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Detection of a novel intergenogroup recombinant Norovirus from Kolkata, India

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ABSTRACT

Mutation and recombination are recognized as important driving forces of evolution among RNA viruses. An intergenogroup recombinant norovirus strain [Hu/Kol/NLV/L8775/*AB290150*/2006/India] was detected in the faecal specimen of a 17 year old male, who had suffered from acute watery diarrhea and severe dehydration. Sequence analysis confirmed that this novel recombinant strain had a polymerase gene fragment that closely resembled a Norovirus (NoV) genogroup-I genotype-3 virus (HuCV/NLV/GI.3/VA98115/*AY038598*/1998/USA) and a capsid gene resembling NoV genogroup-II genotype-4 virus (NoV/Hu/GII.4/Terneuzen70/*EF126964*/2006/NL). The crossing over and recombination was observed at nucleotide (nt) 790 of NoV GI VA98115 strain and nt808 of NoV GII Terneuzen70 strain. In both parent strains conserved nucleotide sequence and hairpin structure (DNA secondary structure) were reported at the junction point of ORF1 and ORF2, exhibiting the mechanism of recombination in these viruses. Thus this novel recombinant NoV is another step in evolution among NoVs, indicating that constant surveillance is important to successfully monitor emergence of these strains.

Introduction

Norovirus (NoV) belonging to family *Caliciviridae* was the first diarrheagenic virus detected by Kapikian et al. (1972). NoVs are nonenveloped, 27 to 40 nm in diameter with single-stranded, positive-sense, polyadenylated RNA genome of 7.4 to 7.7 Kb (Atmar and Estes, 2001). The genome of NoVs comprises of 3 ORFs viz. ORF1 encodes six nonstructural polyproteins [p48, NTPase, p22, VPg, 3CL and RdRp], ORF2 encodes the major capsid protein [VP1] and ORF3 encodes the minor structural protein [VP2] (Hardy, 2005; Jiang et al., 1993; Lambden et al., 1993). NoV is chiefly associated with food borne gastroenteritis, including outbreaks in developed countries (Mead et al., 1999; Okitsu-Negishi et al., 2004). In developing countries, the prevalence rate of NoV associated gastroenteritis is higher and is often associated with severe illness (Atmar et al., 2001; Lopman et al., 2002).

NoVs have been divided into seven genogroups based on genome sequence variability of their RNA dependent RNA polymerase (RdRp) and capsid (Phan et al., 2007), of which 5 genogroups (G) viz. NoV GI, GII, GIV, GVI and GVII have been associated with human gastroenteritis (Ando et al., 2000; Koopmans et al., 2002; Vinje et al., 2004; Phan et al., 2007). To date, molecular characterization of NoVs has revealed 16 genotypes of GI, 23 genotypes of GII, 2 genotypes of GIII and GVI, but only 1 genotype of GIV, GV, and GVII (Phan et al., 2007). Recently Bull et al. (2007) reported intergenotype recombination among 20 different NoV strains. The site of recombination was found mainly at the junction point of ORF1 and ORF2 (Bull et al., 2005; Jiang et al., 1999).

RNA viruses show extremely high mutation rates, owing to lack of proofreading activity in their replicases. The genome of these viruses often undergoes recombination and segmentation (Domingo and Holland, 1997); many genera of positive strand viruses show genome scale ordered RNA structure (GORS), that could play an important role in RNA virus replication and rapid evolution (Simmonds et al., 2004). It has been recognized that these are some of the driving forces of RNA virus evolution that could probably lead to the origin of a new recombinant strains that may be suitable as a multivalent vaccine candidate against these viruses in future (Suzuki et al., 1998). A large number of recombinant strains of NoVs have been reported viz., the NoV strain Arg320 from Norfolk, USA with RdRp region like Lordsdale virus (GII.4) and capsid region of the Mexican NoV (GII.3) strain (Jiang et al., 1999); the NoV strain Mc37 from Thailand showed that its ORF1 sequence has 97.2% nucleotide identity to that of Saitama U1 virus but only 71.3% and 67.9% nucleotide identity in ORF2 and ORF3, respectively (Hansman et al., 2004). The NoV strain MD145-12 has hybrid genome comprising stretches from Lordsdale virus, Gifu96, SaitamaU1, U3, U4, U16, U17, and U25 (Etherington et al., 2006).

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In the course of this study, a recombinant NoV strain [Hu/Kol/NLV/L8775/2006/India] was detected that showed a hitherto unobserved recombination event among NoVs from two different genogroups viz. GI.3/VA98115/1998/USA and GII.4/Terneuzen70/2006/NL. The recombination crossover point was determined by using the RAT program. Furthermore, the DNA secondary structure of both parental NoV strains showed similar secondary structure that affirmed possible mechanism of recombination that gave rise to the L8775 NoV strain.

