Journal of Medical Virology

Group A Rotaviruses circulating prior to a National Immunization Programme in Nigeria: clinical manifestations, high G12P[8] frequency, intra-genotypic divergence of VP4 and VP7.

Journal:	*Journal of Medical Virology*
Manuscript ID	JMV-16-5558.R4
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Japhet, Margaret; Obafemi Awolowo University, Department of Microbiology; Faculty of Science, Ekiti State University, Department of Microbiology; Robert Koch Institut, Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses Famurewa, Oladiran; Faculty of Science, Ekiti State University, Department of Microbiology; Microbiology Unit, Kings University, Department of Biological Sciences Iturriza-Gomara, Miren; University of Liverpool, CIMI, Institute of Infection and Global Health Adesina, Olufisayo; Obafemi Awolowo University, Department of Microbiology Opaleye, Oluyinka; Ladoke Akintola University, Department of Medical Microbiology and Parasitology Niendorf, Sandra; Robert Koch-Institut, Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses Bock, C; Robert Koch Institut, Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses Mas Marques, Andreas; Robert Koch-Institut, Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses
Keywords:	Human rotavirus < Virus classification, Genetic variability < Evolution, Reassortant < Genetics, Genetic variation < Genetics

Group A Rotaviruses circulating prior to a National Immunization Programme in Nigeria: clinical manifestations, high G12P[8] frequency, intra-genotypic divergence of VP4 and VP7

Japhet M.O[1,2,6]., Famurewa O[2,3]., Iturriza- Gomara M[4]., Adesina O.A[1]., Opaleye O.O[5]., Niendorf S[6]., Bock C.T[6]., Mas Marques A[6*]

1. Department of Microbiology, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
2. Department of Microbiology, Faculty of Science, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria.
3. Department of Biological Sciences, Microbiology Unit, Kings University, Ode-omu, Osun State.
4. Institute of Infection and Global Health, University of Liverpool, Liverpool, UK
5. Department of Medical Microbiology and Parasitology, Ladoke Akintola University, Osogbo, Osun state, Nigeria
6. Department of Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses, Robert Koch-Institute, Berlin, Germany.

*Corresponding author

Abstract

Nigeria having approximately 50,000 Rotavirus A (RVA) deaths annually is yet to introduce RVA vaccine into routine national immunization; therefore surveillance of RVA strains circulating before vaccine introduction is essential in evaluating impact of the intervention. Stool samples and sociodemographic data of diarrhoeic children, <5 years were collected between August 2012 and December 2013. While a high prevalence of RVA infection (47.6%; 49/103) was observed by quantitative reverse transcription real time PCR, only 25% (26/103) had high RVA genome concentrations and were antigen positive. G and P types were obtained for 31 and 37 samples respectively. G12P[8] strains were predominant (30.6%; 16/31); Other genotypes found included G9, G3, G2 and P[4], P[6], P[8]. A G12+G2/P[8]+P[6] mixed infection was detected. The P[8] genotype showed divergence with strains distributed in lineage III and IV. Compared to the vaccines, changes in antigenic sites of VP8* and VP7 were found. The finding of the G2P[6] genotype combination and emergence of G12 strains support observations in most of the recent RVA studies from Africa. P[6] is common in many African countries, in contrast to countries in Europe and the Americas. In conclusion, this study shows the circulation of other RVA genotypes compared to the common RVA genotypes in Nigeria. PCR results should be interpreted with caution to avoid significant bias from samples with low RVA genome concentrations.

These findings provide important information on the detection and molecular epidemiology of RVA prior to vaccination and contribute as a baseline for future evaluations after possible vaccine introduction.

Keywords: rotavirus; genotype; lineage; Nigeria; gastroenteritis; children

Introduction

Group A rotavirus (RVA) is the most common cause of severe diarrhea in infants and young children worldwide.[1] RVA gastroenteritis accounts for approximately one third of the total diarrhea deaths worldwide. According to the World Health Organization (WHO), 215,000 (95% CI 197,000-233,000) children die annually from RVA infection and five countries (India, Nigeria, Pakistan, the Democratic Republic of the Congo, and Angola) accounted for more than half of all RVA deaths in children under 5 years of age.[1] In Nigeria, it is estimated that approximately 30,000 RVA deaths occur each year.

Rotavirus forms a genus of the family *Reoviridae* and is characterized by a segmented double-stranded RNA genome.[2] At present, rotaviruses are classified into nine groups (RVA-RVI) based on genetic variability of VP6 protein of which only group A, B, C and H rotaviruses have been found to infect humans;[3-5] RVA is the most common. The two outermost proteins, VP4 (protease sensitive protein) and VP7 (glycoprotein), specify the P and G genotypes, respectively.[2] At present, 32 G and 49 P genotypes have been identified (http://rega.kuleuven.be/cev/viralmetagenomics/virus-classification). The circulating RVA strains detected in Nigeria include G1–G4, G8, G9, G10 and G12 detected in 2012.[6-12]

Two oral live-attenuated RVA vaccines RotaTeq® (RV5) (Merck & Co., Inc., USA) and Rotarix® (RV1) (GlaxoSmithKline Biologicals, Belgium) have been licensed in many countries around the world. Although the WHO recommends universal inclusion of RVA vaccination in national immunization programmes,[13] RVA vaccine is yet to be introduced into the routine immunization in Nigeria but is available in private health sector. Due to the high poverty level in the country, use and impact of the vaccine are negligible.

In preparation for vaccine introduction, many countries initiated surveillance for RVA to document disease burden, to describe the epidemiology and to monitor circulating RVA strains in the respective countries.[14] However, updates on currently circulating RVA strains in Nigeria is scarce. RVA surveillance is critical to determine the burden of disease due to RVA and the characteristics of the RVA strains circulating in Nigeria. This will serve as baseline data against which the impact of RVA vaccines can be assessed when eventually introduced in Nigeria. This information will be useful to guide policy decision making on RVA vaccine introduction.

This study therefore seeks to describe the epidemiology and strain diversity of RVA among children (<5 years) in Ile-Ife, Nigeria before the national introduction of vaccine in the country, and also analyzes RVA infection with respect to available clinical/socio-demographic data.

Material and Methods

Ethics Approval

Permission to conduct this study was sought from the Obafemi Awolowo University Complex Teaching Hospital (OAUTHC) Research Ethics Committee (OAUTHC REC) of the Obafemi Awolowo University Ile-Ife, Nigeria (ERC/2012/10/2).

Study Population and Sample Collection

The study was conducted from August 2012 to December 2013 at the OAUTH Comprehensive Health complex, Ile-Ife as well as the only two State Hospitals within Ile-Ife (State Hospital, Oke-Ogbo and Health Centre, Enuowa). Children, <5 years who presented with diarrhea (3 or more watery stool over a 24hr period, with or without fever, vomiting or dehydration) at the outpatient clinics or admitted to the wards and whose parent signed informed consent form were eligible for inclusion in the study. Demographic data (age, sex), clinical and risk factors (symptoms, source of drinking water, six month exclusive breast feeding, stool description, knowledge about RVA etc. were collected through the use of questionnaire completed by the parent or guardian of the children.

