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Sequencing and phylogenetic analysis of human group A rotavirus genotypes circulating among diarrheic children in Edo State, Nigeria

Osazee Ekundayo Izevbuwa

Department of biological sciences, igbinedion university okada

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ABSTRACT

Group A rotaviruses (RVA) are one of the vital causative agents of acute gastroenteritis (AGE) in young children worldwide. Genotyping of detected RVA strains is needed for a more extensive knowledge of the epidemiology of rotaviral infections. This descriptive cross-sectional study aimed to evaluate the circulation of RVA genotypes in diarrheic children living in Edo State, Nigeria. A total of 400 stool samples collected from children less than five years with acute diarrhea were initially screened for RVA antigen by immunochromatographic method, and the RVA antigen-positive samples were subsequently analysed using reverse transcription polymerase chain reaction (RT-PCR), multiplex PCR and sequencing of the VP7 and VP4 gene segments of the RVA strains. Phylogenetic trees were constructed from the nucleotide sequences using the neighbor joining algorithm in MEGA software, version 6. Seventeen stool samples were confirmed as RVA-positive by the first round RT-PCR out of the twenty RVA antigen-positive samples that were examined. Based on RT-PCR assay, the prevalence of RVA which caused diarrhea in children less than five years was estimated at 4.25%. All the 17 stool samples that were confirmed RVA-positive by first round RT-PCR were successfully genotyped for VP7-G and VP4-P genes. Multiplex PCR revealed that G2[P6] was the most frequently found genotype combination (1.50%) rather than the G1[P8] (1.25%) which occurred most frequently worldwide. G9[P6] strains were responsible for 0.50% of RVA prevalence. Unusual RVA strains carrying genotype G2[P8] accounted for a prevalence of 0.50% while mixed infections caused by the G2G9[P6] strains also accounted for a prevalence of 0.50%. The findings of this study provide baseline data for health authorities to plan public health care strategies that could mitigate the disease burden caused by RVA.

Introduction

Gastroenteritis is a contagious syndrome of the stomach lining and the intestines. Bacterial

toxins, parasites, fungi and viral particles have been implicated as the main causative agents of

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* Corresponding author: Osazee Ekundayo Izevbuwa

E-mail address: osazeeizevbuwa@gmail.com

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gastroenteritis [1-3]. Gastroenteritis caused by viral agents is gradually increasing, particularly in developed countries; although the improvement in sanitation has significantly decreased the incidence of gastroenteritis caused by bacteria and parasites, it, however, has shown little effect on viral gastroenteritis due to the vast mode of viral disease transmissions such as the spread through fomites and respiratory routes which has been implicated in viral gastroenteritis [4-5]. Enteric adenoviruses, rotaviruses and caliciviruses (norovirus and sapovirus) and astroviruses are the causative agents of gastroenteritis of viral origin in humans. Although some other viruses associated with gastroenteritis in humans have been reported, their etiologic roles have not yet been established [4].

Rotaviruses are the most common cause of diarrhea disease among infants and young children [5-7]. Rotavirus is the leading cause of severe gastroenteritis worldwide in infants and young children [1,2, 4, 8-10]. However, there is a significant decline in these values following the introduction of rotavirus vaccination programs in most developed and some developing countries [2,3, 7].

The earliest report of rotavirus-induced diarrhea in Nigeria was conducted in some communities in Oyo State, by **Fagbami et al.** [11]. The earliest report did not examine the circulating genotypes behind the reported outbreaks. The study of **Adah et al.** [12] was the first reported research on the genotypic spread of the rotavirus epidemic among children with acute gastroenteritis in Nigeria. In their study, a prevalence of 14.3% was reported, inclusive of genotypes G1 and G3. The first VP7 sequence analysis from Nigeria was reported in 1996.

Group A rotaviruses are classified into G and P-genotypes, respectively based on the sequence of VP7 and VP4 genes. The gene segment 4 product, VP4, is an exterior capsid protein that is not glycosylated and generates 60 homodimeric spikes on the surface of the virus [13]. The protein VP4 is responsible for cell attachment and rotaviral hemagglutinin's function [14]. VP4 has been linked to virulence in humans, piglets, calves, and mice [15]. The primary outer capsid protein is the glycoprotein VP7 produced by gene segment 9, and forms trimers on the viral surface [16]. A cleavable signal peptide directs VP7 to the endoplasmic reticulum (ER) during morphogenesis. VP7 is co-

translationally glycosylated as it is incorporated into the ER membrane [15].

Due to the gaps in the molecular data on rotavirus infections experienced in some parts of Edo State, Nigeria; as well as certain misconceptions concerning the indigenous rotavirus strains predominant in Nigeria, the present study aimed to evaluate the prevalence of RVA and to carry out the molecular characterization of rotavirus gastroenteritis among hospitalized and out-patient children aged less than 5 years who presented to both private and government-owned hospitals in Edo State, Nigeria.

Subjects and Methods

Study sites

The study sites included hospitals from all the geopolitical zones in Edo State, Nigeria. They were the General Hospital, Agbede, Edo North Senatorial District, Specialist Hospital, Irrua, Edo Central Senatorial District, Central Hospital, Benin City and Igbinedion University Teaching Hospital, Okada, Edo South Senatorial District.

Ethical approval and consent

Informed consent was obtained from the parents of study participants before samplings were carried out. The study participants were diarrheic children less than 5 years of age who met the inclusion criteria. Criteria for the inclusion of study participants were based on the World Health Organization (WHO) case guideline for acute gastroenteritis which recommends an episode within 24 hours of at least three loose stools, and/or two or more vomiting episodes associated with diarrhea, in the 7 days before the medical visit; the episode must have been preceded by a symptom-free period of 14 days. All participants included in the study were also non-vaccinated against rotavirus. Ethical approval was obtained from the Medical Research Ethics Committee of the hospitals.

Sample collection

A total of 400 stool samples, consisting of 100 stool samples from each of the four hospitals selected for this study, were collected from November 2021 to December 2022. Samples were collected aseptically in sterile commercial stool containers adequately labeled (patient ID and date of collection) by the caregivers or the hospital staff from each child with instructions on the proper method of collection. All the stool samples were then transported under a cold chain to the Medical Microbiology Laboratory at

Igbinedion University Teaching Hospital, Okada, Edo State. Upon receipt of the stool samples at the Microbiology laboratory, they were stored at -80°C, until serological analysis.

