ORIGINAL ARTICLE

FIRST MOLECULAR DETECTION AND VP7 (G) GENOTYPING OF GROUP A ROTAVIRUS BY SEMI-NESTED RT-PCR FROM SEWAGE IN NIGERIA

Babatunde Olanrewaju MOTAYO(1), Adekunle Johnson ADENIJI(1,2) & Adedayo Omotayo FANEYE(1)

SUMMARY

Rotavirus is the leading cause of viral gastroenteritis worldwide, and sewage is a major source of the virus dissemination in the environment. Our aim was to detect and genotype rotaviruses from sewages in Nigeria. One hundred and ninety sewage samples were collected between June 2014 and January 2015. The two phase concentration method using PEG 6000 and dextran was used to concentrate sewage samples following WHO protocols. Molecular detection was performed by RT-PCR, and VP7 genotyping by semi-nested multiplex PCR. A total of 14.2% (n = 27) samples tested positive. Monthly distribution showed that June to September had a lower rate (3.7% to 7.4%), while October to January recorded 11% to 26%. Genotype G1 predominated followed by G8, G9, G4 and lastly G2, 7.4% (n = 2) of isolates were nontypeable. This is the first report of rotavirus detection in sewages from Nigeria. Genotype G1 remains the most prevalent genotype. This observation calls for an effort by the governmental authorities to implement a molecular surveillance, both clinical and environmental, in order to provide vital information for the control and the vaccine efficacy not only in Nigeria, but globally.

KEYWORDS: Rotavirus; Sewage; VP7 genotyping; Nigeria.

INTRODUCTION

Viral gastroenteritis resulting from exposure to contaminated drinking and recreational waters have been reported worldwide[1,2]. Many workers have indicated the presence of enteric viruses such as rotavirus and noroviruses in urban human sewages[3,4]. Contamination of surface water with these viruses leading to public health consequences has also been previously reported[5]. Some studies such as the one from Kargar *et al.*[4] have also shown the presence of rotavirus in treated sewage effluents. Globally, rotavirus is the leading cause of acute gastroenteritis in children of less than five years, and over half a million deaths annually[6]. Rotavirus is a non-enveloped RNA virus belonging to the Reoviridae family, the genome consists of 11 double-stranded segments, with six structural proteins forming three concentric layers[1,7]. The outer capsid consists of two neutralizing antigens, the G type (VP7) and a protease-sensitive P type (VP4). Rotaviruses strains have been classified using the molecular and antigenic properties of these two proteins[1,8].

In Nigeria, it has been estimated that over 160,000 deaths are associated with diarrhea in children of less than five years of age[9]. Presently, there are two licensed rotavirus vaccines, the Rotateq, which confers protection against [P5]G1-4 and [P8]G6 strains, and Rotarix, that is protective against [P8]G1[10,11]. In Nigeria, Rotarix vaccine is available but still not included in the National Expanded Immunization Program. Molecular epidemiology has shown the presence of genotypes G1[P8], G3[P8] amongst others in Nigeria[12-15]. Rotaviruses have been shown to persist and survive different physicochemical treatment processes during sewage treatment[16,17]. It is therefore essential to study the role of sewage contamination with rotaviruses, and their possible link to human outbreaks through environmental contamination of rivers, dams and irrigation channels. The aim of this study was to detect the presence of group A rotaviruses in sewage and determine the circulating G genotypes recovered from sewages in Nigeria.

MATERIALS AND METHODS

Study Design

The current study is a prospective analysis of sewage from five states in Nigeria namely, *Sokoto*, *Kano*, *Brono*, *Abuja* and *Lagos*.

Sewage samples were collected by the grab method in a white one liter keg and transported to the Laboratory under reverse cold chain conditions

(1) University of Ibadan, College of Medicine, Department of Virology. Nigeria. E-mail: babatundemotayo@yahoo.com; adek@yahoo.com; adedayoraji@yahoo.com
(2) University of Ibadan, National Poliovirus laboratory, Department of Virology. Nigeria. E-mail: adek@yahoo.com
Correspondence to: Babatunde Olanrewaju Motayo. E-mail: babatundemotayo@yahoo.com

for processing. The cluster sampling technique was adopted following the protocol of the Polio Environmental Surveillance Program[18].

