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Prevalence of, and antigenic variation in, serotype G10 rotaviruses and detection of serotype G3 strains in diarrheic calves: Implications for the origin of G10P11 or P11 type reassortant asymptomatic strains in newborn children in India

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Summary. Previous studies have shown predominant association of G10P11 type bovine rotavirus-derived reassortant strains with asymptomatic infections in newborn children in India. To understand the epidemiological and genetic basis for the origin of these strains in humans, the relative frequencies of different serotypes among bovine rotaviruses (BRVs) isolated from southern, western and central regions of the country were determined by subgroup and serotype analysis as well as nucleotide (nt) sequence analysis of the genes encoding the outer capsid proteins VP4 and VP7. Since the human G10P11 asymptomatic neonatal strain I321 possessed NSP1 from a human rotavirus, to determine its genetic origin in the bovine strains, comparative analysis of partial gene sequences from representative G10P11 strains was also carried out. The following observations were of great epidemiological significance, (i) G10P11 strains predominated in all the three regions with frequencies ranging between 55.6% and 85.2%. In contrast to the high prevalence of G6 strains in other countries, only one G6 strain was detected in this study and G8 strains represented 5.8% of the isolates, (ii) among the G10 strains, in serotyping ELISA, four patterns of reactivity were observed that appeared to correlate with the differences in electropherotypic patterns and amino acid (aa) sequence of the VP7, (iii) surprisingly, strains belonging to serotype G3 were detected more frequently (10.7%) than those of serotypes G6 and G8 combined, while strains representing the new serotype (G15) were observed in a single farm in Bangalore, and (iv) about 3.9% of the isolates were nontypeable as they exhibited high cross-reactivity to the serotyping MAbs

used in the study. Comparative analysis of the VP7 gene sequence from the prototype G3 MAb-reactive bovine strain J63 revealed greatest sequence relatedness (87.6% nt and 96.0% aa) with that of serotype G3 rhesus-monkey strain RRV. It also exhibited high sequence homology with the VP7 from several animal and animal rotavirus-related human G3 strains (Simian SA11; equine ERV316 and FI-14; canine CU-1 and K9; porcine 4F; Feline Cat2 and human HCR3, YO and AU1). Partial nucleotide sequence analysis of the NSP1 gene of J63 showed greatest nt sequence homology (95.9%) to the NSP1 gene allele of the Indian G8 strain, isolated from a diarrheic child, which is likely to have been transmitted directly from cattle and 92.6% homology to that of the bovine G8 strain A5-10 suggesting the likely origin of J63 by gene reassortment between a bovine G8 strain and a G3 animal strain. Prevalence of G10P11 strains in cattle and G10P11 or P11 type reassortant strains in asymptomatic neonates as well as detection of G8P[1] strains in diarrheic children support our hypothesis for bidirectional transmission of rotaviruses between humans and cattle and origin of novel strains catalyzed by the age-old traditions and socio-economic conditions in India.

Introduction

Rotaviruses are recognized as the single most important cause of severe, acute dehydrating diarrhoea in the young of humans and many animal species including calves [36]. High mortality and morbidity among young calves due to rotavirus disease is a serious cause of economic loss to animal farms and dairy industry [14, 30, 50, 61].

Rotavirus is composed of triple-layered protein capsid which encloses a genome of eleven segments of double-stranded (ds) RNA [19]. The genome primarily encodes six structural and five non-structural proteins [20]. VP4 and VP7, encoded respectively by gene segments 4 and 7, 8 or 9 (depending on the strain), specify two distinct serotype specificities termed P and G serotypes [20]. The intermediate capsid protein VP6 possesses the group and subgroup (SG)-specific epitopes. Among the 7 groups (A-G) that have been identified, group A rotaviruses constitute the major pathogens in humans and domestic animals [36]. Four subgroups (I, II, I + II and non I/II) have been observed among group A rotaviruses [24, 32]. Majority of the rotaviruses exhibit either 'short' or 'long' RNA electropherotype based on the slower or faster migration of gene segment 11 in polyacrylamide gels. In general, animal rotaviruses possess 'long' RNA pattern and subgroup I specific VP6. But human strains exhibit both 'long' and 'short' RNA electropherotypes, with the 'short' electropherotype commonly associated with SGI-specific VP6 and 'long' pattern with SGII VP6 [36].

So far 14G serotypes have been identified in humans and animals [36] and a new G serotype has been recently identified in diarrheic calves [49]. Several studies identified three major antigenic determinant regions A (aa 87–101), B (aa 143–152) and C (aa 208–223) on VP7 which together form

complex and interdependent conformational epitopes that significantly differ in sequence, serotype and neutralization specificities among different rotaviruses [17–19, 29, 37, 41, 45, 55].

Based on $> 89.0\%$ aa sequence identity, at least 20 VP4 genotypes (P genotypes) have been observed among human and animal rotaviruses [19, 36]. Proteolytic cleavage of VP4 into amino terminal VP8* and carboxy terminal VP5* domains results in enhancement of rotavirus infectivity and the VP8* region (aa 1–247) contains the P serotype specific determinants of VP4 [19, 39, 40]. Because of the paucity of appropriate antibody reagents, so far only 11 P serotypes have been characterized [19, 36].

Among the 14G serotypes, G1–G4 predominate infections in humans. Serotypes G5, G6, G8 and G10 are mostly encountered in animals. While strains belonging to serotypes G11, G13 and G14 are restricted to pigs and horses, G7 strains are mostly observed in birds. G12 strains are so far detected only in humans [36]. Although VP7 belonging to specific G serotypes is shared between human and some animal rotaviruses, serotype G3 viruses appear to exhibit broadest host range, but rarely observed in cattle [36]. To date, only two G3 strains have been detected in cattle in a single study [31].

Strains belonging to serotypes G6, G8 and G10 are the major pathogens in cattle, though rare occurrence of strains belonging to G1, G2, G7 and G11 has been reported [6, 31, 34]. The predominant bovine serotypes G6, G8 and G10 possess either of the three VP4 types P6[1], P7[5] and P8[11] [19, 53].

In recent years, rotaviruses with G8 and G10 and/or P11 specificities have been isolated in significant numbers from diarrheic children as well as asymptomatic neonates [4, 8, 11, 13, 21, 22, 27, 35, 36, 52]. In India, strains having G10 and/or P11 type outer capsid proteins similar to those found in BRVs have been shown to be predominantly associated with asymptomatic infections in newborn children [12, 13, 16, 21]. Such an unusual epidemiological situation suggested the likelihood of predominance of G10P11 strains in cattle in India and the asymptomatic strains I321 (P8[11],G10) and 116E (P8[11],G9) could have originated in nature by gene reassortment between human and bovine rotaviruses.

Though rotavirus diarrhoea in cattle in India has been reported, there exists only a single report on the prevalence of bovine rotavirus G and P types among a limited number of strains isolated from a single location in the northern region of India [23]. Because of the predominance of G10 and/or P8[11] type BRV-like strains in neonates in India, study of the distribution of BRV G and P types on a wider scale is of clinical importance.

