

Novel Recombinant Norovirus Causing Outbreaks of Gastroenteritis in Santiago, Chile

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Capsid and polymerase (RdRp) genes of 13 norovirus outbreak strains from Chile were compared. The genes sequences were discordant for five strains, and recombination was confirmed for two of them by amplification of a 1,360-bp gene segment containing a fragment of both genes. These strains belonged to a novel genogroup by RdRp sequence and to genogroup GII/3 by capsid sequence. Determining the clinical and epidemiological impact of human calicivirus recombination will require future studies.

Human caliciviruses (HuCVs), particularly noroviruses (NVs), are a major cause of gastroenteritis outbreaks in humans (4). The NV genome is organized into three open reading frames (ORFs), of which ORF-1 encodes a polyprotein that includes the RNA polymerase (RdRp), ORF-2 encodes the capsid protein, and ORF-3 encodes a protein of unknown function (2). Genetic characterization of HuCVs has detected a significant degree of diversity among these viruses, more in the capsid gene than in the RdRp gene (6, 13). This diversity has been recognized taxonomically with the designation of four calicivirus genera. Within the NV genus are phylogenetic genogroups that are further divided into genetic clusters which might represent distinct serotypes (1). Serotyping has not been possible, because HuCVs cannot be cultured, hampering the development of conventional neutralization assays. Due to this limitation, genetic characterization of the capsid gene has been used as an approximation of serotyping.

Comparisons between phylogenetic analyses of ORF-1 and ORF-2 have led to the identification of the presence of NV RNA recombination in a small number of reports (5, 7, 10, 15). A previous study characterized the RdRp gene of 13 NV strains from 13 gastroenteritis outbreaks in Chile (18). Two strains grouped in genogroup GI, eight grouped in GII, and three grouped in one genetic cluster that differed by more than 40% in nucleotide identity from sequences in GenBank, suggesting that they could constitute a new genogroup (18). Of the strains that grouped in GII, five grouped together in a new genetic cluster.

The purpose of this study was to characterize genetically the capsid of the NV strains detected in gastroenteritis outbreaks in Santiago, Chile, and to compare the phylogenetic analyses of both genes. The genetic characterization of the capsid is im-

portant both to support the potential new genetic group postulated by the RdRp gene characterization and to determine the possibility of recombination, which is important for immune diagnosis and vaccine purposes. If recombination were to be a common event, the genetic characterization of capsid genes may prove to be more useful than RdRp gene characterization in Chile.

This study was developed under the experimentation guidelines of the University of Chile and approved by the ethical committee of the Faculty of Medicine, University of Chile.

Stool extraction for reverse transcription (RT)-PCR was performed using the TRIzol extraction method (Gibco BRL, Gaithersburg, MD). cDNA was obtained with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), and PCRs were performed with *Taq* DNA polymerase (Promega, Madison, WI). A pool of degenerate primers of last generation, 289 h and 289 i for RT and 290 h, 290 i, 290 j, and 290 k for PCR, were used to detect conserved sequences on the polymerase region of norovirus (8). Amplification of the norovirus capsid gene was done using the specific primer R₂ for RT; for PCR amplification, we used two primer pairs, F₁-G1SK for GI strains and Mon 381-Mon 383 for GII strains (Table 1) (11, 16). The primers R₂ and F₁ were designed by us from sequences available in the GenBank database by using OMIGA 2.0 software for alignment and the Primer3 program for primer design. In order to prove the existence of recombination in two discordant strains, we used RT-PCR that generated a 1,360-nucleotide consensus sequence containing 785 nucleotides of RdRp and 575 nucleotides of the S domain of the norovirus capsid at the 5' end. The pool of degenerate sense primers 290 h, 290 i, 290 j, and 290 k and primer Mon 383 for antisense were used for this amplification. For this reaction, reverse transcription was performed at 42°C for 1 h using random primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). PCR was performed by using *Taq*

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TABLE 1. List of primers used for RT-PCR amplification of the norovirus capsid genes

Primer	Polarity	Genogroup	Primer sequence (5' to 3')	Location	Size (bp)	Reference
Mon 381	Positive	GII	CCAGAATGTACAATGGTTATGC	5362–5383 ^a	321	13
Mon 383	Negative		CAAGAGACTGTGAAGACATCATC	5661–5683 ^a		
F ₁ ^c	Positive	GI	ATGATGATGGCGTCTAAGGA	5348–5377 ^b	323	This study
G1SKR	Negative		CCAACCCARCCATTRTACA	5653–5671 ^b		
R ₂ ^c	Negative		ACCCAHGKHNHCAAANDVIAAHWD	6855–6877 ^b 6621–6643 ^a		This study

^a In Lordsdale virus genome (X86557).

