

Full Length Research Paper

Detection and partial characterization of norovirus among children with acute gastroenteritis in Lagos, Nigeria

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Norovirus (NoV) has captured increasing attention as an agent of childhood diarrhea, but its incidence in developing countries such as Nigeria has been underreported. This study was conducted to investigate the role of NoVs in sporadic cases of acute diarrhea among hospitalized children. One hundred and eighty-eight (188) specimens comprising 161 diarrheic and 27 non-diarrheic stools were randomly selected from 668 stools previously screened for rotaviruses. These specimens were collected from children under 5 years of age who were hospitalized between November 2007 and May 2008 in Lagos, Nigeria. The specimens were examined for NoV antigen using monoclonal antibody-based enzyme immunoassay (EIA), and the positive specimens were further characterized for norovirus genogroups using reverse transcription-Polymerase chain reactions (RT-PCR) technique. NoV was detected in 60/161 (37.3%) of diarrhoeal and 1(3.7%) of non diarrhoeal children tested. Noroviruses were detected throughout the study period with most patients infected in age group 6 - 12 months. More males were infected with NoV than females however, the difference was not significant ($\chi^2 = 0.983$, $p > 0.05$). Sixteen among the positive samples were amplified by PCR using specific primers, of these, 5 (31%) were typed to be GI, 8 (50%) were GII and three samples (19%) showed a mixed infection of norovirus GI and GII genogroups. This study confirmed the relevance of NoV as a causative agent of pediatric diarrhoea and reported norovirus genogroup GII as the predominant type in circulation in Lagos, Nigeria.

Key words: Diarrhoea, norovirus, RT-PCR, children, Nigeria.

INTRODUCTION

Acute gastroenteritis remains the leading cause of morbidity and mortality worldwide, especially among those up to five years old. The global mortality is estimated at two million per year, solely in developing countries (Okitsu-Negishi et al., 2004). Many infectious agents (bacteria, parasites, viruses) are implicated but viruses are mainly responsible for endemic and epidemic gastroenteritis mostly represented by group A rotaviruses (RV) Astroviruses, human Adenoviruses and noroviruses (NoVs).

NoVs are the major cause of non-bacteria gastro-

enteritis and are associated with outbreaks of diarrhoea illness. Norovirus is one of the two genera belonging to Caliciviridae family which causes human infection. It is a small non-enveloped, single stranded RNA virus that requires special cell systems for in vitro replication (Straub et al., 2007). Human norovirus strains belong to GI, GII or GIV genogroups from the five existing ones. NoVs are the major cause of epidemic gastroenteritis worldwide and are related to the consumption of food and water or aquatic recreational activities attacking persons of all ages [de Wit et al., 2003] as well as outbreaks in nurseries, hospitals, daycare centers and hotels (Gallimore et al., 2004; de Wit et al., 2007). However, molecular procedures have over a decade, recognized this virus as a sporadic self-limiting gastroenteritis (de Wit et al., 2001; Marshall et al., 2003, Oh et al., 2003).

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Table 1. Oligonucleotide primer sequences used for norovirus genogrouping.

Primers	Sequence (5 -3)
G1FFN	GGAGATCGCAATCTCCTGCC (21 mer)
G1SKR	CCAACCCARCCATTRTACA (19 mer)
G1IFBN	TGGGAGGGCGATCGCAATCT (20 mer)
G1ISKR	CCRCCNGCATRHCCRTRTACAT (23 mer)

IUPAC – Ambiguity Codes for Nucleotides.
R = A or G; H = A or C or T; N = A or C or G or T.

NoVs have been studied extensively in the developed world but only little work have been conducted on this subject in developing countries such as Nigeria. Therefore, this study investigates the frequency of sporadic norovirus infection among diarrhoea children admitted to some hospitals in Lagos, Nigeria and partially characterized the isolates to know the prevalent genogroup in circulation in Lagos, Nigeria.

MATERIALS AND METHODS

Patients and samples

A total of one hundred and eighty- eight (188) stool specimens comprising 161 diarrheic and 27 non-diarrheic stools were randomly selected from 668 stools previously screened for rotaviruses. The specimens were collected from children presenting with diarrhoea under 5 years of age who attended Massey children hospital and the Pediatrics' section of Orile Agege General Hospital in Lagos, Nigeria, between November 2007 and May 2008. These hospitals in which samples were collected serve patients of different educational and socio-economic backgrounds living in neighborhoods with distinctly different level of sanitation. The samples were collected into sterile universal bottles and stored at -20°C until transported on ice to Noguchi Memorial Institute for Medical research, Ghana for analyses.