Detection of group A human rotavirus by immunochromatographic assay

Stool suspensions of 10 to 20% were prepared in phosphate-buffered saline (pH 7.2). Each faecal suspension was then analyzed by immunochromatographic method using a commercially available test kit (Aria, USA) specific for rotavirus to demonstrate the presence of rotavirus antigens in the stool samples. Aliquots of the stool samples positive for rotavirus, after the preliminary screening, were aseptically dissolved into sterile RNA shield solution (Zymo, USA) in a ratio of 1:3, and stored at -20°C, until molecular analysis.

RNA extraction

Stools positive for group A rotaviruses were shipped in the cold chain to Inqaba Biotec West Africa limited for RNA extraction and other molecular characterizations. RNA was extracted from the samples using the Quick-RNA Miniprep Plus Kit (Zymo, USA). 800ul of DNA/RNA Shield (1X) was added to an appropriate amount of the fecal samples, followed by homogenization by vigorous vortex mixing. 15ul of Proteinase K and 30ul of PK digestion buffer were added to 300ul of the homogenized mixture, and the resultant mixture was incubated at room temperature for 30 minutes; followed by centrifugation at maximum speed to obtain a clear supernatant that was transferred to a nuclease-free tube. RNA lysis buffer was then added to the supernatant in a 1 to 1 ratio, and subsequently transferred to a Spin-Away™ Filter in a collection tube; followed by centrifugation to remove the majority of genomic DNA. The flow-through was mixed with 95% ethanol in a 1:1 ratio and thereafter transferred into a Zymo-Spin™ IICR Column in a collection tube, followed by centrifugation and discarding of the flow-through. The column was washed with 400µl of RNA wash buffer and centrifuged, with the flow-through discarded. 80ul of DNase I-DNA digestion buffer mix was then added directly to the column matrix and allowed to incubate at 20-30°C for 15 minutes. 400µl of RNA prep buffer was also added to the column and centrifuged, followed by discarding the flow-through. 700µl of RNA wash buffer was thereafter added to the column and centrifuged. The flow-through was discarded and 400µl of RNA wash

buffer was added to the column. The column was centrifuged afterward for 1 minute to ensure the complete removal of the wash buffer. The column was carefully transferred into a nuclease-free tube and 50 -100ul of DNase/RNase-free water was added, followed by centrifugation.

Reverse transcription (RT) PCR and sequencing of VP7 and VP4 genes

The dsRNA-extracted sample was employed as the template for reverse transcription to synthesize cDNA copies from both strands. The extracted RNA was converted to cDNA using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, USA). The protocol was performed with 20 µl reaction mixture containing 4 µl template RNA, 2 µl D(T)₂₃VN, 10 µl ProtoScript II Reaction Mix (2X), 2 µl ProtoScript II Enzyme Mix (10X) and 2 µl nuclease-free water. The mixture was incubated at 42°C for 1 hour. Thereafter, the reverse transcriptase enzyme was inactivated by raising the temperature to 80°C for 5 minutes. Amplification of the VP7 and VP4 genes was according to previously described protocols [17-21]. The first round of PCR amplification of the VP7 genes was performed using OneTaq® Quick-Load® 2X Master Mix (New England Biolabs, Inc.). Specific primers used for amplifying the VP7 genes were *Beg9-F* (5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3') and *End9-R* (5'-GGTCACATCATAACAATTCTAATCTAAG-3'), with amplicon size of 1062 base pairs. The 25 µl reaction mixture contained 2 µl template cDNA, 0.5 µl 10µM *Beg 9* primer, 0.5 µl 10 µM *End 9* primer, 12.5µl One Taq Quick Load 2X Master Mix with standard buffer, and 9.5 µl nuclease-free water. The first round of amplification of the VP7 gene, using the GeneAmp PCR system 9700 (Applied Biosystems), consisted of the following thermal cycling conditions: initial denaturation for 5 minutes at 94°C followed by 35 cycles of denaturation, with each cycle consisting of denaturation at 94°C for 30 seconds; annealing at 55°C for 1 minute; extension at 68°C for 90 seconds; and a final extension step at 68°C for 10 minutes. Specific primers used for first round amplification of the VP4 genes were *VP4-F* (5'-TATGCTCCAGTNAATTGG-3') and *VP4-R* (5'-ATTGCATTTCTTTCCATAATG-3'), with amplicon size of 663 base pairs. An identical PCR protocol was also used to perform the first round amplification of the partial VP4 gene except that the annealing temperature was set at 48°C. 10 µl portions of the amplified products were analyzed by

gel electrophoresis on a 2% agarose prepared in Tris-Borate-EDTA buffer containing 0.5 µg/ml of ethidium bromide at 100 V for 1 hour. The DNA band in the gel was subsequently visualized and documented on the gel documentation system (Applied Biosystems). A molecular marker (100 base pair ladder) was run concurrently.

DNA sequencing of the VP7 and VP4 amplicons was performed with the dideoxy-chain termination method [22]. The amplicons were cleaned up with ExoSAP-IT (ThermoFisher Scientific), and then subjected to cycle sequencing with the Big Dye Terminator version 3.1 (Applied Biosystems) using standard cycling conditions. The purified cycle sequencing product was separated by capillary electrophoresis on an ABI 3730×I DNA analyzer. The sequence was then quality-checked and proofread with Sequencher version 4.10.1 (Gene Codes Corporation, USA). Query sequence comparison with reference sequences in standard databases was performed with the BLASTN 2.8.0 + program [National Center for Biotechnology Information (NCBI)].

Typing of amplified VP7 and VP4 genes by multiplex PCR

The multiplex PCR for typing of the VP7 and VP4 genes was performed according to previously described methods [17-21]. PCR amplicons from the RT-PCR served as templates for a second round amplification which was performed using a cocktail of specific primers which amplified variable regions of the VP7 gene, G types and VP4 gene, P types. The VP7 and VP4 amplicons derived from the first round of RT-PCR were used directly or excised and extracted from the agarose gel as purified DNA, upon completion of electrophoresis. Primer cocktail specific to VP7, types G8 or aAT8 (GTCACACCATTGTGTAATTTCG); G1 or aBT1 (CAAGTACTCAAATCAATGATGG); G2 or aCT2 (CAATGATATTAACACATTTTCTGTG); G4 or aDT4 (CGTTTCTGGTGAGGAGTTG); G3 or aET3 (CGTTTGAAGAAGTTGCAACAG); G9 or aFT9 (CTAGATGTAACACTACAACACTAC) and consensus primer RVG9 (GGTCACATCATAACAATTCT) was employed for VP7, type G PCR. The amplicon sizes for G8, G1, G2, G4, G3 and G9 were 885 bp, 749 bp, 652 bp, 583 bp, 374 bp and 306 bp, respectively. Primer cocktail specific to VP4, types P[4] or 2T-1 (CTATTGTTAGAGGTTAGAGTC), P[6] or 3T-1 (TGTTGATTAGTTGGATTCAA), P[8] or 1T-1D

