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Norovirus based viral gastroenteritis in Chennai city of southern India - An epidemiological study

S. Anbazhagi*, S. Kamatchiammal and D. Jayakar Santhosh

National Environmental Engineering Research Institute Chennai Zonal Laboratory, CSIR Madras Complex, Taramani, Chennai – 600 113, India.

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The study evaluated the incidence of norovirus associated gastroenteritis in India. During the year 2005 to 2006, stool samples from communicable disease hospital were collected from subjects suspected for non – bacterial gastroenteritis and were examined for norovirus. Samples were analyzed by reverse transcriptase polymerase chain reaction and further confirmed by hybridisation and sequencing. In addition the epidemiology of norovirus was studied by statistical analysis. 44.4% of the samples were positive for norovirus, the isolated strain represented the Genogroup I of norovirus. The study observed that norovirus infection is not only a winter vomiting disease it can also occur in the summer.

Key words: Gastroenteritis, norovirus, reverse transcriptase polymerase chain reaction, stool.

INTRODUCTION

Diarrheal disease is a major cause of childhood morbidity and mortality, especially in developing countries (Bern et al., 1992). Several different groups of viruses have been shown to be responsible for the high incidence of acute viral diarrhea among children during their first few years of life. Rotavirus is the single most important etiological agent in severe dehydrating diarrhea (Parashar et al., 2003). Recent studies investigating calicivirus in sporadic cases of gastroenteritis in children have concluded that caliciviruses comprise the second cause of viral diarrhea after rotavirus (Simpson et al., 2003; Subekti et al., 2002). Direct electron microscopy is used to screen fecal specimens for enteric viruses in the public health laboratories of many countries and it is clear that talented electron microscopists who receive fecal samples collected early in the course of an infection can often detect viruses (Wright et al., 1998). Detection of enteric viruses in stool specimens using direct electron microscope requires virus concentrations of at least 10^6 per ml of stool (Doane et al., 1994). The small numbers of viral particles present in fecal samples make direct electron microscopy studies even after concentration, relatively insensitive. However, this method requires highly highly skilled microscopists and expensive equipment, making it not feasible for large epidemiological or clinical studies.

Norovirus is an important cause of acute nonbacterial gastroenteritis in children and adults worldwide. Noro-viruses belong to the family caliciviridae. Calicivi-ruses are small non-enveloped viruses approximately 27 to 35 nm in diameter with a positive-sense, single stranded RNA genome (Green et al., 2001). The first discovered norovirus was associated with a human outbreak of gastroenteritis in Norwalk, Ohio, which gave the name Norwalk Virus to the prototype strain of norovirus, in 1968 (Adler and Zickl et al., 1969). Norovirus is also associating with sporadic and outbreak cases of gastroenteritis in individuals of all ages, with a distinct seasonality linked to the winter months (O'Neill et al., 2002). Infection is characterized by acute onset of nausea, vomiting, abdominal cramps, and diarrhoea, which generally last for about 48 h. Transmission occurs predominantly through ingestion of contaminated water, food (particularly oysters); and person by the fecal-oral route, airborne transmission and contact with conta-minated surfaces (Anbazhagi and Kamatchiammal, 2010; Girish et al., 2002; Parashar and Monroe et al., 2001). Caliciviruses are characterised by stability in the environ-ment (Rzezutka and Cook, 2004) and relative resistance to inactivation (Duizer et al., 2004).

*Corresponding author. E-mail: sanbazhagi@gmail.com. Tel: +91 44 – 22541250. Fax: +91 44- 22541964

The ease with which norovirus is transmitted as well as the low infectious dose required to establish an infection results in extensive outbreaks in numerous environments such as hospitals, hotels, schools, nursing homes, and cruise ships (Marks et al., 2000; 2003; Cheesbrough et al., 2000). Several comprehensive reviews on norovirus infections, including recommenda-tions on outbreak control, have been reported (Parashar et al., 2001; Chadwick et al., 2000).

