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Emergence of Noroviruses homologous to strains reported from Djibouti (horn of Africa), Brazil, Italy, Japan and USA among children in Kolkata, India

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Abstract. – *Aim:* **A total of 625 faecal specimens of diarrheic cases (n-313) and non diarrheic controls (n-312), were screened by RT-PCR to detect Noroviruses in children aged below 5 years in Kolkata, India.**

Materials and Methods: **Out of the 313 fecal specimens (cases) screened using CDC primer set, 10 (3.19%) showed amplification in reverse transcription-polymerase chain reaction (RT-PCR) for Norovirus. These included 5 of 260 (1.92%) from hospitalized and 5 of 53 (9.43%) from out patients departament (OPD) cases.**

Results: **Nine (90%) of Norovirus positive cases belonged to genogroup GII and one specimen (10%) was positive for genogroup GI. Among the 312 non diarrheic controls 2 (0.63%) were positive for Norovirus GII. Partial RNA dependent RNA polymerase gene (RdRp) sequences corresponding to the six Norovirus GII positive samples showed homology to the sequences of Djibouti (horn of Africa), Brazil, Italy, Japan and US norovirus strains.**

Conclusion: **This study shows the detection of newly emerging Norovirus strains among diarrheic and non diarrheic children in Kolkata.**

Key Words:

Emerging Noroviruses, Diarrhoea case, Asymptomatic control, Reverse transcription-polymerase chain reaction.

Introduction

Norovirus (NoVs) belongs to the *Caliciviridae* family. The virion is 27 to 32 nm in diameter, nonenveloped with icosahedral symmetry and has a linear, positive-sense, single-stranded RNA (ssRNA) genome (7.5-7.7 kb)[1]. NoVs are the major cause of nonbacterial epidemic gastroenteritis, a disease that usually occurs in family or community-wide outbreaks[2]. NoVs have also been associated with food borne gastroenteritis at large, leading to outbreaks in developed countries[3]. Currently five major phylogenetic clades, or *genogroups*, designated GI through GV of NoVs are documented that are subdivided into 32 genetic clusters[4]. Genogroups I, II, IV are associated with human gastroenteritis outbreaks[5]. The genome is organized into three major *open reading frames* (ORF1, ORF2, and ORF3) with polyadenylated 3'end[6]. Furthermore, ORF1 encodes a large polyprotein that is proteolytically processed into the mature nonstructural protein[7], ORF2 encodes the *major capsid protein*, VP1 and ORF3 encodes a *minor structural protein*, VP2. Various molecular epidemiologic studies have shown marked genetic diversity among circulating NoVs documenting distinct genetic clustering of strains viz. GI (8 clusters); GII (19 clusters); GIII (2 clusters); GIV (2 clusters) and GV (1 cluster) to date[2]. The GII noroviruses, particularly those of the GII.4 cluster, were the predominant viruses detected world wide and 7 distinct sub clusters are documented[4]. Like all enteric viruses noroviruses are also spread by several modes of transmission. The predominant modes of transmission are person-to-person contact and

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food- or water-borne spread[8]. Clinical manifestations associated with NoVs include the nausea, vomiting, diarrhea, abdominal cramps, headache, fever (subjective), chills, myalgias and sore throat[9]. Reverse transcription-polymerase chain reaction (RT-PCR), coupled with sequence analysis of the amplicons, has been used extensively to detect and characterize NoVs in various outbreaks. The objective of this study was to monitor the emergence and/or genetic diversity of NoVs circulating among diarrhoeic children and asymptomatic controls, aged below five years in Kolkata, India.

Materials and Methods

Study Subjects and Design of Study

In course of norovirus surveillance during May 2008 to May 2009, 313 faecal specimens were collected from acute watery diarrhea cases (under 5 years) at [a] Diarrhea Therapy Unit of NICED in Dr B. C. Roy Memorial Hospital for Children (n=53) and [b] Infectious Diseases and Beliaghata General Hospital (n=260), Kolkata, India. A total of 312 fecal specimens were also collected from the asymptomatic children from [a] Dr B. C. Roy Memorial Hospital for Children (n=261) and [b] Infectious Diseases and Beliaghata General Hospital (n=51), Kolkata, India. Patient information and clinical details were recorded in diarrhea case/ control reporting forms respectively. Informed consent was also obtained from parents or child guardians. Inclusion criteria for cases included any child suffering from acute gastroenteritis, hospitalized for the purpose of supervised oral rehydration or intravenous rehydration for at least 6 hours and aged below 5 years. Inclusion criteria for the controls included any child not suffering from acute gastroenteritis currently or for the preceding 4 weeks, hospitalized for less than 48 hours for an illness not related to the gastrointestinal tract and age group was same as cases.

