Original Research Article

Molecular Detection and Sequencing of Rotavirus VP4 among Children aged 0-5 years with Gastroenteritis in 2 Selected Healthcare Centres in Keffi, Nigeria

ABSTRACT

Aims: This study was conducted to detect and sequence Rotavirus VP4 among children aged 0-5 years with gastroenteritis in 2 selected healthcare centres in Keffi, Nigeria.

Study Design: The study was a cross sectional study.

Place and Duration of Study: Keffi, Nasarawa State, between March and June 2019.

Methodology: Stool samples were collected from 303 (203 from FMC Keffi and 100 from PHC Angwan Waje, Keffi) children with gastroenteritis and information about them were obtained by structured questionnaires. All collected samples were screened for the presence of Rotavirus antigen using Aria Rotavirus antigen detection test kit (CTK Biotech, Inc, San Diego, USA). VP4 was detected from Rotavirus positive samples by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using specific primers. The sequences of the amplified VP4 genes were verified using MEGA software version 7 and Rotavirus strains were determined by pasting the FASTA (Text based format for representing nucleotide sequence) format into the Basic Local alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Data collected were analysed using Smith's Statistical Package (version 2.8, California, USA) and *P* value of ≤ 0.05 was considered statistically significant.

Results: Of the 303 children screened, 54(17.8%) were positive for Rotavirus infection. Highest prevalence of the viral infection was recorded among males (19.7%) aged 0-12 months (24.8%). Gender was found to be associated with rate of Rotavirus infection in this study ($P<0.05$). However, age was not significantly associated with the viral infection ($P>0.05$). Furthermore, based on the RT-PCR carried out, 3(5.6%) out of the 54 Rotavirus positive samples were positive for the VP4 gene and sequences of this gene were all found to be of type P [11] Strain N115.

Conclusion: This study reveals the presence of infection with type P [11] Strain N115 (5.6%) of Rotavirus in the study population. The detection of this rare rotavirus strain in this study is a cause for concern and hence there is an urgent need for the Nigerian health authorities to implement a nationwide surveillance system for monitoring rotavirus molecular epidemiology.

Keywords: VP4; Rotavirus; Gastroenteritis; Children; Keffi; Nigeria

1. INTRODUCTION

Rotavirus is the leading cause of severe gastroenteritis particularly among infants and young children worldwide with over 70% of cases occurring in Nigeria and other endemic sub-Saharan African countries [1-4]. It is believed that almost every child in the world is infected with rotavirus at least once by the age of five [5]. However, Immunity develops with each infection, so subsequent infections are less severe and hence adults are rarely affected [6].

Rotavirus belongs to the virus family *Reoviridae,* it is a non-enveloped and has an icosahedral nueleocapsid structure, enclosing a double stranded (ds) RNA genome segmented into 11 compartments. The genome codes for six structural proteins, (VP1 to VP4, VP6 and VP7) and five nonstructural proteins (NSP1 to NSP5) [7]. VP4 is on the surface of the virion that protrudes as a spike [8]. It binds to molecules on the surface of cells called receptors and drives the entry of the virus into the cell [9]. It has to be modified by the protease enzyme trypsin, which is found in the gut, into VP5 and VP8 before the virus is infectious [10]. It also determines how virulent the virus is and determines the P-type of the virus [11].

There are at least 10 distinct species/groups of Rotavirus (A- I, J), differentiated by their VP6 antigenic properties [12]. Groups A, B and C are found to cause infection in both humans and animals, whereas groups D, E, F, G and I have been found only in animals [13]. There are 32 G (VP7) genotypes and 47 P (VP4) genotypes identified through molecular epidemiology [2].

Rotaviruses are transmitted by the fæcal-oral route, via contact with contaminated hands, surfaces and objects, and possibly by the respiratory route [3, 4, 14]. The viral infection is usually associated with fever, nausea and vomiting, followed by abdominal cramps, frequent watery diarrhea, which may last for 3-8 days, cough and runny nose occurring mostly among infants [3, 15].

Rotaviruses are stable in the environment and can survive for long periods of time on toys and ordinary surfaces found in most homes. They also are relatively resistant to most soap and commonly used disinfectants, hence preventing a child from exposure can be difficult [3]. Therefore, vaccination still remains the best hope for preventing infection with Rotavirus especially in resource constraint nations such as Nigerian [1, 16]. Additionally, most hospitals and diagnostic centres in such nations do not routinely diagnosed Rotavirus infection probably due to it high cost and may be because it share similar signs and symptoms with other infantile gastroenteritis [14, 15, 17].This consequently usually leads to misdiagnosis and mistreatment of the viral infection.