The frequent occurrence of such outbreaks highlights the difficulties and challenges in effective control of norovirus infection in paediatric setting (Isakbaeva et al., 2005). In India the role of noroviruses in causing gastroenteritis is not well defined. Very few studies evaluated the role of viral agents in childhood diarrhoea in India (Preeti and Shobha, 2008). This study was conducted to find out the parameters responsible for norovirus infection by analysing the stool samples collected from the diarrhoeal patients.

MATERIALS AND METHODS

Standard norovirus strain

Norovirus positive stool isolates were obtained from Christian Medical College, Vellore, India. The isolates were confirmed by polymerase chain reaction using respective primers (Green et al., 1995) and were used as positive template. PCR reactions were carried out on a Perkin-Elmer Cetus DNA Thermo cycler 2400 (Perkin- Elmer Cetus Corp., USA).

Stool samples

Fecal specimens were collected in sterile plastic containers from cases of acute nonbacterial gastroenteritis at the Communicable Disease Hospital, Chennai between July 2005 and November 2006 and stored at −20°C. 81 ill subjects who were suspected for non bacterial gastroenteritis were chosen for the present study. A close-ended questionnaire was given to the subjects and was used for analysis purpose. Stratified sampling design was adopted for the study purpose.

RNA extraction

Viral RNA was extracted from 200 µl of a 10 to 20% stool PBS/MEM suspension by binding to size-fractionated silica particles in the presence of guanidinium isothiocyanate as described by Boom et al. (1990). The RNA was eluted in 49 µl of RNAse free distilled water and 1 µl of RNAsin (Biocorporals, India). RNA was either used directly in RT-PCR or stored at -70°C.

Reverse transcriptase PCR evaluation

cDNA synthesis

cDNA synthesis was carried out using cDNA synthesis kit/RevertAid kit (Biogene UK/Fermentas, USA). 5 µl of purified template was added to 1 µl of 10 mM of random hexamer and 0.3 µM each of dATP, dCTP, dGTP and dTTP in a final reaction volume of 12 µl and incubated at 65°C for 5 min and then snap-cooled. 5X RT buffer (components containing 1× First Strand Buffer® 0.01 M dithiothreitol) 40 units of Rnase inhibitor, were added to the chilled primer/template mixture making a final volume of 19 µl. This mixture was incubated at 42°C for 2 min before the addition of 1 µl of reverse transcriptase (Fermentas, USA). The reactions were then incubated for 50 min at 42°C followed by 70°C for 15 min.

Conventional RT-PCR

5 µl cDNA was added to 35 µl of PCR master mix (10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer (NI forward primer and E3 reverse primer) (Green et al., 1995), 1 U of Taq polymerase). After an initial dena-turation at 94°C for 2 min, 30 amplification cycles of 95°C for 1 min, 40°C for 1 min and 72°C for 1 min were performed followed by a final extension of 72°C for 10 min. RT-PCR amplicons were analyzed by electrophoresis of 20 µl of reaction mix in agarose at 10 V/cm for 1.5 to 2 h. PCR amplifies a 113 bp region of the RNA polymerase gene. Molecular weights were determined by comparison with a 100 bp DNA ladder (Genei Bangalore, India).

Gel electrophoresis and slot blot analysis of PCR products

The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized with UV light. 15 µl of the PCR product was denatured with equal volume of 0.5 N NaOH and spotted onto the hybridization membrane using a vacuum filtration manifold. The DNA transferred to the membrane was cross-linked in a UV Cross-linker (Foto/Prep from Fotodyne). Hybridization was performed with a cDNA (norovirus positive stool samples) labelled with dioxigenin – 11 dTTP was replaced by a mixture of digoxigenin-11 dUTP and dTTP in the molar ratio of 0.35:0.65 and were used as probes. Hybridization was performed as described by Boehringer- Mannheim (Genius TM Kit, Boehringer Mannheim, Indianapolis, IN). The labelled probe (100 µl) was denatured by heating at 95°C for 5 min and snap cooled on ice. The probe was added to the bag containing the membrane and kept at 42°C overnight. Hybridization and chromogenic detection of the hybrids with nitroblue tetrazolium (NBT) and BCIP (5-bromo-4-chloro-3-indolylphosphate) were carried out according to the protocols recommended by the manufacturer.