RNA Extraction

The fecal specimens collected were prepared as 10% fecal suspensions in phosphate-buffered saline (pH 7.0) for viral RNA extraction, vortexed for 15 min and then centrifuged for 10 min 20,000 x g. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (QIAgen Hilden, Germany) on QIAgen Qiacube machine according to manufacturer's protocols. A total of 60 µl of purified viral RNA was obtained and stored at -20^{o}C until further analysis.

Denaturation, RT and Virus Detection by Quantitative Reverse Transcription Real Time PCR

Denaturation at 95^{o}C for 1 min followed by chilling immediately on ice for about 2 min was performed on 2.2 µl RNAse free water and 2.6 µl RNA template mixture. Reverse transcription and quantitative real time polymerase chain reaction (qRT-PCR) specific for NSP4 of RVA was then carried out in a single-step approach using SuperScript III/Platinum Taq OneStep kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Briefly, 8.3 µl master mix was placed into all the wells of 96 well micro plate. Master mix contained buffer, RVA sense (5'-GCTTTTAAAAGTTCTGTTCCGAG) and antisense (5'-

ACTCAATGTGTAGTTGAGGTCGG) primers, RVA probe (5'-VIC-ATCTTTCCGCACGC-MGB) and Platinum Enzyme mix. Then, 3.7 µl denaturated template was added. The reaction plate was placed into a LightCycler® 480 device (Roche, Mannheim, Germany) and cycling conditions were as follows: 15 min 50°C, 2 min 95°C [15 sec 94 °C, 32 sec 55 °C] 45 cycles. The efficiency of the RT and the qRT-PCR was evaluated by control RNA (RVA positive control, internal control RNA from MS2 phage) and DNA (plasmids dilutions containing the respective PCR target sequence). The qRT-PCR assay displayed a dynamic range from 10^1 to 10^6 target copies with a detection limit of 20 plasmid DNA copies per reaction whereas the analytic sensitivity of the PCR for weak positive RNA-dilutions was 95.2%. For the determination of the reproducibility, intra- and inter-assay variability was done using isolated RNA of patients' feces. The range of the inter-assay Ct-values varied with coefficients from 0.58% to 6.12% in correlation to the sample, the overall coefficient of intra-assay variation was between 1.54% and 5.53%. The analytic specificity was 100%.

All RVA positive samples from qRT-PCR were subjected to G and P typing.

Rotavirus G and P typing

Samples pretested by qRT-PCR were genotyped as previously described using published methods and primers.[15,16] Briefly, G and P type-specific amplification of VP7 and VP4 fragments was performed in two separate PCR assays using QIAgen One-step RT-PCR kit (QIAgen, Hilden, Germany). For the first round PCR primers used were VP7-F and VP7-R for the VP7 gene while VP4-F and VP4-R were used for the VP8* region of the VP4 gene. A second semi-nested multiplex PCR was performed using G1-, G2-, G3-, G4-, G8-,G9-, G10- and G12-specific sense primers for G types, while P-typing included P[4]-, P[6]-, P[8]-, P[9]-, P[10]-, and P[11]-specific antisense primers together with VP7-R and VP4-F, respectively. Cycling conditions were used as published previously.[16] For first quality control, the PCR products were visualized by electrophoresis on 1.5% agarose gel, stained with GelRed DNA stain (Biotium, Hayward, USA) on a BioDocAnalyze Live device (Biometra, Göttingen, Germany). For high resolution fragment length analysis, a ABI 3500xL Dx device and GeneMapper 5 software (Applied Biosystems, Foster City, USA) were used. All results were subsequently verified by sequencing of the VP4 and VP7 fragments.

ELISA

In addition to characterization by PCR methods, rotavirus antigen detection of all samples was performed by ELISA using RIDASCREEN® Rotavirus kit (R-Biopharm, Darmstadt, Germany) according to manufacturer's instruction. Briefly, a disposable pipette was used to aspirate 1 ml RIDASCREEN® sample dilution buffer and added to a sample of stool (approx. 100 µl). The stool suspension was aspirated and homogenized. Thereafter, 100 µl of the positive control, the negative control and the stool sample suspension were added to appropriate wells. Subsequently, 100 µl of biotin-conjugated antibody was added and mixed by tapping lightly on the side of the plate which was then incubated for 60 min. at room temperature. The plate was washed 5 times with the use of a microplate washer. A second incubation for 30 min. at room temperature was done after the addition of 100 µl conjugate 2 and the washing process was repeated. A third incubation for 15 min. in darkness at room temperature was carried out later. Lastly, 50 µl stop reagent was added and the result was read on an ELISA plate reader at dual wavelengths of 450/620 nm.

Nucleotide Sequencing

For sequencing of VP4 and VP7 genes, the first round PCR products and primers were used. The PCR product was cleaned up using EXOSAP-IT (USB Corporation, United States). For this, 5 µl PCR product were mixed with 2 µl EXOSAP-IT and incubated at 37°C for 15 min. The EXOSAP-IT reaction was then inactivated by heating to 80°C for 15 min. Purified PCR products were sequenced based on Big Dye 3.1 chemistry (Applied Biosystems, Foster City, USA), using 0.5 µl primer (10 µM), 1 µl BigDye and 1.5 µl buffer each with the following cycling profile: 1 min 96°C, [30 sec 96°C, 15 sec 50°C, 4 min 60°C] 25 cycles.

Sequence Analysis of the VP7/VP4 RVA Genes

Sequencing raw data files were analysed and edited manually using Sequencher 5.2.4 software (Genecodes). The web-based automated RVA genotyping tool, RotaC 2.0, available at http://rotac.regatools.be, was used for quick assignment and confirmation of genotype of the study strains.[17]

The sequences were also compared to GenBank reference strains using the Basic Alignment Search Tool (BLASTn) analysis to screen for related strains, which were included in subsequent phylogenetic analysis. Multiple sequence alignments were created using Geneious version 8.1 (Biomatters, Auckland, New Zealand). Phylogenetic analysis was performed

using MEGA6 with the Hasegawa-Kishino-Yano substitution model and maximum-likelyhood method to infer phylogenetic relationships among relevant strains. Bootstrapping included 500 replicates. The respective trees with the highest log likelihoods were selected. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. The methods and parameters were chosen considering model testing available in MEGA6 with the options as follows: automatic neighbor joining tree, substitution type: nucleotide, gaps: complete deletion, all codon positions, branch swap filter: very strong. The same models for VP8* and VP7 were chosen based on lowest Bayesian Information Criterion values.

.

The partial nucleotide sequences for the VP7 and the VP4 genes were submitted to GenBank (accession numbers KT952018-KT952047).

Statistical analysis

The RVA positive and negative specimens were compared in terms of gender, age group, seasonal distribution and risk factors by use of chi-square and Fischer exact tests, using two-tailed significance. SPSS version 20.0.1 for Windows was used for statistical analysis.