(TCTACTGGRTTRACNTGC), P[9] or 4T-1 (TGAGACATGCAATTGGAC), P[10] or 5T-1 (ATCATAGTTAGTAGTCGG), P[11] (GTAAACATCCAGAATGTG) and VP4F (TATGCTCCAGTNAATTGG) was used for VP7, type P PCR. The amplicon sizes for P[4], P[6], P[8], P[9], P[10] and P[11] were 362 bp, 146 bp, 224 bp, 270 bp, 462 bp and 191 bp, respectively. Conditions for the multiplex PCR were similar to those for the RT-PCR.

Phylogenetic analysis

Multiple sequence alignments were implemented with the MUSCLE algorithm in MEGA software, version 6 [23]. Phylogenetic trees were constructed using the neighbor joining algorithm in MEGA software. The statistical significance of the clusters in the trees was estimated by bootstrap iterations (1000 replications).

Statistical analysis

Descriptive statistics of prevalence datasets were done with NCSS ver. 12 data analysis software. Shapiro–Wilk normality test, Levene test of homogeneity and non-parametric Kruskal–Walis test was also performed with NCSS ver. 12 data analysis software. The test of the hypothesis was considered statistically significant if the achieved level of significance (*p*) was less than 0.05.

Results

Prevalence of RVA

Twenty RVA antigen-positive samples were detected from the 400 stool samples examined, respectively, corresponding to eight, six, two and four RVA antigen-positive samples obtained from General Hospital, Agbede, Specialist Hospital, Irrua, Central Hospital, Benin City and Igbinedion University Teaching Hospital, Okada, Edo State, Nigeria. Upon first round RT-PCR analysis (**Figures 1, 2**) of the 20 RVA antigen-positive samples, 17 stool samples were confirmed as RVA positive, resulting in an overall prevalence of 4.25% RVA, causing diarrhea among children who are less than five years in Edo State, Nigeria. Based on the geopolitical zones in Edo State, RVA prevalence in Edo North, Edo Central and Edo South Senatorial Districts was confirmed as 7.00% (7/100), 5.00% (5/100) and 2.50% (5/200), respectively.

Prevalence of RVA genotypes

All the 17 stool samples that were confirmed RVA-positive by first round RT-PCR were successfully genotyped for VP7, G and VP4, P genes. G-typing

generated amplicons with sizes of 306 bp, 652 bp and 749 bp which indicated the presence of G9, G2 and G1 genotypes, respectively (**Figure 3**). P-typing generated amplicons with sizes of 146 bp and 224 bp, revealing the presence of P[6] and P[8] genotypes, respectively (**Figure 4**). Two of the stool samples were confirmed to possess mixed G genotypes. G2 and P[6] were found to be the main G and P genotypes circulating among children who are less than five years in Edo State, Nigeria. Representative strains RVA/Human-wt/NGR/IZEVBUWA-201/2023/G2, RVA/Human-wt/NGR/IZEVBUWA-203/2023/G2, RVA/Human-wt/NGR/IZEVBUWA-206/2023/G2, RVA/Human-wt/NGR/IZEVBUWA-202/2023/G1 and RVA/Human-wt/NGR/IZEVBUWA-204/2023/G9 isolated from the stool samples in this study, which produced G proteins (glycoproteins), have been respectively assigned accession numbers OQ737680, OQ737682, OQ737685, OQ737681, OQ737683 at the United States NCBI GenBank database. Representative strains RVA/Human-wt/NGR/IZEVBUWA-207/2023/G2[P6], RVA/Human-wt/NGR/IZEVBUWA-209/2023/G2[P6], RVA/Human-wt/NGR/IZEVBUWA-212/2023/G2[P8], RVA/Human-wt/NGR/IZEVBUWA-208/2023/G1[P8] and RVA/Human-wt/NGR/IZEVBUWA-210/2023/G9[P6] isolated from the stool samples in this study, which produced P proteins (protease-sensitive proteins), have been respectively assigned accession numbers OQ737686, OQ737688, OQ737691, OQ737687, OQ737689 at the NCBI GenBank database. Representative strains RVA/Human-wt/NGR/IZEVBUWA-205/2023/G2G9 and RVA/Human-wt/NGR/IZEVBUWA-211/2023/G2G9[P6] obtained from the present study which produced mixed G proteins have been assigned accession numbers OQ737684 and OQ737690, respectively.

Distribution of G and P genotypes

The distribution of different combinations of genotypes reported in this study is presented in **table (1)**. The distribution of G types revealed that a total of 2.00% G2 genotype circulated among diarrheic children less than 5 years in Edo State, Nigeria; followed by G1 genotype (1.25%), G9 genotype (0.50%) and G2G9 mixed genotypes (0.50%). The P[6] genotype (2.50%) occurred most frequently among diarrheic children less than 5 years in Edo State, Nigeria; followed by the P[8] genotype

(1.75%). Overall, G2[P6] was the most abundant genotype combination (1.50%) circulating among diarrheic children less than 5 years in Edo State, Nigeria; followed by the G1[P8] genotype combination (1.25%) and G9[P6] (0.50%). G2[P8], an unusual genotype combination, was found circulating in 0.50% of diarrheic children less than 5 years in Edo State, Nigeria. Mixed genotype G2G9[P6] was also found circulating in 0.50% of children less than 5 years of age. Shapiro-Wilk test revealed that the prevalence datasets of all the detected genotypes were non-normally distributed ($p < 0.05$) with equal variance ($p > 0.05$), as indicated by the Levene test. Kruskal-Wallis test showed that there was no significant difference ($p > 0.05$) in the prevalence datasets (median values = 0.00) of the genotypes circulating among diarrheic children less than five years from all four selected hospitals in Edo State, Nigeria.

Phylogenetic analysis

Partial genome sequences of VP7 and VP4 gene segments were selected from the present study and the United States NCBI GenBank database to construct phylogenetic trees using the neighbor joining algorithm.