Norovirus cloning, sequencing and phylogenetic analysis

Each norovirus-positive RT-PCR product was cloned and selected for sequence analysis. PCR products were purified with Minelute PCR purification kit (QIAGEN, Germany). The purified PCR products were cloned using the TOPO TA Cloning® system (Invitrogen, India) according to the manufacturer's instructions. After transformation, at least five positive colonies were selected. Selected colonies were grown in LB medium containing 50 to 100 µg/ml ampicillin for overnight. Plasmid DNAs were isolated and were sequenced to confirm the presence of the insert using automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems) (MWG, Bangalore, India). Sequences were compared with other sequences in genbank using basic local alignment search tool (BLAST) family of programs on the World Wide Web service of National Centre for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov) and were read using Tree view.

Statistical analysis

Results obtained from the virological determinations were subjected to statistical analysis using the SPSS package (SPSS Version 11,

GmbH software, Munich, Germany).

RESULTS AND DISCUSSION

Detection of norovirus by conventional RT-PCR

Increasing attention has been paid to viral gastroenteritis outbreaks during the last few years. The development of molecular methods has shown that norovirus is one of the most common causes of gastroenteritis outbreaks in adults and children (Höhne and Schreier, 2004). The increasing number of outbreaks leads to increasing numbers of samples that have to be handled in diagnostic laboratories. Therefore, RT-PCR seems to be the method of choice because it combines a potentially high throughput with reproducibility, sensitivity, and specificity. PCR products of 81 stool samples were analysed by agarose gel electrophoresis. 113 bp product specific for NI/E3 primers confirms the presence of noro-virus among 36 number of stool samples. The debate over, whether RT-PCR is a legitimate tool for measuring infectious virus is well rehearsed; fundamentally the detection of nucleic acid does not directly indicate the presence of infectious virus. However, the norovirus capsid is environmentally robust, it is likely that any naked RNA would be quickly inactivated and replication is efficient. Hence detection of norovirus cDNA in a sample strongly suggests its origin from an infectious virion (Battacharya et al., 2004).

An interesting study by Battacharya et al. (2004) using hepatitis A virus, demonstrated that signals generated after RT-PCR amplification of viral genome correlated well with infectivity substantiates the present hypothesis.

Hybridization of PCR products

PCR products of 81 stool samples were analysed by Slot blot analysis. The RNA extracted from the stool samples were amplified using the norovirus specific primers NI/E3, blotted on a nylon membrane and probed using the digoxigenin labelled norovirus positive stool. Analysis of the PCR products by hybridization using the specific probes gave the same result as agarose gel electrophoresis.

PCR amplicon cloning

Representative RT-PCR amplicons for NoV generated from stool samples were cloned using a TA cloning system (TOPO®, Invitrogen, UK) as previously described by Leoni et al. (2003). Duplicate plates of the transformant were made and the colonies were picked and processed directly for PCR. The PCR was performed using the insert specific primers NI and E3. The expected PCR products of 113 bp were obtained.