Results

Prevalence of Rotavirus Infection

A high prevalence of RVA infection was observed during the study. From the 103 children with diarrhea included in the study, 49 (47.6%) were positive for RVA by qRT-PCR method. In 27 samples (26.2%) RVA genome concentrations between 9×10^6 and 1.3×10^{11} genome equivalents (GE) per ml stool suspension were measured. The remaining 22 samples (21.4%) contained quantities that were at least two orders of magnitude lower (between 4.5×10^2 and 5.7×10^4 GE per ml stool suspension). Comparison of the viral load with ELISA results shows that 26 out of the 27 RVA positive samples with high RVA genome concentration were RVA antigen positive by ELISA while all the 22 samples containing lower RVA concentration were RVA antigen negative by ELISA.

Rotavirus genotypes

Notably, 16 (32.7%) of the 49 RVA positive children were infected with RVA G12P[8], with one being a mixed infection (see table 1). This is 51.6% of the 31 samples for which G types could be determined. All of the G12 strains were associated with P[8]. Among the P types observed in the study, P[8] was the most frequent with 61.2% (30/49) detection rate (81.1% of the 37 for which P types could be determined), followed by P[6] (n=4; 8.2%) and P[4] (n=2; 4.1%).

RVA prevalence by age and gender

None of the children enrolled was found to be up to 5 years of age. RVA infection was more prevalent in children less than one year compared with children older than one year (P =0.026; Table 2). Children in the age group 0-6 months accounted for 44.9% of the total RVA infection (22/49) followed by age group 6-11 months with 36.7% (18/49). Of note is the fact that 81.6% (40/49) of the total RVA infection occurred in children less than 1 year. Of the 103 children included in the study, 51 were female and 52 were male, RVA prevalence by sex was 44.9% and 55.1%, respectively (Table 2).

Temporal distribution of RVA infection

RVA infection was detected continuously in a 6-month period lasting from August through January but infections increased most rapidly and sharply during October to January which

corresponded with the dry season in Nigeria (Figure 1). In January, the RVA prevalence was 100% though the total sample collected for the month was limited. Samples were not collected between February 2013 and July 2013 due to intermittent strike by health workers which made the parents not to visit hospital regularly and when they did, they usually came for ailments other than diarrhea.

Patient characteristics

Table 3 shows the clinical details of the patients as provided on the questionnaire by the parent/guardian. Only the the group of 27 samples with higher RVA genome concentrations (>10^6 GE/ml stool suspension) was included in this analysis. The 22 samples with low quantities (<10^5 GE/ml stool suspension) were excluded. Generally, knowledge about RVA seems to be lower than expected as only 55.7% of the parents know about the virus. However, there is no statistical significance between knowledge about RVA and RVA infection (P= 0.22). The data shows that RVA positive stools were either watery (55.8%; n= 29/52), loose (50%; n=7/14), mucoid (40%; n=8/20) but not bloody (0%; n 0/2). Majority (73/103) of the children were breastfed exclusively for six month. The RVA infected children had mild, moderate or severe dehydration. Mild or moderate dehydration was also found in few RVA negative children, however the few cases of severe dehydration were found to be associated with RVA infection (100%; n=4). There was neither statistical correlation between RVA prevalence and level of maternal education, nor source of drinking water (P= 0.33 and 0.26 respectively).

Phylogenetic analysis of the VP4 and VP7

In the present study the sequences of the VP4 (n=37, 505 nucleotides sequence length within VP8*) and VP7 (n=32; 715 nucleotides sequence length) PCR products of the RVA positive specimens were analyzed. Only the 31 samples (including 1 mixed infection) that were successfully sequenced in both genome regions, were included in the phylogenetic trees, corresponding with 32 VP7 (figure 2) and 32 VP4 (figure 3) sequences. RVA positive samples that could not be sequenced in one or both genome regions had much lower viralconcentrations (median: 2x10^3 genome equivalents/ml stool suspension) compared to samples with sequences from both regions (median: 6x10^8 genome equivalents/ml stool suspension), rather reflecting the limit of sensitivity than a lack of specificity. Distribution of G and P types was not biased between higher or lower RVA genome concentrations.

The VP7 sequences of the G2 and G9 strains segregated into two different clusters according to the P type associated with them (P[6] vs. P[4] or P[8] vs. P[4], respectively). A similar pattern of clustering was observed among the P[6] sequences: The 3 VP4 sequences of the G2P[6] strains (0990, 1049 and 1075) clustered in a separate group form that associated with the G3 (1089). There was no distinct clustering among the P[8] sequences corresponding to the G type association (G12 vs G9). All of the VP7 sequences derived from the G12 strains were found in the same lineage (lineage 3). Most of the G12 sequences from this study were closely related (≥99% identity) with sequences from strains MRC-DPRU5144 from Togo and R27 from a rural community in Nigeria [Japhet et al., 2012], which were detected in samples from 2010 and 2011, respectively. However, the G12 sequence of RVA/Human-wt/NGA/14-G1048/2013/G12G2P[6]P[8] was more similar to other strains from Sub-Saharan Africa (RVA/human-wt/BFA/BF74/2011/G12P[8], RVA/Human-wt/CMR/ES283/2010/G12P[6], RVA/Human-wt/NGA/R54/2010/G12P[8], RVA/Human-wt/TGO/MRC-DPRU5171/2010/G12P[8]) and also shared ≥99% identity with sequences from France, India and the United States. While RVA/Human-wt/NGA/14-G1034/2013/G9P[4] was clustered with strains from Kenya, Mauritius and Lebanon, most G9 sequences from the present study were more closely related to RVA/human-wt/PAK/NIBGE-42/2010/G9P[8] from Pakistan. Similarly, one of the G2 sequences (RVA/Human-wt/NGA/14-G0999/2013/G2P[4]) clustered separately from the G2P[6] strains, which were similar to different strains from Africa. In contrast, the G2P[4] strain did not cluster with African strains but with strains from Bangladesh, Belgium and the United States. Sequences similar to the G3 strain RVA/Human-wt/NGA/14-G1089/2012/G3P[6] were found in Nigeria, Ethiopia, Belgium, Italy and the United States.

The P[8] sequences were much more divergent showing 86.6 - 100% nt identity among each other, with sequences clustering in lineages 3 and lineage 4. G9 strains from the present study were found in both, P[8] lineage 3 and 4. Sequences of all other P types and of all G types clustered within the same lineage of their respective genotype. The P[6] sequences clustered with strains from Nigeria, South Africa and the United States. Regarding P[4], RVA/Human-wt/NGA/14-G1034/2013/G9P[4] and RVA/Human-wt/NGA/14-G0999/2013/G2P[4] were found in two different clusters with strains from Asia but none from Africa. In contrast, most P[8] sequences were closely related to sequences from strains from Nigeria, Togo, South Africa, Belgium and the United States. The OP354-like sequences (lineage 4) clustered with strains from Nigeria, South Africa, India and Belgium.