Sewage concentration

Sewage samples were concentrated by using polyethylene glycol 6000, dextran, NaCL using a two-phase concentration method described by the guidelines for environmental surveillance for Poliovirus circulation[18]. Briefly each sewage sample was centrifuged at 1,500 g for 10 min, then 400 mL of the upper supernatant was concentrated with a mixture of polyethylene glycol 6000, dextran 20% and NaCL 5M. Overnight incubation at 4 °C was carried out in a separation funnel, and a standard volume of 4 mL was harvested combining the bottom and the hazy interphase[18].

RNA extraction and cDNA synthesis

Viral RNA was extracted beginning with PEG 6000 and dextran-concentrated sewage samples using a kit based on silica gel spin columns, Total RNA purification Kit by Jena Bioscience® GmbH, Frankfurt, Germany. Extracted RNA was transcribed into cDNA using Red Load Taq Master/high yeild by Jena Bioscience® GmbH, Frankfurt, Germany, according to manufacturer's instructions. Briefly 5 μL of template RNA was added to 8 μL of the cDNA synthesis mixture containing 4 μL of RT buffer, 1 μL of each dNTPs, DTT, RNase inhibitor, 0.5 μL each of random hexamers and the RT enzyme (200 units). To this, 7 μL of nuclease-free H2O was added to a final volume of 20 μL. The reaction mixture was incubated at 42 °C for 10 min then, at 50 °C for 60 min.

Rotavirus detection and VP7 genotyping by semi-nested multiplex PCR

Rotavirus detection was performed using the primers VP7f and VP7r[19]. Genotyping was performed according to the protocol described by Rodriguez-Daiz et al.[19]. For the PCR detection, 4 μL of cDNA was added to a reaction mixture of 21 μL consisting of 5 μL of the Red load Taq Master/high yeild mix from Jena Bioscience® GmbH, Frankfurt, Germany, 0.2 μM of each of the first-round primers (Table 1) and 14 μL of nuclease-free H2O. The second round of amplification consisted of the same volume of the red load PCR mix and 1 μL of each of the genotype-specific primers, and the reverse primer of the first-round reaction. Primer positions and sequences are shown in Table 1. Cycling conditions were performed according to the protocol of Rodriguez-Diaz et al.[19]. All the samples were tested in duplicate before genotypes were assigned.

Amplicon purification and sequencing

Representative samples of the amplification products corresponding to each of the identified rotavirus genotypes were purified using PCR Purification Kit by Jena Bioscience® GmbH, Frankfurt, Germany amplicon purification Kit, following the manufacturer's instructions. Purified amplicons were shipped to Inqaba Bioscience Inc, Pretoria, South Africa, for DNA sequencing performed using the second-round PCR primers.

Sequence assembly and Phylogenetic analysis

Sequences were verified using the Chromas Lite software, version 2 (www.techelysium.com.au), and assembled into contigs using the Bioedit software (www.mbio.ncsu.edu/bioedit/). Strains were determined by BLAST (Basic Local Alignment Search Tool) http://blast.ncbi.nlm.nih.gov/Blast.cgi from the National Center for Biotechnology Information (NCBI) and genotyped using the Rota C software, version 2.0 (www.rotac.regatools.be). Sequences were aligned along with other reference rotaviruses sequences retrieved from GenBank, using the CLUSTAL W program of the MEGA 5 software (www.megasofware.net). A neighbor joining tree was constructed with 1,000 bootstrap replicates using the Mega 5 software (www.megasoftware.com).

