 strains alone accounted for more than 50% of rotavirus diarrhoea in the Malawi study. Further, seroepidemiological surveys have demonstrated the presence of G8-specific antibody in South American and Indian children [13, 39]. Serotype G8 strains have also been detected in horse [35] and pigs [27]. Table 2 describes the G8

MP409	D	MEKFTDLNYTLGVITLNMNSTLHTLLEDPGMAYLPIYASVLTFLHKSASPTMKIALKTSKCSYKVVKCCIVTIFNTLLKLAGYKEQITTKDEIEKQMD	100
1076	N	F	Y T
KUN	S	F	L
S2	S	F	L
E210	S	F	L
RV5	S	F	L
UK	S	F	I
NCDV	SS	F	V
I321	S	F	G A N
SA11	S	F	G A N
OSU	S	F	S I Q
RV4	S	F	S I
WA	S	F	S I Q
ST3	S	F	S I
M37	S	F	S I
YM	S	F	S I Q
AU1	S	F	M
RRV	S	F	M
FRV64	S	F	M
EC	LA	E	N E
EHP	LA	E	N E
EW	LA	E	N E
MP409	R	M	R
1076	R	M	R
KUN	Y	R	R
S2	Y	R	R
E210	Y	R	D
RV5	Y	M R	R
UK		MIRVVD	R
NCDV		MIRAVD	R
I321	C	Y M RTAD	R
SA11	Y	T T	R
OSU	AAR	VDA	S F
RV4	N	TRPVDV	S F
WA	N	TRPVDV	S F
ST3	N	TRPVNV	SM F
M37	N	TRIVDV	S F
YM	PR	VTRPVDV	S F
AU1		IKPVDK	SQ F
RRV		IKPVDK	SQ F
FRV64		AT VDK	T Q F
EC	L	Q A E	S T R AF
EHP	L	Q T E	S T AFN
EW	L	Q A E	S T AF
101	R	M	R
EFVKEMRRQLEMIDKLTTRTREIEQVELLKRHDKLIVQSTGEIDMTKKEINOQNVKLTLEEWESGRNPPYEKCVTAAM	175		
RV	R		
KUN	Y	R	R
S2	Y	R	R
E210	Y	R	D
RV5	Y	M R	R
UK		MIRVVD	R
NCDV		MIRAVD	R
I321	C	Y M RTAD	R
SA11	Y	T T	R
OSU	AAR	VDA	S F
RV4	N	TRPVDV	S F
WA	N	TRPVDV	S F
ST3	N	TRPVNV	SM F
M37	N	TRIVDV	S F
YM	PR	VTRPVDV	S F
AU1		IKPVDK	SQ F
RRV		IKPVDK	SQ F
FRV64		AT VDK	T Q F
EC	L	Q A E	S T R AF
EHP	L	Q T E	S T AFN
EW	L	Q A E	S T AF

Fig. 4. Comparison of the deduced amino acid sequence of MP409 NSP4 with the NSP4 sequences belonging to the KUN, AU-1 and Wa alleles and from murine rotaviruses. Refer to the footnote to Table 1 for accession numbers of the gene sequences

Table 2. Properties of G8 serotype strains identified so far

Strain	Species origin	Electro-pherotype	Subgroup	Country of origin	Serotype/genotype	Reference
J2538	Bovine	Long	I	Scotland	P6[1], G8	[56]
A5	Bovine	Long	I	Thailand	P6[1], G8	[59]
NCDV-cody	Bovine	Long	I	US	P6[1], G8	[16, 43]
BRV-14 & BRV-15	Bovine	Long	I	Japan	P6[1], G8	[55]
26/94	Equine	Long	–	UK	P[1], G8	[35]
678	Bovine	Long	I	Scotland	P7[5], G8	[47]
69M, B37 & B38	Human	Supershort	I	Indonesia	P4[10], G8	[3, 44]
HAL1166, HAL1271 & HAL8590	Human	Long	I	Finland	P[14], G8	[26]
PA171	Human	Long	II	Italy	P?, G8	[26]
HMG89	Human	Short	I	Nigeria	P?, G8	[1]
Malawi isolates	Human	Short	–	Malawi	P[6], G8 & P[4]G8	[17]
EGY1850 & EGY2295	Human	Long	I	Egypt	P[14], G8	[30]
MP409 & MP480	Human	Long	I	India	P[1], G8	Present study

strains characterized so far from humans and animals in different regions of the world.

Analysis of some of the human G8 strains revealed significant genetic relatedness to bovine rotaviruses regardless of their differences in G serotype specificities suggesting that bovine rotaviruses and human G8 strains could have originated from a common ancestor or that the human G8 strains could have been derived from bovine strains by genome reassortment with G2 human strains in nature [12, 46]. P serotype/genotype analysis of some of the G6 and G8 human strains revealed that the VP4s in human strains are different from those found in the bovine counterparts. Thus the Italian G6 type human isolates PA151 and PA169 contained P3[9] and P11[14] type VP4, respectively [36, 43, 47, 55, 56]. In contrast, the G8 human strains 69M, B37 and B38 with supershort RNA electropherotype, isolated in Indonesia, possessed P4[10] type VP4 [42] and the Finnish G8 isolates HAL1166, HAL1271 and HAL8590 as well as the Egyptian G8 isolates EGY1850 and EGY2295 contained VP4 of P11[14] type (Table 2) [30, 33]. The P types of human G8 strains PA171 isolated in Italy [26] and HMG89 isolated in Nigeria [1] are yet to be established. The large number of G8 serotype strains belonging to two P genotypes, P[6] and P[4], isolated in Blantyre, Malawi and possessing 'short' RNA electropherotypes, further support the hypothesis of the origin of human G8 strains by reassortment between human G2 and bovine G8 strains. Based on the present results, MP409 and MP480 likely represent isolates of a single P6[1], G8 bovine strain being transmitted from cattle to humans.