The derivative VP8* amino acid sequences of the OP354-like P[8] strains from this study showed several changes in antigenic epitopes compared to RV1 and RV5, with S145G,

N149S, S187G, A191S, N192D and N194T in epitope 8-1, R182G in epitope 8-2, and N113D in epitope 8-3 (residue numbering based on vaccine strains). The derivative VP7 amino acid sequence of G2 strains differed from the RV5 G2 strain in epitopes 7-1a (A87T, D96A, N130D) and 7-1b (S213) with strain 14-G0999 having additional amino acid changes in epitope 7-1a (N130D) and 7-1b (S242N). The G3 strain 14-G1089 differed in epitope 7-1b (K238N, D242N) and 7-2 (A146V, A 221D) from the RV5 G3 strain.

Discussion

In the present study, the diversity of the circulating RVA strains in children with diarrhea in Ile-Ife, a South-western part of Nigeria, before national vaccine introduction was analyzed. Furthermore, the correlation between clinical/sociodemographic factors and rate of RVA infection among the study population was examined.

A high prevalence of RVA G12P[8] strains with close relation to strains from other Sub-Saharan countries was observed and this made up half of the RVA positive samples identified in this study. Since its first detection in the Philippines in 1987, G12 strains have been detected in many parts of the world and may be regarded as the sixth most important human genotype.[18-20] Furthermore, G12 strains have been found with high frequency in most of the recent African studies.[12,21,22] Of note is the fact that all the G12 strains observed in this study were associated with P[8] exclusively. Most African G12 isolates are G12P[8] [19,23,24] and more recent studies show increasing number of G12P[8].[20,25,26] Detection of G12P[8] is also common with studies from the USA and Europe.[27-29]

In addition to G2P[4], also G2P[6] was detected which is in accordance with previous data from African countries but contrary to studies from Europe and the Americas where the G2P[4] genotype combination is highly predominant and G2 with P[6] is unusual.[19,20,23,24,27,30,31] This difference was also reflected in the present study by the VP4 and VP7 sequences of RVA/Human-wt/NGA/14-G0999/2013/G2P[4] that were most similar to strains from Asia and Europe, while the VP4 and VP7 sequences of G2P[6] were closely related to African strains.

Genotype P[8] exhibited a high diversity, with sequences clustering in two different lineages. While the P[8] sequences of the G12 and some G9 strains belonged to lineage 3, the OP354-like sequences in lineage 4 comprised only G9 strains. This may possibly have been due be a recent reassortment, since some G9 sequences were identical despite of their differential association with P[8] lineage 3 and lineage 4 sequences (e.g. 10-G1059 and 10-G1061). However, no sequence data on other genome segments was available for further analysis of the possible reassortant strains.

Compared to RV1 and RV5, the amino acid changes in antigenic epitopes of OP354-like strains, G2 strains and the G3 strain found in the present study were also detected in studies from Brazil, Belgium, Nicaragua and Tunisia.[32-35] While some of the amino acid changes are known to escape neutralization by monoclonal antibodies,[36] an impact on the protection by the vaccines has not been shown.

Among the strains usually associated with most infections in children globally, G1 and G4 strains were not detected in this study while only one G3 and five G2 were detected. The second most common circulating strain in this study was G9. G8 strains which were detected with high frequency in earlier African studies [19,20,37] were not detected in this study, nor in other recent studies in Africa, seemingly coinciding with the increase in detection of G12 strain in the African continent. This shift has been observed with and without rotavirus immunization programs, and therefore the evidence suggests that it represents natural genotype distribution fluctuations, and is not related to vaccine introduction.

Genetic diversity is recognized to be generally greater in developing countries than in industrialized countries.[37-39] The heterogeneity of strains leading to high inter- and intragenotypic divergence, as observed with G2, G9 and P[8] in the present study, emphasizes the need for regular G and P typing and sequencing, which should not be seen as multiplicity of data, but a vital information to determine the heterogeneity of circulating strains which may have implication for the vaccine efficacy (VE) in the long term. Both available vaccines, RV1 and RV5, have comparable efficacy and confer significant degree of cross protection against heterotypic strains.[40] Despite this, there is evidence from both developed and developing countries, that VE is lower against some heterotypic strains, and in particular early assessment following the roll out of RV1 in Malawi suggests that VE is lower against G2 and G12 strains than against G1 strains.[41] An influence of vaccination on circulating RVA genotypes has also been reproduced in mathematical models that consider a combination of natural and vaccine-induced immunity against homo- and heterotypic strains, and explain observed changes after introduction of mass vaccination.[42] Therefore strain surveillance before and during the implementation of mass vaccination programs remains important in order to verify and fully understand this phenomenon. Over time, significant drifts or shifts in strain distribution may result in changes in the efficacy of the vaccination programs and may result in real or perceived fears of increases in vaccine failures.

In this study, a correlation between RVA infection and age (P= 0.026) was found. Majority of the RVA infections occurred in children <1 year compared with children >1 year, in agreement with other reports from the region [10,12,43-46] and data from the African Rotavirus Surveillance Network.[19] Nigeria, like many other African countries have two main seasons in a year, namely wet (warm, rainy) and the dry (cool, dry months) seasons. An increase of RVA within the dry season was observed in this study supporting previous findings from the few

data available on the seasonal pattern of RVA infection in West Africa and specifically in Nigeria.[10,12,44,47]

Considering the importance of RVA and the fact that almost every child by the age of 3 to 5 years will have been infected by the virus, there is the need for RVA awareness in Nigeria, since only about half (55.6%) of the respondents have knowledge about RVA. Awareness of the importance of RVA could probably increase the number of parents that will be ready to pay for the RVA vaccine which is available in private health sector but the use and the impact in the country are negligible due to the high poverty level in the country. No difference is observed in the proportion of RVA infection between children breastfed exclusively for six months and those bottle fed; however, possibility exists that breastfeeding reduced the severity of the diarrhea since 70.8% (n=73/103) of the mothers breastfed their children for the first six months of life during which maternal antibodies would have been transferred although not able to prevent infection. Previous studies have also suggested that the passive protective effect of maternal antibodies, transferred through placenta or through breast milk, may have role against RVA infection, at least during early months of breastfeeding.[48,49] However, further studies with larger population size and where breastfeeding is not common is needed to confirm this observation.

Previous studies have reported correlation between ELISA and real time PCR results [50-54] and low Ct-values (high viral load) being related to more severe disease.[55] While ELISA and PCR results overall appear to be useful to screen populations for vaccine effectiveness, samples from patients with AGE that were positive in qRT-PCR but negative by ELISA did not show a significant correlation.[52] This is likely due to the fact that RVA vaccination efficiently reduces cases of severe disease.[56] but does not completely prevent infection, similar to natural rotavirus infections.[57] This would explain why the percentage of cases with low RVA genome concentrations in vaccinated children with AGE were not significantly lower than in unvaccinated healthy children.[52] Supported by the high proportion of samples with low levels of RVA shedding in the present study, a significant bias appears to be likely when PCR results are interpreted without considering genome concentration or additional antigen testing. This corroborates data from Malawi on the use of qRT-PCR to distinguish symptomatic and asymptomatic rotavirus infections.[58]

The main limitations of the present study are the small sample size and the incidental interruption of sample collection in the first half of 2013. No data on the onset of diarrhea

were available which would have helped to identify samples where the cause of low RVA genome concentrations could have been sampling at the end of the acute phase when RVA shedding might have decreased already. In other cases, low RVA quantities of $<10^5$ GE/ml stool suspension (cycle threshold >30 in the present qRT-PCR), could indicate asymptomatic RVA infection [58,59] and the involvement of a different aetiological agent than RVA. However, RVA genotype distribution was not associated with genome concentration and the results were broadly in line with other studies from the region, which suggests that there were no significant biases.