Results

Detection of recombinant human Norovirus

The faecal specimen (L8775/2006/Kolkata/India) was obtained from a 17 year old male hospitalized for treatment of acute watery diarrhea and severe dehydration at the Infectious Diseases and Beliaghata General Hospital, Kolkata, India. Acute watery diarrhea was associated with other clinical symptoms such as abdominal pain and vomiting. The specimen was negative for *Escherichia coli*, *Vibrio cholerae*, *Shigella,* Rotavirus, Adenovirus, Astrovirus, Sapovirus and Picobirnavirus. The specimen L8775 was positive for NoV RNA by RT-PCR. The monoplex PCR was carried out with different primers (Table 1) that showed the following results: (1) NV1F-NV1R amplified 814 bp of the RdRp gene which was confirmed as a GI nucleotide fragment after sequencing, and (2) JV24–JV21 amplified 300 bp of the capsid gene which was confirmed as a GII nucleotide fragment after sequencing. The NV1F-JV21 amplified 1098 bp of the partial RdRp, ORF1–ORF2 overlap and the partial capsid gene of the L8775 strain which was confirmed after sequencing. The primer pair NV1F-JV21 eliminates the possibility of co-infection with two different NoV genogroups and locates the crossover site in the nucleotide sequence of L8775 NoV. The PCR amplicons of the L8775 strain were sequenced and the partial nucleotide sequence was aligned with other NoV reference strains available in GenBank using the BLAST program. The RdRp region showed 86% similarity to the US NoV strain (Hu/NLV/GI.3/VA98115/98/US), 85% to that of other NoVs viz. NoV/Hu/Vesoul576/03/France, NLV/LittleRock/316/94/US, and NoV Hu/GI/Otofuke/79/JP, and 83% with Desert Shield virus DSV395. The capsid region showed 99% similarity with the Netherland strain (NoV/Hu/GII.4/Terneuzen70/06/NL) and 98.9% similarity to the Japanese strain (NoV/Hu/GII.4/Isumi/060936/06/JP). The comparison of nucleotide sequence similarity between different NoVs and the partial sequence of the L8775 strain is shown in Table 2. Moreover, the nucleotide sequence (1098 bp) of the L8775 strain (the nt1–nt820) showed similarity with NoV GI.3 strains (VA98115, Otofuke and DSV), whereas the nt544 to nt1095 (551nt) stretch closely resembled that of GII.4 NoVs (Oxford, Isumi, and Terneuzen70).

Detection of breakpoint

The RAT program was executed by comparing the percentage nucleotide similarity of seven strains [GI.3/NLV/VA98115/98/US, GI.3/Otofuke/79/JP, GII.4/Terneuzen70/NL, GII.4/Chiba/04-899/04/JP, GII.4/Oxford/B2S16/02/UK, GII.4/FarmingtonHills/02/USA, GII.4/Isumi/060936/06/JP] with the L8775 strain as a query; a recombination point was observed at nt881 in the L8775 NoV strain that was 62 bases downstream from junction point of ORF1–ORF2. But the GII strain shows similarity with the L8775 strain before the recombination point, suggesting that the point is significantly earlier than nt881 which is located at nt819 and 6nt downstream from the junction of ORF1–ORF2. The recombination point was observed at nt790 of NoV VA98115 strain reported from USA and at nt808 of NoV Terneuzen70 strain reported from Netherlands, which are 6nt and 3nt downstream from the overlapping region respectively. Before the breakpoint, the L8775 NoV strain showed maximum similarity with NoV GI, but beyond this point maximum similarity was observed with NoV GII (Fig. 1) which affirms the occurrence of a prominent recombination between two genogroups of NoVs.

Table 1
Primers used for detection of different NoV genogroups during the study

Primer	Sequence (5′–3′)	Ref
NV1F(+)	G A T G C A G A T T A T A C A G C A T G G G A	This study*
NV1R(−)	C T T K G A C G C C A T C W T C A T T R AC	This study*
NV2F(+)	T C A G C T C T A G A A A T C A T G G T T	This study**
NV2R(−)	T T C G A C G C C A T C T T C A T T C A C A	This study**
JV22(+)	G T A A A T G A T G A T G G C G T C T A	de Bruin et al. (2006)
JV23(−)	A T A T T I C C M A C C C A R C C A T T	de Bruin et al. (2006)
JV24(+)	G T G A A T G A A G A T G G C G T C G A	de Bruin et al. (2006)
JV21(−)	C C N R C M Y A A C C A T T R T A C A T	de Bruin et al. (2006)

K-G or T, W-A or T, R-G or A, I-inosine, M-A or C, N-A or T or G or C, Y-T or C.
*The position is indicated relative to Noroviruses *M87661* for NV GI from 4562–4584 to 5354–5375 and ** indicate position related to *AY587984* for NV GII from 4340–4360 to 5080–5101.