Nucleotide sequence accession numbers

Sequenced amplification products generated from this study have

Table 1
Molecular weight of amplification products and primer sequences for rotaviruses VP7 (G) genotyping by semi-nested RT-PCR according to Rodriguez-Diaz et al.[19], in sewage samples from Nigeria

Genotype	Primer name	Primer sequence 5'-3'	Nucleotide position	Molecular weight (bp)
VP7 (G) 1st rnd	VP7 F	ATGTAGGGTATTGAATATACCAC	49-71	
VP7 (G) 1st rnd	VP7 R	AACTTGCCACCATTTTTTCC	914-933	884
VP7(G)	VP7 R	AACTTGCCACCATTTTTTCC	914-933	
G1	aBT1	CAAGTACTCAATGAATGATGG	314-335	619
G2	ACT2	CAATGATATTAACACATTTTCTGTG	411-435	522
G3	G3	ACGAACTCAACACGAGAGG	250-269	683
G4	aDT4	CGTTTCTGGTGAGGAGTTG	480-499	453
G8	aAT8	GTCACACCATTTGTAAATTGC	178-198	755
G9	aFT9	CTAGATGTAACTACAACTAC	757-776	176
G10	G10	ATGTCAGACTACARATACTGG	666-687	267

Rnd – round; bp – base pairs

been deposited in GenBank with the accession numbers KU 88451 and KU 88454.

Statistical analysis

Data were analyzed and tested for significance using the Chi-square test. The statistical software SPSS version 18.0 (IBM, New York, USA) was used for all the analysis and a *p* value of $p < 0.05$ was considered significant.

RESULTS

Detection and seasonal distribution

A total of 190 sewage samples were processed and tested. Rotavirus was detected in 14.2% (n = 27) of the samples by RT-PCR. Monthly distribution of rotavirus showed a significant difference between the rainy months from June October, and the early dry months from November to January ($p < 0.05$) (Fig. 1). The months of June and July recorded a 3.7% isolation rate, August and September 7.4%, and there was a sharp rise in the detection rate in November (18.5%), December (22.2%) and January (26%). The regional distribution of rotaviruses is shown in Figure 2, with the northern region, comprising *Kano* and *Borno* States recording the highest rates (16.2%; n = 17), followed by southern region, composed of *Lagos* State (13.6%; n = 6), and lastly Central Nigeria *Abuja* (12.2%; n = 4).
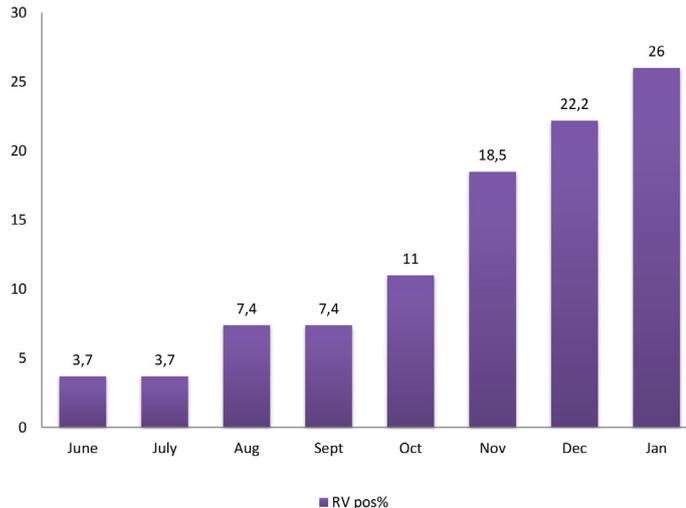

Fig. 1 - Rotavirus distribution from sewage in Nigeria June 2014 to January 2015.

Genotype distribution

The genotype distribution of rotaviruses strains showed isolates with single genotype specificity that were responsible for 70.4% (n = 19), isolates with mixed genotype specificity accounting for 22.2% (n = 6), while 7.6% (n = 2) were nontypeable isolates as shown in Table 2.