This paper describes relative frequencies of different G and P types including detection in significant numbers of serotype G3 strains in cattle in three different geographical locations in India as determined by serological assays and nt sequence analysis of the outer capsid proteins VP7 and/or VP4 as well as the gene encoding the nonstructural protein NSP1 which has been implicated in determining the host range phenotype of rotaviruses [7, 15, 38, 62].

Materials and methods

Stool specimens

Fecal samples from 3 to 90-day old calves were collected from cattle farms in three different geographical locations of India during 1994 and 1996; Bangalore (southern region), Mumbai (western region) and Jabalpur (central region). Specimens collected from Mumbai region were numbered B1 to B101 and those from Jabalpur were denoted as J1 to J75. In Bangalore area, samples from two isolated cattle farms as well as from inside the city were collected. Samples from the military farm were numbered MF1 to 116, those from Hesaraghatta farm as Hg1 to Hg114 and those from inside the city were represented as Bg1 to Bg50. A 20 percent dilution of stool specimens was made in phosphate-buffered saline, clarified by centrifugation and the supernatants were stored at $-20\,^{\circ}C$ as described previously [54].

Virus strains and cultivation

The following strains were grown in culture in MA104 cells and were used as controls in ELISA and electropherotype analysis. The prototype symptomatic human strains used are Wa (SGII, P1A[8],G1), ST3 (SGII, P2A[6],G4) and I321 (SGI, P8[11],G10). RRV (SGI, P5[3],G3), NCDV (SGI, P6[1],G6) and OSU (SGI, P9[7],G5) represent the cell culture-adapted animal strains. MP409 (SGI, P6[1],G8) is a G8 strain isolated from diarrheic child and signifies direct transmission from cattle to humans [35]. DxRRV(G1), DS1xRRV(G2), PxUK(G3) and ST3xSA11(G4) are single gene reassortants in which the VP7 gene of the animal strain was substituted by that of the human strain and were described previously [28, 43]. These reassortant strains were used as they are easy to grow in cell culture. Cell culture-adapted Hg18 and Hg23 (P[21],G15) [49] were also used for ELISA. 1040 is a prototype strain representing a large number of isolates possessing SGI specificity and short RNA electropherotype. These strains exhibited cross reactivity to MAbs specific for several serotypes [2] and were 20-fold less susceptible in neutralization assays in comparison to DS1 using hyperimmune serum raised against DS1. The strains, though reacted with the G2 MAb 1C10 in ELISA, were not susceptible to the MAb in neutralization assays. VP7 of these strains contained several amino acid substitutions, including in the antigenic regions A and B, and probably represent a subtype of serotype 2 (unpublished data).

Extraction of viral genomic dsRNA and analysis by PAGE

Presence of rotavirus in the fecal samples was determined by analysis of the viral genomic dsRNA by polyacrylamide gel electrophoresis (PAGE). Viral dsRNA was purified by binding to and elution from CC41 cellulose matrix (Whatman) as previously described [12]. Viral dsRNA segments were separated by PAGE and detected by staining with silver nitrate [26, 54].

Subgrouping and serotyping ELISA of bovine isolates

Subgroup and serotype specificities of bovine stool samples positive for rotavirus, as identified by PAGE, were determined by ELISA as previously described [25, 46, 54]. MAbs specific for human serotypes 1 to 4, porcine type 5 and bovine serotypes 6 and 10 which were available in the laboratory were used in serotype ELISA. All the MAbs used in this study were kindly provided by Dr. Harry B. Greenberg, Stanford University, Stanford, CA, USA. The MAbs used are: 5E8 (serotype G1), ICI0 and 2FI (serotype G2), 159 (serotype G3), S2-2G7 (serotype G4), IC3 (serotype G6) and B223/N7 (serotype G10). These MAbs have been evaluated in serotyping ELISA in previous studies [2, 25, 46, 60]. An isolate was assigned a subgroup or serotype if the absorbance reading with the specific MAb was at least twice that observed for MAbs specific for other subgroup or serotypes as described earlier [2, 25, 46].

Stool sample negative for rotavirus as well as cell lysate of uninfected MA104 cells were used as negative controls. Absorbance readings lower than 0.200 was not considered positive. The serotype specificity of strains that were positive by PAGE, but could not be assigned to a serotype by ELISA, was determined by nucleotide sequence analysis of the VP7 gene from representative strains. Since the G8 MAb was not available in the laboratory, the presence of G8 strains was determined by nucleotide sequence analysis of the VP7 gene from among the nontypeable strains.

Cloning of VP7, VP4 and NSP1 genes of bovine G10 and G3 strains

The genomic dsRNA purified directly from the stool samples was used for reverse transcription by AMV-reverse transcriptase. dsDNA was synthesized by polymerase chain reaction (PCR) using Taq-DNA polymerase as described [48]. The primers used for cDNA synthesis and PCR amplification were designed by incorporating degeneracy at specific positions such that the corresponding genes from several serotypes could be amplified. The sequences of the 5′ and 3′ primers for VP7 gene are: 5′-ATCCCGGGGCTTTAAAAGA/CGAGAAT-3′ AND 5′-ATCCCGGGTCACATCA/GT/AA/CCA-3′ and the sequences correspond to nt positions 1 to 18 (5′ primer) and 1062–1049 (3′ primer), respectively, of the gene. The 5′ and 3′ primers of the VP4 gene are: 5′-CTAAGCTTCCCGGGGCTATAAAATGG/CC/GTTC-3′ and 5′-CTAAGCTTCCCGGGTCACATCC/TT-3′, respectively and correspond to the 5′ and 3′ termini of the VP4 gene. The P type specificity of the strains was determined by carrying out nested PCR using a common 5′'primer (Bov4com5) and 3′ primers that are type specific [23]. The nucleotide positions and sequences of the primers (5′ to 3′) were as follows : Bov4com5 (nt 1067 to 1085), ATTATTGGGACGATTCACA; P6[1] (nt 1505–1525), TAAATTCATCTCTTAGTTCTC; P7[5] (nt 1723 to 1704), CCGCATCGGATAAA GAGTCC; P8[11] (nt 1393 to 1373), TGATGCCTCATAATATTGTTG. The P8[11] VP4 from the G10 bovine strains was further confirmed by nested PCR of the VP8* region using the 3′ primer: 5′-GCAGGTCGACTTAGGATCCCGCAATTTCTTGACTCCT-3′. The sequence is located from nt position 733–750 of the I321 gene 4 [12]. The P type specificity was confirmed by analysis of the nt sequences of the 5′ and 3′ termini (∼250 nt) of the full length VP4 gene.

