

Molecular Study of Aetiology of Acute Gastroenteritis in Children of South Mumbai

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ABSTRACT

Introduction: Globally, Acute Gastroenteritis (AGE) is the leading cause of morbidity and mortality among children under five years of age. Majority of diarrhoeal illness in childhood are of viral aetiology. In the era of Rotavirus vaccine, Norovirus is emerging as an important cause of AGE in children.

Aim: To evaluate the aetiology of diarrhoea in children of South Mumbai.

Materials and Methods: A prospective study was carried out during July 2013 at a tertiary care hospital and research centre for evaluating diarrhoea in children ≤ 12 years of age. Clinical details of the patients were recorded on a case record form. Severity of disease was assessed by the modified Vesikari scoring pattern. Stool samples were collected from outpatients and inpatients. Stool routine microscopy and culture were done at tertiary care hospital. Aliquots of stool samples were tested at the Research Centre for Rota Virus,

Adenovirus, Norovirus and Enterovirus by Polymerase Chain Reaction (PCR).

Results: A total of 51 patients were included in the study. Of these, 45.10% (23/51) were positive for viruses by real time PCR. Routine stool culture was positive for only one patient for *E. coli* O157. One patient had *Entamoeba histolytica* infection. Of 23 patients positive for viral aetiology, Norovirus was detected in 41.18% (21/51). Of the Norovirus positive children, 42.85% were ≤ 1 year of age. Diarrhoea followed by fever was the most common presentation. Among the Norovirus positive children, 33.33% (7/21) had moderately severe disease while 66.66% (14/21) had mild disease.

Conclusion: Norovirus was the leading cause of acute gastroenteritis in the children included in the study. This study emphasizes the need to include Norovirus in the routine diagnostic algorithm of children with AGE and paves the way for syndromic approach based testing.

Keywords: Diarrhoea, India, Norovirus, Polymerase chain reaction

INTRODUCTION

Gastroenteritis is defined as the inflammation of the mucus membranes of the gastrointestinal tract and is characterized by diarrhoea or vomiting. Though it is a common and self-limiting childhood disease, it can lead to complications such as dehydration associated with electrolyte imbalance and acidosis, diminished growth, and impaired cognitive development particularly in resource-limited countries [1]. In developing countries, diarrhoea causes an estimated two million deaths annually among children aged < 5 years [2].

Agents causing gastroenteritis include viruses, bacteria and protozoa. In children, 70% of cases of AGE are caused by viruses [1]. Four viral families are commonly associated with AGE: *Reoviridae* (Group A Rotaviruses), *Caliciviridae* (Noroviruses), *Adenoviridae* (Adenoviruses 40/41), and *Astroviridae* (Astroviruses), rarely Toroviruses, Picobirnaviruses, Picornavirus and Enterovirus 22 may also be associated [3].

Globally, Rotavirus AGE leads to 453,000 deaths and over two million hospitalizations among children under the age of five years [1]. In countries that have added Rotavirus vaccine to their routine childhood immunization policy the incidence and severity of Rotavirus infections have declined significantly [1,3]. Norovirus is now being recognized as most frequent cause of childhood gastroenteritis causing sporadic cases and outbreaks [1]. Each year Norovirus cause 64,000 episodes of diarrhoea requiring hospitalization and 900,000 clinic visits among children in industrialized countries, and up to 200,000 deaths of children < 5 years of age in the developing countries [4].

Noroviruses are named after the original Norwalk strain, which caused an outbreak of AGE in a school in Norwalk, Ohio in 1968.

Human Noroviruses belong to the family *Caliciviridae*, which is divided into four genera, *Norovirus* and *Sapovirus*, which cause human infections and *Lagovirus* and *Vesivirus*, which are associated with veterinary infections [5]. Noroviruses are small, single-stranded, nonenveloped, positive sense Ribonucleic Acid (RNA) viruses with a genomic size of approximately 7.5 kb [6]. Noroviruses are currently classified into five different genogroups (G) GI – GV, of which GI, II, and IV infect humans. GIII and GIV infect bovine and murine species, respectively [6].

Globally, Rotavirus is the predominant pathogen associated with gastroenteritis in children [1,7-9]. However, the prevalence of Norovirus in developing countries may be underestimated due to limitation of diagnostics tests available [10]. In India, there is limited data on Noroviruses associated diarrhoea in children [11]. The present study aims to evaluate pathogens associated with AGE in children of south Mumbai over a period of one month. Knowledge on the current epidemiology of the agents associated with AGE in the given geographic area will help in better management and timely practice of infection control measures.