To conclude, a high prevalence of RVA infections with genotypes G12 and G9 was found in the pediatric population with acute gastroenteritis. The common G1-G4 strains were either not detected or very few in number. No G1 strain was found, which is of relevance as baseline data since different studies have suggested that VE is lower against G2 and G12 strains than against G1 strains. Also, the high divergence in P[8] sequences detected in this study and changes in antigenic epitopes of some of them compared to RV1 and RV5 may provide relevant baseline information for the assessment of effectiveness of RVA vaccination when eventually introduced into the Nigerian national immunization program. Furthermore, significant bias from asymptomatic cases with low levels of RVA shedding is possible and should be considered when interpreting PCR results.

REFERENCES

1. Tate JE, Burton AH, Boschi-Pinto C, Parashar UD, World Health Organization-Coordinated Global Rotavirus Surveillance N. Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. *Clin Infect Dis.* 2016;62 Suppl 2:S96-S105.
2. Estes MK, Greenberg HB. Rotaviruses. In: Virology. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013. 1347–1401.
3. Matthijnssens J, Otto PH, Ciarlet M, Desselberger U, Van Ranst M, Johne R. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. *Arch Virol.* 2012;157:1177-1182.
4. Ghosh S, Kobayashi N. Exotic rotaviruses in animals and rotaviruses in exotic animals. *Virusdisease.* 2014;25:158-172.
5. Mihalov-Kovacs E, Gellert A, Marton S, et al. Candidate new rotavirus species in sheltered dogs, Hungary. *Emerg Infect Dis.* 2015;21:660-663.
6. Pennap G, Peenze, De Beer M, et al. VP6 subgroup and VP7 serotype of human rotavirus in Zaria, northern Nigeria. *J Trop Pediatr.* 2000;46:344-347.
7. Adah MI, Wade A, Taniguchi K. Molecular epidemiology of rotaviruses in Nigeria: detection of unusual strains with G2P[6] and G8P[1] specificities. *J Clin Microbiol.* 2001;39:3969-3975.
8. Audu R, Omilabu SA, de Beer M, Peenze I, Steele AD. Diversity of human rotavirus VP6, VP7, and VP4 in Lagos State, Nigeria. *J Health Popul Nutr.* 2002;20:59-64.
9. Steele AD, Nimzing L, Peenze I, et al. Circulation of the novel G9 and G8 rotavirus strains in Nigeria in 1998/1999. *J Med Virol.* 2002;67:608-612.
10. Aminu M, Ahmad AA, Umoh JU, Dewar J, Esona MD, Steele AD. Epidemiology of rotavirus infection in north-western Nigeria. *J Trop Pediatr.* 2008;54:340-342.
11. Esona MD, Steele D, Kerin T, et al. Determination of the G and P types of previously nontypeable rotavirus strains from the African Rotavirus Network, 1996-2004: Identification of unusual G types. *J Infect Dis.* 2010;202 Suppl:S49-54.
12. Japhet MO, Adesina AO, Famurewa O, Svensson L, Nordgren J. Molecular epidemiology of rotavirus and norovirus in Ile-Ife, Nigeria: high prevalence of G12P[8] rotavirus strains and detection of a rare norovirus genotype. *J Med Virol.* 2012;84:1489-1496.
13. WHO. Rotavirus vaccines WHO position paper: January 2013 - Recommendations. *Vaccine.* 2013;31:6170-6171.
14. Mwenda JM, Tate JE, Steele AD, Parashar UD. Preparing for the scale-up of rotavirus vaccine introduction in Africa: establishing surveillance platforms to monitor disease burden and vaccine impact. *Pediatr Infect Dis J.* 2014;33 Suppl 1:S1-5.
15. Iturriza-Gomara M, Kang G, Gray J. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. *J Clin Virol.* 2004;31:259-265.
16. Mas Marques A, Diedrich S, Huth C, Schreier E. Group A rotavirus genotypes in Germany during 2005/2006. *Arch Virol.* 2007;152:1743-1749.
17. Maes P, Matthijnssens J, Rahman M, Van Ranst M. RotaC: a web-based tool for the complete genome classification of group A rotaviruses. *BMC Microbiol.* 2009;9:238.
18. Matthijnssens J, Heylen E, Zeller M, Rahman M, Lemey P, Van Ranst M. Phylodynamic analyses of rotavirus genotypes G9 and G12 underscore their potential for swift global spread. *Mol Biol Evol.* 2010;27:2431-2436.
19. Mwenda JM, Ntoto KM, Abebe A, et al. Burden and epidemiology of rotavirus diarrhea in selected African countries: preliminary results from the African Rotavirus Surveillance Network. *J Infect Dis.* 2010;202 Suppl:S5-S11.
20. Seheri M, Nemarude L, Peenze I, et al. Update of rotavirus strains circulating in Africa from 2007 through 2011. *Pediatr Infect Dis J.* 2014;33 Suppl 1:S76-84.
21. Nakagomi T, Nakagomi O, Dove W, et al. Molecular characterization of rotavirus strains detected during a clinical trial of a human rotavirus vaccine in Blantyre, Malawi. *Vaccine.* 2012;30 Suppl 1:A140-151.