The NSP1 gene was amplified using gene-specific primers. The sequences and nucleotide positions of the 5′ and 3′ oligonucleotides were: 5-ATCCCCGGGAGTCTTGTG TGAGCCATG-3′ (nt 18–35) from the 5′ end and 5′-ATCAAGCTTCTGCCTAGGCGCTA CTCTAG-3′ located from nt position 16 to 36, upstream from the 3′-end of the gene [15], respectively. These primers will amplify the NSP1 gene alleles present in the bovine strains RF, UK, B223 and A5 as well as I321. The I321 NSP1 gene 5′ sequence differed at nt positions 27, 28. While the B223 gene has a T at position 28, the I321 gene has G and A at positions 27 and 28 instead of T and G, respectively. These changes located in the middle of the primer did not affect amplification of the gene from I321. To confirm the specificity of the I321 gene, another primer was also used. The sequence of the primer is 5′-ATCCCCGGGCATTTCCTCTG TTCGCTAAT-3′ (from nt 696 to 677 in I321 gene). The gene specific sequences in all the primers are underlined. The DNA products obtained from PCR amplification were cloned either directly into pGEM-T vector (Promega Biotech) or into pBluescript KS^+ (PBS^+) after digestion of the inserts and the vector with restriction endonucleases for which the sites have been incorporated in the primers [48].

Nucleotide sequence analysis of VP7, VP4 and NSP1 genes of serotype G10 bovine rotaviruses and VP7 and NSP1 genes from G3 bovine strains

The nucleotide sequence of the cloned rotaviral genes was determined by the dideoxy nucleotide-mediated chain termination method [51]. Nucleotide sequence of both strands

was determined using vector- and gene-specific primers. The nt and deduced amino acid sequences were analyzed using the sequence analysis package from the Genetics Computer Group (GCG), University of Wisconsin. The nucleotide sequences have been submitted to GenBank and the GenBank accession numbers for the VP7 sequences of Indian bovine strains are: J63, AF386914; Hg2, 386916; B69, AF386917; B75, AF386918; MF53, AF386919 and B8, AF386928 and that of human G3 strain MP126 is AF386915. Since the sequences of other strains belonging to a specific electropherotype were highly similar only the sequences of representative strains were submitted to GenBank.

Results

Electropherotypes

All the bovine isolates, without exception, showed 'long' RNA electropherotype (Fig. 1) and subgroup I specificity, a common characteristic of animal rotaviruses. Among the bovine strains, eight different electropherotypes were observed. Electropherotype I was represented by B8, B12 and J58; electropherotype II by B67, B69 and B75; electropherotype III by MF49, MF53 and MF66; electropherotype

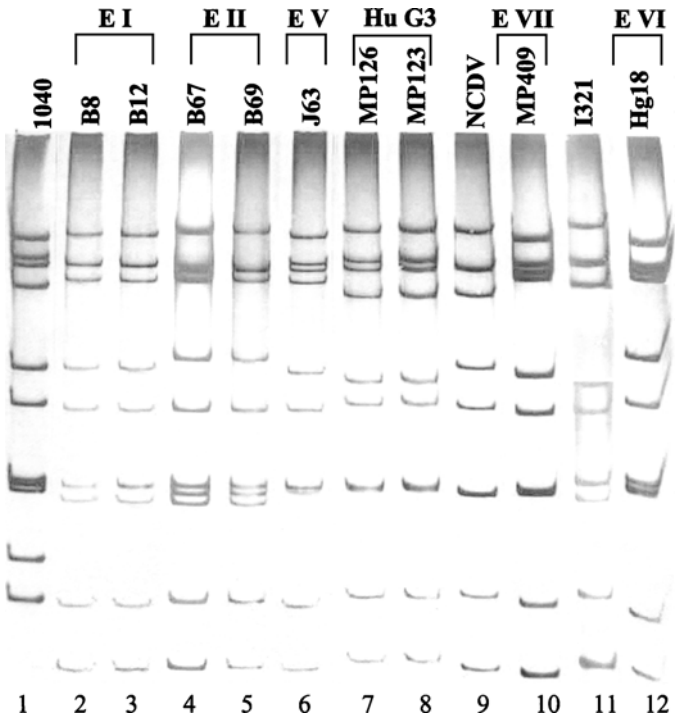

Fig. 1. Analysis of viral genomic dsRNAs by polyacrylamide gel electrophoresis (PAGE). *1* Serotype G2 strain 1040; *2*, *3* electropherotype I bovine strains B8 and B12; *4*, *5* electropherotype II strains B67 and B69; *6* electropherotype V strain J63; *7*, *8* human serotype G3 strains MP126 and MP133 isolated in Mysore, India; *9* bovine serotype G6 strain NCDV; *10* Indian human G8 strain MP409 which shows similar electropherotypic pattern as exhibited by the bovine G8 strains (VII); *11* serotype G10 human asymptomatic strain I321 and *12* the serotype G15 strain Hg18 exhibiting electropherotype VI. Electropherotypes III, IV, VIII are not shown due to limiting quantity and poor quality of the RNA. *E* Electropherotype

IV by Hg2 and Hg13; electropherotype V by J39, J63, J72 and B74; electropherotype VI by Hg18 and Hg23; electropherotype VII by the G8 strains J73 and B73 and electropherotype VIII by the highly cross-reactive strains represented by J7, J23 and J43. The RNA electropherotypic patterns III, IV, VII and VIII are not shown because of the limitation of the quality and quantity of these RNAs compared to others. Major differences among different electropherotypes were noticed in the migration patterns of RNA segments 4, 5, 6, 7, 8 and 9. The bovine G8 strains were very similar to the G8 human strains MP409 and MP480, reported recently from diarrheic children [35].

Serotypes

In serotyping ELISA, the bovine strains exhibited five reactivity patterns (Table 1). Majority of the bovine isolates which reacted with the serotype G10-specific MAb also reacted with either G1 or G3 MAb or both (Table 1). The serotype specificity of these isolates as well as those that did not show significant reactivity to any of the MAbs used in this study, (including the G10, G8 and G15 strains), was determined by nucleotide sequence analysis of the VP7 and/or VP4 genes. Employing serotyping ELISA and nt sequence analysis of the VP7 gene, it was observed that serotype G10 predominated in all the three geographical locations of the country. Serotype G10 strains accounted for 55.6% (10/18) of the isolates in Jabalpur, 70.8% (17/24) in Mumbai and 68.9% (42/61) on the average in Bangalore farms (Table 2). In Bangalore, while two of the farms accounted for 80.0% (8/10) and 85.2% (23/27), in another farm, the G10 strains represented only 45.8% of the isolates. The low prevalence of G10 strains in this farm was due to the presence of strains belonging to the new serotype G15 [49] which constituted 50.0% (12/24) of the isolates in that farm (Table 2). It is of interest to note that the G15 strains were restricted to a single farm in Bangalore and were not detected in other farms in Bangalore or in other regions of the country (Table 2).

Only one sample from Bangalore reacted with the G6-specific MAb, but the viral RNA was barely detectable by PAGE. G6 strains were not detected in Central and Western locations of the country. Nucleotide sequence analysis of the nontypeable strains revealed that serotype G8 strains accounted for just 5.8% (6/103) of the total BRVs, but were detected in all the regions.