In the G1-VP7 phylogeny (**Figure 5**), Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-202/2023/G1P[8] isolated from children under five years in this study was found clustering with rotavirus A strain RVA/Human-wt/GMB/MRC-DPRU3174/2010/G1P[8] and Rotavirus A strain RVA/Human-wt/SEN/MRC-DPRU2130-09/2009/G1P[8]. There was a 67% likelihood that the G1-VP7 strain obtained from this study and the other clustered reference G1 strains from the Gambia and Senegal originated from the same ancestry. All the reference G1 strains and the G1 strain from this study used for constructing the G1 phylogenetic tree significantly diverged from the Australian G3 strain (Rotavirus A strain RVA/Human-wt/AUS/RCH272/2012/G3P[14]) used as an out-group.

The G2-VP7 phylogenetic tree (**Figure 6**) was constructed with nucleotide sequences of three Nigerian G2 strains obtained from this study seven reference G2 strains deposited in the NCBI GenBank. Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-201/2023/G2[P6] and human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-203/2023/G2[P6] isolated from children under five years in this study were

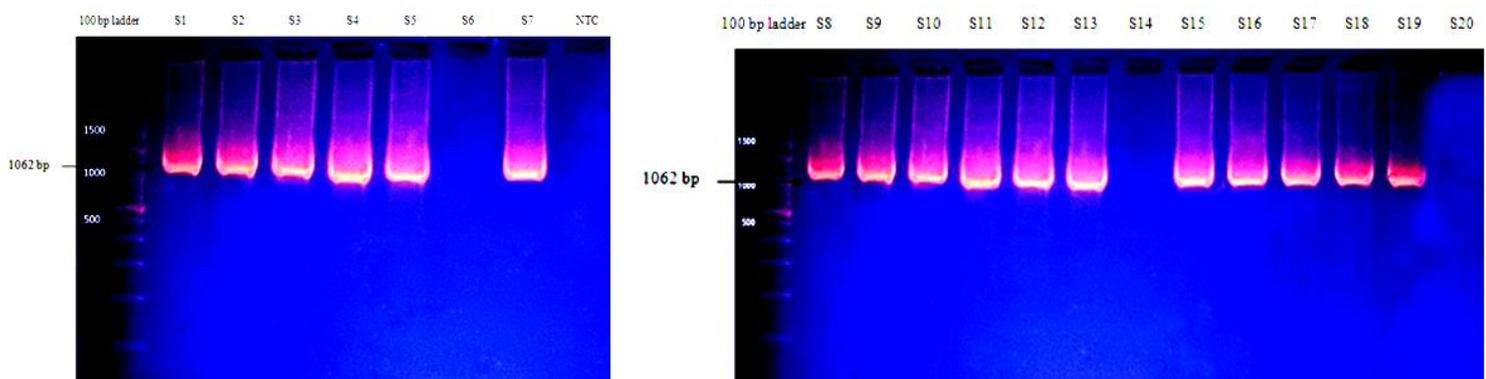
closely related to rotavirus A strain RVA/Human-wt/GHA/Ghan-052/2008/G2P[6] and rotavirus A strain RVA/Human-wt/GMB/MRC-DPRU3165/2008/G2P[6] detected in Ghana and Gambia, respectively; with 50% likelihood that these closely-related strains shared the same ancestry. Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-205/2023/G2G9 obtained from this study was also found to share the same cluster with rotavirus A strain RVA/Human-wt/GHA/Ghan-108/2009/G2P[6], rotavirus A strain RVA/Human-wt/GHA/Ghan-009/2009/G2P[6], rotavirus A strain RVA/Human-wt/GMB/MRC-DPRU3180/2010/G2P[6] and rotavirus A strain RVA/Human-wt/CMR/MRC-DPRU3016/XXXX/G2P[6] detected in Ghana, Gambia and Cameroon, respectively; with 85% likelihood that the clustered strains originated from the same ancestor. All the reference G2 strains and the G2 strains isolated in this study used for constructing the G2 phylogenetic tree significantly diverged from the Australian G3 strain (Rotavirus A strain RVA/Human-wt/AUS/RCH272/2012/G3P[14]) used as an out-group.

As observed in [P6]-VP4 phylogeny (Figure 7), there was a 60% likelihood that the clustered Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-209/2023/G2[P6] and human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-210/2023/G9[P6] obtained from this study and the reference Ghanaian rotavirus A strain RVA/Human-wt/GHA/Ghan-052/2008/G2P[6] originated from a common ancestor. Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-207/2023/G2[P6] obtained from this study was closely related to reference Malawian rotavirus A strain RVA/Human-

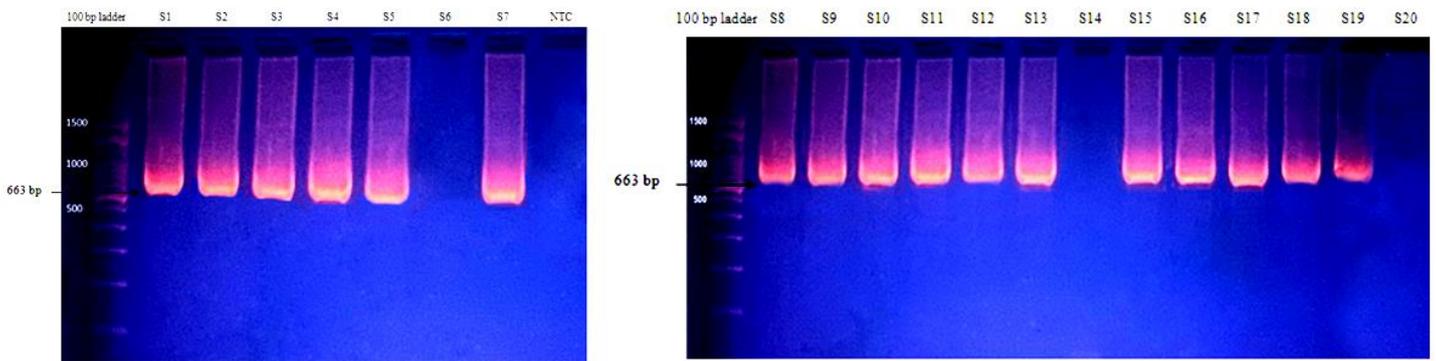
wt/MWI/BID1AW/2012/G2P[6] and rotavirus A strain RVA/Human-wt/MWI/BID15I/2012/G2P[6]; with a 63% likelihood that they shared a common ancestor. Human rotavirus A strain RVA/Human-wt/NGR/IZEVBUWA-211/2023/G2G9[P6] obtained from this study was also found to share the same cluster with rotavirus A strain RVA/Human-wt/MLI/Mali-029/2008/G2P[6], rotavirus A strain RVA/Human-wt/GMB/MRC-DPRU3180/2010/G2P[6] and rotavirus A strain RVA/Human-wt/GHA/Ghan-053/2009/G2P[6] detected in Mali, Gambia and Ghana, respectively; with 72% likelihood that the clustered strains originated from a common ancestor. All the reference [P6] strains and the [P6] strains isolated in this study used for constructing the [P6] phylogenetic tree significantly diverged from the Australian G3 strain (rotavirus A strain RVA/Human-wt/AUS/RCH272/2012/G3P[14]) used as an out-group.