Thus the aim of this study was to detect and sequence Rotavirus VP4 among children aged 0-5 years with gastroenteritis in 2 selected healthcare centres in Keffi, Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

This study was conducted at Federal Medical Centre (FMC) and Primary Healthcare Centre (PHC), Angwan Waje in Keffi Local Government Area, Nasarawa State, Nigeria. Keffi town, where the 2 centres are located is approximately 68 km from Abuja, Nigeria's Federal Capital Territory and 128 km from Lafia, the capital of Nasarawa State. It is located geographically between latitude 8°3'N of the equator and longitude 7°50'E and situated on an altitude of 850 m above sea level [18].

2.2 Study Population

The study participants were male and female children with gastroenteritis aged 0-5 years accessing health care in the 2 selected healthcare centres in Keffi, Nasarawa State, Nigeria. Their socio-demographic and other required information were obtained from their parents/guardians by the use of a designed questionnaire.

2.3 Ethical Approval and Consent

Formal ethical approval to conduct this study was obtained from the Research Ethics Committee of Federal Medical Centre, Keffi (FMC/KF/HREC/207/17). Permission was also obtained from the management of Primary Healthcare Center Angwan Waje, Keffi. In addition, prior to collection of samples, consent was sort from parents/guardians of the participating children.

2.4 Sample Size Determination

To determine the sample size, the formula by Naing *et al.* [19] for sample size calculation at 0.05 level of precision was used;

$$n = \frac{Z^2 pq}{d^2}$$

Where:

n = required sample size

Z = standard normal deviation at the required confidence interval (1.96) which corresponds to 95% confidence interval.

P = prevalence of Rotavirus infection from previous study (25.0%) (0.2) [20].

Q = 1 – p = 0.9

d = degree of precision expected (0.05)

$$n = \frac{(1.96)^2(0.2)(0.9)}{(0.05)^2} = \frac{0.76832 \times 0.9}{0.0025} = \frac{0.691488}{0.0025} = 276.6$$

$$n = 277$$

To ensure minimum error however, this was rounded up to 303 samples.

2.5 Sample Collection and Storage

A total of 303 stool samples (203 from FMC keffi and 100 from PHC Angwan Waje, Keffi) were collected from children with gastroenteritis aged 0-5 years. The samples were collected into sterile wide mouth universal containers, labeled accordingly and stored at -20^{o}C until ready for use [21].

2.6 Laboratory Analysis

2.6.1 Detection of Rotavirus antigen

All collected stool samples were screened for the presence of Rotavirus antigen using Aria Rotavirus antigen detection test kit (CTK Biotech, Inc, San Diego, USA). The tests procedure and results interpretation were done according to the instructions of the manufacturer.

2.6.2 Test procedure

The specimen was brought to room temperature and the test device was removed from the pouch and placed on a clean flat surface and labeled accordingly. Two drops of the watery stool was added into the labeled sample dilution vial followed by vigorous shaking to mix. Thereafter, 2 drops of the mixture was dispensed into the sample well of the test device and a timer was set up for 15 minutes after which the results were read and interpreted as positive, negative or invalid according to the manufacturer's instructions.

2.6.3 Molecular detection of Rotavirus VP4

The VP4 was detected by a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) system previously described by Gentsch *et al.* [22] using specific primers (consensus primers VP4F/VP4R) adopted from the work of Simmonds *et al.* [23].

2.6.4 Rotavirus RNA extraction

Viral RNA was extracted and purified from 10% faecal suspensions in phosphate-buffered saline using the TRIzol method (Gibco BRL, Invitrogen, Burlington, Canada).

2.6.5 Rotavirus VP4 RT-PCR

The VP4 gene was reversely transcribed and amplified using the following primers as adopted from the work of Simmonds *et al.* [23]:

Con2 Forward (5′-ATTTCGGACCATTTATAACC-3′)

Con3 Reverse (5′-TGGCTTCGCTCATTTATAGACA-3′)

The complementary DNA was generated by reverse transcription at 45^{o}C for 30 minutes with initial denaturation at 95^{o}C for 5 minutes followed by 35 cycles of denaturation at 95^{o}C for 1 minute.