^b In Norwalk virus genome (M87661).

^c Primers designed for amplification of capsid genes (R2 and F1) were obtained from sequences with the following GenBank accession numbers: AF320625, AF436881, AF439267, AF472623, AF542090, AY030098, AY030312, AY030313, AY038598, AY038599, AY038600, AY054299, AY054300, CVORFS, CVXRNA, HCA277606, HCA277607, HCA277608, HCA277609, HCA277610, HCA277611, HCA277612, HCA277613, HCA277614, HCA277615, HCA277616, HCA277617, HCA277618, HCA277619, HCA277620, HCA277621, HCA313030, HCU07611, HCU46500, HCU75682, and SRSV4864.

DNA polymerase (Promega, Madison, WI). The amplification program conditions were an initial hot start at 96°C for 3 min; 39 cycles of 94°C for 30 s, 49°C for 1 min, and 72°C for 1 min 30 s; and a final extension at 72°C for 10 min.

RT-PCR products were cloned by using a pGEM-T Easy vector system (Promega, Madison, WI) following the supplier's instructions, and inserts were sequenced (MWG Biotech, High Point, NC). The 293-base nucleotide sequence encoding RdRp and the 276- and 278-base nucleotide sequences encoding GI and GII capsids, respectively, were aligned by using OMIGA 2.0 (Oxford Molecular, Madison, WI) software and compared with 21 reference sequences retrieved by BLAST searches of the GenBank database. The alignments excluded the primers. Phylogenetic distances were calculated by the p-distance method, and a phylogenetic tree was plotted by the neighbor-joining method using MEGA, version 2.1 (12). Bootstrap values were based upon 1,000 generated trees.

We successfully amplified both RdRp and capsid genes for the 13 strains as shown in the corresponding phylogenetic trees (Fig. 1A and B). The prototype strains segregated into five genogroups that were concordant in both RdRp and capsid phylogenetic trees.

By RdRp analysis after amplification with the pool of degenerate primers, two strains belonged to GI, eight to GII, and three to a potential new genogroup (Fig. 1A). The two GI strains by RdRp sequence (B66 and B76) displayed 70 to 74% and 73 to 83% capsid sequence identity with five GenBank GI strains and only 30 to 32% and 33 to 35% identity with four GII strains, confirming their GI status by capsid analysis. The eight strains that grouped with GII according to their RdRp sequence also grouped with GII strains after capsid analysis, with 67 to 91% identity. Strains B72, B26, and B27, which represent the novel genogroup by RdRp sequence, grouped together after capsid analysis with the Toronto virus strain, a prototype of the genetic cluster GII/3, with 91% identity. Alignment of the 1,360 nucleotides obtained for two of the three potentially recombinant strains (B26, B27) confirmed the genogroup discordance between their RdRp and capsid sequences (Fig. 1). By detecting the seven consensus amino acids that initiate the norovirus capsid sequence (17), we were able to detect the overlapping between the RdRp 3' end and the capsid 5' end, represented by 20 nucleotides that join the end and the beginning of ORF-1 and ORF-2, respectively. This

represents the probable site of recombination for both strains (Fig. 2). Cocirculation of diverse NV strains in the same region, and coinfection with more than one strain, favors the emergence of recombinant strains by recombination events during dual infections (7, 19). Evaluation of a larger number of strains in broader areas and for longer time periods may help identify strains that are the origin for RdRp with recombinant capsid lineages.