Wt/NGR/IZEVBUWA-208/2023/G1[P8] isolated from diarrheic children under five years in this study was found clustering with Human rotavirus A strain RVA/Human-wt/CHN/Fuzhou18-195/2018/G1P[8], Human rotavirus A isolate RVA/Human-wt/CHN/E5365/2017/G1P[8] and Human rotavirus A RVA/Human-wt/JPN/MU17-02/2017/G9P[8]. There was a 66% likelihood that the strain obtained from this study and the other clustered reference [P8] strains from China and Japan originated from a common ancestor. All the reference [P8] strains and the [P8] strain from this study used for constructing the [P8] phylogenetic tree significantly diverged from the Australian G3 strain (rotavirus A strain RVA/Human-wt/AUS/RCH272/2012/G3P[14]) used as an out-group.

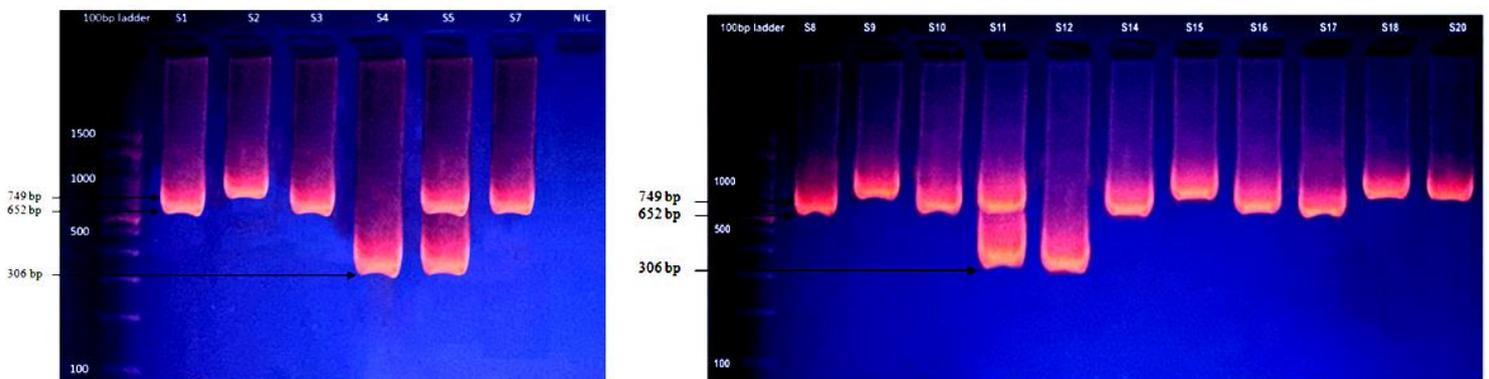
Figure 1. First round PCR amplification of the VP7 gene from RVA cDNA using Beg9 and End9 primers.



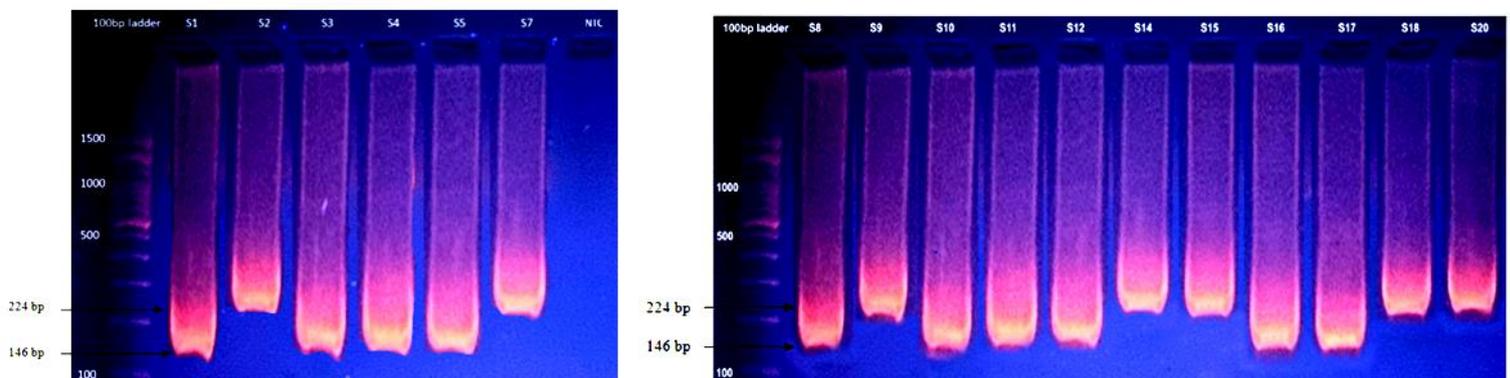
S1 – S20 represent the RVA antigen-positive samples examined. NTC represents the no template control (sterile water). PCR Primers: Beg9- GGCTTTAAAAGAGAGAATTTCCTGCTGG; End9- GGTCACATCATAACAATTCTAATCTAAG. Amplicon size (1062 bp)

Figure 2. First round PCR amplification of the VP4 gene from RVA cDNA using VP4F and VP4R primers.