Table 2
Comparative nucleotide sequence similarity (%) of the RdRp and capsid region of Kolkata strain L8775 with different Genogroup I and Genogroup II NoVs*

		RdRp								
	IND	A_GI	B_GI	C_GI	D_GI	E_GII	F_GII	G_GII	H_GII	I_GII
IND		**86.2**	**85.4**	**84.5**	**76.9**	62.6	64.8	63.9	64.7	63.9
A_GI	65.7		84.9	97.3	75.6	64.3	64.5	64.7	64.5	62.1
B_GI	65.2	85.8		84.7	78.3	64.2	65.6	65.0	65.9	65.4
C_GI	66.0	98.3	86.8		90.2	64.7	65.1	65.3	65.1	46.9
D_GI	65.2	89.2	83.0	73.9		58.7	60.0	59.9	60.5	59.9
E_GII	**99.0**	64.3	64.5	64.6	64.5		96.3	98.4	95.9	98.5
F_GII	**98.2**	65.0	65.2	65.3	65.2	97.5		97.2	99.4	96.2
G_GII	**97.9**	64.6	64.5	65.0	64.5	98.9	96.7		96.3	98.3
H_GII	**97.5**	64.3	64.5	64.6	64.5	98.6	98.9	98.2		95.8
I_GII	**98.9**	65.0	65.2	65.3	65.2	98.9	98.6	90.6	90.2	
		Capsid								

* IND = Hu/NLV/L8775/06/*AB290150*/IND; A_GI = Hu/NLV/VA98115/*AY038598*/1998/USA; B_GI =Hu/GI/Otofuke/*AB187514*/1979/JP; C_GI = Hu/NLV/Little Rock/316/*AF414405*/1994 /USA; D_GI =Hu/GI/DSV395/DesertShield/*U04469*/2000/USA; E_GII = Hu/GII.4/Terneuzen70/*EF126964*/2006/NL; F_GII = Hu/NoV/Farmington Hills/*AY502023*/2002/USA; G_GII = Hu/Chiba/04-899/*AB220924*/2004/JP; H_GII = Hu/NLV/Oxford/B2S16/*AY587989*/ 2002/UK; I_GII = Hu/NLV/Isumi/060936/*AB294790*/2006/JP.

Phylogenetic analysis of the partial sequence of RdRp and Capsid region

The phylogenetic tree generated with the 814 bp nucleotide sequence of RdRp region showed that the L8775 strain clustered with Otofuke, DSV, Djibouti, Vesoul and Mougon strains of NoVs which belong to GI and genotype-3 (GI.3) (Fig. 2A). However the phylogenetic tree of capsid sequences with 300 bp fragments showed that the L8775 strain clustered with Terneuzen70 and Isumi strains that belong to GII and genotype-4 (GII.4) (Fig. 2B). The phylogenetic analysis of 1098 bp nt sequences [spanning the partial sequence of RdRp region as well as the capsid region, together with its junction point] of L8775 NoV strain and several NoV GI and GII strains, as proposed by Zheng et al. (2006), revealed two distinct clades — A and B and relationship among them near the overlapping region. The clade A was found to comprise of few genotypes of NoV GI (GI.5, GI.7 and GI.8) and NoV GII (GII.5, GII.6, GII.7, GII.8, GII.10, GII.12, GII.13, G.II14 and GII.15), while in clade B a sub cluster was quite distinct with GI.3 NoV strain DSV that appeared to be distantly related to other NoV strains. The Kolkata strain L8775 clustered with GI.3/VA98115 strain to form a distinct sub cluster I; within the sub cluster II GII.1, GII.2, GII.3, GII.4, GII.16, GII.17 and GII.18, were observed and within the sub cluster III GII.9, GII.11, and GII.19 NoVs were observed. The sub cluster IV consisted of GI.1, GI.2, GI.4 and GI.6. Clade B shows the relationship