Nucleotide sequencing

Plasmid DNAs were isolated and were sequenced to confirm the presence of the insert. NoV amplicon (101 bp segment) was characterized for genogroup, genotypes and genetic relationship with the reference strains based on their capsid regions classification scheme (Stephen et al., 1997). Their partial nucleotide sequences were compared to each other as well as to reference NoV strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5' ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. All of the NoV sequences showed highest identity of 86 to 94% and were classified into genogroup I. The Phylogenetic tree illustrating the genetic relationship of norovirus isolates from stool samples of subjects, India is given in Figure 1. Sequenced NoVs were very similar at the amino acid level and were most closely related to the Hu/NLV/GI/684/US, NV/Saitama T83GI/02/JP, NV/Saitama T67GI/02/JP, NV/Saitama T62GI/02/JP, NV/Saitama T61GI/02/JP, NV/Saitama T59GI/02/JP and NV/Saitama KU4aGI/99/JP strain.

Clinical features of norovirus -positive and norovirus-negative patients

Clinical data of the 81 subjects infected with diarrhoea were listed in Table 1. Out of 81 patients admitted for diarrhoeal infection it was found that 44.4% were found positive for norovirus and 55.6% were negative. The most common clinical symptoms were diarrhoea (92%), vomiting (83%) and fever (67%). The percentage of diarrhoea in norovirus-positive patients was higher than norovirus-negative patients. It was found that more patients had symptoms of severe diarrhoea (92%) than vomiting (83%), which is in agreement with Wyatt et al. (1974). It was also found that NoV-infected persons developed symptoms of watery diarrhoea and vomiting typically remain symptomatic for 2 to 3 days.

Seasonal distribution of norovirus

The seasonal pattern of NoV infection is shown in Figure 2. During the study period between July 2005 and November 2006, prominent numbers of NoV infection was observed on April 2006 (6 norovirus-positive) followed by January 2006 (5 norovirus-positive). Whereas in September 2005 and May 2006 showed 4 cases of positive stool samples. Among the 36 positive samples collected throughout the study, fifty two percent (19 of 36) of positive samples were notified during this peak period of infectious time. Norovirus is generally referred to as "winter-vomiting disease", but this study on contrary identified "norovirus" in the summer season especially during April.