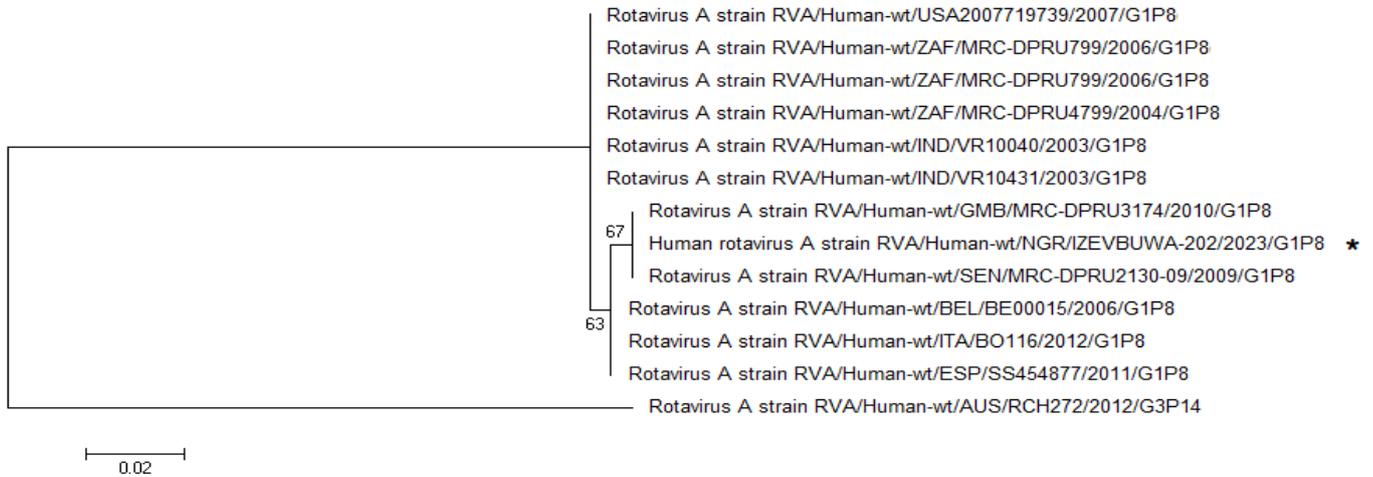
S1 – S20 represent the RVA antigen-positive samples examined. NTC represents the no template control (sterile water). PCR Primers: VP4F-TATGCTCCAGTNAATTGG; VP4R- ATTGCATTCTTTCCATAATG. Amplicon size (663 bp).

Figure 3. VP7-G-typing multiplex PCR.

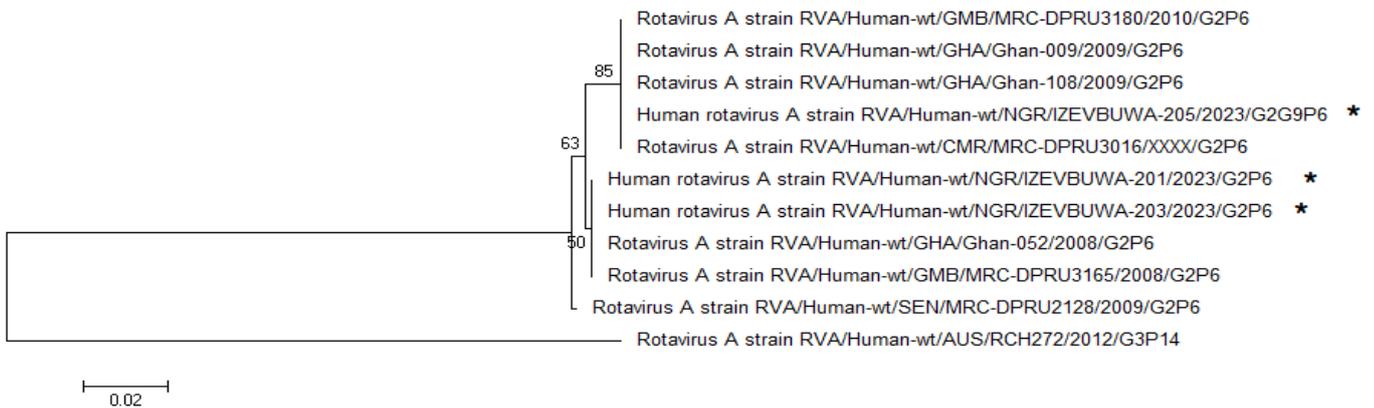
S1 – S5, S7 – S12, S14 – S17 as well as S18 and S20 represent the first round RT-PCR-positive samples examined. NTC represents the no template control (sterile water). Primer cocktail specific to six serotypes G8 or aAT8 (GTCACACCATTGTAAATTCG); G1 or aBT1 (CAAGTACTCAAATCAATGATGG); G2 or aCT2 (CAATGATATTAACACATTTTCTGTG); G4 or aDT4 (CGTTTCTGGTGAGGAGTTG); G3 or aET3 (CGTTTGAAGAAGTTGCAACAG); G9 or aFT9 (CTAGATGTAACTACAACACTAC) and consensus primer RVG9 (GGTCACATCATACAATTCT). Amplicon size (G8 = 885 bp, G1 = 749 bp, G2 = 652 bp, G4 = 583 bp, G3 = 374 bp, G9 = 306 bp).

Figure 4. VP4-P-typing multiplex PCR.

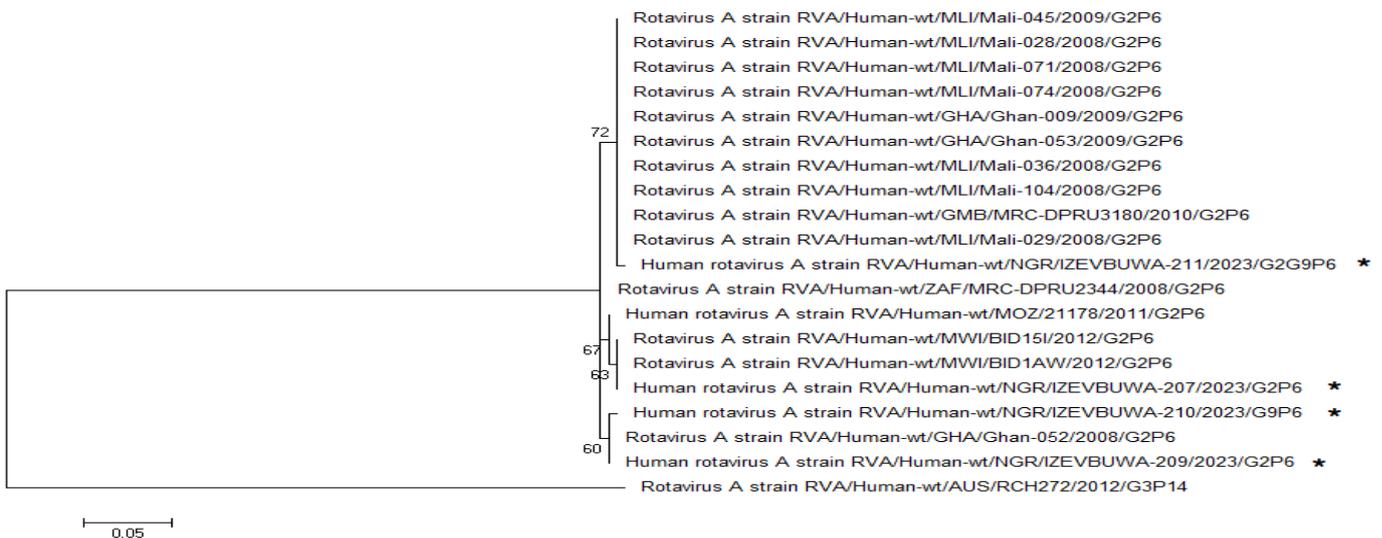
S1 – S5, S7 – S12, S14 – S17 as well as S18 and S20 represent the first round RT-PCR-positive samples examined. NTC represents the no template control (sterile water). Primer cocktail specific to six serotypes P[4] or 2T-1 (CTATTGTTAGAGGTTAGAGTC), P[6] or 3T-1 (TGTTGATTAGTTGGATTCAA), P[8] or 1T-1D (TCTACTGGRTRTRACNTGC), P[9] or 4T-1 (TGAGACATGCAATTGGAC), P[10] or 5T-1 (ATCATAGTTAGTAGTCGG), P[11] (GTAAACATCCAGAATGTG) and VP4F (TATGCTCCAGTNAATTGG). Amplicon size (P[4] = 362 bp, P[6] = 146 bp, P[8] = 224 bp, P[9] = 270 bp, P[10] = 462 bp, P[11] = 191 bp).