Norovirus antigen detection by EIA

The stool specimens were screened for human noroviruses using commercially available norovirus genus-specific kits (Norovirus RIDASCREEN® r-Biopharm, Germany). The test was run according to the manufacturer's instructions. Briefly, a 10% dilution of each specimen was prepared using the sample diluent (v/v 1:11), and spun at 5000 rpm for 3 min. Approximately, 100 µl of each diluted specimen (supernatant) was dispensed into each microwell of a 96-well microtitre plate pre-coated with mouse monoclonal antibody (MAb) against group specific antigen of norovirus, leaving out first and second wells as negative control (sample diluent) and positive control (inactivated adenovirus strain), respectively. This was followed by adding 100 µl of enzyme conjugate 1 (norovirus specific mouse MAb conjugated to horseradish peroxidase) to each microwell and incubated for 1 h at room temperature. After a total of five washes with diluted wash buffer (v/v 1:10), 100 µl enzyme conjugate 2 was added to each well and was incubated at room temperature for 30 min. A second round of five washes with diluted wash buffer (v/v 1:10) was done after which 100 µl of substrate was added into each microwell, followed by incubation at room temperature for 15 min in the dark. Reactivity was read visually and

also determined by spectrophotometry at 450 nm after adding 50 µl stop solution (1 N sulfuric acid). The cut off was calculated as the sum of the extinction for the negative control and 0.15. Samples were considered positive if their extinction is more than 10% above the calculated cut-off.

Norovirus genogrouping by RT-PCR

Norovirus single-stranded RNA was extracted from selected samples that were EIA positive by phenol/chloroform method as described by Steele and Alexander (1987) and purified using the RNaid kit (BIO101 systems, QBIogene Carlsbad, USA). Extracted RNA samples were then reversed transcribed as described by Kojima et al. (2002) with some slight modifications. Briefly, 0.5 µl of hexanucleotide random primers (20 mU; PdN6; Pharmacia Biotech) (Iturriza-Gomara et al., 1999) was added to 5 µl ssRNA template. A master mix (19.5 µl) consisting of (4 µl 5 x buffer; 0.5 µl avian myeloblastosis reverse transcriptase; 1 µl each of 10 mM dATP, 10 mM act, 10 mM dGTP, 10 mM dTTP; 11 µl of RNase free water) was used. The RT assay was carried out in the water bath at 42°C for 4 h to produce cDNA.

The cDNA generated was then amplified by PCR in a 45 µl reaction mixture containing (0.25 µl each of 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP; 10 µl 5 x Green Go Taq Buffer; 0.25 µl Taq Polymerase; 30.75 µl RNase free water; 5 µl cDNA template). For GI noroviruses typing 2 and 1 µl 20 pmol of specific primers G1SKR and G1FFN were used while for genotyping GII noroviruses, 1 and 2 µl 20 pmol of specific primers G1IFBN and G1ISKR, respectively, were used in an RT-PCR analysis (Table 1) (Armah et al., 2006). A 40 cycles of PCR (10 min initial denaturation at 95°C, 30 s annealing at 48°C, 5 min for extension at 72°C) and a final extension cycle at 72°C for 5 min was performed using Primus 25 system cyler, Germany. The PCR products were loaded unto 2% agarose gel with 0.5 µg/ml ethidium bromide and electrophoresed in Tris acetic EDTA (TAE) buffer at 100 V for 1 h. The products were visualized on UV Tran illuminator and photographed using Polaroid camera.

RESULTS

Sixty-one (32.5%) of the 188 stool specimens from young children were positive for presence of norovirus antigen (Plate 1). Thirty seven percent were positive from the diarrhoea group, while in the control group only one (3.7%) who was a female tested positive for norovirus. Thirty- four of the diarrhoea positive cases (37.4%) were males while 26 (37.1%) was recorded for female (Table 2). Diarrhoea and vomit were observed to be common

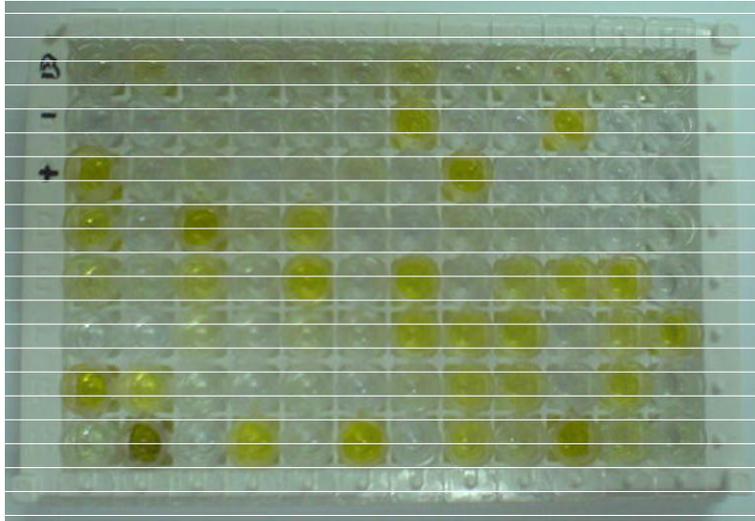


Plate 1. Microtitre plate showing EIA result for norovirus. Positive wells (yellow) and Negative wells (colourless).