A surprising observation was the detection in significant numbers of serotype G3 strains in cattle in all the three regions which accounted for 10.7% of the isolates. Several strains (J39, J63, J72 and B74) showed high reactivity only with the cross-reactive G3 MAb 159 (Table 1). Significantly, bovine G3 strains exhibited a pattern of migration of genes 7, 8 and 9 that is very similar to that of human G3 strains (Fig. 1). G3 strains are more frequently encountered in central and western regions of the country than in the southern region. In fact, serotype G3 represented the third most abundant serotype in Indian calves (Table 2). The recently identified new bovine serotype G15 [49] represented the second most prevalent type (11.7%) though its presence is restricted to Bangalore area.

Table 1. Serotype analysis of representative bovine strains by ELISA using MAbs

Rotavirus strain	Reactivity of strains to MAbs specific for different serotypes									Serotype by ELISA/nt sequence
	Electro-pherotype	5E8 (G1)	1C10 (G2)	2F1 (G2)	159 (G3)	4F8 (G3)	S2-2G7 (G4)	5B8 (G5)	IC3 (G6)	B223/N7 (G10)	
B8	I	0.093	0.049	ND	**0.478**	ND	0.075	ND	0.055	**0.477**	10
B12	I	0.128	0.000	ND	**0.885**	ND	0.049	ND	0.011	**0.926**	10
J58	I	0.015	0.000	ND	**0.278**	ND	0.060	ND	0.006	**0.369**	10
B67	II	**2.559**	0.000	ND	**2.931**	ND	0.090	ND	0.009	**3.019**	10
B69	II	**1.058**	0.000	ND	**2.285**	ND	0.055	ND	0.000	**2.188**	10
B75	II	0.040	0.000	ND	**0.243**	ND	0.028	ND	0.000	**0.194**	10
MF49	III	**1.350**	0.081	0.076	**>3.000**	0.091	0.112	0.046	0.110	**1.560**	10
MF53	III	**0.324**	0.028	0.013	**1.160**	0.011	0.025	0.027	0.027	**1.035**	10
MF66	III	**0.880**	0.262	0.208	**>3.000**	0.265	0.213	0.157	0.144	**>3.000**	10
Hg2, Hg13	IV	0.021	0.023	0.029	0.041	0.004	0.001	0.001	0.000	0.021	10
J39	V	0.021	0.017	ND	**0.803**	ND	0.054	ND	0.000	0.008	3
J63	V	0.044	0.031	ND	**2.362**	ND	0.091	ND	0.012	0.023	3
Hg23, Hg18	VI	0.034	0.050	0.093	0.010	0.002	0.022	0.006	0.004	0.029	15
J73, B73	VII	0.000	0.000	0.002	0.100	0.003	0.043	0.012	0.000	0.005	8
J7, J23, J43	VIII	1.028	0.927	ND	1.182	ND	0.734	ND	0.908	0.536	ND
WA		**2.673**	0.204	0.151	0.385	0.214	0.146	0.164	0.091	0.101	1
DXRRV		**>3.000**	0.110	0.149	0.412	0.112	0.166	0.270	0.150	0.153	1
DS1XRRV		0.146	**0.677**	**1.520**	0.402	0.195	0.233	0.240	0.324	0.292	2
PXUK		0.137	0.105	0.113	**>3.000**	**>3.000**	0.127	0.316	0.135	0.129	3
ST3XSA11		1.055	0.150	0.162	0.359	0.104	**>3.000**	0.279	0.110	0.105	4
OSU		0.174	0.103	0.112	0.279	0.126	0.108	**1.675**	0.123	0.132	5
NCDV		0.133	0.104	0.158	0.281	0.111	0.189	0.296	**>3.000**	0.212	6
I321		0.120	0.155	0.174	0.142	0.132	0.189	0.170	0.120	**0.680**	10

ND Not determined; Data are shown as average absorbance at 410 nm of two wells. Though more than one strain belonging to electropherotypes IV, VI, VII and VIII are studied the values corresponding to only one of the strains are given in the table as the values obtained for other strains belonging to that electropherotype are very similar

Table 2. Frequency of rotavirus serotypes in diarrheic calves

	No. of samples	No. positive	Percent positive	G serotypes					
				G3	G6	G8	G10	G15	NT
Bangalore	280	61	21.8	2	1	2	42 (68.9%)	12	2
Farm 1	116	27	23.3	1	1	1	23 (85.2%)	0	1
Farm 2	114	24	21.1	0	0	1	11 (45.8%)	12	0
Farm 3	50	10	20.0	1	0	0	8 (80.0%)	0	1
Mumbai	101	24	23.8	5	0	2	17 (70.8%)	0	0
Jabalpur	75	18	24.0	4	0	2	10 (55.6%)	0	2
Total	456	103	22.6	11 (10.7%)	1 (1.0%)	6 (5.8%)	69 (67.0%)	12 (11.7%)	4 (3.9%)

NT Non typeable

Isolates represented by J7, J23 and J43 exhibited high cross-reactivity with all the MAbs used in this study and represented 3.9% of the isolates. These could not be assigned to any serotype as the VP7 gene is yet to be characterized.

Correlation between serotypic MAb reactivity and electropherotype

The eight electropherotypes detected in this study exhibited five different reactivity patterns in serotyping ELISA. Strains exhibiting electropherotype I (B8, B12, J58) showed reactivity with G3- and G10-specific MAbs 159 and B223/N7, respectively. Strains with electropherotype II (B67, B69, B75) and III (MF43, MF49, MF53, MF66) reacted with MAbs specific for G1 (5E8), G3 (159) and G10 (B223/N7). Strains belonging to electropherotype V (J39, J63, J72, B74) reacted only with the G3 MAb 159 (Table 1).

While two strains belonging to electropherotype IV (Hg2 and Hg13) and twelve strains belonging to electropherotype VI (represented by Hg18, Hg23) and six strains of electropherotype VII (J73, B73) did not react with any of the MAbs, those of electropherotype VIII (represented by J7, J23, J43) exhibited high cross reactivity with all the MAbs used in this study. The lack of reactivity to MAbs by two strains (Hg2 and Hg13) may be attributed due to lack of sufficient virus in the sample as observed by very faint RNA bands in PAGE. The low reactivity of the electropherotype II strain B75 is also reflected by the low amount of virus in the sample as evident from faint RNA bands (data not shown). Thus a correlation, in general, appeared to exist between electropherotypic patterns of the strains and serotypic MAb reactivities.