Recombination within the same genogroup is suggested for two strains, B77 and B85, that resulted in GII/4 in the ORF-1 tree but that are closely related to Hawaii virus, a GII/1 prototype, and distantly related to Bristol virus, a GII/4 prototype strain in ORF-2. Interestingly, this same recombination pattern has been reported for the prototype strain Saitama U1 (Sai U1), which was described in Japan in 1998 and has been confirmed to be a recombinant between ORF-1 and ORF-2 (10); B77 and B85 were closely related to this strain. One explanation for the similarity between Sai U1, B77, and B85 could be that these recombinants may have a global distribution, although, to our knowledge, similar strains have not been described in countries other than Japan and Chile. Another possibility is that they derive from parental strains with biological properties that make them prone to recombine. Recombination may have important implications in immunodiagnosics. Predominance of GII/4 strains worldwide has favored the development of GII/4-specific diagnostic kits (3). Strains B77, B85, and Sai U1 may not be detected by these kits, despite being classified as GII/4 strains based upon their RdRp sequences (14).

Five of the six strains that formed a potentially new GII genetic cluster in the RdRp gene phylogenetic tree also formed a new cluster in the capsid gene phylogenetic tree analysis. One of these strains, B86, differed by 27% in capsid identity from B64, B78, B62, and B71 and, thus, belongs to a different cluster by capsid analysis. The finding of new genetic clusters is not uncommon, and different groups that have attempted to characterize NV strains circulating in a single region have reported new genotypes (3, 10, 14). Whether this new genetic cluster represents a distinct serotype is difficult to determine because of the inability to perform neutralization assays and the lack of specific antibodies for enzyme-linked immunosorbent assay. Additional studies of these strains are ongoing with a 9-valent and type-specific enzyme-linked immunosorbent assay (9).