Genotype G1 was the overall most predominant isolate accounting for 40.7% (n = 11) of the total, when both mono and mixed isolates were considered (Table 2), followed by G8 (26%; n = 7), G9 and G3 (14.8%; n = 4), G4 (11%; n = 3) and lastly G2 (7.4%; n = 2), (Table 3).
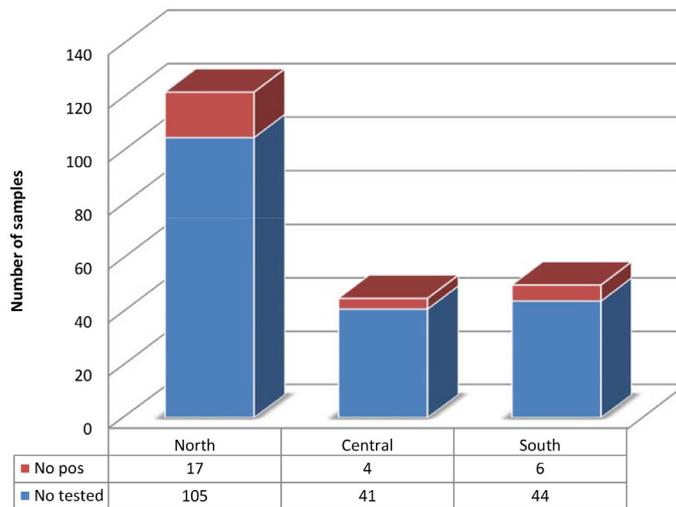

Fig. 2 - Stacked bar chart showing number of samples tested for rotavirus RNA versus number of rotavirus RNA positive samples according to geographical region. North represents *Sokoto, Bornu* and *Kano* States. Central represents Abuja, and south represents *Lagos* State.

Nontypeable strains corresponded to two out of the 27 (7.4%) isolates (Table 3). The genotype distribution in relation to seasonality showed that all the genotypes except for genotype 4 and 3 displayed a higher level of circulation during the dry season than the rainy season (Fig. 3).

Sequence and Phylogenetic analysis

Samples representative of each genotype were sequenced, then submitted to sequence assembly and BLASTn search: two genotypes were distinctly identified, genotype G1, and genotype G3, samples representing genotypes G9, G3 and G4 were not readable due to a low yield of amplified DNA. Phylogenetic analysis shown in Figure 4 revealed that G1 isolates have clustered with a recently identified Nigeria isolate, as well with a Chinese isolate. It has also been shown that the G3 isolate clustered closely with G3 isolates from Ghana.

DISCUSSION

The current study detected the presence of rotaviruses in sewage from Nigeria for the first time. A total of 14.2% of the sewage samples collected during the study period tested positive, this result is similar to that of a recent study from Shiraz, conducted in Iran, in which 25% (n = 15) of urban and hospital sewage samples tested positive for group A rotaviruses[4]. Other studies have reported detection rates from 11% to 42% [20,21]. In our study we used molecular methods rather than Enzyme Immunoassays (EIA) because of the higher sensitivity and specificity of PCR to detect fewer copies of enteric viruses such as rotaviruses from environmental sources[2,17].

In a study from Argentina, a very high detection rate was reported using qPCR technique, with 32 out of 35 samples (91.4%) testing positive[2]. This is one example of the high level of efficiency of molecular methods to detect enteric viruses from environmental sources.

The virus concentration method used in this study, the two-phase concentration method using PEG 6000 and dextran as resins with NaCL is

Table 2
Monthly isolation rate and genotype distribution of group A rotaviruses detected in sewage from Nigeria

Month	Positive/ total number	G genotype							
		G1	G2	G3	G4	G8	G9	Nt	Mixed G
June	1/21	+	–	–	–	–	–	–	–
July	1/18	–	+	–	–	–	–	–	–
Aug.	2/23	+	+	–	–	–	–	–	–
Sept.	2/25	–	–	+	–	–	+	–	–
Oct.	3/24	–	–	–	–	+	–	+	+
Nov.	5/28	+	–	+	+	–	+	–	+
Dec.	6/26	+	–	+	–	–	+	+	+
Jan.	7/25	+	–	+	+	–	–	–	+

represents rotaviruses positive samples; [+] represents positive results; [–] represents negative results; (N) represents the number of samples tested per month. [Nt] = nontypeable strain; [Mixed G] represents mixed genotypes.