Sequence analysis of the VP7 gene of the G10 MAb-reactive bovine rotaviruses

Since the bovine strains exhibited five patterns of reactivity in serotyping ELISA and majority of the strains that reacted with the G10 MAb also reacted with the G1 and/or G3 MAbs, the serotype specificity of the strains was determined by

Table 3. Percentage nucleotide and aminoacid identities of G10 serotype VP7 from bovine and human rotaviruses

	Hg2	MF53	B8	B75	B69	Mc35	A64	B223	KK3	61A	A44	I321
Hg2		97.6	96.6	86.1	85.9	96.1	89.0	86.5	85.5	85.7	85.4	84.1
MF53	97.2		95.5	85.1	85.0	95.1	87.9	85.6	84.7	84.9	84.7	83.1
B8	97.5	96.6		86.2	85.0	95.9	89.1	86.7	85.7	85.6	85.0	83.9
B75	96.0	95.1	96.9		99.2	86.3	86.4	93.0	92.7	97.6	96.6	89.3
B69	94.8	93.9	95.1	98.2		86.2	86.3	92.7	92.5	97.2	96.4	88.9
Mc35	96.3	95.4	97.5	95.1	93.9		88.6	86.2	85.4	87.1	86.3	84.0
A64	96.0	95.1	96.0	94.8	93.6	95.1		86.4	85.9	86.6	86.6	84.1
B223	96.0	95.4	96.0	97.5	96.3	94.8	95.7		94.6	93.2	92.5	91.0
KK3	95.4	95.1	95.4	96.3	95.1	94.8	93.9	96.9		93.5	93.6	90.2
61A	95.1	94.5	95.7	97.2	96.3	95.7	94.2	96.6	96.6		98.2	89.0
A44	94.8	94.2	95.4	96.9	95.7	95.4	93.9	96.3	96.3	97.9		89.0
I321	92.0	91.4	92.3	95.3	92.0	91.1	91.7	94.5	92.3	92.6	92.3	

Percentage nucleotide homology is given in the upper triangle and the percentage aminoacid homology is given in the lower triangle. For simplicity, sequence identities of only one strain representing each of the four electropherotypes, except B75 which showed a few differences in the coding region with reference to B69, are shown. The accession numbers of the VP7 gene sequences used in this study are: Mc35, Q08779; A64, X63156; B223, X57852; KK3, D01056; 61A, X53403; A44, D01055; I321, L07658

analysis of partial or complete nucleotide sequences of the VP7 and/or VP4 genes from a few strains representative of each of the serotypic MAb reactivity patterns and the corresponding electropherotypes. Comparison of the VP7 gene sequences of the bovine strains that reacted with either the G10 MAb alone or showed cross reactivity with G1 and/or G3 MAbs revealed that the gene from all these strains is highly homologous to that from G10 rotaviruses (Fig. 2), the nt homology ranging from 83.1% to 98.2% depending on the strain (Table 3). The percent VP7 amino acid sequence homologies of the Indian bovine G10 strains with the published G10 VP7 sequences ranged between 91.1 and 98.2 (Table 3). These results indicated that all the strains that showed reactivity with the G10 MAb, irrespective of their reactivity with other serotyping MAbs, belonged to G10 serotype.

Sequence analysis of VP7 gene from strains that did not react with serotyping MAbs

Since twenty strains belonging to electropherotypes IV, VI and VII did not react with any of the typing MAbs in serotyping ELISA, to determine the serotype specificity of these strains, partial or complete nt sequence of the VP7 gene from two strains representative of each of these electropherotypes was determined. Comparative analysis revealed that while both the strains of electropherotype IV (Hg2 and Hg13) possessed G10 type VP7 (Fig. 2), those of electropherotype VI (Hg18 and Hg23) possessed VP7 representing the new serotype G15 [49].

Analysis of 300 nt from the 5′ and 3′ ends of the VP7 gene from strains belonging to electropherotype VII (J73, B73) revealed greatest homology to serotype

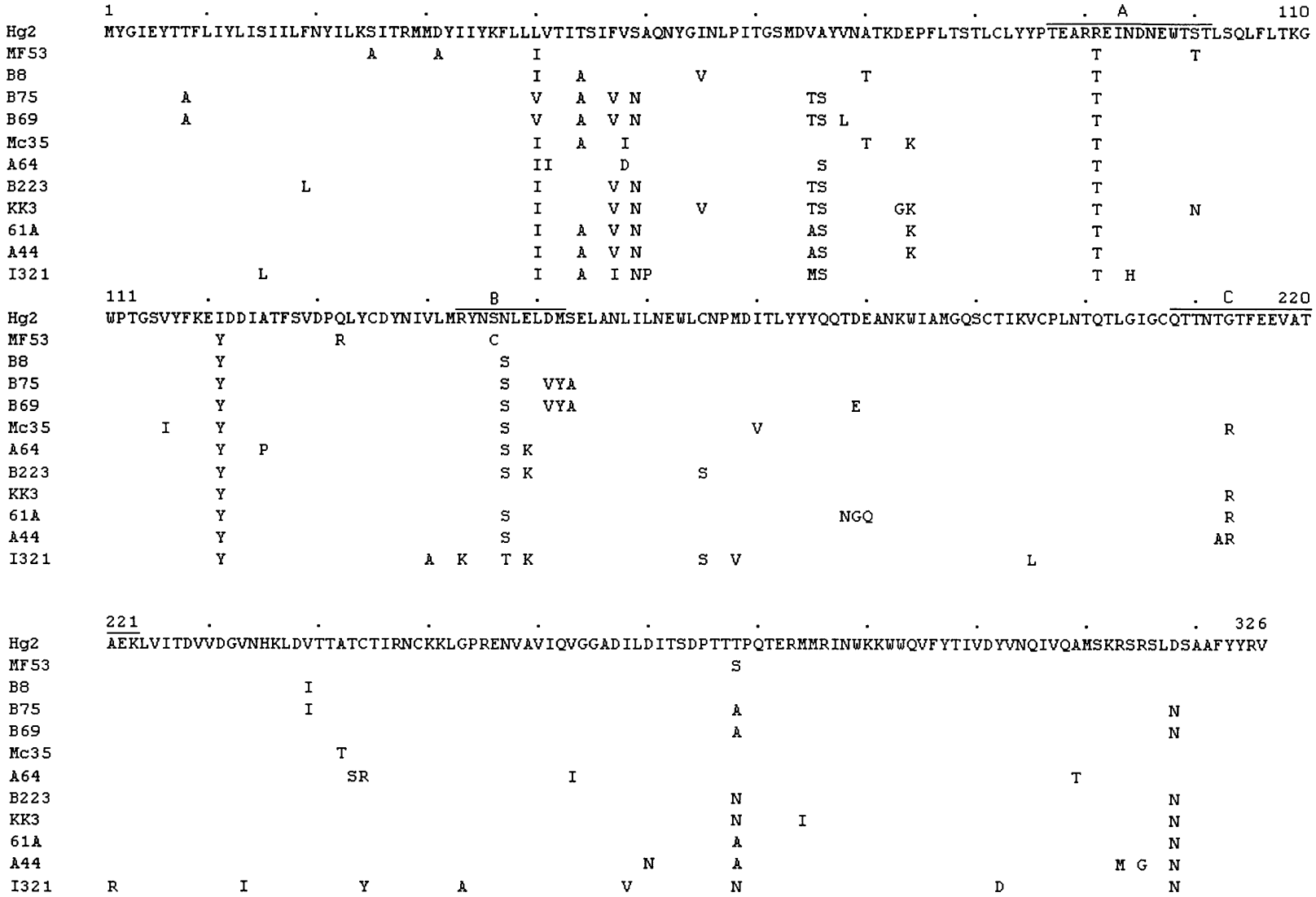

Fig. 2. Comparison of the VP7 sequence of representative Indian G10 bovine strains with that of other bovine and human G10 strains. Major antigenic regions A, B and C are overlined. As the VP7 sequences of strains belonging to a particular electropherotype are highly related, for simplicity, the sequence of only a single representative strain is shown. Accession numbers of sequences used for comparison are given in footnote to Table 3

G8 VP7 exhibiting 98% identity at nucleotide level with that of the human G8 strain MP409 which was recently shown to represent direct transmission from cattle to humans [35] (data not shown). The strains belonging to electropherotype VIII that showed cross reactivity with several MAbs are yet to be characterized.