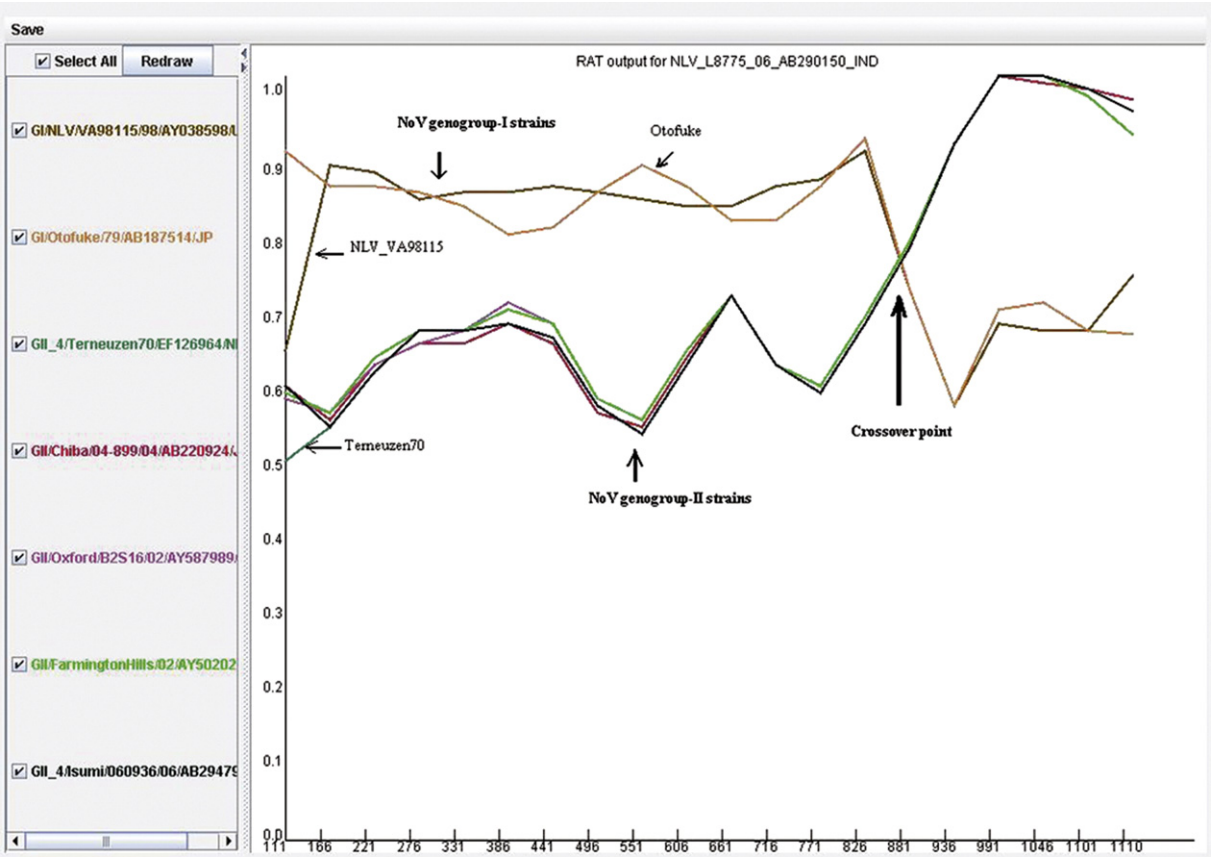

Fig. 1. RAT output for 1098 bp nt sequence [*AB290150*] of Kolkata L8775 NoV strain showing recombination crossover point around nt881. Before crossover point the Kolkata strain shows similarity with NoV GI in RdRp region and thereafter it matches with NoV GII in capsid region. The lines on the graph represent the genetic distance (*y*-axis) of each sequence present in list pane (left). The *x*-axis represents the location on the sequence.

between some strains of GI and GII which is very significant (Fig. 3). The overlapping region of ORF1 and ORF2 was found between nt797 and nt813 in the L8775 strain. The sequence alignment of Kolkata strain L8775 with closely related strains of GI and GII showed that the junction point of ORF1 and ORF2 was highly conserved and a characteristic motif **AATGA-GATGGCGTC-A—GACGC** was found which is highly significant (Fig. 4). The L8775 strain showed similarity to that of NoV GII from the end point of this motif.

Secondary structure of DNA at junction point of ORF-1 and ORF-2

The junction point of ORF1–ORF2 of NoVs, is referred to as a 'hot spot' (Jiang et al., 1999) where recombination occurs frequently; so this overlapping region of VA98115 and Terneuzen70 NoV strain was taken to analyse their DNA secondary structure because these strains are considered as the parental strains of the L8775 strain. The DNA secondary structure was predicted and the most energetically stable

Fig. 2. Phylogenetic analysis of the partial nucleotide sequence [*AB290150*] of Kolkata NoV strain (Hu/NLV/Kol/L8775/2006/India) and other NoVs. The tree [A] has been constructed from an 814 bp nt sequence fragment of RdRp region. The tree [B] has been constructed from a 300 bp nt sequence of capsid region. The percentage bootstrap values were determined from 1000 replicates. Each phylogenetic tree was rooted with the Dresden strain of Sapovirus defined as the outgroup. The arrow indicates the position of Kolkata strain on each tree.