Table 3
Distribution of rotaviruses G genotype specificities in sewage samples from Nigeria

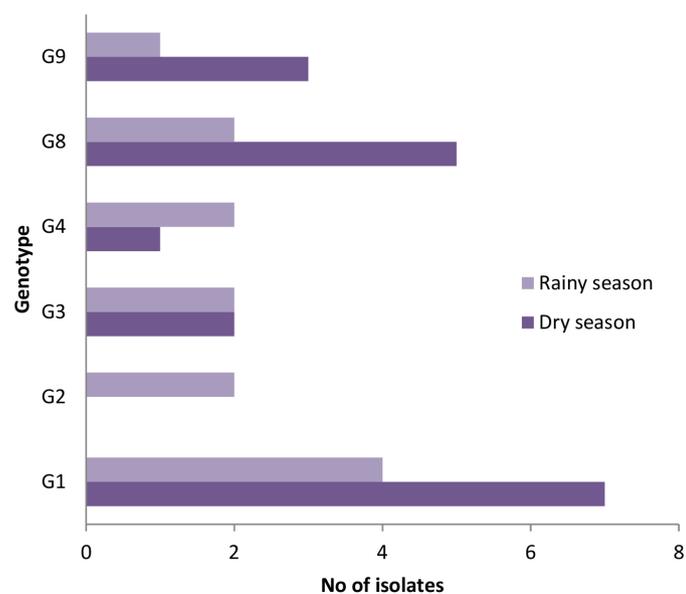|	Single genotype distribution						
G-type	G1	G2	G3	G4	G8	G9	G10
N (%)	7 (26)	2 (7.4)	2 (7.4)	1 (3.7)	3 (11)	4 (14.8)	0 (0)
	Mixed genotype distribution						
G-type	G1;G3	G1;G8	G4;G8	G1;G3	G8;G1	G9;G1	Nt
N (%)	2 (7.4)	2 (7.4)	2 (7.4)	0 (0)	0 (0)	0 (0)	2 (7.4)

Nt = nontypeable isolates

Fig. 3 - Seasonal distribution of rotavirus genotypes isolated from sewage from in Nigeria.
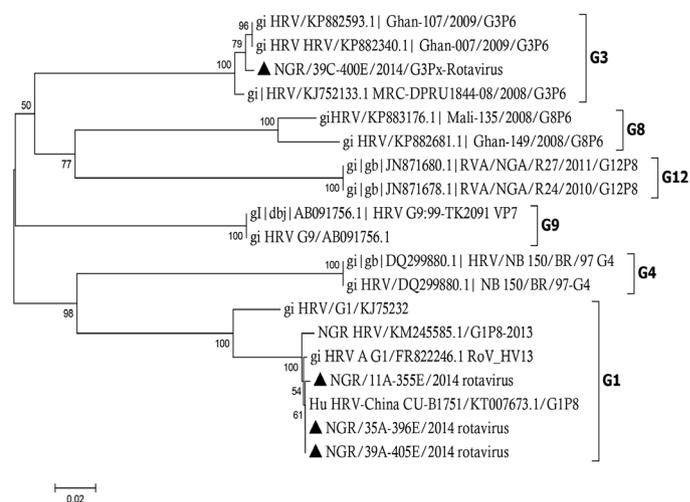

Fig. 4 - Phylogenetic relationship of partial VP7 gene sequences of rotaviruses. The newly sequenced strains from this study are indicated in black triangles, genotype assignments are indicated in bold letters by the right. GenBank accession numbers of the reference rotavirus sequences are indicated in the tree.