Correlation between amino acid sequence variation in G10 VP7 and serotype MAb reactivity

The G10 strains from Mumbai belonging to electropherotype I (B8, B12) that cross reacted only with the G3 MAb differed from those of electropherotype II (B67, B69 and B75) which cross reacted with both G1 and G3 MAbs, primarily in the antigenic region B. In the electropherotype II strains, the consecutive aas Asp, Met and Ser from position 151–153 that are conserved in other G10 strains were replaced by Val, Tyr and Ala at the respective positions. Though the G10 strains belonging to electropherotypes II and III cross reacted with both G1 and G3 MAbs, the type III strains (MF49, MF53 and MF66) besides differing at positions 151 to 153, also contained substitutions at aa positions 100 (Ser $\rightarrow$ Thr) in antigenic region A, 146 (Ser $\rightarrow$ Cys) in region B and 132 (Gln $\rightarrow$ Arg) between regions A and B (Fig. 2). The Bangalore G10 strains MF49, MF53 and MF66 (electropherotype III) and Hg2 and Hg13 (electropherotype IV) differed from majority of the G10 strains in that they contained Asn at position 147 in region B instead of Ser. Further, while electropherotype III strains differed from other G10 strains in having a Cys at aa position 146 in place of a conserved Ser, the G10 strains belonging to electropherotype IV that did not react with any of the serotype specific MAbs used in this study differed from other G10 strains at position 91 in the antigenic region A at which the highly conserved Thr was substituted by Arg. Also in these MAb-nonreactive strains, a conserved Tyr at aa position 121 was replaced by Ile between the antigenic regions A and B. Substitution of a conserved Thr by Arg at aa position 91 in region A, Ser by Asn at position 147 in region B and Tyr by Ile at position 121 between the two antigenic regions appear to result in loss of reactivity to MAbs by Hg2 and Hg13. But this lack of reactivity may also be reflected by low amount of virus particles in the sample as observed by relatively faint RNA bands in PAGE. These results suggest that aa substitutions at quite different positions in VP7 belonging to the G10 serotype could result in similar or different reactivity patterns with serotyping MAbs in ELISA.

Sequence analysis of VP4 and NSP1 genes from bovine G10 strains

To determine the P type specificity of the G10 bovine strains, the VP4 gene was amplified by RT-PCR using primers common to, as well as specific for P11, P5 and P6 VP4 alleles. Gene 4 from the Indian bovine G10 strains could be amplified, in nested PCR, only with the primers specific for P11 type VP4 gene. The P11 specificity of the bovine G10 strains was further confirmed by amplification of a 740 nt region corresponding to the VP8* region using 3′ primer specific for P11 type VP4 (data not shown).

Previous studies on the asymptomatic neonatal strains represented by I321 in our laboratory revealed that I321 had originated in nature by reassortment between a G10P11 bovine rotavirus and a human virus with all the genes, except for the genes encoding the nonstructural proteins NSP1 and NSP3, derived from the bovine parent [12, 16, 48]. Large scale infection of newborn children by I321, its persistence in newborn nurseries and hospitals in Bangalore during the last 12 years and its ability to replicate in neonatal calves (unpublished data) suggested that I321 might be transmitted from cattle owing to the close association of majority of the Indian population with cattle as well as through contaminated milk. In order to determine if some of the G10 bovine strains could be similar to the human asymptomatic neonatal reassortant strain I321 having the NSP1 gene derived from human rotavirus, we have amplified the gene from representative strains (B8, B67, MF53, Hg2) of the 4 electropherotypes by RT-PCR using primers common for B223, RF, UK and A5 and I321 gene alleles. The gene could be amplified from all the isolates. Comparative sequence analysis of the 5′ and 3′ terminal 234 and 242 nt, respectively, with the corresponding gene sequences from different rotaviruses revealed that the NSP1 gene from the Indian G10 bovine strains to be highly related to that from B223 gene allele. While the 5′ and 3′ terminal sequences of the Indian strains exhibited 91.5 to 92.0%, and 95.0 to 95.5% sequence identities with the corresponding sequences of the bovine B223 NSP1 gene, the respective percent homologies with the human I321 NSP1 gene were 76.8 and 64.8 and with that of bovine UK gene allele were 88.0 and 74.0. Since the NSP1 gene sequences of the Indian bovine strains are highly related sharing (98.5–99%) sequence identity among themselves, alignment of the 3′ terminal sequence of only the B8 gene with that of other strains is shown in Fig. 3.