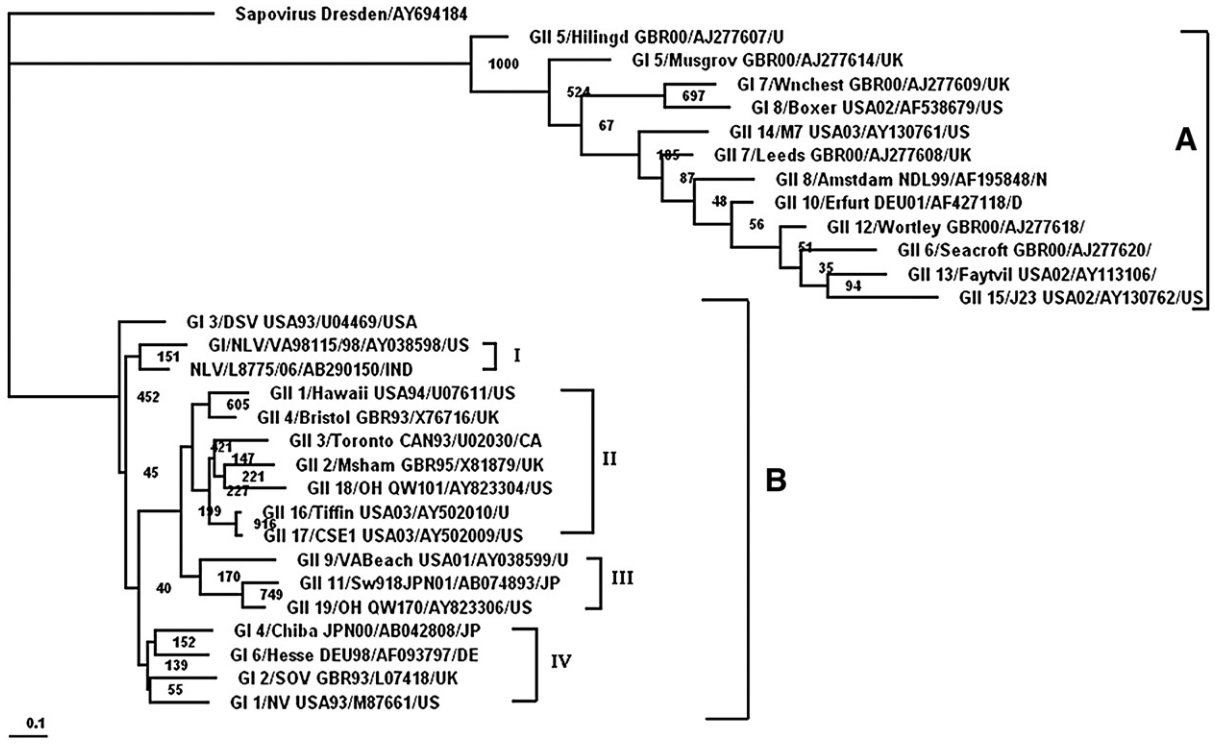

Fig. 3. Phylogenetic relationship of combined fragment (1098bp) of RdRp and Capsid gene that includes the junction point of ORF1 and ORF2 of the recombinant L8775 Kolkata strain (*AB290150*) to 28 known NoV strains and prototype strains based on the classification of Zheng et al. (2006). The phylogenetic tree was rooted with the Dresden strain of Sapovirus defined as the outgroup. The percentage bootstrap values were determined from 1000 replicates. The different clades, subclusters are shown within brackets.

structure was selected for study. It was observed that the secondary structure showed a hairpin loop structure at the junction site of ORF1 and ORF2. This hairpin was found to show >90% similarity in both the NoV strains. The hairpin loop was formed by four unpaired nucleotides which are referred to as 'tetraloop'. The strain *AY038598*_NVGI (VA98115) shows 'TAAG' whereas *EF126964*_NVGII (Terneuzen70) shows 'GAGT' as tetraloop (Figs. 5A and B). An asymmetrical internal loop was observed having 4/2 free nt between '60 and 95' position and a 'triloop' at the top in VA98115 DNA secondary structure, whereas Terneuzen70 shows an asymmetrical internal loop having 2/1 free nt between position '10–30' and a 'triloop' at the top. In VA98115 the asymmetrical internal loop lies after the hairpin structure but in Terneuzen70, the asymmetrical internal loop was formed before the hairpin structure with a 'triloop' at the top. In the VA98115 DNA secondary structure a hairpin with a 'hexaloop' between position '1 to 20' was observed whereas in Terneuzen70 the position lies between

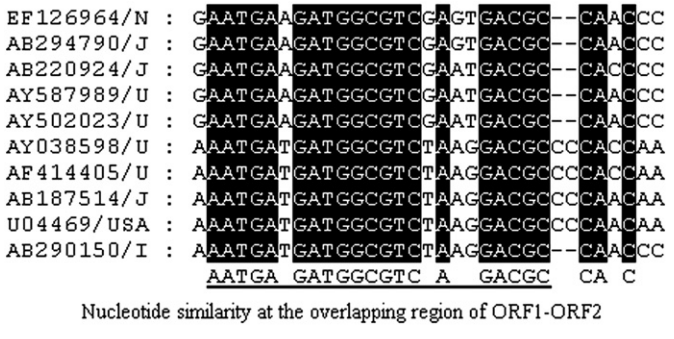

Fig. 4. Nucleotide sequence alignment of the recombinant Kolkata L8775 NoV strain [*AB290150*], with other NoVs [*AY038598* (NoV GI), *AB187514* (NoV GI), *AF414405* (NoV GI), *U04469* (NoV GI), *AY502023* (NoV GII), *AB220924* (NoV GII), *AY587989* (NoV GII), *AB294790* (NoV GII) and *EF126964* (NoV GII)] showing the conserved sequence AATGA-GATGGCGTC-A—GACGC at the junction point of ORF1 and ORF2 which has been underlined.

'80 to 98'. Moreover, VA98115 showed another hairpin with a 'tetraloop' between '20 to 40' whereas Terneuzen70 showed a hairpin with a 'pentaloop' between position '58 to 80'. The strain VA98115 and Terneuzen70 showed similar DNA secondary structure at the junction of ORF1–ORF2, that explains the mechanism of recombination between these two NoVs.