Figure 5. A phylogenetic tree of G1-VP7 genes in RVA strains constructed with the neighbor-joining method.

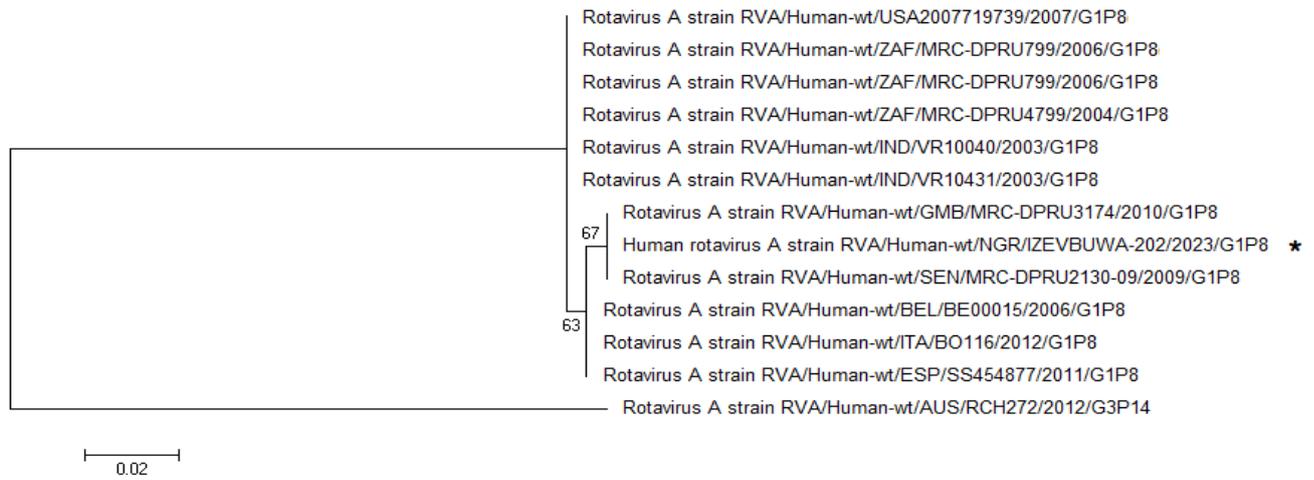
* is used to indicate the human rotavirus A strains isolated from children under five years that were examined in this study. The tree was rooted on midpoint and bootstrap values displayed on the branches were above 50 %.

Figure 6. A phylogenetic tree of G2-VP7 genes in RVA strains constructed with the neighbor-joining method.

* is used to indicate the human rotavirus A strains isolated from children under five years that were examined in this study. The tree was rooted on midpoint and bootstrap values displayed on the branches were above 50 %.

Figure 7. A phylogenetic tree of [P6]-VP4 genes in RVA strains constructed with the neighbor-joining method.

* is used to indicate the human rotavirus A strains isolated from children under five years that were examined in this study. The tree was rooted on midpoint and bootstrap values displayed on the branches were above 50 %.

Figure 7. A phylogenetic tree of G1-VP7 genes in RVA strains constructed with the neighbor-joining method.

* is used to indicate the human rotavirus A strains isolated from children under five years that were examined in this study. The tree was rooted on midpoint and bootstrap values displayed on the branches were above 50 %.

Table 1. Distribution of RVA G and P genotypes among diarrheic children less than five years in Edo State, Nigeria.

Hospitals from the different geo-political zones	G genotypes	P genotypes				All 4 hospitals visited in the 3 Senatorial Districts				
		P[6]		P[8]		G genotypes	P[6]		P[8]	
		Mean H ± SE	95% CI	Mean H ± SE	95% CI		Mean H ± SE	95% CI	Mean H ± SE	95% CI
		N = 100	N = 100	N = 100	N = 100	N = 400	N = 400	N = 400	N = 400	
		%	%	%	%	%	%	%	%	
General Hospital, Agbede (Edo North Senatorial District)	G1	0.00 ± 0.00	0.00 – 0.00	1.00 ± 1.00	0.00 – 2.96	G1	0.00 ± 0.00	0.00 – 0.00	1.25 ± 0.56	0.16 – 2.34
	G2	3.00 ± 1.72	0.00 – 6.36	1.00 ± 1.00	0.00 – 2.96	G2	1.50 ± 0.61	0.31 – 2.69	0.50 ± 0.35	0.00 – 1.19
	G9	1.00 ± 1.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00	G9	0.50 ± 0.35	0.00 – 1.19	0.00 ± 0.00	0.00 ± 0.00
	G2G9	1.00 ± 0.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00	G2G9	0.50 ± 0.35	0.00 – 1.19	0.00 ± 0.00	0.00 ± 0.00
Specialist Hospital, Imua (Edo Central Senatorial District)	G1	0.00 ± 0.00	0.00 – 0.00	1.00 ± 1.00	0.00 – 2.96					
	G2	1.00 ± 1.00	0.00 – 2.96	1.00 ± 1.00	0.00 – 2.96					
	G9	1.00 ± 1.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00					
	G2G9	1.00 ± 1.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00					
Central Hospital, Benin City (Edo South Senatorial District)	G1	0.00 ± 0.00	0.00 – 0.00	1.00 ± 1.00	0.00 – 2.96					
	G2	1.00 ± 1.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00					
	G9	0.00 ± 0.00	0.00 – 0.00	0.00 ± 0.00	0.00 ± 0.00					
	G2G9	0.00 ± 0.00	0.00 – 0.00	0.00 ± 0.00	0.00 ± 0.00					
Igbinedion University Teaching Hospital, Okada (Edo South Senatorial District)	G1	0.00 ± 0.00	0.00 – 0.00	2.00 ± 1.41	0.00 – 4.76					
	G2	1.00 ± 1.00	0.00 – 2.96	0.00 ± 0.00	0.00 ± 0.00					
	G9	0.00 ± 0.00	0.00 – 0.00	0.00 ± 0.00	0.00 ± 0.00					
	G2G9	0.00 ± 0.00	0.00 – 0.00	0.00 ± 0.00	0.00 ± 0.00					