22. Ndze VN, Papp H, Achidi EA, et al. One year survey of human rotavirus strains suggests the emergence of genotype G12 in Cameroon. *J Med Virol.* 2013;85:1485-1490.
23. Cunliffe NA, Ngwira BM, Dove W, et al. Serotype g12 rotaviruses, Lilongwe, Malawi. *Emerg Infect Dis.* 2009;15:87-90.
24. Page NA, de Beer MC, Seheri LM, Dewar JB, Steele AD. The detection and molecular characterization of human G12 genotypes in South Africa. *J Med Virol.* 2009;81:106-113.
25. Ouedraogo N, Kaplon J, Bonkoungou IJ, et al. Prevalence and Genetic Diversity of Enteric Viruses in Children with Diarrhea in Ouagadougou, Burkina Faso. *PLoS One.* 2016;11:e0153652.
26. Uzoma EB, Chukwubuikem C, Omoyibo E, Tagbo O. Rota virus genotypes and the clinical severity of Diarrhoea among children under 5 years of age. *Niger Postgrad Med J.* 2016;23:1-5.
27. Iturriza-Gomara M, Dallman T, Banyai K, et al. Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined by EuroRotaNet, a pan-European collaborative strain surveillance network. *Epidemiol Infect.* 2011;139:895-909.
28. Cilla G, Montes M, Gomariz M, et al. Rotavirus genotypes in children in the Basque Country (North of Spain): rapid and intense emergence of the G12[P8] genotype. *Epidemiol Infect.* 2013;141:868-874.
29. Mijatovic-Rustempasic S, Teel EN, Kerin TK, et al. Genetic analysis of G12P[8] rotaviruses detected in the largest U.S. G12 genotype outbreak on record. *Infect Genet Evol.* 2014;21:214-219.
30. Bucardo F, Nordgren J. Impact of vaccination on the molecular epidemiology and evolution of group A rotaviruses in Latin America and factors affecting vaccine efficacy. *Infect Genet Evol.* 2015;34:106-113.
31. Clark HF, Lawley D, DiStefano D, et al. An unusual outbreak of rotavirus genotype G2P[6] during the 2005-2006 epidemic season in Philadelphia. *Diagn Microbiol Infect Dis.* 2011;70:218-222.
32. Bucardo F, Rippinger CM, Svensson L, Patton JT. Vaccine-derived NSP2 segment in rotaviruses from vaccinated children with gastroenteritis in Nicaragua. *Infect Genet Evol.* 2012;12:1282-1294.
33. Zeller M, Patton JT, Heylen E, et al. Genetic analyses reveal differences in the VP7 and VP4 antigenic epitopes between human rotaviruses circulating in Belgium and rotaviruses in Rotarix and RotaTeq. *J Clin Microbiol.* 2012;50:966-976.
34. Gomez MM, Carvalho-Costa FA, Volotao Ede M, et al. Prevalence and genomic characterization of G2P[4] group A rotavirus strains during monovalent vaccine introduction in Brazil. *Infect Genet Evol.* 2014;28:486-494.
35. Mouna BH, Hamida-Rebai MB, Heylen E, et al. Sequence and phylogenetic analyses of human rotavirus strains: comparison of VP7 and VP8(*) antigenic epitopes between Tunisian and vaccine strains before national rotavirus vaccine introduction. *Infect Genet Evol.* 2013;18:132-144.
36. Dormitzer PR, Sun ZY, Wagner G, Harrison SC. The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO J.* 2002;21:885-897.
37. Todd S, Page NA, Duncan Steele A, Peenze I, Cunliffe NA. Rotavirus strain types circulating in Africa: Review of studies published during 1997-2006. *J Infect Dis.* 2010;202 Suppl:S34-42.
38. Gentsch JR, Laird AR, Bielfelt B, et al. Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. *J Infect Dis.* 2005;192 Suppl 1:S146-159.
39. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Rev Med Virol.* 2005;15:29-56.
40. Desselberger U. Rotaviruses. *Virus Res.* 2014;190:75-96.

41. Bar-Zeev N, Kapanda L, Tate JE, et al. Effectiveness of a monovalent rotavirus vaccine in infants in Malawi after programmatic roll-out: an observational and case-control study. *Lancet Infect Dis.* 2015;15:422-428.
42. Pitzer VE, Bilcke J, Heylen E, et al. Did Large-Scale Vaccination Drive Changes in the Circulating Rotavirus Population in Belgium? *Sci Rep.* 2015;5:18585.
43. Chan-it W, Thongprachum A, Dey SK, et al. Detection and genetic characterization of rotavirus infections in non-hospitalized children with acute gastroenteritis in Japan, 2007-2009. *Infect Genet Evol.* 2011;11:415-422.
44. Bonkoungou IJ, Sanou I, Bon F, et al. Epidemiology of rotavirus infection among young children with acute diarrhoea in Burkina Faso. *BMC Pediatr.* 2010;10:94.
45. Nitiema LW, Nordgren J, Ouermi D, et al. Burden of rotavirus and other enteropathogens among children with diarrhea in Burkina Faso. *Int J Infect Dis.* 2011;15:e646-652.
46. Abebe A, Teka T, Kassa T, et al. Hospital-based surveillance for rotavirus gastroenteritis in children younger than 5 years of age in Ethiopia: 2007-2012. *Pediatr Infect Dis J.* 2014;33 Suppl 1:S28-33.
47. Cunliffe NA, Kilgore PE, Bresee JS, et al. Epidemiology of rotavirus diarrhoea in Africa: a review to assess the need for rotavirus immunization. *Bull World Health Organ.* 1998;76:525-537.
48. Clarke E, Desselberger U. Correlates of protection against human rotavirus disease and the factors influencing protection in low-income settings. *Mucosal Immunol.* 2015;8:1-17.
49. Krawczyk A, Lewis MG, Venkatesh BT, Nair SN. Effect of Exclusive Breastfeeding on Rotavirus Infection among Children. *Indian J Pediatr.* 2016;83:220-225.
50. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, Gray J. Diagnosing rotavirus A associated IID: Using ELISA to identify a cut-off for real time RT-PCR. *J Clin Virol.* 2009;44:242-245.
51. Dung TT, Phat VV, Nga TV, et al. The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. *J Virol Methods.* 2013;187:138-143.
52. Tate JE, Mijatovic-Rustempasic S, Tam KI, et al. Comparison of 2 assays for diagnosing rotavirus and evaluating vaccine effectiveness in children with gastroenteritis. *Emerg Infect Dis.* 2013;19:1245-1252.
53. Corcoran MS, van Well GT, van Loo IH. Diagnosis of viral gastroenteritis in children: interpretation of real-time PCR results and relation to clinical symptoms. *Eur J Clin Microbiol Infect Dis.* 2014;33:1663-1673.
54. Fumian TM, Leite JP, Rocha MS, et al. Performance of a one-step quantitative duplex RT-PCR for detection of rotavirus A and noroviruses GII during two periods of high viral circulation. *J Virol Methods.* 2016;228:123-129.
55. Kang G, Iturriza-Gomara M, Wheeler JG, et al. Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: correlation with clinical severity in children in South India. *J Med Virol.* 2004;73:118-122.
56. Koch J, Wiese-Posselt M, Remschmidt C, et al. Background paper to the recommendation for routine rotavirus vaccination of infants in Germany. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz.* 2013;56:957-984.
57. Velazquez FR, Matson DO, Calva JJ, et al. Rotavirus infection in infants as protection against subsequent infections. *N Engl J Med.* 1996;335:1022-1028.
58. Bennett A, Bar-Zeev N, Jere KC, et al. Determination of a Viral Load Threshold To Distinguish Symptomatic versus Asymptomatic Rotavirus Infection in a High-Disease-Burden African Population. *J Clin Microbiol.* 2015;53:1951-1954.
59. Mukhopadhya I, Sarkar R, Menon VK, et al. Rotavirus shedding in symptomatic and asymptomatic children using reverse transcription-quantitative PCR. *J Med Virol.* 2013;85:1661-1668.