2.6.6 Agarose Gel Electrophoresis

The PCR products were analyzed by running a 1% agarose gel stained with ethidium bromide. The sizes of PCR products were estimated in relation to the migration pattern of a 100bp to 1000bp increments plus DNA molecular marker (BIONEER Daejeon, North Korea).

2.6.7 Rotavirus VP4 gene sequencing

The amplified VP4 genes obtained were sent to Inqaba Biotec, South Africa for sequencing. The method described by Sanger *et al*. [24] with modification was used. Briefly: 10µL of the amplified VP4 genes

were cleaned by mixing with 2.5µL EXO/SAP master mix and incubated at 37^{o}C for 15 minutes. The reaction was stopped by heating the mixture at 80^{o}C for 15 minutes. Sequencing was then done on the mixture using Nimagen; Brilliant Dye™ terminator cycle sequencing kit version 3.1 BRD. The labelled products were then cleaned using ZR-96 DNA sequencing clean-up kit and analysed using Applied Biosystems ABI3500XL Genetic analyser yielding sequence chromatogram. Sequences were verified using MEGA software version 7. The strains were determined by pasting the FASTA (Text based format for representing nucleotide sequence) format into the Basic Local alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

2.7 Data Analysis

The data obtained were analyzed using Smith's Statistical Package (version 2.8, California, USA). Chi-square test was conducted at 95% confidence interval and *P* values ≤ 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

This present study was conducted to detect and sequence Rotavirus VP4 among children aged 0-5 years with gastroenteritis in 2 selected healthcare centres in Keffi, Nigeria. A total of 303 children (203 from FMC Keffi and 100 from PHC Angwan Waje, Keffi) majority of which were males (157/303) aged 0-12 months (153/303) were recruited and screened for Rotavirus antigen. Overall, 54 (17.8%) children tested positive for Rotavirus infection giving a total prevalence of 17.8% (Table 1).

Table 1: Prevalence and distribution of Rotavirus infection in relation to age and gender among children between ages 0-5 years with gastroenteritis in 2 selected healthcare centres in Keffi, Nigeria

Risk factor	No. Examined (N=303)	No. Positive (N=54)	Prevalence (%) (Overall=17.8)	p-value
Age (months)				
0–12	153	38	24.8	0.7792
13–24	71	14	19.7	
25–36	37	1	2.9	
37–48	21	1	4.8	
49–60	21	0	0.0	
Gender				
Male	157	31	19.7	0.0004*
Female	146	23	15.8	

**Statistically significant*

The 17.8% prevalence of Rotavirus infection recorded in this study was higher than the 6.0% reported among children in Kwara State [25], 7.4% among children with diarrhoea in Kano State [Suleiman *et al.,* 2020] and 12.5% among infants in Calabar [26]. It was However lower than the 24.8% reported among

children with gastroenteritis in Akure [27], 25.0% among children less than five years of age in Abuja satellite towns [28] and 56.0% among children with diarrhea in Enugu State [29]. Interestingly, researchers from other parts of Africa and the world also reported varying rates of the viral infection. For instance, it was 14.5% in Kenya [30], 16% in Sudan [31], 18% in India [32], 39.2% in Benin Republic [33], 44.8% in Indonesia [34] and 56% in Portugal [35]. The differences observed in the prevalence rates from different studies were possibly due to differences in testing methods used, location of the studies, time and season of sampling and study population types with different associated risk factors [2].

This study did not record significant association between Rotavirus infection and age of the participants ($P>0.05$). However, Most of the children infected were between the age group 0-12 months (24.8%), followed by 13-24 months (19.7%), 37-48 months (4.8%) and 25-36 months 1(2.9%) while none was detected in age group 49-60 months (Table 1). This observation agrees with the report of Theophilus *et al.* [20], Okebugwu *et al*. [27] and Dhital *et al*. [36] among children with gastroenteritis in Abuja, Akure and Eastern India respectively but disagrees with that of Mohammed *et al*. [37] and Aliyu *et al*. [38] who reported higher prevalence of the viral infection among Nigerian children within the age group 25-50 months. The higher prevalence of the infection recorded among younger children in this present study may be attributed to the absence of well-developed immune system among them as older children are expected to acquire protective immunity during repeated exposures to the virus and therefore, subsequent infections are mild or asymptomatic [39].