Discussion

NoVs are recognized as the most common cause of food borne gastroenteritis and outbreaks spread very rapidly through contaminated food, water or from person to person contact (Parashar et al., 2001). As NoVs are shed in high numbers within 48 h, RT-PCR has become the most sensitive technique for their identification (Lopman et al., 2002). In diagnostic PCR, an amplicon spanning the overlapping region of RdRp and capsid genes has been used for NoV detection (Vinje et al., 2003). As it is difficult to characterize the human NoVs by any single immunological method (serotyping), sequencing and phylogenetic analysis is the method of choice for detailed molecular characterization. Molecular phylogeny traces relationships of an individual in a family, and to determine how the individual may have evolved. A recombinant NoV is apparent when its polymerase sequence and capsid sequence forms distinct clusters with closest neighbors in the phylogenetic tree. In our analysis, the nt1–nt820 of Kolkata NoV strain L8775, showed similarity with NoV GI.3 strains (VA98115, Otofuke and DSV), whereas the nt544 to nt1095 (551nt) stretch was found to closely resemble GII.4 NoVs (Oxford, Isumi, and Terneuzen70) and clustered with them on the phylogenetic tree. The RAT Program analysis showed a distinct crossover point in the NoV strain L8775. The recombinant strain WUG1 shows maximum similarity with Southampton virus before the same crossover point and with Norwalk BS5 after the point, with both parent strains being GI NoVs (Etherington et al., 2006). Similar phenomenon was observed in L8775 strain where maximum similarity was observed with NoV GI before crossover point and with NoV GII after the point, forming a

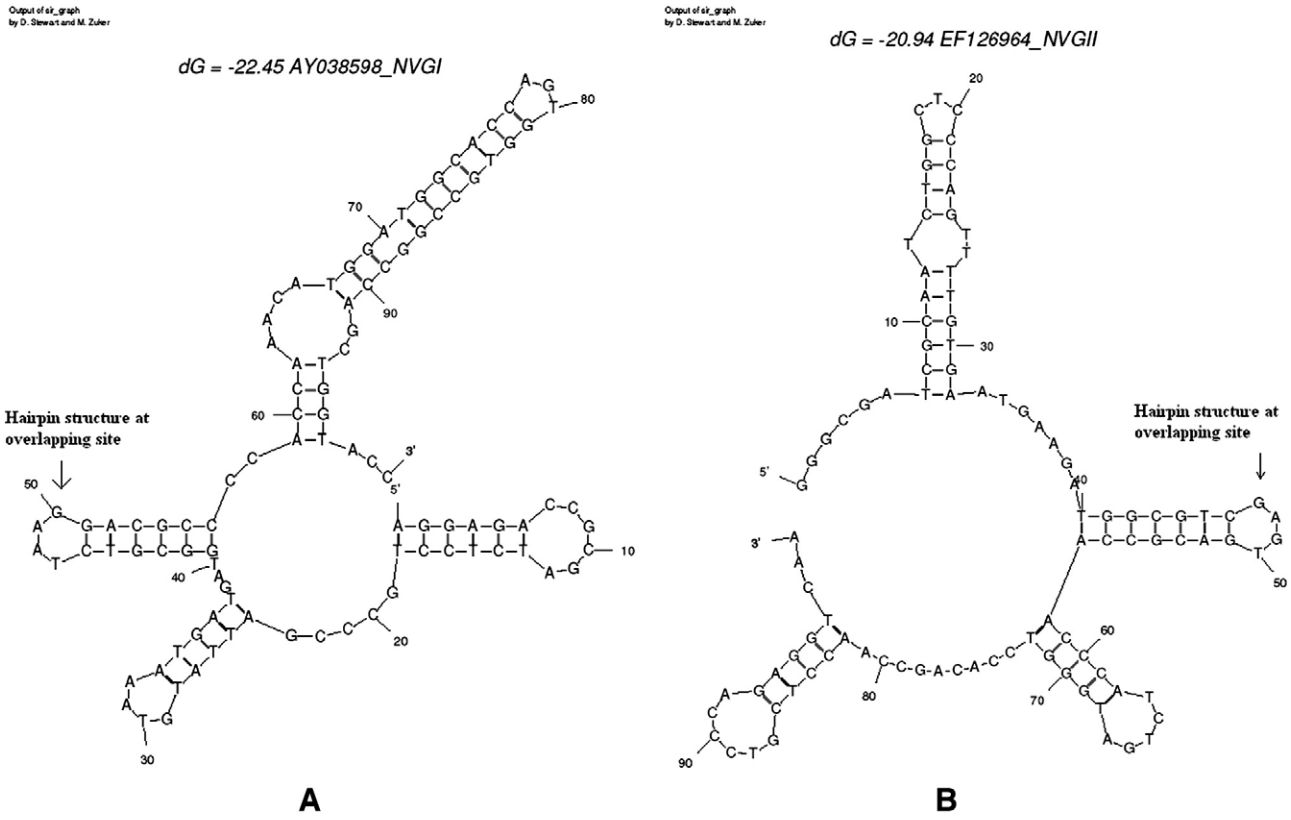

Fig. 5. DNA secondary structure showing maximum stability with least free energy and hairpin formation at the junction site (overlapping region of ORF1 and ORF2) in Mfold analysis for (A) NoV GI [*AY038598*] (Hu/NLV/VA98115/1998/USA) (B) NoV GII [*EF126964*] (Hu/NLV/GII.4/Terneuzen70/2006/NL).