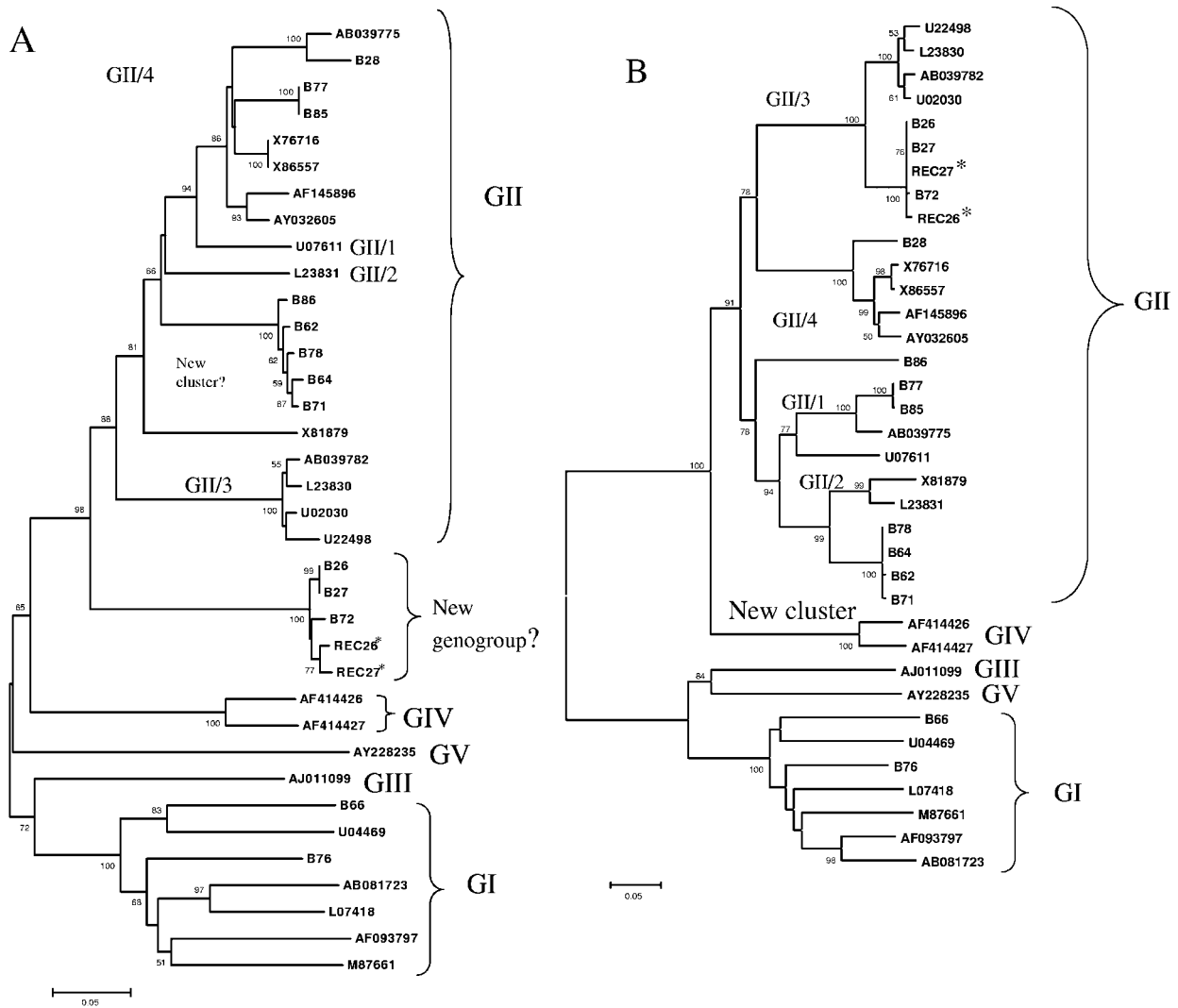


FIG. 1. RdRp and capsid phylogenetic trees. Phylogenetic distances were calculated by the p-distance method and by using MEGA, version 2.1. Bootstrap values were based upon 1,000 generated trees. (A) NV phylogenetic tree based upon a 293-bp region of ORF-1 using 13 sequences obtained from norovirus isolated from Chilean outbreaks and 21 sequences of GenBank reference NV strains representative of known genogroups, named according to GenBank accession numbers. (B) NV phylogenetic tree based upon a region of 274 bp for GI and 277 bp for GII in ORF-2, using the same sequences obtained in Chile and the 21 prototype sequences as for panel A. GenBank accession numbers correspond to Norwalk (M87661), Saitama U1 (AB039775), Saitama U201 (AB039782), WUG1 (AB081723), Schreier (AF093797), Camberwell (AF145896), Fort Lauderdale (AF414426), Saint Cloud (AF414427), Jena (AJ011099), Maryland (AY032605), murine NV (AY228235), Southampton (L07418), OTH25 (L23830), Snow Mountain (L23831), Toronto (U02030), Desert Shield (U04469), Hawaii (U07611), Mexico (U22498), Bristol (X76716), Melksham (X81879), and Lordsdale (X86557) viruses. *, REC26 and REC27 correspond to the RdRp (A) or capsid (B) sequence of outbreak strains 26 and 27 obtained by amplification of the 1,360-bp fragment that includes both ORF-1 (3' end) and ORF-2 (5' end).

This new genetic cluster included an important number of strains from these outbreaks (i.e., B62, B64, B71, and B78). Future research should determine if this cluster predominates in Chile over time; if so, such persistence would be of potential importance in the local development of detection assays and in the design of potential vaccines.

Strains B86 and B62 differed by 1% in a relatively large, 293-bp segment of the RdRp gene. If we had used a smaller segment, as other investigators have reported, analysis of these strains would have found them to be identical. Capsid analysis, however, would have clearly distinguished them, in

support of capsid gene characterization in outbreak investigation.

In conclusion, outbreaks of gastroenteritis occurring in Santiago, Chile, between 2001 and 2003 were caused by genetically diverse NV strains that grouped mainly into GII. Simultaneous analysis of capsid and RdRp sequences identified five possible genetic recombinants. For two strains, a recombination event from two parental strains belonging to different polymerase and capsid genogroups was confirmed by the amplification of a large segment containing a fragment of both genes. The circulation of genetically diverse strains and of genetic re-