MATERIALS AND METHODS

A prospective study was carried out in July 2013 at a tertiary care hospital to investigate the cause of diarrhoea in children along with molecular testing at a reference center. Informed consent was obtained from parents of each child before inclusion in the study. This study was approved by the Hospital Ethics Committee. Children ≤ 12 years of age, having AGE were included in the study. Children with history of antibiotics treatment or hospitalization in the previous month were excluded from the study. Diarrhoea was defined as the passage of three or more loose or liquid stools per day accompanied with or without vomiting and/or other symptoms

such as fever, nausea, abdominal pain and cramps [12]. Clinical history was recorded in a case record form. Severity of disease was assessed by the modified Vesikari scoring pattern [13].

Stool samples were collected from both OPD and hospitalized children having diarrhoea in a wide mouth container provided by the hospital and was processed within one hour of sample receipt. Collected samples were processed for stool routine microscopy and culture as per hospital laboratory protocol. Gross examination of the stool samples included colour, consistency and reporting of occult blood. Routine microscopy was performed under 10x and 40x (Saline and Iodine preparation) for Red Blood Cells (RBCs), Pus cells, and Parasites (Ova and Cysts). For routine bacterial cultures MacConkey agar and *Salmonella Shigella* agar were inoculated and incubated aerobically at 35°C. Enrichment was done in Selenite F broth and after incubation for two hours at 35°C subculture was done on MacConkey agar and *Salmonella Shigella* agar. Identification and susceptibility testing of suspected bacterial pathogens was performed on Vitek 2 Compact (BioMérieux Pvt., Ltd.).

A part of fresh stool sample was frozen immediately and stored at -20°C. Maintaining the cold chain, the stored samples were transported to reference centre in batches for molecular testing (PCR) for Rota Virus, Adenovirus, Norovirus and Enterovirus.

Molecular Testing for Viral Aetiology

RNA extraction and reverse transcription: Viral RNA was extracted from 30% (w/v) stool suspensions in Minimal Essential Medium (MEM) using TRIzol (Invitrogen, USA) according to manufacturer's instructions and the viral RNA obtained was dissolved in RNase free water and stored at -20°C until used. A cDNA was synthesized at 37°C using random primers pd(N)₆ (Roche, Diagnostics) and 100UM-MLV reverse transcriptase (Invitrogen).

Detection and characterization of Norovirus strains: Norovirus GI and GII were detected by PCR using cDNA as the template with primers Mon 432 and Mon 434 and primers Mon 431 and Mon 433 respectively [14]. The PCR amplicates (213 bp) were analysed on the 2% agarose gel.

Detection of Enteroviruses: The cDNA was used as the template for the detection of the 5'NCR of the Enterovirus gene by using panEV PCR primers EV1 and EV2 [15]. The amplification products were analysed on 10% polyacrylamide gels after staining with 0.5 µg/mL ethidium bromide to visualize PCR amplification bands using UV Transilluminator (BioRad).

Enterovirus Isolation: The stool samples were treated with chloroform before inoculating in the Rhabdomyosarcoma (RD) cells for virus isolation according to World Health Organization (WHO) protocol [16].

The cultures were incubated at 36°C and the Cytopathic Effect (CPE) as observed for five days. Samples not showing CPE were passaged again and the CPE was observed for another five days. If the CPE was observed, tissue culture material was harvested for RNA extraction for virus identification. Samples were scored negative when three serial passages did not produce CPE. Tubes showing CPE were harvested after two freeze-thawing cycles. Virus isolates were identified by partial VP1 sequencing as suggested by Oberste et al. [17].

Partial VP1 sequencing and Enterovirus serotype identification:

The cDNA was used for partial VP1 PCR using 222/224 or AN88/ AN89 primer pairs [18]. The PCR products were used for Enterovirus serotype identification by sequencing on automated DNA sequencer ABI 3130x/ using BigDye Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystem, Forster City, CA) as per the manufacturer's instructions. The primers used for PCR amplification were used as the sequencing primers. The Enterovirus serotypes were identified by comparison of the VP1 sequences obtained with database of VP1 sequences of all EV serotypes from GenBank using BLASTn programme.

Detection of Rotavirus: The cDNA was used as template for the detection of Rotavirus using NSP4F and NSP4R PCR primers [19]. The Amplification products were analysed on 1% agarose gel.

