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Molecular characterization of three novel intergenotype norovirus GII recombinant strains from western India

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ABSTRACT

The phenomenon of recombination has been widely described among noroviruses (NoVs) in the past few years. In a NoV surveillance study conducted in western India, 3 novel and 3 known combinations of RNA-dependent RNA polymerase (RdRp) and capsid genes were identified in genogroup (G) II NoV strains. The present study pertains to the characterization of three novel intergenotype NoV GII recombinant strains. RT-PCRs were carried out for the amplification of nearly complete RdRp and complete capsid genes spanning ORF1/2 overlap of three strains followed by sequencing of the amplicons. The recombination event was confirmed by phylogenetic analysis using Bayesian MCMC approach, SimPlot analysis and Maximum χ^2 method. Three novel intergenotype (GII) recombinations of GII.b/GII.18, GII.1/GII.12 and GII.3/GII.13 specificities were identified respectively in the strains PC03, PC24 and PC25 for the first time. The breakpoint in the novel recombinants was placed in the vicinity of the 20 bp ORF1/2 overlap, a common hotspot known to exist in NoV recombinants. The capsid genes of all of the 3 recombinants were closely related to their counter parts in reference strains however, a high degree of variation emerged in the polymerase genes especially of PC24 and PC25 in comparison to the reference strains.

1. Introduction

Noroviruses (NoVs), classified under family *Caliciviridae*, possess a single-stranded RNA genome of 7.5–7.7 kb. The genome consists of three open reading frames (ORFs) 1, 2 and 3. Based on amino acid sequence diversities of the ORF2 (VP1 gene), classification of NoVs has been proposed into five major groups Genogroup I (GI) to Genogroup V (GV) (Zheng et al., 2006). GI, GII and GIV infect primarily humans; GIII and GV infect bovine and murine species, respectively. GI, GII and GIII have been subdivided into 8, 17 and 2 genetic clusters, respectively, whereas GIV and GV have 1 cluster each (Zheng et al., 2006).

Genotyping based solely on the capsid sequences is not sufficient to characterize the recombinant NoVs occurring naturally. A recombinant NoV displays separate genetic specificities when different regions of its genome are subjected to phylogenetic analysis (Bull et al., 2005). Among NoVs, three types of recombination viz. intergenogroup, intergenotype and intragenotype have been reported of which intergenotype is the most commonly detected recombination (Bull et al., 2007; Phan et al., 2007). To date, recombinant NoVs have been described within GI, GII and GIII (Katayama et al., 2002; Han et al., 2004; Rohayem et al., 2005; Phan et al., 2006, 2007; Etherington et al., 2006; Bull et al., 2007; Nayak et al., 2008, 2009). The complexicity of recombination events in NoVs is elevated with the addition of new recombinants with known capsid types in combination with unclassified polymerases (GII.a, GII.b, GII.c and GII.d) (Hardy et al., 1997; Jiang et al., 1999; Bull et al., 2005; Phan et al., 2007).

One intergenogroup and three intergenotype recombinations have been reported to date from Kolkata, eastern India (Nayak et al., 2008, 2009). The aim of the present study was to characterize GII NoV strains with novel combinations of RNA-dependent RNA polymerase (RdRp) and capsid genes, circulating in western India.

2. Study design

2.1. Specimens

In a study conducted on NoV surveillance among acute gastroenteritis cases from western India during 2005–2007, 6 different combinations of RdRp and capsid genes were identified among 40 of 89 (44.9%) NoV infections (Chhabra et al., 2009). These included 3 novel (GII.b/GII.18, GII.1/GII.12, and GII.3/GII.13) and 3 known combinations (GII.b/GII.3, GII.b/GII.4, and GII.d/GII.3) (Table 1). A 874 bp nucleotide stretch covering ORF1/2 overlap and flanking partial polymerase (611 bp) and capsid (282 bp) genes was ampli-

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Table 1
Distribution of different combinations of RdRp and capsid genes identified in the NoV strains from western India.

RdRp		ORF2		No. of strains	Related strains (Accession No.)
Genotype	Reference strains (Accession No.)	Genotype	Reference Strain (Accession No.)		
GII.b	VannesL169 (AY773210)	GII.3	VannesL169 (AY773210)	22	VannesL169 (AY773210)
GII.b	VannesL169 (AY773210)	GII.4	DenHaag89 (EF126965)	1	Nyiregyhaza/1057 (AY672564)
GII.d	Saitama T66e (AB112321)	GII.3	Saitama T66e (AB112321)	1	Saitama T66e (AB112321)
GII.1	Hawaii virus/71 (U07611)	GII.12	Saitama U1 (AB039775)	1	Novel
GII.3	Saitama U18 (AB039781)	GII.13	Fayetteville/98 (AY113106)	5	Novel
GII.b	Sydney C14 (AY845056)	GII.18	IF 1998 (AY675554)	10	Novel

fied in a single RT-PCR reaction (QIAGEN, UK) for all 40 strains using specific primers SR46 and G2SK (Table 2). Thirty (75%) of the 40 strains showed amplification confirming recombination event in sequencing and phylogenetic analysis (data not shown). In the present study, one strain each (PC03, PC24, and PC25) from the 3 novel combinations (GII.b/GII.18, GII.1/GII.12, and GII.3/GII.13) was selected to authenticate the existence of potential recombination event in NoVs.