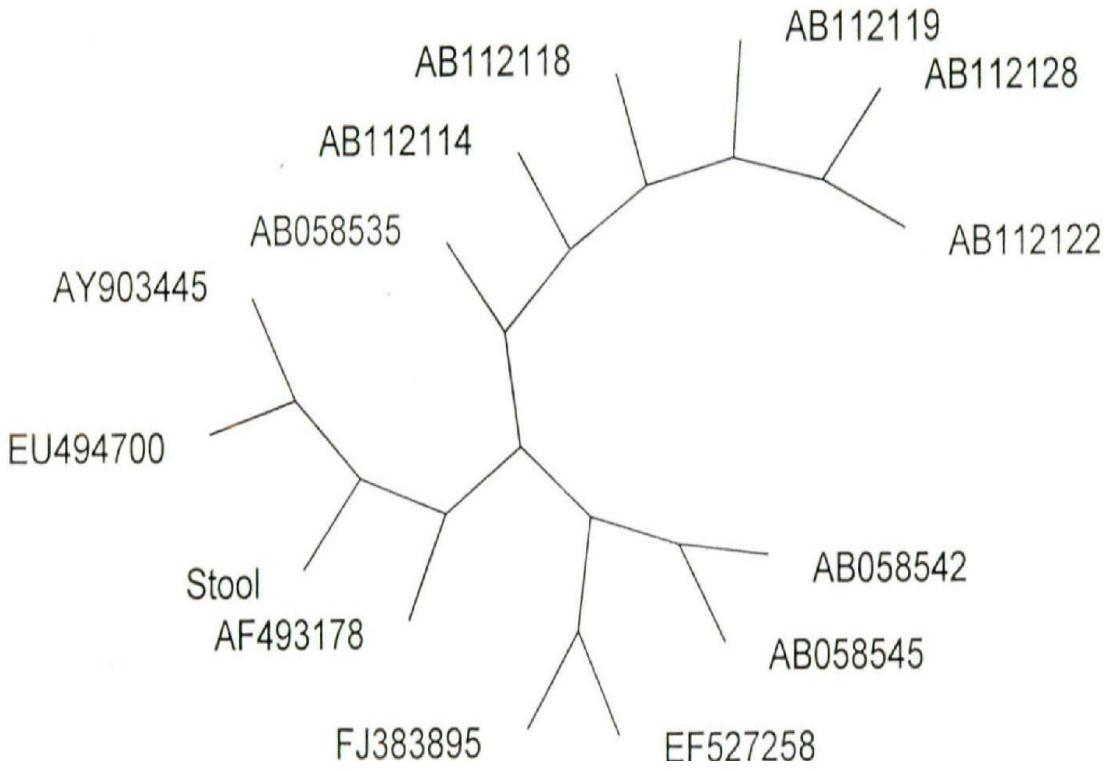

Figure 1. The Phylogenetic tree illustrating the genetic relationship of norovirus isolates from stool samples of gastroenteritis subjects, India.

Statistical analysis of the clinical samples

Theoretically a large number of variables are at force in determining the “norovirus” among subjects admitted due to diarrhea. The list includes nature of vomiting, source of water, number of days hospitalized, vegetable, nature of diarrhoea, fresh or preserved food, dysentery, sex and milk products. It may not be necessary that all the ariables shall have its influence in norovirus uniformly. A certain set of variable may be dominant while certain other is more influencing. This can be a hypothetical situation as to which type of variable exerts its influence significantly as a determinant factor is a matter to be taken for hypotheses testing. The nine variables have been identified to run the correlation with “norovirus” which include dummy (coding) variable such as sex, vegetable, milk produce, fish, source of water, nature of diarrhoea, dysentery and nature of vomiting and the other independent variable namely, number of day hospitalized. Correlation matrices were applied to understand and shortlist the number of variables, which correlated with the norovirus. Of the nine variables, nature of vomiting, number of days hospitalized, fresh or preserved food, dysentery, sex and milk were chosen as variables those which exhibit a positive correlation and rest of the factors like source of water, vegetable, nature of diarrhoea turned negative with norovirus. The short listed factors were considered for running the regression against norovirus.

Tables 2a and b shows the regression and coefficients for the prediction of norovirus from the collected stool samples. The regression analysis brings forth the factors like number of days hospitalized due to diarrhoea and sources of drinking water turned out to be significant ($P<0.05$). The R square value turns at to be 0.732. This shows that 73% of the variations in dependent factor are explained by these two independent variables.

Conclusion

From the study, it was observed that out of 81 stool samples collected from diarrhoeal patients, 44.4% were positive for norovirus and 55.6% were negative for Norovirus. Presence of norovirus among the stool samples were detected by reverse transcriptase PCR and were confirmed by both agarose gel electrophoresis and by hybridization. This study has monitored norovirus distribution in peak summer month (April) also which is in contradiction with norovirus outbreaks which are generally more prevalent in winter.

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Table 1. Details of the stools samples collected from the patients infected with diarrhoea.

Patient No.	Sex	Age (years)
2941/c	M	52
1282/m	M	0.67
1284/m	M	24
2950/c	M	35
2940	F	25
1285/m	M	1
3257/c	F	32
3251/c	M	38
3252/c	M	20
3244/c	M	21
3246/c	M	24
2984/c	M	48
1299/m	F	22
1298/m	F	28
2942	M	1.5
3472/c	M	7
1569/m	F	1.25
3421/c	M	1
3254/c	M	22
3439	F	70
3507/c	F	1.5
1621/m	F	40
3516/c	M	1.17
3528/c	F	36
3480/c	M	1.25
3501/c	M	75
3499/c	M	1.5
23/m	M	30
17/m	F	37
25/m	M	1
58/m	M	0.92
71/m	M	4
114/c	F	52
127/m	F	25
157/m	F	1.5
31/m	F	49
33/c	F	40
266/m	F	35
270/c	M	1.5
422/c	F	63
614/c	F	24
1038/c	F	47
1036/c	M	20
876/c	F	60
883/c	M	33
1507/c	M	0.92
1459/c	M	21
642/m	F	0.67
643/m	F	40
706/m	F	50
1407/c	M	30

Table 1. Cont.