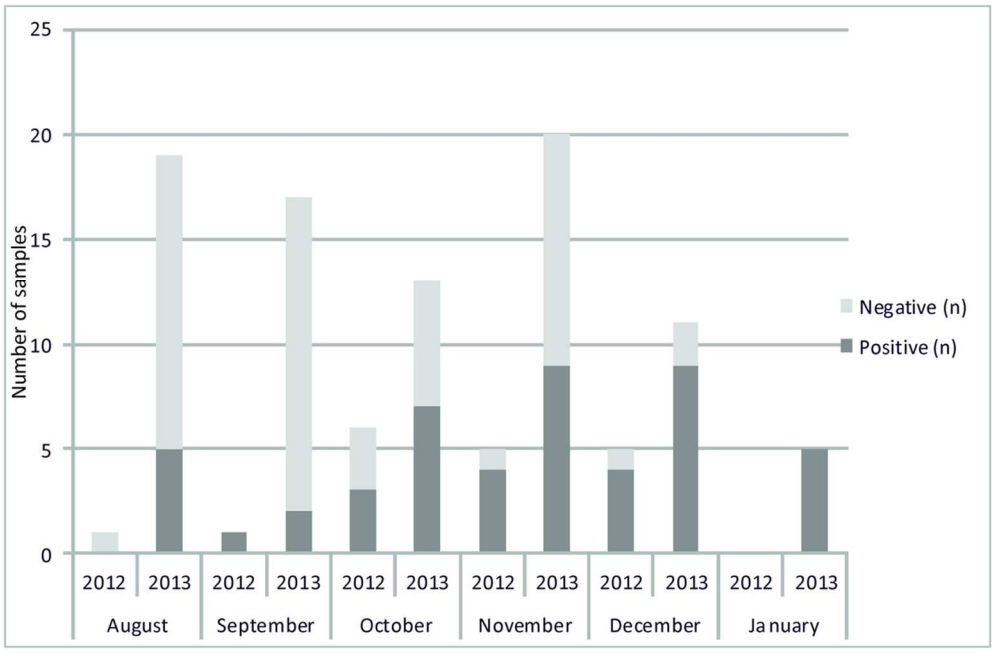

Figure 1: Seasonal distribution of RVA from August 2012 to December 2013

105x69mm (300 x 300 DPI)

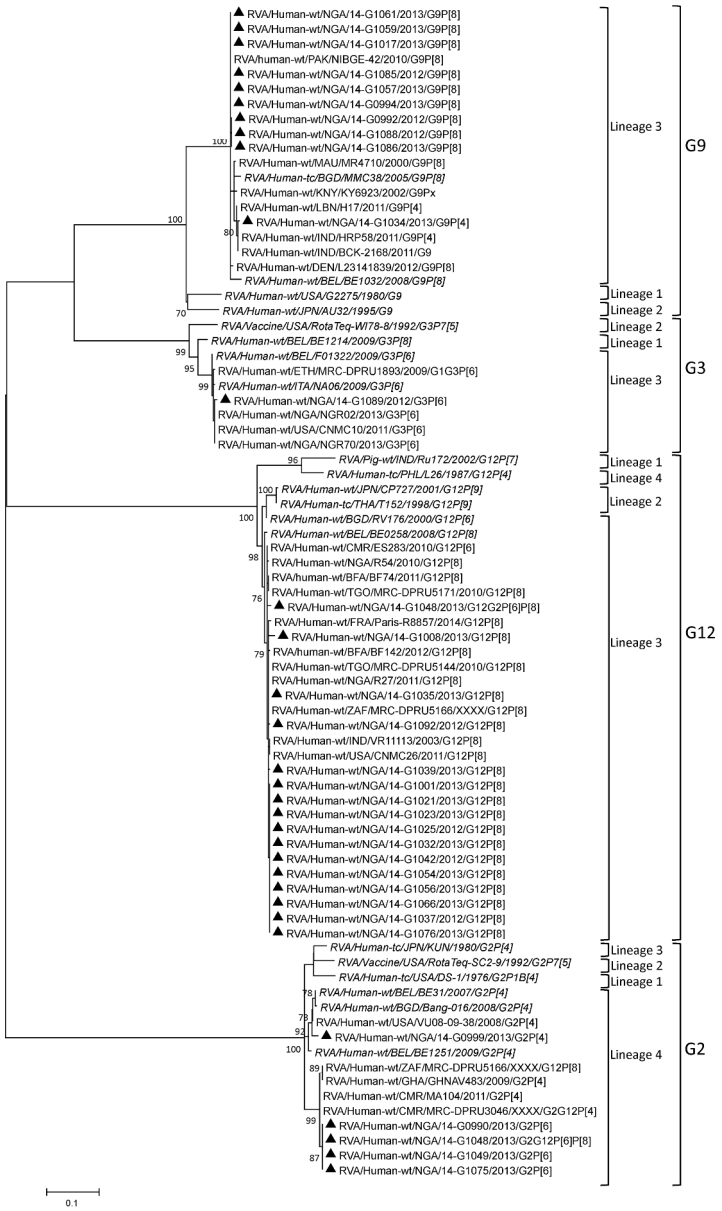

Figure 2: Phylogenetic tree of the partial VP7 gene (nt position 51- 932) strains from Nigeria (with asterisk, sample ID and year of sample collection) and different G type reference strains. The tree was constructed using the maximum-likelihood method and HKY substitution model available in MEGA6 (scale bar: nucleotide substitutions per site). Reference sequences were obtained from the GenBank database. Reference sequences for genotype lineages are printed in italic font. To avoid duplication of sequences in GenBank, only representative sequences of different clusters were submitted. G2: KT952018 (14-G0990), KT952019 (14-G1048), KT952020 (14-G0999), G9: KT952021 (14-G1086), KT952022 (14-G1088), KT952023 (14-G1085), KT952024 (14-G1017), KT952025 (14-G1034), G3: KT952026 (14-G1089), G12: KT952027 (14-G1025), KT952028 (14-G1037), KT952029 (14-G1035), KT952030 (14-G1039), KT952031 (14-G1092), KT952032 (14-G1048).

249x404mm (300 x 300 DPI)