Though G and P type analysis is useful in characterization of rotaviruses, detailed sequence analysis of the genomes of naturally selected unusual rota-

viruses/reassortants is required to identify genes that determine host range restriction and pathogenicity phenotype between bovine and human strains. The observation that the so far characterized human G6 and G8 strains possessed VP4s that are of non-bovine origin suggests an association of VP4 with host range restriction and/or pathogenicity phenotype. However, both the outer capsid proteins of the Indian asymptomatic neonatal strain I321 and of the symptomatic strains MP409 and MP480 are derived from bovine rotaviruses belonging to different serotype specificities. It is of significance to note that the VP4 sequence in MP409 is not identical to other P6[1] type VP4s from A5 and NCDV and contained several amino acid substitutions. In the context of the findings that VP4 determines pathogenicity and host restriction and that single amino acid substitutions in porcine rotavirus VP4 markedly influence the pathogenicity phenotype of the virus [10, 14, 15, 37, 49], the observed amino acid substitutions at several positions in the VP4 of MP409 and MP480 could contribute to the host range restriction and pathogenicity properties of these predominantly bovine strains in humans.

NSP4 has been identified as the intracellular receptor for subviral particles during virus morphogenesis with membrane destabilizing properties and has been implicated as possible viral enterotoxin, inducing diarrhoea by stimulating chloride ion secretion through a calcium-dependent signalling pathway involving interaction with an yet to be identified putative receptor on the intestinal epithelial cells [5, 6, 8, 48, 61]. A peptide spanning a region overlapping the Ca^{2+} -binding and VP4-interaction domains (position 114–135) was suggested to be sufficient to cause diarrhoea in a newborn mouse model system [6] and certain mutations in this region and the adjacent region between amino acid positions 135 and 140 were suggested to correlate with the virulence phenotype of the strains presumably due to the changes in the structure of the C-terminal region that affects interaction with yet unidentified host cell proteins [6, 64]. However, there have been conflicting reports regarding the enterotoxigenic activity of NSP4. Comparison on a wider scale of sequences of NSP4 from various strains failed to show an absolute correlation between the sequence and the symptomatic/asymptomatic character of the virus [18, 31, 32, 40]. In this context, as seen in Fig. 4, the NSP4 of the asymptomatic strain 1076 differed only at 3 positions in the entire carboxy terminal half of the protein from that of the virulent MP409. Further, the attenuated phenotype of a vaccine strain derived from the virulent wild-type human rotavirus (strain 89-12) did not correlate with mutations in the NSP4 gene [62]. In addition, Angel et al. [4] have concluded that a homologous NSP4 of strain EC did not play a dominant role in inducing diarrhoea in mice.

Newton et al. [48] have identified a cationic amphipathic helical domain between amino acid residues 55 and 72 that exhibited membrane destabilization activity in cells *in vitro* and proposed this region to be critical for cytopathic effect of NSP4. The apparent viral virulence mediated by NSP4 may result from its membrane destabilization function during outer capsid assembly and virus budding into the lumen of the endoplasmic reticulum and from its effects on cellular

calcium levels at late stages of the viral replication cycle, rather than from its putative role as an enterotoxin.

Our previous studies on the P8[11], G10 type asymptomatic neonatal strain I321 as well as genetic and comparative sequence analysis studies by others showed a clustering of NSP1 sequences according to species origin and suggested a role for NSP1 and NSP3 as determinants of host range restriction of rotaviruses [11, 19, 21, 41, 51, 63]. Further, heterologous NSP1 did not affect replication kinetics *in vivo* [9]. The present observation that NSP1 and NSP3 in MP409 and MP480 are of bovine origin suggests that both these proteins are not major determinants of host specificity, at least in this study between human and bovine rotaviruses. Further, NSP1 is unlikely to be associated with virulence phenotype since asymptomatic neonatal strains do not have a common NSP1 sequence [50].

To date, rotaviruses having P6[1] type VP4 have not been reported in humans. The present study shows for the first time that P6[1], G8 strains commonly found in cattle can replicate in humans and cause disease. Detailed epidemiological studies on rotaviruses in India, though limited, and restricted to large cities, revealed persistent predominance of unique bovine-human reassortant rotaviruses in nurseries [20, 22, 57]. Considering the close association of majority of the rural population with cattle and extensive use of cattle waste as manure and fuel as well as in various Indian traditions in rural settings in India, the present study is of epidemiological importance and signifies the need for study of rotaviruses in rural population.

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