Detection of Adenovirus: The cDNA was used as the template for the detection of Adenovirus using Ad40 and Ad41 PCR primers [20]. The Amplification products were analysed on 1% agarose gel.

Details of primers used are given in [Table/Fig-1] [14,15,18-20].

RESULTS

Fifty-one children ≤12 years of age with AGE were included in the study. All the patients included in the study were vaccinated for Rotavirus. Of 51 patient tested, 45.10% (23/51) were positive for viruses by real time PCR. Routine stool culture was positive for only one patient for pathogenic *E. coli* O157. One patient had *Entamoeba histolytica* infection. Of 23 patients positive for viral aetiology, Norovirus were identified in 41.18% patients (21/51), Enterovirus was seen in 3.92% (2/51) patients and two patients had mixed infection with Norovirus, Enterovirus and Rotavirus [Table/Fig-2].

Viruses	Primers	Sequences (5'-3')	Region	Nucleotide positions	PCR product size (bp)
Norovirus [14]	Mon 432	TGGACICGGYGGICCYAAAYCA	B (GI)	5093 – 5112	213 bp
	Mon 434	GAASCGCATCCARC GGGAACA		5285 – 5305	
	Mon 431	TGGACIAGRGGICCYAAAYCA	B (GII)	5093 – 5112	
	Mon 433	GAAYCTCATCCAYCTGAACA		5285 - 5305	
Enterovirus [15,18]	EV1	ACACGGACACCCAAAGTAGTCGGTTCC	5'NCR	539-565	114 bp
	EV2	TCCGGCCCCCTGAATGCGGCTAATCC	5'NCR	452-476	
	224	GCIATGYTIGGIACICAYRT	VP3	1977-1996	762 bp
	222	CICCIGGIGGIAYRWACAT	VP1	2969-2951	
	AN89	CAGCACTGACAGCAGYNGARAYNGG	VP1	2602-2627	372 bp
	AN88	TACTGGACCACCTGGNGNAYRWACAT	VP1	2977-2951	
Rotavirus [19]	NSP4F	GGCTTTTAAAAGTTCTGTTC CG	NSP4F	1-22	743 bp
	NSP4R	GTCACTAAGACCATTC	NSP4R	753-732	
Adenovirus [20]	Ad40	GCCGCAGTGGTCTTACATGCACATC	Hexon	18858-18882	300 bp
	Ad41	CAGCACGCCGCGGATGTCAAGT	Hexon	19136-19158	

[Table/Fig-1]: Primers used for PCR amplification and sequencing [14,15,18-20].

Standard IUB nucleotide ambiguity codes are used:

I=Deoxyinosine; N=G, A, T or C; Y=C or T

W=A or T; R=A or G

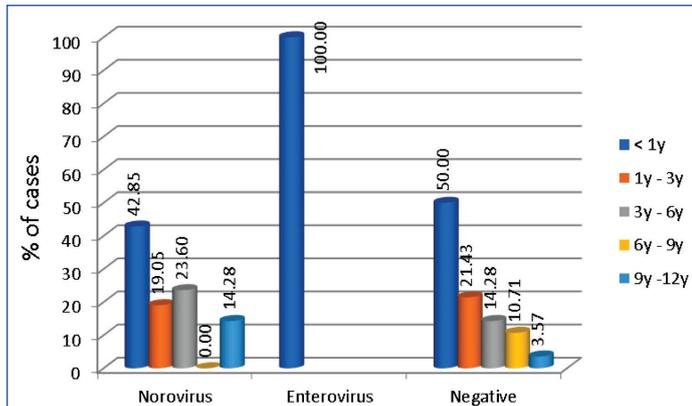
The locations of all primers are those relative to the genome of PV1 Mahoney (GenBank accession number J02281).

Patients positive for Viral Aetiology	Norovirus (NV) Genogroup			Rotavirus (RV)	Enterovirus (EV)	Mixed Infection	
	GI	GII	GI + GII			RV+NoVGI+EV	NoVGI+GII+EV
23	9	8	2	0	2	1	1

[Table/Fig-2]: Virus aetiology and their Genotypes.