Collection and Processing of Faecal Specimens for Molecular Characterization

The faecal samples were collected under the guidance of an experienced physician in sterile plastic containers and transported to Division of Virology of National Institute of Cholera and Enteric Diseases. Viral RNA was extracted from virus suspension using TRIzol LS (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and viral RNA obtained was dissolved in RNase free water and stored at –20°C. Reverse transcription (RT) reaction for NoV was preformed by using 100ng of random hexamers and Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (New England Biolabs, Ipswich, MA, USA). The partial RNA dependent RNA polymerase (RdRp) region B (partial ORF1) of NoV genome was amplified by polymerase chain reaction (PCR) using genogroup GI and GII specific primers described previously[10] by Ando et al., 1995 (Table I).

Processing of Amplicons, Sequencing and Analysis of Sequence Data

The amplicon was purified using QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) 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 Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). The sequence data was read using Finch TV (Version 1.4) and deduced amino acid data was documented using DNASIS (Version 2.1). To determine the relative sequence homology of the Kolkata strains with other NoVs strains the se-

Table I. Primers used for amplification of Norovirus during the study.

Region	Primer Name	Primer sequence 5′- 3′	Sense	Product size(bp)	Specificity
B	Mon432	TGGACICGYGGICCYAAYCA	+	213	GI
B	Mon434	GAASCGCATCCARCGGAACAT	–	213	GI
B	Mon431	TGGACIAGRGGICCYAAYCA	+	213	GII
B	Mon433	GAAYCTCATCCAYCTGAACAT	–	213	GII

A = Adenosine; C = Cytidine; G = Guanosine; T = Thymidine; I[Inosine]; Y[T/C]; S[C/G]; R[G/A]

quence data was run on the BLAST program[11]. LALIGN program[12] was used to study similarity among NoVs using the parameter of global alignment without end gap penalty. Multiple sequence alignments of norovirus GI and norovirus GII were generated by CLUSTALW (version 1.8.3)[13] and bootstrapped phylogenetic tree was constructed by neighbor joining method with 1000 bootstrap replicates, defining Sapovirus as an outgroup strain using the Molecular Evolutionary Genetics Analysis version 4.0 (MEGA 4.0)[14].

Sequence Submission and Accession Numbers

The partial nucleotide sequences covering RdRp region of the NoV strains from Kolkata [present study] was submitted to DNA Data Bank of Japan (DDBJ), under the following accession numbers viz. **AB514586** (NVKOL**277**), **AB514587** (NVKOL**290**), **AB514589** (NVKOL**N21**), **AB514590** (NVKOL**N26**) **AB514591** (NVKOL**N34**) and **AB514592** (NVKOL**NC14**).

Results

Out of the 313 fecal specimens (cases) screened using CDC primer set, 10 (3.19%) samples showed amplification in RT-PCR for NoVs. These included 5 of 260 (1.92%) from hospitalized and 5 of 53 (9.43%) from Out Patient Department (OPD) cases. Nine (90%) NoVs positive cases belonged to genogroup GII and one specimen (10%) was positive for genogroup GI. Among the 312 non diarrheic controls 2 (0.63%) were positive for norovirus GII. Among ten diarrheic cases 8 were below 2 year age group indicating maximum prevalence among <2 years age group. Both the non diarrheic controls belonged to <1 year age group. NoVs prevalence among the male children (8) was more compared to female children (2). Among non diarrheic controls one patient was male and one patient was female. Onset of diarrhea among cases was one or two days and frequency of diarrhea ranged from 6-20 times in a day and vomiting was seen in 3 cases. Most of the NoVs positive cases underwent oral rehydration therapy (ORT) and only one case was treated with intravenous fluid [IVT] on the first day of illness and later treated with ORT. The peaks of NoVs positivity were observed in September and December months. Clinical details of the positive samples are shown in Table II.