a very efficient recovery method to be applied to viruses from water and environmental sources such as sewage[22]. This method has been reported to enhance the yield of intact virions[4]. Hovi *et al.*[23] have demonstrated that the virus concentration from water samples can be increased by up to 100 fold using the two-phase method. The monthly distribution of rotaviruses in sewage from Nigeria revealed a low detection rate in the months of June to September (Fig. 1), with June and July having the lowest rates of all (3.7%). These months coincides with the peak of the rainy season in Nigeria when humidity and precipitations are very high. There are also heavy rainfalls during these months. However detection has increased steadily from September to January of the next year, coinciding with the beginning of the dry season otherwise known as *harmattan* in Nigeria. This observation shows that rotaviruses transmission can occur in both the rainy season and the dry season in Nigeria. This seasonality in rotaviruses detection has also been reported in the context of other viruses, particularly measles and influenza[24,25], however detection rates more than doubled during the dry months. This finding is an indicator of a higher risk of transmission during the dry season. Since rotaviruses can be shed by both symptomatic and asymptomatic people[1], this observed pattern does not necessarily suggest a higher level of rotaviruses outbreaks during the dry months. Some other factors such as general hygiene, nutrition and heard immunity also play a role before disease manifestation occurs. Previous studies in other parts of the world such as the study carried out in Argentina showed a similar seasonal pattern of rotavirus detection in sewage, although there was no statistically significant association[2]. This observed trend in seasonality has been proposed by Bishop[26], so that the virus might be spread by ingested aerosolized particles. The consequence of the currently observed trend in rotaviruses seasonal detection is that the disease transmission is most likely to increase during the dry season because of the poor sanitary conditions resulting in scarcity of water particularly in rural areas. The geographical distribution results have found a higher detection rate in the northern states compared with the central and southwestern state of *Lagos* (Fig. 2). This finding does not necessarily mean that Rotavirus burden is higher in Northern Nigeria, because a nationwide survey has never been carried out and cases of rotaviruses leading to diarrhea continue to be reported across the country. However, our results have shown that rotavirus transmission occurs across the country.

The genotype distribution revealed that genotype G1 was the predominant circulating genotype. This is in line with previous reports from Nigeria which have shown G1 to be the main circulating strain among diarrheic children from different parts of Nigeria[12,14,15,27,28]. The global genotype distribution has also identified G1[P8] to be the predominant circulating strain[7]. Genotypes G2, G3, G4, and G8 that have been previously reported, have also been identified among the typed strains[9,12,14,27]. A significant number of mixed genotypes (22.2%; n = 6) were identified in the present study, this is in line with other studies on clinical samples from Nigeria[15,30]. In addition, genotype G9 isolates, that have already been reported, were also identified in this study[13,15,28]. Apart from the few episodes in which G9 has been retrieved, G9 rotaviruses have been rarely identified in Nigeria, despite their clinical and epidemiologic global importance. The number of nontypeable strains recorded was 7.4% (n = 2) and this can be attributed to a high level of rotaviruses diversity in Nigeria, as previously reported[13,15]. This finding has also been associated with an immune evasion pressure in countries where rotavirus vaccination has been included in the expanded program for immunization (EPI)[29]. This is however not the case in Nigeria, as rotavirus vaccines have not yet been included in the EPI program, and are available only to those who can afford the vaccination. The seasonal distribution of G genotypes showed a higher number of G1, G8 and G9 genotypes identified during the dry season than during the rainy season (Fig. 3). Genotype G4 and G2, however, displayed a different pattern with the rainy season having a higher distribution. Analysis of representative rotaviruses sequences has correctly identified two genotypes, G1 and G3. Blast search results for the sequences presenting high bootstrap similarity values to Asian G1 rotaviruses isolates, showed that our G3 isolate had a high similarity bootstrap value in comparison with other G3 strains from Asia. The phylogenetic analysis showed a distinct clustering of our G1 isolates with other representative G1 isolates from China and Nigeria (Fig. 4). Our G3 isolate, however, has clustered with other G3 isolates from Ghana, West Africa, with a high (79%) bootstrap value (Fig. 4). One limitation of this study was our inability to analyze sequences of other representative rotaviruses genotypes, due to unreadable sequences resulting from a low yield of the amplification products after purification, however, all the samples had been retested twice before genotypes were assigned.

The results from our study demonstrated, for the first time, the diversity of rotaviruses genotypes in sewage from Nigeria. Rotavirus G1 remains the predominant genotype circulating in Nigeria, genotype G9 was also detected. There were also a number of nontypeable rotaviruses strains, which may correspond to highly divergent genotypes. This observation calls for an effort by the governmental authorities to implement a molecular surveillance, both clinical and environmental, in order to provide vital information for the control and the vaccine efficacy not only in Nigeria, but globally.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding this research.

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