Sequence analysis of the VP7 gene from serotype G3 bovine strains

To determine whether strains that reacted only with the cross-reactive G3 MAb 159 in serotyping ELISA truly represented serotype G3, we have cloned the VP7 gene from two representative strains J39 and J63 and the complete nt sequence of J63 and 5′ and 3′ terminal sequences (250 nt) of J39 were determined. The 3′ and 5′ terminal nt sequences of both strains were found to be identical indicating that both strains belonged to the same serotype. The J63 VP7 gene was 1062 nts in length and contained an open reading frame encoding a polypeptide of 326 aa in length. Comparison of J63 VP7 sequence with the published VP7 sequences representing the 15 known G serotypes revealed greatest relatedness to serotype G3 VP7. Comparative sequence analysis of J63 VP7 revealed greatest sequence relatedness to the G3 VP7 of rhesus monkey strain RRV with 87.6% and 96.0% homologies at nt and aa levels, respectively (Table 4). It is also highly related to the equine G3 strains ERV316, and FI-14; simian virus SA11, canine strains CU-1 and K9; porcine strain 4F; feline strain Cat2 and human strains HCR3, YO and AUI (Table 4). These human strains containVP7 closely related to that of animal strains [45]. Though the SA11 strain exhibited relatively less homology at nt level (83.0%) compared to that of RRV, HCR3, ERV316 and K9, it shared

```
                .         .         .         .         .         .         .         .         .      1423
A5-10 1324 TTCCGAGGATATTAAATTTAAATGACATTCGATTGATATTGAGCTCGTTAATATATGATTGGTTTGACATAAGATACATGAGAGAGACGCCAATGATAAC
B8    1323  G  A AA  CC G G    G        AA AA    A  G  G AA    T              TG G          G     A   G A  TGT
B223  1323  G  A  A  CC G G   TG     G  AA AA    A  G TG AA    T              TG G     T    G     A   G A  TGT
UK    1323  A  A  A  T  G  CA       T CAAAGAA      A  TA AA      T             G               A          CG
RF    1324  A  A  A  T  G   A G     T CAAAGAA    C    TG AA G    T   C         C      T        A  A       CG
I321  1311  A  ACAA       C      AA TG   TTACA  TA  CAA  TA T    C   A        T             GA T  A      GCT


          1424     .         .         .         .         .         .         .         .         .      1523
A5-10     ATCAACCACGAATGAATTGCGTAAATTAAATAGAAAGAATGAGCTGATTGATGAATATGATCTAGAACTATCAGACATTGAGTAAACAGTCATACATGGT
B8        G     A     C   C AA A  GC G  C A G T   A T  C  G    GG      TAT  G     T  T     A  G ..... CG   T
B223      G  G  A     C   C  A A  GC G  C A G T  CA T  C  G    GG  C   TAT  G  G  T  T     A  G .....  G   T
UK        G     A      C GC TA A C C G    A  G         C  A     G  C  CT       T     TG    A     .....TATGG CAC
RF        G     A  A  CC  C T  A C C G  C A  G         C` A     G  C   T       T     TG          .....TATGG CAC
I321        TT  A TTG  A    AA GCG   GC  .. C     T .AA  C  T          TCT GCG    T  TG    A  G GGAAA G G .


          1524     .         .         .         .         1573
A5-10     CACAACTTAGCCAACACAAGACCGTCTGCACTGGAGTAGCGCCTAGGCAG
B8        ...  T AT                          A                1564
B223      ...  T AT    G                     A                1564
UK        ...  A AC    G                     A                1564
RF        ...    AC    G                     A                1565
I321       GT ..  T    C       TTCC          A                1554
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Fig. 3. Comparison of the 242-nt sequence near the 3′ region of the NSP1 gene of the serotype G10 bovine strain B8 with that of other strains. Note an 8 nt insertion between nt positions 1498 and 1518 in the NSP1 gene of A5-10 in comparison to other NSP genes. The nt positions of different genes are indicated. The accession numbers of sequences used for alignment are:A5-10, D38147; UK, L11575; RF, M22308; B223, Z12105 and I321, U08418. As the NSP1 gene sequences of the Indian G10 strains are highly related (98.5–99% homologous), only the sequence of strain B8 is used for comparison

Table 4. Percentage of nucleotide and deduced amino acid identities between the VP7 Gene of bovine G3 strain J63 and of G3 strains from other species

Strain	Species origin	Percent identity with bovine G3 strain J63	
		Nucleotide	Amino acid
RRV	Rhesus monkey	87.6	96.0
SA11	Vervet monkey	83.0	95.7
HCR3	Human	84.9	95.4
CU-1	Dog	ND	95.4
FI-14	Horse	ND	94.5
ERV316	Horse	96.0	95.4
K9	Dog	84.3	94.5
4F	Porcine	82.0	92.0
Cat2	Cat	ND	91.4
AU1	Human	81.4	91.4
YO	Human	82.2	91.1
107E1b	Human	81.2	91.1
EB	Mouse	77.3	90.5
MP126/MP133	Human	80.9	90.2
EC	Mouse	76.8	89.6
MO	Human	80.9	89.3

ND Not determined

107E1b, MP126 and MP133 are G3 type human Indian strains. 107E1b is from the northern region and MP126 and MP133 are from Mysore in the southern region of the country. The accession numbers of VP7 gene sequences compared in this study are: RRV, M21650; SA11, J02354; ERV316, L49043; HCR3, L21666; YO, D86284; K9, U97199; AU1, D86271; 10731b; U04350; EB, U08420; MO, D86289; EC, Q83443; 4F, L10360. The aa sequences of strains CU-1, Cat2 and FI-14 were from reference Nishikawa K, Hoshino Y, Taniguchi K, Green KY, Greenberg HB, Kapikian A, Chanock RM, Gorziglia M (1989) Virology 171: 503–515

95.7% homology at aa level with J63 VP7. The VP7 of J63 showed homologies of only 81.1% and 80.9% at nt and 91.1% and 90.2% at aa levels with those of Indian human G3 strains 107E1b and MP126/MP133, respectively (Table 4). It also showed significant homology to the G3 murine strains EB and EC (Table 4).

Partial nucleotide sequence analysis of the NSP1 gene of the bovine G3 strain J63

Previous studies have shown clustering of the NSP1 gene by species origin suggesting that the NSP1 gene could determine host range restriction, though in recent studies interspecies relationships have also been observed [15, 38, 62]. Since the bovine G3 strain J63 contained VP7 closely related to that from simian, canine, equine, feline, porcine and murine strains (as discussed in the previous section), we sought to determine the nt sequence of the NSP1 gene in order

```
                     .         .         .         .         .         .         .         .         .      1429
A5-10 1330  GGATATTAAATTTAAATGACATTCGATTGATATTGAGCTCGTTAATATATGATTGGTTTGACATAAGATACATGAGAGAGACGCCAATGATAACATCAAC
J63          A    C                                                      T  G            A                G
MP409 1331                G    T                                C        T  G                             G
UK    1329   A  T  G  CA       T CAAAGAA      A  TA AA      T             G                A          CG  G
RF    1330   A  T  G   A G     T CAAAGAA    C    TG AA G    T   C          C      T        A  A       CG  G
B223  1329   A  CC G G   TG     G  AA AA    A  G TG AA    T              TG G     T     G     A   G A  TGTG  G
69M   1317  AA     G C   G      G  ATT CA  T  AC TATAA     G             T     G TT      A C         TCT  G TC

            1430     .         .         .         .         .         .         .         .         .      1529
A5-10       CACGAATGAATTGCGTAAATTAAATAGAAAGAATGAGCTGATTGATGAATATGATCTAGAACTATCAGACATTGAGTAAACAGTCATACATGGTCACAAC
J63                     A           C           AT                   G           TG    A   G      C            T
MP409                   A           C           AT          G        G            G    A   G    T              T
UK          A      C GC TA A C C G    A  G         C  A     G  C  CT       T     TG    A   ........ T          A
RF          A  A  CC  C T  A C C G  C A  G         C  A     G  C   T       T     TG        ........ T
B223        A     C   C  A A  GC G  C A G T  CA T  C  G    GG  C   TAT  G  G  T  T     A  .....GA   G  T   ... T
69M         AGTC   A     AAGC     T  GA    G  A A... C  CA         TC  GTA    C  TG    A    ..TT  G G AAT  ...TG