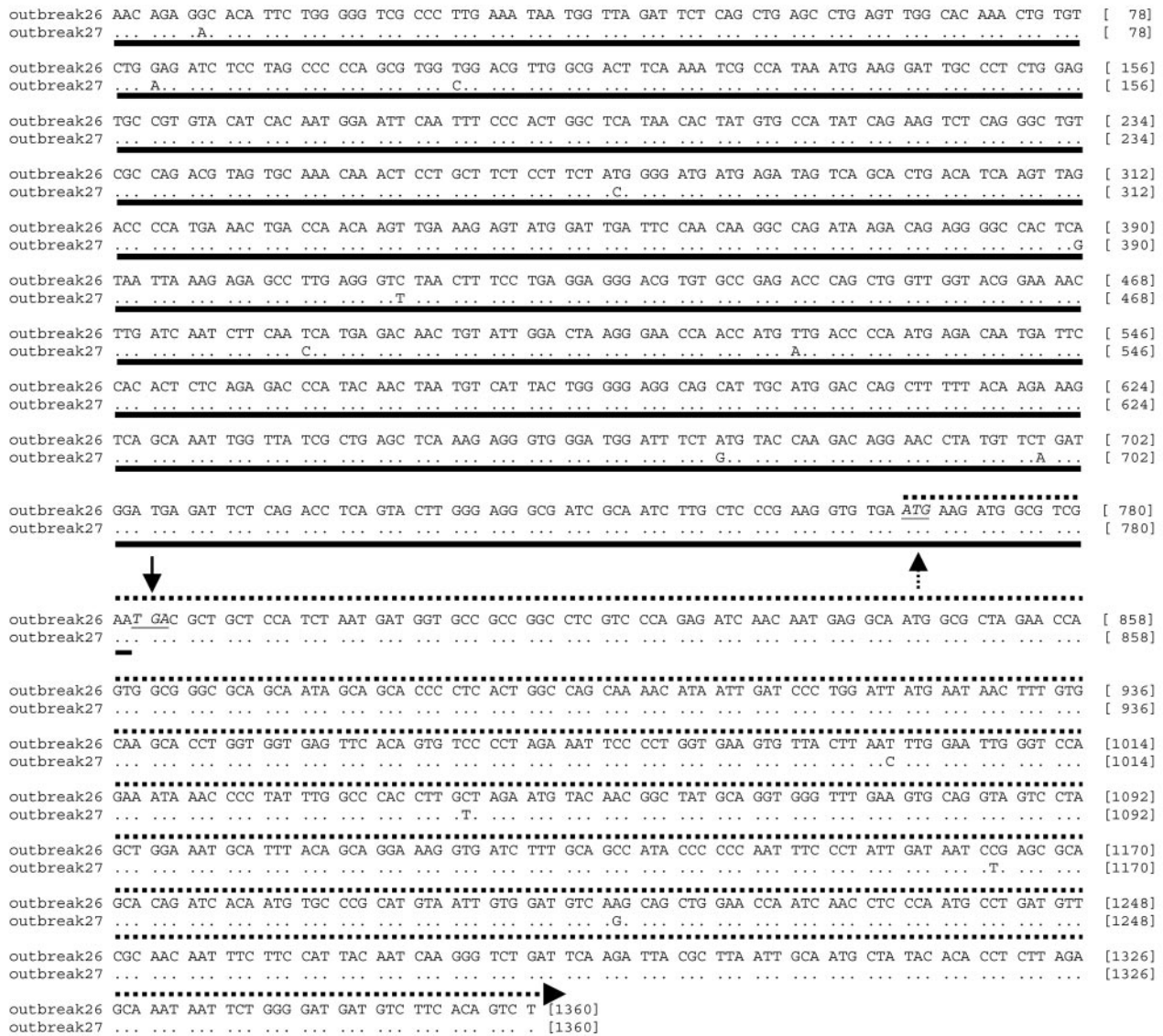


FIG. 2. The genome recombination site of outbreaks 26 and 27 identified at the ORF-1 (solid line)–ORF-2 (dotted line) overlap. The site number at the end of the line indicates the nucleotide position in the 1,360-nucleotide sequence obtained using PCR primers 290 h, 290 i, 290 j, 290 k, and Mon 383, which allow amplification of the 3' end of the RdRp and the 5' end of the capsid S domain. The ORF-1 termination codon and the ORF-2 initiation codon are shown in italic underlined text (and are also indicated by arrows).

combinants supports the need for future studies aimed to correlate this genetic variability with clinical and epidemiological findings.

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