Table 2. Sex distribution of NoV infection among children in Lagos, Nigeria.

Sex	Diarrhoeal cohort			Control cohort		
	n	Positive	%	n	Positive	%
Female	70	26	37.1	13	1	7.7
Male	91	34	37.4	14	0	0.0
Total	161	60	37.3	27	1	3.7

$\chi^2 = 0.983, p = 0.05.$

Table 3. Distribution of NoV infection according to age among under 5years old Nigerian.

Age group (month)	NoV (n = 61)		
	Frequency	Percentage	Cumulative (%)
0 - 6	9	14.9	14.8
>6-12	29	47.5	62.3
>12-18	15	24.6	86.9
>18 - 24	4	6.6	93.5
> 24+	4	6.6	100

$\chi^2 = 0.008, p = 0.05.$

symptoms among NoV positive cases. NoVs were detected in all age group tested. Ninety-four percent of the norovirus infection occurred in children of about two years of age and the highest infections rate was observed between 6 - 12 months (Table 3). Norovirus was detected throughout the study period with two peaks recorded in February and April (Figure 1).

Among the 30 samples randomly selected from the sixty one EIA norovirus positive samples amplified by RT-PCR, 16 (53%) yielded norovirus specific *gene* amplicon while 14 (47%) showed amplification failure after gel

electrophoresis using Open Reading Frame (ORF) 1/ 2 primers. Eight (50%) of the NoV positive by RT- PCR were identified as belonging to GII genogroup (Plate 2), 5 (31%) to GI and the remaining 3 (19%) were mixed infection of GI + GII.

DISCUSSION

Gastroenteritis is one of the most popular sicknesses among children and can be caused by many infectious

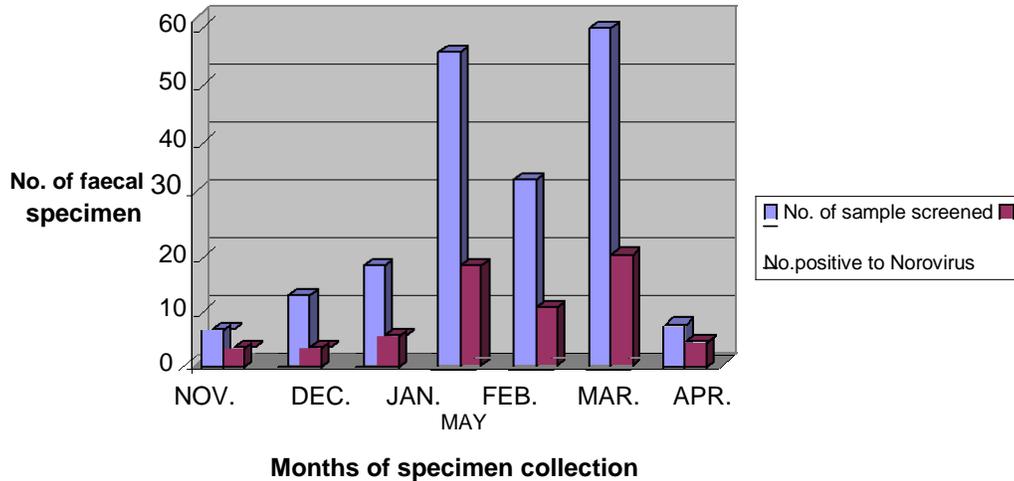


Figure 1. Monthly detection of norovirus among children in Lagos State.