Partial RNA polymerase sequences, corresponding to the first six NoVs positive samples (five from cases, one from control) detected with the CDC primers, were derived and analyzed to obtain potential strain variation. BLAST analysis confirmed the homology of the sequences to those of previously reported human NoVs strains deposited in GenBank. On BLAST analysis Kolkata strains NVKOL277 and NVKOLNC14 showed homology of 96% and 98% with strains from Brazil (8040/2004/BRA), and Djibouti (DjiboutiVdG66/2003/Djibouti) respectively. Strain NVKOL290 showed homology of 98% with strain from Italy (998/2007/ITA). Strain NVKOLN21 showed 100% homology with previously reported Indian strain (V1772/07/IND) and Italy strain (976/2007/ITA). Strains NVKOLN26 and NVKOLN34 showed 95% and 86% identity with Japanese strain (OC04043/2004/JP) and USA strain (PortCanaveral/301/1994/US) respectively. Amino acid sequence identities between the six Kolkata NoV strains ranged from 63.2-98.2%. The Kolkata strains shared amino acid sequence identities ranging from 63.2-98.2% with Djibouti, Brazil, Italy, India, Japan and US strains. The Kolkata strain NVKOLNC14 shared 100% aa identity with India, Japan and US strains. Phylogenetic analyses, based on the deduced amino acid sequence of the 172 nucleotide region of the RNA polymerase gene showed a clustering of the Kolkata NoV strains with their counterparts from Djibouti, Brazil, Italy, India, Japan and US (Figure 1).

Discussion

Studies on NoVs associated acute gastroenteritis in children from India are limited. Recent studies indicated NoVs prevalence among hospitalized and community cases from India. These studies were from Vellore in South India[15], Pune in Western India[16], New Delhi[17] and Kolkata[18] in Eastern part of India. A study from Kolkata shown 21 NoV cases among children (n=111) visiting outpatient department to a hospital with acute gastroenteritis[18]. In a study from western India (Pune, Nagpur, and Aurangabad cities)[16]

Table II. The patient information and clinical symptoms associated with the Norovirus positive cases.

Sample No	Norovirus characterization	Date of sample collection	Age of the children (months)	Sex of the children	Diarrhea	Onset of diarrhea (hours)	Frequency of diarrhea	Dehydration	Vomiting	Frequency of vomiting	Treatment
NVKOL277	GII	02.05.08	18	M	+	48	09 times	Some	+	3	ORT
NVKOL290	GII	07.05.08	54	M	+	24	08 times	Some	–	–	ORT
NVKOLN07	GI	16.07.08	08	M	+	48	06 times	Some	–	–	ORT
NVKOLN21	GII	21.08.08	11	M	+	48	05 times	Some	–	–	ORT
NVKOLN26	GII	05.09.08	10	M	+	48	20 times	Some	–	–	ORT
NVKOLN34	GII	22.09.08	09	M	+	48	08 times	Some	+	6	ORT
NVKOLN51	GII	14.11.08	06	F	+	24	10 times	Some	–	–	ORT
NVKOL721	GII	13.12.08	07	M	+	24	14 times	Some	–	–	IVT
NVKOL737	GII	05.12.08	12	F	+	48	12 times	Some	–	–	ORT
NVKOL740	GII	05.12.08	42	M	+	24	06 times	Some	+	3	ORT
NVKOLNC14	GII	11.09.08	11	F	–	–	–	–	–	–	–
NVKOLNC91	GII	14.12.08	02	M	–	–	–	–	–	–	–

ORT = Oral rehydration therapy; IVT = Intravenous therapy.