2.2. RNA extraction and RT-PCR

The viral RNA was extracted from 30% fecal suspensions using Trizol, LS reagent (Invitrogen, USA) according to the manufacturer's instructions. A 1345 nucleotide stretch covering ORF1/2 overlap and flanking polymerase (766 bp) and capsid (598 bp) regions was amplified in a single RT-PCR reaction (QIAGEN, UK) according to the manufacturer's instructions using specific primers NV4611 and Mon383 (Table 2). Other RT-PCRs were carried out for amplification of nearly complete RdRp and complete capsid genes using primers specific for both genes (Table 2). Briefly, reverse transcription was carried out at 45 °C for 30 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 70 °C for 2.5 min with final extension at 70 °C for 7 min. The amplified products were analyzed on 2% agarose gels stained with ethidium bromide.

2.3. Nucleotide sequencing

All RT-PCR products were excised from the gel for purification using QIAquick gel extraction kit (QIAGEN, UK). This was followed by cycle sequencing using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and nucleotide sequences were detected using an ABI 3130 sequencer (Applied Biosystems). The GenBank accession numbers for the nucleotide sequences reported in this study are EU019230, EU921353 and EU921354.

2.4. Phylogenetic analysis

Sequence identity was determined through BLAST (www.ncbi.nlm.nih.gov/blast) and multiple sequence alignment was carried out with ClustalX 1.83 program (Thompson et al., 1997). The phylogenetic trees were constructed by Bayesian MCMC approach implemented in the software MrBayes 3.2 (Huelsenbeck et al., 2001). The evolutionary model (GTR + I + Γ4) used for phylogenetic analysis was selected on the basis of Akaike Information Criterion (AIC) as implemented in the software ModelTest 3.7 (Posada and Crandall, 1998). All of the remaining parameters carried their default values. The convergence of the analysis was confirmed by the PSRF (Potential Scale Reduction Factor) value approaching 1. The program SimPlot 3.5 was used to carry out recombination analysis (Lole et al., 1999). BootScan analysis and Maximum χ^2 method were used to identify the putative breakpoint (Smith, 1992; Salminen et al., 1995). The *p*-value for each breakpoint was calculated by using Fisher's exact test based on the phylogenetically informative sites supporting alternative tree topologies.

3. Results

The strain PC24 with GII.1/GII.12 specificity clustered with the strain Hawaii Virus/71 (first strain in BLAST search) of GII.1 speci-

Table 2
List of primers used in the study.

Primer	Sequence (5′-3′)	Sense	Genome locations	Reference
SR46	TGGAATTCCATCGCCCACTGG	+	4493–4513[a]	Ando et al. (1995)
G2SK	CCRCNGCATRHCCRTTRTACAT	–	5389–5367[a]	Kojima et al. (2002)
NV4611	CWGCAGCMCTDGAAATCATGG	+	4338–4358[a]	Yuen et al. (2001)
Mon383	CAAGAGACTGTGAAGACATCATC	–	5661–5683[a]	Noel et al. (1997)
NV6F	AGCACCAAGACGAAATTCTGGAG	+	3638–3660[a]	This study
NV6R	ATGGAGTTCCATTGGGAGGTGCA	–	4481–4503[a]	This study
NV8F	GGTGAGCAACTTCTTTTCTT	+	6363–6382[a]	This study
NV8R	TTTGTCATGGGGGCGTTGATT	–	7004–7023[a]	This study
NV12.7F	CTTCCAGATGTTAGGAACAA	+	5556–5575[b]	This study
NV12.7R	TCAGTGCTACATCTGTTTGTG	–	6472–6492[b]	This study
NV13.7F	ATGCTTCCCCATTTGATTGT	+	418–437[c]	This study
NV13.7R	TATTCCCTTAGCATTTTTGGC	–	1042–1062[c]	This study
NV13.8F	CTCCAGCTGACCTATCCAAAT	+	907–927[c]	This study
NV13.8R	CCAACAGCAGAAGTCAACAT	–	2822–2840[d]	This study
IC7F	GCTCAGCCCTGCTCAAATCACA	+	390–411[e]	This study
IC7R	AGTCCTGCGTGCCAAAAG	–	960–976[e]	This study
IC8F	GCTGATGTAGTTGTCCAACCC	+	757–777[e]	This study
IC8R	TTACTGAATCCTTCTGCGCCC	–	1594–1614[e]	This study

[a] Genomic locations of primers are given as per Lordsdale Virus genome (X86557).
[b] Genomic locations of primers are given as per Saitama U1 (AB039775).
[c] Genomic locations of primers are given as per Fayetteville/98 (AY113106).
[d] Genomic locations of primers are given as per Goulburn Valley G5175B (DQ379714).
[e] Genomic locations of primers are given as per IF1998 (AY675554).