N represents the number of stool samples examined. H represents prevalence. SE represents standard error of the mean prevalence. CI represents confidence interval of the mean prevalence. % represents percentage prevalence

Discussion

RVA gastroenteritis is regarded to be a principal cause of infant and childhood morbidity and mortality, especially in developing countries [24]. The present study reported the prevalence and characteristics of RVA genotypes circulating among children less than five years in Edo State, Nigeria. Based on the RT-PCR assay, the prevalence of RVA in this study was estimated at 4.25%. The prevalence rate of 4.25% by RT-PCR was due to the inability of the assay to detect RVA in three out of the twenty RVA antigen-positive samples preliminarily detected by the immunochromatographic method. The low prevalence rate reported in this study was at variance with the prevalence rates reported in most studies conducted in different localities in Nigeria [25-30], but agreed with the study of **Kuta et al.** [31] who reported a low prevalence of RVA in children under five years from Kwara State, Nigeria. High RVA prevalence rates have been reported in countries like Pakistan, India, Cambodia, Thailand and Mongolia [32-39]. There might be several reasons for these observed prevalence differences in Nigeria and other countries. These may include contrasting study designs and sampling intervals.

The diversity of rotavirus genotypes and strains prevailing worldwide provides beneficial insight into rotavirus evolution. The rotavirus molecular epidemiology differs from country to country based on socio-economic status and meteorological conditions [37]. The usual G-genotypes seen in humans are G1, G2, G3, G4, G9 and G12 while the usual human P-genotypes are P[4], P[6] and P[8] [38]. The six major epidemiologically significant genotypes combinations circulating the globe are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] [38-39]. In earlier studies, the seven most prevalent G genotypes reported in Nigeria were G1, G2, G3, G8, G9, G10, and G12 while the three most frequent P genotypes were P[4], P[6] and P[8] [25, 28]

The G2 strains were the most frequently detected VP7 RVA strains in this study. Such results are not unexpected since G2 strains have been rapidly evolving and proliferating during the last decade. The G2 strains were also the most frequently detected in the study of **Patić et al.** [40] which characterized RVA infections in children and adults with gastroenteritis from Serbia. However, the findings in the study of **Sadiq et al.** [39], who characterized human RVA genotypes circulating in

Pakistan, indicated that G3 strains were the most frequently detected rather than the G2 strains. The findings of the present study were also at variance with most studies carried out in Europe, North America and Australia that have reported that the G1 strains were the most frequently detected, and accounted for 70% of gastroenteritis [41]. Phylogenetic analysis revealed that all G2 strains from this study clustered with G2 strains from Ghana, Gambia and Cameroon. The G1 strains from this study also clustered with G1 strains from other African countries such as Gambia and Senegal. The human G9 RVA genotype has acquired epidemiological significance and is currently accepted as the fifth main human RVA genotype [37, 39]. Many studies have shown a close genetic link between human and pig G9 RVA strains, indicating that interspecies transmissions in association with reassortment have brought about the emergence of this genotype in humans [42-43]. In this study, the G9 strains were found circulating among children under five years. The detected G9 genotype was found in combination with P[6] genotype. G9 strains have been reported in Pakistan [39], Taiwan [44] and France [45].

The RVA P[6]-VP4 genotype has been detected worldwide in association with different types of G genotypes [46]. In humans, the P[6] genotype is regarded to be the most widespread genotype in South Asia and Sub-Saharan Africa [47]. In this study, P[6] genotype, in combination with G2 genotype, was the most frequently detected in children under five years in Edo State, Nigeria. Phylogenetic analysis indicated that the P[6] strains from this study clustered with P[6] strains from other African countries such as Ghana, Mali, Gambia and Malawi.

The P[8]-VP4 genotype originated from South and East Asia, and has migrated to European, North American and African countries [48]. In the present study, the P[8] strains were found circulating in children under five years. Based on phylogenetic analysis, the P[8] strains from the present study were found to be closely related to P[8] strains from China.

RVA mixed infections have been reported worldwide [49-50]. Mixed G2G9[P6] strains responsible for mixed infections were found to circulate among the study participants in the present study. The 0.5% prevalence of G2G9[P6] reported in the present study agreed with previous

reports from Pakistan and Nigeria [39-51]. Unusual G2[P8] strains were also found circulating among the study participants in the present study. The unusual genotypes and mixed genotypes arise due to mixed infections that lead to the re-assortment and evolution of novel genotypes [37].

Even though the datasets of the present study satisfactorily discriminated the RVA genotypes, it could not differentiate among sub-genotypic lineages because the sequencing techniques used in this study could only partially sequence the VP7 and VP4 gene segments of the RVA. Nevertheless, this study provided substantial evidence of genetic and potential antigenic differences associated with the VP7 and VP4 genes in RVA strains circulating in Edo State, Nigeria. The present study has presented preliminary data for further research that could include sequencing of the whole RVA genome.

Conclusion

This study revealed that G2[P6] RVA strains were the dominant type responsible for rotavirus infections in children under five years in Edo State, Nigeria. It also provided evidence of the contribution of unusual RVA strains in causing diarrhea. Complete molecular characterization of the isolated RVA strains in the present study will be advantageous in identifying additional components that may be required in the evolution of rotavirus vaccines.

Conflicts of interest

None.

Financial disclosure

None.

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