1408/c	F	38
1406/c	F	6
732/m	M	25
734/m	M	4
746/m	M	31
1503/c	F	0.83
752/m	M	55
1505/c	M	39
751/m	M	35
769/m	F	1.5
1697/c	M	52
842/m	F	35
789/m	F	50
794/m	F	38
793/m	M	56
1602/c	F	25
2144/c	M	35
2487/c	M	45
2516/c	F	55
4089/c	M	15
4064/c	M	62
1766/m	M	20
1793/m	F	50
1844/m	F	46
1848/m	F	35
3784/c	F	51
4267/c	M	23
4317/c	M	38
4523/c	M	0.75
796/m	M	17

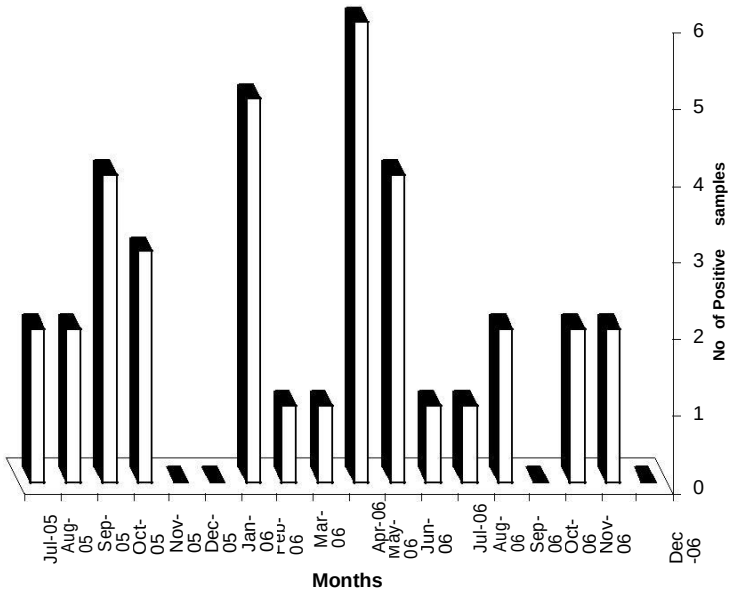

Figure 2. Seasonal distribution of norovirus positive stool samples collected from July ’05 to November ’06.

Table 2a. Prediction of norovirus – Regression results model summary.

Model	R	R square	Adjusted R square	Standard error of the estimate
1	0.855	0.732	0.698	0.275

Predictors: (constant), nature of vomiting, source of water, no. of days hospitalized, vegetable, nature of diarrhoea, fresh or preserved fish, dysentery, sex and milk.

Table 2b. Coefficients.

	Unstandardized coefficients		Standardized coefficients	t	Sig.
	B	Std. error	Beta		
(Constant)	-0.708	0.169		-4.181	0.000
Sex	3.088E-02	0.067	0.031	0.462	0.646
No. of days hospitalized	0.315	0.025	0.823	12.512	0.000
Vegetable	-4.728E-02	0.042	-0.074	-1.113	0.269
Milk	2.529E-02	0.040	0.044	0.628	0.532
Fresh/Preserved fish	2.279E-02	0.043	0.036	0.527	0.600
Source of water	-0.104	0.053	-0.129	-1.962	0.054
Nature of Diarrhoea	-1.040E-02	0.021	-0.033	-0.500	0.618
Dysentery	2.976E-02	0.027	0.072	1.095	0.277
Nature of vomiting	6.948E-02	0.060	0.078	1.153	0.253

Dependent variable: norovirus.

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