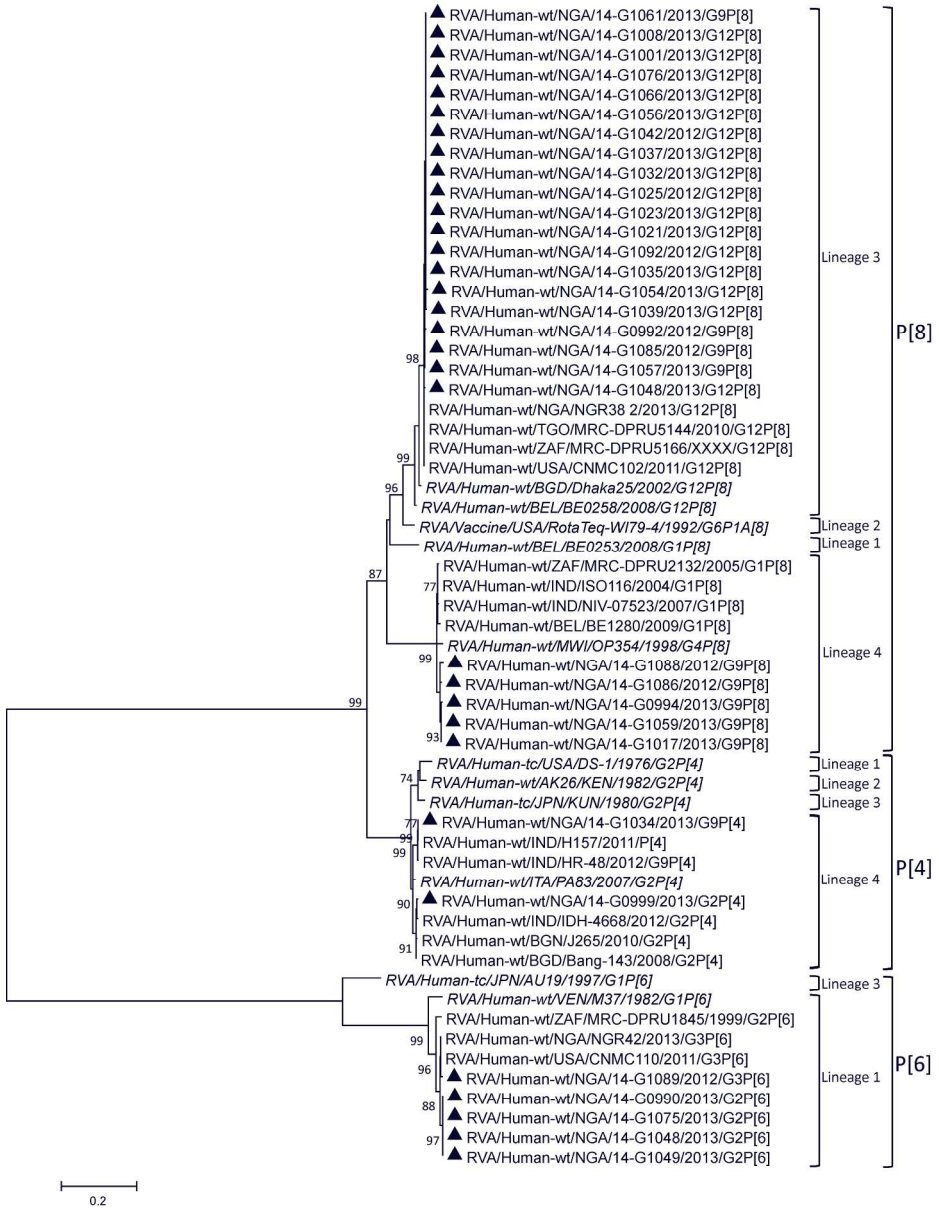

Figure 3: Phylogenetic tree of the partial VP4 gene (nt position 132- 795) strains from Nigeria (with asterisk, sample ID and year of sample collection) and different P type reference strains. The tree was constructed using the maximum-likelihood method and HKY substitution model available in MEGA6 (scale bar: nucleotide substitutions per site). Reference sequences were obtained from the GenBank database. Reference sequences for genotype lineages are printed in italic font. To avoid duplication of sequences in GenBank, only representative sequences of different clusters were submitted. P[6]: KT952033 (14-G0990), KT952034 (14-G1048), KT952035 (14-G1089), P[8]: KT952036 (14-G0992), KT952037 (14-G1085), KT952038 (14-G1008), KT952039 (14-G1035), KT952040 (14-G1042), KT952041 (14-G1076), KT952042 (14-G1054), KT952043 (14-G1048), KT952044 (14-G1086), KT952045 (14-G1088), P[4]: KT952046 (14-G0999), KT952047 (14-G1034).

216x280mm (300 x 300 DPI)

Table 1: Distribution of G and P genotype combination of RVA strains detected among children (0-5years) with gastroenteritis in Ile-Ife, Nigeria.

Rotavirus genotype	No. (%) of strains					
	P[4]	P[6]	P[8]	P[8] P[6] (mixed)	P n/t	Total
G2	1	3	0	0	0	4 (8.2)
G3	0	1	0	0	0	1 (2.0)
G9	1	0	9	0	0	10 (20.4)
G12	0	0	15	0	0	15 (30.6)
G2/G12 (mixed)	0	0	0	1	0	1 (2.0)
G n/t	0	0	6	0	12	18 (36.7)
Total	2 (4.1)	4 (8.2)	30 (61.2)	1 (2.0)	12 (24.5)	49(100)

n/t= not typeable

Table 2: Age and Sex Profile of RVA Infection

Characteristics	RVA detected	RVA Not detected	Total
(a) Age			
<6 months	22	13	35
6-11 months	18	28	46
1-2 years	7	11	18
3-4 years	2	2	4
Total	49	54	103
(b) Sex			
Male	27	24	52
Female	22	30	51
Total	49	54	103

Table: 3 Clinical/sociodemographic factors and Rotavirus infection

Clinical characteristics/manifestation	RVA positive (%)	RVA negative (%)	Total (%)
(a) Source of Drinking water			
Well	14 (48.3)	15 (51.7)	29 (35.8)
Tap	4 (21.1)	15 (78.9)	19 (23.5)
Bore hole	1 (11.1)	8 (88.9)	9 (11.1)
Sachet water	7 (36.8)	12 (63.2)	19 (23.5)
Bottled water	0 (0.0)	2 (100)	2 (2.5)
Rain	1 (33.3)	2 (66.7)	3 (3.7)
Total	27 (33.3)	54 (66.7)	81 (100)
(b) Stool description			
Watery	16 (41.0)	23 (59)	39 (48.2)
Loose	5 (41.7)	7 (58.3)	12 (14.8)
Mucus	4 (25.0)	12 (75.0)	16 (19.8)
Bloody	0 (0.0)	1 (100)	1 (1.2)
No data	2 (15.4)	11 (84.6)	13(16.0)
Total	27 (33.3)	54 (66.7)	81(100)
(c) Symptoms (diarrhea with)			
Mild or no dehydration	16 (24.6)	49 (75.4)	65 (80.2)
Moderate dehydration with/without vomiting	9 (64.3)	5 (35.7)	14 (17.3)
Severe dehydration with/without vomiting	2 (100)	0 (0.0)	2 (2.5)
Total	27 (33.3)	54 (66.7)	81 (100)
(d) Level of maternal Education			
None	0 (0.0)	2 (100)	2 (2.5)
Low	4 (44.4)	5 (55.6)	9 (11.1)
Middle	12 (40.0)	18 (60.0)	30 (37.0)
High	7 (20.0)	28 (80.0)	35 (30.8)
No data	4 (80.0)	1 (20.0)	5 (6.2)
Total	27 (33.3)	54 (66.7)	81 (100)
(e) Knowledge about RVA			
Yes	13 (28.9)	32 (71.1)	45 (55.6)
No	12 (37.5)	20 (62.5)	32 (39.5)
No data	2 (50.0)	2 (50.0)	4 (4.9)
Total	27 (33.3)	54 (66.7)	81 (100)
(f) 6 month exclusive breastfeeding			
Yes	21 (36.2)	37 (63.8)	58 (71.6)
No	2 (33.3)	4 (66.7)	6 (7.4)
Not sure	4 (23.5)	13 (76.5)	17 (21.0)
Total	27 (33.3)	54 (66.7)	81 (100)