'chimerical' character among 2 genogroups depicting a novel type of intergenogroup recombination among NoVs.

In RNA viruses, recombination is a driving force for viral evolution that leads to emergence of a new strain. The recombinant NoV strains have also been reported to cause outbreaks in human population (Symes et al., 2007). Moreover in Hungary, the emerging recombinant NoV became the second most common NoV variant after GII.4 (Reuter et al., 2006). Over the past few years' new variants of NoVs have often been reported (Ike et al., 2006; Lopman et al., 2004; Waters et al., 2006). Thus, NoVs showing distinct genetic diversity form different clusters in the phylogenetic tree, which may have possibly resulted through the accumulation of point mutations and recombination in course of their evolution. During viral replication, mutants are generated quite often and their accumulation is responsible for overall viral adaptability. This could possibly explain the tremendous difficulty encountered in designing effective vaccines (Domingo and Holland, 1997), yet instances of efficient control have been documented, with multivalent vaccine in case of some RNA viruses (Holmes et al., 1999; Suzuki et al., 1998). Molecular epidemiological studies in different geographical locales should include vital information on the genetic nature of ORF1 and ORF2 of NoV strains, so that efforts can be directed towards the development of a suitable vaccine.

Genetic recombination involves the exchange of genetic material between two viruses which can lead to increased genetic variation between viral strains (Smith et al., 1997; Hirst, 1962). The detection of the breakpoint in a recombinant strain is important to understand the origin of the recombinant strain. Although the recombinant strain MD 145-12, isolated from Maryland, USA showed homology with Lordsdale virus from UK, its origin was traced back to the parental NoV strain from Japan viz. Saitama U1 or Gifu'96 (Etherington et al., 2006). The NoV GII strain Saitama U1 represents an intergenotype recombination with polymerase gene of GII.4 NoV and capsid of GII.12 NoV (Katayama et al., 2002). Recombinant GIIb NoV was detected for the first time in France in August 2000 with RNA polymerase gene that did not resemble any known genotype, but the capsid gene was closely related to that of NoVs belonging to three different genotypes, viz. Hawaii (*U07611*/GII.1), Toronto (*U02030*/GII.3), and Snow Mountain (*AY134748*/GII.2) respectively (Ambert-Balay et al., 2005). In Japan, another recombinant GIIb was identified which showed recombination between GII.12 polymerase and GII.3 capsid gene (Phan et al., 2006a,b,c). Vidal et al. (2006) reported a novel recombinant NoV from Chile which showed novel RdRp sequence and GII.3 capsid sequence. The NoV strain IrlN05771 showed novel recombination in its polymerase gene having maximum similarity with Hokkaido133 strain whereas the rest of the sequence showed maximum identity to Lordsdale GII.4 NoV (Waters et al., 2007). Some NoVs show recombination which is restricted to their capsid region only (Rohayem et al., 2005). Phan et al. (2007) reported three types of recombination viz. intergenogroup, intergenotype and intersubgenotype among NoVs. The strain Mex 7076/99 is an intergenogroup recombinant NoV strain where the polymerase gene was of GII and novel genotype, whereas the capsid region belonged to GVI (Phan et al., 2007). This is the first instance of intergenogroup recombination that has been reported to date. In this study we report a recombinant strain of NoV [L8775/Kol] from Kolkata, India having polymerase sequence of GI and genotype 3 (GI.3), whereas the capsid sequence belongs to GII and genotype 4 (GII.4) which has not been reported to date among NoVs. Most recombinant strains of NoVs showed that the breakpoint was found to lie between ORF1 and ORF2 of the genome, which is referred to as a recombination 'hot spot' (Ambert-Balay et al., 2005; Bull et al., 2005; Jiang et al., 1999). The strain 146/Kunming/04/China showed recombination at the ORF1–ORF2 overlap region between GII.6 polymerase and GII.7 capsid (Phan et al., 2006a,b,c) whereas in the strain L8775 the recombination point was observed near (6 bases downstream) the junction point of ORF1–ORF2.

It is characteristic of single-stranded RNA to form secondary structure for its stability, which acts as an enhancer for RNA replication, by forming a binding site for the replicase (Nagy et al.,

1999). The 'hairpin loop' provides the site for interaction with protein and nucleic acid, and serves as a nucleation site for RNA folding (Nowakowski and Tinoco, 1997). The RNA replication enhancers promote RNA recombination directly by constituting recombination hot spots through binding of the replicase-aborted nascent strand complex during the crossover event (Nagy and Simon, 1997). The overlapping region (ORF1–ORF2) was observed as the 'hot spot of recombination' in both the parental strains of NoV L8775, due to the presence of a hairpin structure in this region (Figs. 5A and B), which was also observed earlier in *Turnip crinkle virus* (TCV) by Nagy et al. (1999).