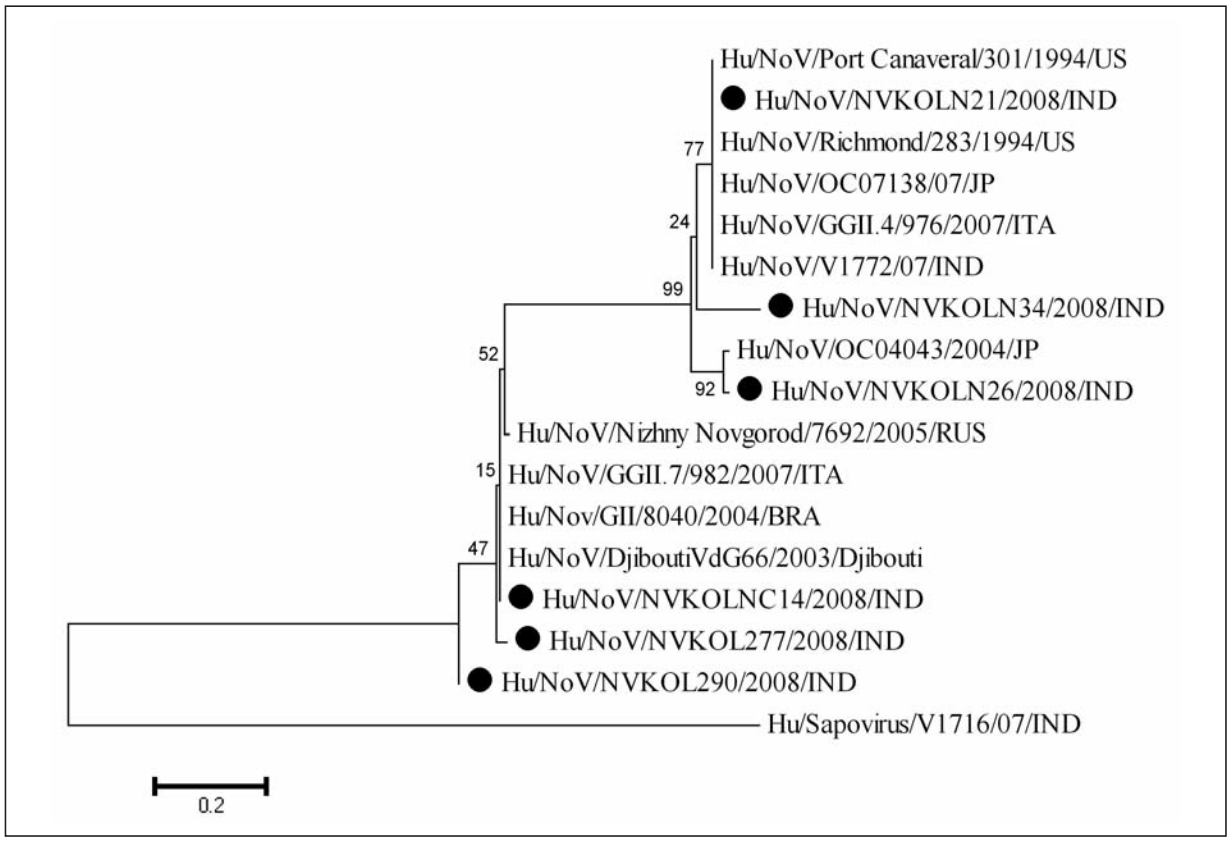

Figure 1. Phylogenetic analysis based on deduced amino acid sequences corresponding to 172bp nucleotide fragment of the RNA dependent RNA polymerase gene (RdRp) of 6 Kolkata NoV strains shown as • and other NoVs. The accession numbers of the RdRp fragment from other NoV strains shown on the tree are as follows: AF414421 (Port Canaveral/301); AF414419 (Richmond/283); AB434770 (OC07138); FM212863 (ITA/976/); AB447423 (IND/V1772); AB279559 (OC04043); FJ268597 (Nizhny Novgorod/7692); FM212875 (ITA/982); DQ397313 (BRA/8040); EF190920 (Djibouti/VdG66); AB447416 (outgroup strain Sappovirus SV/V1716).

children ≤7 years of age were reported to be suffering from acute gastroenteritis and NoVs positivity varied between 6.3% and 12.6% with the predominance of GII (96.6%) and NoVs infections were very common in the patients ≤2 years of age. Our study shows 3.63% and 0.63% positivity among diarrheic cases and non diarrheic controls below 5 year age group respectively. Earlier studies show the predominance of genogroup GII among the diarrheic patients and our study also showed the predominance of genogroup GII (90%). Increasing data showed emergence of novel recombinant NoVs reported from various parts of the world[19,20]. The NoVs circulating in different parts of India were genetically diverse as seen in a report from Kolkata[21]. The present study enabled detection of hitherto unreported genogroup GII strains NVKOL277, NVKOL290, NVKOLN21, NVKOLN26 and NVKOLN34 from diarrheic children and NVKOLNC14 from an asymptomatic child. It is important to note that NoVs strains reported earlier from Djibouti (horn of Africa), Brazil, Italy, Japan and US are now being detected among children in Kolkata. The phylogenetic analysis shows the clustering of Kolkata strains with NoVs from different parts of the world. This implies the emergence of NoVs as another important public health threat. Hence, in the light of our recent findings it is imperative that stringent surveillance and constant monitoring of emerging NoVs should be continued to keep track of the evolution in Norovirus.

Ethical Approval

The research project was submitted for ethical approval and carried out after being approved by the Ethic Committees.

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