            1530     .         .         .             1572
A5-10       TTAGCCAACACAAGACCGTCTGCACTGGAGTAGCGCCTAGGCA
J63                                   A
MP409                                 A                1573
UK           AC    G                  A                1563
RF           AC    G                  A                1564
B223         AT    G                  A                1563
69M           C    C  TG    TC        A                1551
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Fig. 4. Alignment of the 243 nt sequence near the 3′ region of the NSP1 gene of the serotype G3 bovine strain J63 with those of bovine strains. Positions of nucleotides of the gene alleles are indicated. Note that the NSP1 gene of other strains had a deletion of 8 nt in the 3′ region between nt position 1498 and 1518 with respect to that of A5-10, MP409 and J63, the exact positions of deletion are different in different strains. The accession numbers of NSP1 gene sequences of MP409 and 69M are AF141916 and D38151, respectively. For the accession numbers of other sequences refer to the legend of Fig. 3

to understand the bovine specificity of the strain. The gene was amplified by RT-PCR, cloned and the sequence of the 3′ terminal 243 nt from position 1572 to 1330 (corresponding to the bovine strain A5-10) was determined. This sequence corresponds to nt position 1563/4 to 1329/30 of other bovine strains UK, A44 and B223. Comparison of the 3′ terminal NSP1 gene sequence of J63 with published NSP1 gene sequences showed greatest sequence identity (95.9%) to that of the human Indian G8 strain MP409 [35]. The percent identity with that of the bovine A5-10 strain was 92.6. The percent nt identities with other strains were less than 74.5, with the NSP1 of human G8 strain 69M exhibiting identity of only 68.2. Further, while alignment of the J63 NSP1 gene sequence with that of MP409 and A5-10 gene sequences did not show any gaps, those of other strains showed a deletion of 8 nucleotides in the region between position 1507 and 1527 corresponding to the A5-10 sequence (Fig. 4).

Discussion

The present studies on prevalence of different rotavirus serotypes and characterization of representative strains from Indian calves revealed a very interesting epidemiological situation in India.

The present finding that strains belonging to serotype G10P11 constituted the largest proportion of BRVs in cattle throughout India including the northern region [23] is of significance in the context of the evolutionary origin of the G10P11 neonatal asymptomatic strain I321 in the southern region [12, 16, 54] and G9P11 strain 116E [13, 21] in the northern region. But I321-like reassortant strains which are predominantly bovine in their genetic make-up, except for only two genes encoding NSP1 and NSP3 [12, 15, 16, 48], could not be detected among the G10 bovine strains collected from cattle farms as confirmed by partial nt sequence analysis of the NSP1 gene. Significantly the VP7 gene of the human neonatal strain I321 showed relatively low homology at nt level ($< 91.0\%$) with that of the bovine G10 strains (Table 3). The comparatively higher divergence of I321 from the Indian bovine strains might reflect either distant origin of the virus and/or host-specific genetic drift associated with adaptation to newborn children. It is likely that I321 is well established in the human population and is not transmitted from bovine sources though I321 is capable of replicating in newborn calves (unpublished data).

It is of significance to note that the bovine G10 strains that either reacted with G1, G3 and G10 MAbs or G3 and G10 MAbs or did not react with any of the MAbs used, contained aa substitutions in the antigenic regions A and B, though at different positions (Fig. 2). Thus aa substitutions at different positions in region A and B could result in quite contrasting reactivity patterns of the G10VP7 in ELISA. There is also evidence that single nucleotide substitutions could result in electropherotype differences [17]. A correlation between reactivities based on ELISA and neutralization specificities of the MAbs has not been carried out as N-MAb escape mutants in G10 strains have not been yet reported.

Comparison of VP7 gene sequences from bovine and human G10 rotaviruses revealed that the Indian bovine strains could be classified into two lineages typified by Hg2/MF49/MF53/MF66/B8/B12 (electropherotypes I, III and IV) and B67/B69/B75 (electropherotype II) based on nt sequence homology (Table 3). While strains belonging to electropherotypes I, III and IV are more closely related to the human symptomatic strain Mc35, strains belonging to electropherotype II showed relatively greater relatedness to the bovine strains 61A and A44 (Table 3). In spite of the sequence divergence at nt level, a clear distinction among the G10 strains at aa level was not apparent. Among the G10 strains, I321 showed relatively low homology to the Indian bovine strains though it is more closely related to B67/B69/B75 strains (electropherotype II) than to those of electropherotypes I, III and IV.

Detection of G3 strains in cattle in significant numbers is unusual since G3 strains were not commonly observed in cattle though they have been isolated from different species [36]. To date, only two isolates of G3 type were reported in cattle in USA [31], but characterization of these strains has not been reported yet. The observation that the NSP1 gene of the Indian G3 bovine strain J63 is highly related to that of the Indian human G8 strain MP409 which signified direct transmission from cattle to humans [35] and the bovine G8 strain A5-10 [56] strongly suggests the likelihood of the origin of J63 by genetic reassortment between an animal G3 strain (simian, canine, or equine) and a G8 bovine strain. In this context, it is of interest to note that G8 strains having different VP4 alleles have been frequently isolated in humans and appear to have evolved by gene reassortment [1, 3, 11, 22, 27]. An equine G8 strain has also been reported [33]. Further, two different VP4 gene alleles P6[1] and P7[5] have been reported among Bovine G8 strains [44]. Thus G8 strains appear to have high propensity to undergo gene reassortment and cause disease in humans and animals. Characterization of the VP4 gene of J63 should reveal the genetic origin of the parental G3/G8 strain.

Detection of strains exhibiting high degree of cross-reactivity to MAbs specific for several serotypes is also of significance since strains (1040, MP312, MP415) with similar properties in humans possessed ‘short’ RNA electropherotype and P[4], G2 genotype specificities [2, 42] and represented the second most abundant type of strains in humans as revealed by our previous epidemiological study [2]. These strains suffered several aa substitutions in the antigenic regions of VP7 and VP4 and were several fold less susceptible in neutralization assays with hyperimmune serum against DS1 and were not neutralized by the G2-specific MAb 1C10 in comparison to the G2 strain DS1 (unpublished data). Characterization of these cross-reactive bovine strains is needed to ascertain the G and P genotypes.

The present observation that serotype G10 predominated over other bovine serotypes in Indian calves provides conclusive epidemiological basis for the evolution of the asymptomatic neonatal strains I321 [12, 16] and 116E [13, 21] in different regions of the country. The recently characterized G8P[1] strains MP409 and MP480 from diarrheic children further provided evidence for direct transmission of bovine rotaviruses from cattle to humans in rural setting

where cattle constitute part and parcel of the human habitat. Extensive use of cattle waste in age-old Indian traditions and as manure and firewood and close association of majority of the Indian population with cattle might be playing a facilitating role in the transmission of bovine strains from cattle to humans and subsequent origin and evolution of novel reassortant strains. Although bidirectional transmission of rotaviruses between humans and cattle is expected to occur under this unique situation, we do not have conclusive evidence, at present, for the presence of human strains or human rotavirus-derived reassortants among bovine rotaviruses collected in this study from isolated farms. Characterization of bovine rotaviruses isolated from calves in the rural setting, where contamination of field water and grazing fields with human excreta is high, should provide information on the extent of transmission of human strains to cattle. Considering the high likelihood of bidirectional transmission of rotaviruses between humans and cattle, novel strategies are needed to be employed towards development of an effective vaccine against rotavirus disease under the Indian context.

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