In the phylogenetic tree GI.3NoVs and GII.4NoVs are present in the same clade suggesting a high level of sequence homology exists between them near the overlapping region. Moreover, the presence of a conserved nucleotide motif at the overlapping site, as well as a hairpin structure suggests the possibility of gene rearrangement and recombination, by homologous mechanisms of recombination (Rohayem et al., 2005; Beekwilder et al., 1995; Lai, 1992). In this mechanism, the viral RdRp complex switches mid replication from one RNA molecule to another. It is presumed that strain L8775 might have evolved by recombination of genes between two NoV genogroups near the overlapping region of ORF1–ORF2 (nt790 for the VA98115 strain and nt808 for the Terneuzen70 strain) and owing to sequence similarity between the two parent strains of L8775, the recombination point was observed at nt864 for NoV G1.3 strain VA98115 and nt870 for NoV GII.4 strain Terneuzen70 (Fig. 1). This explains the 62nt downstream shift of the recombination site for L8775 strain, now located in ORF2, showing a new recombination event in the genus NoV.

NoVs infect a large number of mammals beside humans, viz. bovine, porcine and murine species and have been recognized as one of the major causes of nonbacterial gastroenteritis. Thus it is important to maintain stringent surveillance to monitor their prevalence and immediately report the emergence of new NoV strains in different settings.

Materials and methods

Faecal specimens and preparation of virus suspension

Faecal specimens collected from acute watery diarrhea cases requiring admission to the Infectious Diseases and Beliaghata General Hospital were routinely examined in the Molecular Virology Laboratory for screening of diarrheagenic viruses. The 20% faecal suspension was prepared for RNA extraction as described by Bhattacharya et al. (2006).

RNA extraction and RT-PCR for detection of Noroviruses

The viral RNA was extracted from the virus suspension using QIAamp viral RNA kit (QIAGEN) according to the manufacturer's instructions. The RT reaction was carried out which consisted of 100 ng of random hexamers 0.7 μl (7U) of AMV RT, 3.0 μl of 5x enzyme buffer, 0.5 μl (20U) of RNasin® Plus RNase Inhibitor, and 2.5 μl of 2.5 mM of dNTPmix. In each PCR reaction 5 μl cDNA was added to 20 μl of PCR reaction mix consisting of 1.5 U of Taq polymerase, 2.5 μl of 10× PCR buffer (-$MgCl_2$), 2.0 μl of 25 mM $MgCl_2$, 10 pmol of forward and reverse primer, dNTPs 0.5 μl (2.5 mM each) (Promega Corpn, USA). The primer sets NV1F-NV1R and NV2F-NV2R were used to amplify the RdRp region of NoVGI and NoVGII respectively and the primer sets JV22–JV23 and JV24–JV21 were used to amplify the capsid gene of NoVGI and NoVGII respectively (Table 1). The PCR experiments were also performed with primer set NV1F-JV21, NV1F-JV23, NV2F-JV23 and NV2F-JV21 to cross check for any recombination and co-infection.

Nucleotide sequencing and cloning

The amplicon from the RT-PCR was purified using QIAquick PCR purification kit (QIAGEN) according to manufacturer's instructions. Sequencing PCR reaction was carried out separately with the forward and reverse primer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits, version 3.1 (Applied Biosystems). For the cross checking of sequence the PCR amplicons were cloned in pDrive cloning vector (QIAGEN) according to manufacturer's instructions, and inserts were sequenced in automated sequencer using M13 forward and M13 reverse primers.

Sequence analysis

The sequences of the Kolkata strain were read using Sequencher program (Gene Codes Corporation, version 4.0.5) and amino acid prediction was carried out using DNASIS program (Version 2.1). The sequence data was then run on the BLAST program (Altschul et al., 1990; Ye et al., 2006) to determine the relative sequence homology of the Kolkata strain with other NoV strains. LALIGN program (Huang and Miller, 1991) was used to study similarity among Noroviruses using the parameter of global alignment without end gap penalty. Multiple sequence alignments of NV GI and NV GII were generated by ClustalX (1.8) and phylogenetic trees were observed by using TreeView 1.6.1. The bootstrapped phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates using the Molecular Evolutionary Genetics Analysis version 4.0 (MEGA 4.0) software (Tamura et al., 2007) and genetic distances among human Norovirus genogroups were calculated by Kimura's 2 parameter method (Kimura, 1980). The possible recombination and breakpoint was detected using the Recombination Analysis Tool (RAT) program (Etherington et al., 2005). RNA secondary structure of 100 nucleotide bases that included the overlapping region of ORF1 and ORF2 was computed using the Mfold version 3.2 web servers for RNA folding, to analyse the predicted structure at the junction site of recombination (White and Morris, 1995; Zuker, 2003).

Nucleotide sequence accession number

The partial 1098 bp nucleotide sequence covering RdRp and capsid region of the Kolkata recombinant strain was submitted to DNA Data Bank of Japan (DDBJ), http://www.nig.ac.jp under the accession number *AB290150*.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. This article did receive clearance through the appropriate channels at the CDC prior to submission.

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