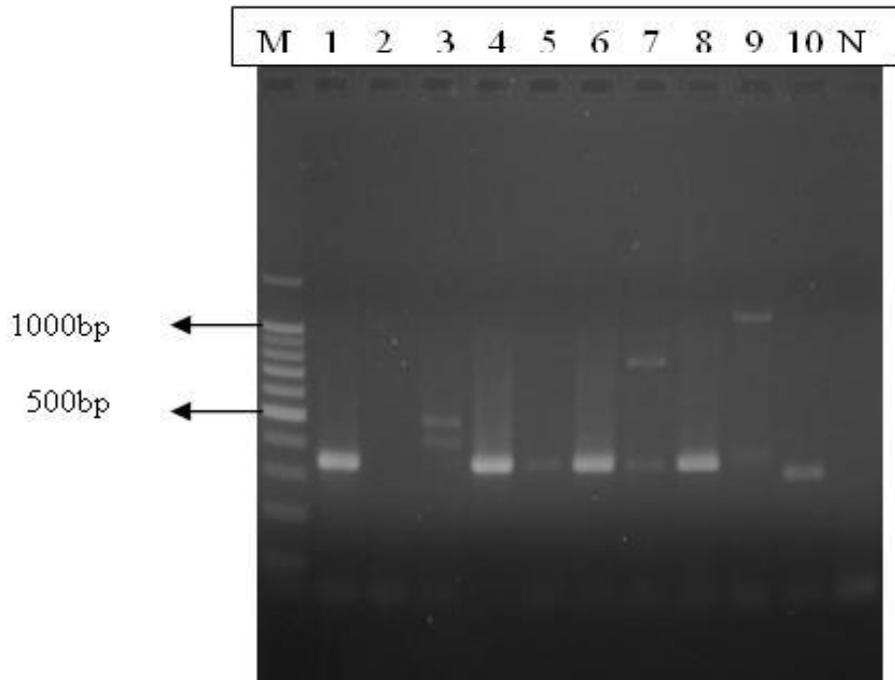


Plate 2. Different NoV GII amplicons as determined by RT-PCR. Amplification products were electrophoresed on 2% agarose gel stained with 0.5 g/mL ethidium bromide at 100 V for 60 min. Lanes 1, 4, 5, 6, 8 10 show GII amplicon (350 bp); lane 3 shows GII amplicons (400 and 500 bp); lane 7 shows GII bands (350 and 800 bp) and lane 9 shows GII bands (350 and 1000 bp); lane 2 shows genotyping failure; M: 100 bp DNA ladder, N: negative control.

agents, varying from bacteria, parasites and viruses, whose etiology and prevalence varies among developing and developed countries. However, enteric viruses (Rotaviruses, Noroviruses enteric, Adenoviruses and astroviruses) have been discovered as important agents, independent of improvements in basic sanitation and hygiene (Clark and Mc Kendrick, 2004). Noroviruses has

been shown to be an important agent of sporadic gastroenteritis in the developed part of the world [Kirkwood et al., 2005], however, little or non existence of epidemiological information is available on burden of the disease associated with sporadic norovirus infection in clinical or hospitalized patients in developing countries (Gallimore et al., 2004; Victoria et al., 2007; Borges et al.,

2006) especially in Africa (Armah et al., 2006). It is interesting to state here that this is the first report on NoV in hospitalized children with gastroenteritis in Nigeria.

The prevalence rate of (37.3%) recorded for norovirus infection in this study is rather high and is close to the findings of Ribeiro et al. (2008) in which they reported (39%) in hospitalized paediatric patients in Victória, Espírito Santo, Brazil. However, the high prevalence of NoV reported in this study is contrast to other reports elsewhere on sporadic cases in hospitalized children which vary from 5.4 - 30% in paediatric patients (Subekti et al., 2002; Hansman et al., 2004). Likewise, the rate observed here is much higher than the one reported in the neighbouring country (Ghana) where Armah et al. (2006) reported 15% among children presenting with diarrhoea. However, prevalence as high as 48.4 and 53% have also been reported in Italy and Australia, respectively (Colomba et al., 2007; Kirkwood and Bishop, 2001). NoVs were detected in 37.3% of the diarrhoea and in 3.7% of the non diarrhoea specimens. This high prevalence observed in diarrhoea group establishes norovirus as an important agent in hospitalization requiring admission due to gastroenteritis. Although, the period of sample collection was not up to a full year, to make a definitive statement on the seasonal variation however, norovirus was seen to occur throughout the study period and was observed to peak in February and April.

The age distribution of children shedding norovirus was significant in that shedding predominantly occurred in younger children and the majority of the infections occurred in children between 6 - 12 months of age ($\chi^2 = 0.008$, $p < 0.05$). This is not surprising as this is the age group in which children are usually wean from maternal antibody and introduced to semisolid foods which may be contaminated due to improper handling. Likewise, it is the period in which they crawl around picking anything they see into the mouth. This finding is consistent with the observation in Ghana (Armah et al., 2006) and in Bangladesh (Black et al., 1982) where infection was found common in children under 2 years of age. NoV genogroups GI, GII and GI+GII were detected, with GII predominated. The detection of GII strain as the predominant strains was in agreement with observations reported from studies in Canada, France and Ghana where the predominance of GII strains in children was between 77 and 90% (Armah et al., 2006; Al Mashhadani et al., 200; Pang et al., 1999). However, this report is in contrast to the finding of Kojima et al. (2002) in which they observed that GII strains are more predominant in contaminated food while GI are predominant in faecal contaminated specimens.

In conclusion, the present study which is the first report on this subject in Nigeria, has shown that NoV is an important agent causing diarrhoea leading to hospitalization in children under 5 years old in Nigeria. Occurrence of NoV infection was common in the first two years of life and norovirus GII predominated. However, there is need to accomplish further studies on norovirus

strains genotyping, to better determine the impacts of this infection as there is data scarcity on this subject.

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