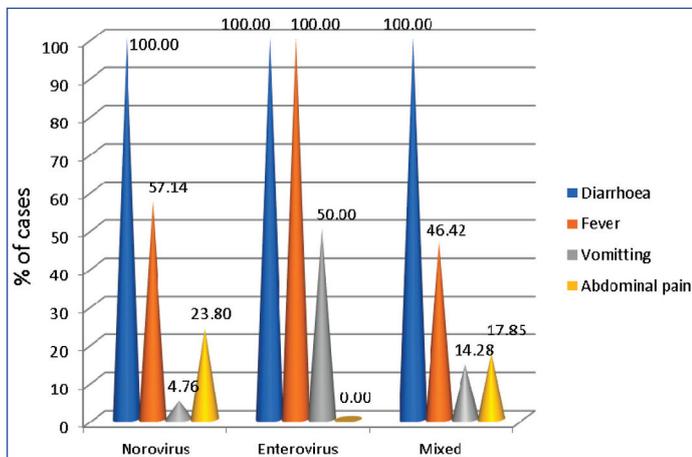
Our study group included 43 outpatients and 8 inpatients. Norovirus positivity was 41.86% (18/43) and 37.5% (3/8) among outpatients & inpatients respectively. Both the patients positive for Enterovirus were out patients.

In our study, 65.21% (15/23) of children under three years of age had viral diarrhoea. Age distribution of patients who were Norovirus positive is given in [Table/Fig-3].



[Table/Fig-3]: Age-wise distribution of patients positive and negative for Viral aetiology.

Overall diarrhoea was the most common presentation followed by fever in both those tested negative and those positive. Vomiting and abdominal pain were less common presentations [Table/Fig-4]. Clinical severity of disease was assessed by using modified Vesikari scoring system by (Ruuska and Vesikari (1990) [Table/Fig-5,6] [12].



[Table/Fig-4]: Clinical symptoms of the patients included in the study.

DISCUSSION

In present study, Norovirus were identified in 41.18% patients (21/51). Chhabra P et al., reported a prevalence of 6.3% -12.6 % in children < 7yrs of age suffering from AGE from Western India [6]. In India, the prevalence varies from 6.6 to 25.7% from Southern and Northern India [8,21]. Some studies have reported lower prevalence of 1.4 % to 2.3% [22,23]. In Tunisia, the prevalence of Norovirus in sporadic cases was found to be 9.3% [24]. A prevalence of 25% was reported by Zeng M et al., in China [25].

Globally and in India, GII is the predominant genogroup causing gastroenteritis in all age groups [1,8,9]. Contrary to this, in our study we observed that genogroup I (47.61%) was predominant. Further molecular studies are needed to confirm this variation in the epidemiology observed in our study.

Global epidemiology of AGE indicates that Rotavirus is the predominant pathogen causing diarrhoea in children [1,8]. Contrary

Severity category			
Mild	Moderate	Severe	Maximum score
<7	7 -10	>=11	20
Score			
Parameter	1	2	3
Diarrhoea			
Maximum stools per day	1-3	4-5	>=6
Diarrhoea duration (days)	1-4	4	>=6
Vomiting			
Maximum number vomiting episodes per day	1	2-4	>=5
Vomiting duration (days)	1	2	>=3
Temperature			
	37.1 -38.4	38.5 -38.9	>=39
Dehydration			
	N/A	1 -5 %	>=6%
Treatment			
	Rehydration	Hospitalisation	N/A

[Table/Fig-5]: Vesikari clinical severity scoring system severity rating scale and scores. N/A: Not applicable. Lewis K. Vesikari Clinical Severity Scoring System manual. Seattle: PATH, 2011.

Clinical severity score		Norovirus (n=21)	Enterovirus (n=2)	Negative (n=28)
<7	MILD	66.66	50	78.57
7-10	MODERTAE	33.33	50	14.28
>11	SEVERE	0	0	7.14

[Table/Fig-6]: Severity of disease among study group.

to this, in our study Norovirus was the predominant viral pathogen. Clinical history reveals all the patients included in our study were vaccinated against Rotavirus. Studies have shown that in the Rotavirus vaccine era, Norovirus is emerging as an important pathogen causing enteric infection in children [1,23]. Present study points out to this changing epidemiology of acute viral gastroenteritis.

Globally, the prevalence of mixed infections reported from various studies ranges from 4.4% to 14% [26-28]. Studies from India have shown mixed infections in the range of 0.6% to 23.2% [8,9,22], while in our study co-infections were seen in 3.92% of patients [Table/Fig-5]. Coinfection is commonly reported with Rotavirus and Norovirus or Adenovirus or Enterovirus or Astrovirus [8,9,27,28].

In this study, Norovirus was detected in 41.86% (18/43) and 37.5% (3/8) outpatients and inpatients respectively. In a study from south India, 9.4% and 15.1% positivity were seen among outpatients & hospitalized patients in western India was 9% and 12.5% respectively [11]. Gupta S et al., reported 2.3% of hospitalized patients positive for Norovirus [8]. Higher rates of 34% in hospitalized children similar to our study have been reported from Japan [29]. China and Italy report, 25.6% and 47% of hospitalized children with Norovirus infections respectively [25,30].

Although Norovirus were detected in all age groups, the infection with Norovirus was more commonly seen in children less than one year of age (42.85%). Chhabra P et al., in a study from western India also reports 40% of children ≤ one year of age to be infected by Norovirus [6]. In a study from Delhi and Himachal Pradesh Norovirus infection was seen in children < 2 year of age [8,22]. In China, it is seen in children < 3 years of age [25]. In Tunisia, Norovirus was frequently seen in children more than 35 months [24].

Vomiting along with diarrhoea and fever is the most common presentation of Norovirus infection. Sai L et al., reported 67.5% children had vomiting and 46.3% had fever [31]. Similarly, in Taiwan, vomiting was reported as a more common presentation compared to fever and diarrhoea [32]. In our study, only 4.76% of children presented with vomiting. Study from Western India has reported 32% of hospitalized and 43% outpatients did not have vomiting [6]. Gupta S et al., reported increased in duration of vomiting for Norovirus infected patients, though not statistically significant (p -value=0.076) [8]. Study by Ramirez S et al., has also reported absence of vomiting in 51% of Norovirus infected patients [30].

Norovirus infection is called as “Winter Vomiting Disease” in West. In present study, maximum cases were observed during the monsoons. In Indian studies, Norovirus infection was reported in summer as well as winter season [6,8]. In Taiwan, higher incidence was seen in winter months [32] whereas in China, season of Norovirus associated disease varies with the region [25].

According to modified Vesikari scoring system, 66.66% of patients with Norovirus diarrhoea had mild disease and 33.33% had moderate disease and no cases of severe illness were reported. Chhabra P et al., reported, 29%, 57% and 14% of outpatients with severe, moderate and mild disease respectively [6]. In our study, 14% of Norovirus affected children required hospitalization. Contrary to this, Chhabra P et al., reported >50% hospitalized patients with Norovirus infection having severe disease [6]. Another study from western India reported 29.2% and 70.8% of the children having moderate and severe disease respectively [11].

Gonzalez-Galan V et al., in Spain reported 20% of patients with Norovirus infection had co-infection with *Salmonella* spp. [33]. In present study, one patient with Norovirus infection was co-infected with *E. coli* 0157.

Worldwide, Norovirus is the leading cause of non-bacterial gastroenteritis among individuals of all age groups [34]. Apart from symptomatic disease, Norovirus infection can lead to asymptomatic infections which serve as reservoirs of infection [21]. Norovirus has very low infecting dose and transmission takes place by multiple modes such as directly, from person to person; via contaminated food or water; via airborne droplets of vomitus and through contaminated environmental surfaces. Its ability to resist desiccation and disinfection favours its survival in the environment for long period of time allowing transmission via fomites. All these features add to its potential to cause outbreaks in institutional settings e.g., nursing homes, hospitals, military camps, cruise and schools [35,36]. Norovirus generally causes mild to moderate disease however severe disease is seen in the vulnerable population like children, elderly or immunocompromised patients [37,38]. There is no specific treatment available for Norovirus gastroenteritis, the therapy is only supportive [36].

Currently there are no licensed vaccines available for Norovirus [36,38]. Challenges associated with development of vaccine include inability to grow the virus in culture, genetic/antigenic diversity, limited knowledge on Norovirus immunology and assessment of vaccine performance in naïve individuals. NoV-Virus-Like Particles (VLPs) produced by recombinant technology have been identified as potential vaccine candidates and have shown good serum response [36,38,39]. But the success of a commercially available NoV vaccine, in developing countries will depend upon factors like cost, need for maintenance of a cold chain and integration into an already crowded immunization program.

LIMITATION

A limitation of study is the small size of the subjects as the study was conducted during a period of one month. Multicentric studies are needed to understand the epidemiology of the virus in India and to put measures for infection prevention and control, particularly in the era of Rotavirus vaccine.

CONCLUSION

In the era of Rotavirus vaccine, Norovirus could become an important pathogen causing AGE. As Norovirus disease is difficult to distinguish clinically from other causes of AGE, a syndromic approach based testing of AGE may be considered in the diagnostic algorithm.

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