There was significant association between gender and prevalence of Rotavirus infection in this study ($P<0.05$) as males were more infected (19.7%) compared to their female counterparts (15.8%) (Table 1). This is consistent with the reports of most other previous studies conducted in Nigeria [3, 15, 18, 26, 28, 29, 37] and other parts of the world [30, 31, 34, 40]. The higher prevalence of the infection among male subjects in most previous studies may be connected to the fact that females mount stronger humoral and cellular immune responses to infection or antigenic stimulation than the males [41].

Furthermore, based on the RT-PCR carried out in this study, 3(5.6%) out of the 54 samples that tested positive with the lateral flow immunoassay kit were positive for the VP4 gene (Figure 1.1). The low rate of rotavirus VP4 gene detected by RT-PCR in this present study could possibly be as a result of PCR inhibitors in the faeces that were carried over into the RNA extracts [42]. It could also be attributed to prolonged storage of the stool samples, method of RNA extraction and effects of freezing and thawing [43].

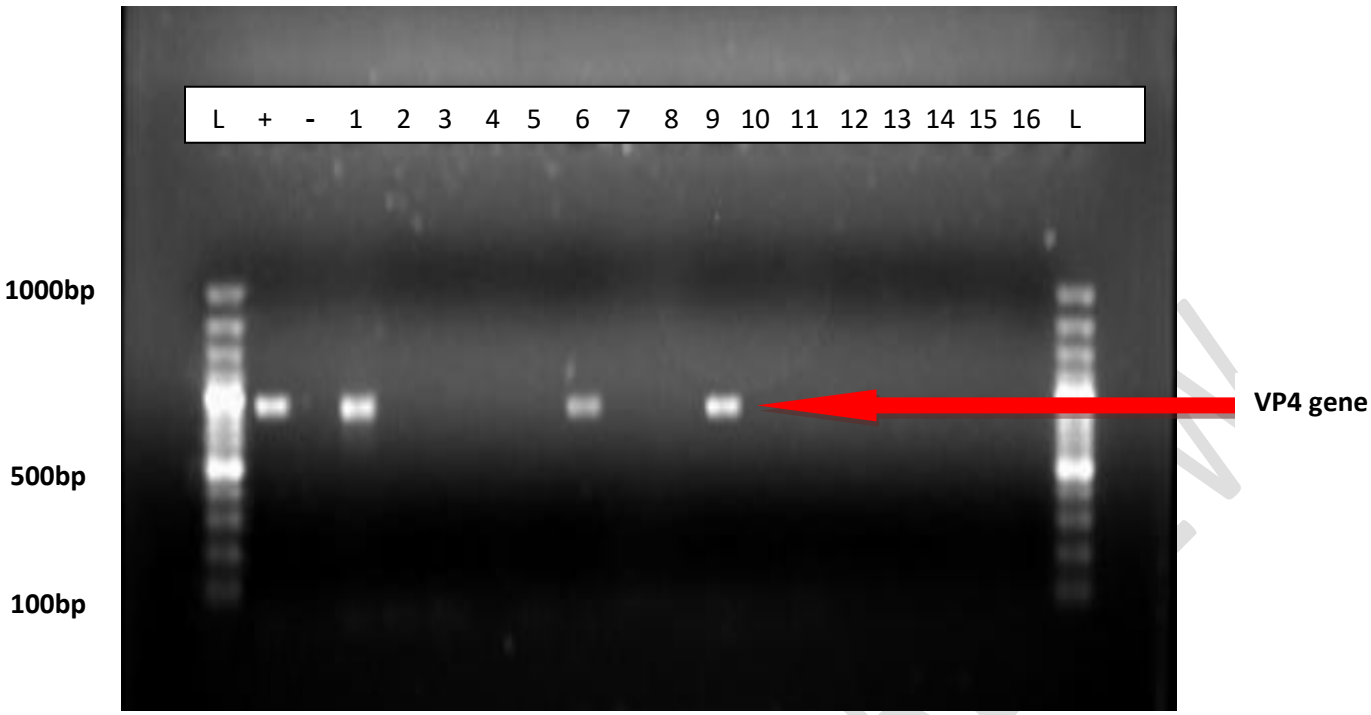

Figure 1: Agarose gel electrophoretogram of Rotavirus VP4 amplified gene. Samples 1, 6 and 9 were positive for Rotavirus VP4 gene while samples 2, 3, 4, 5, 7, 8, 10, 11, 12, 13, 14, 15 and 16 were negative. *L represents the molecular ladder, ' −'is the negative control while ' + ' is the positive control.

The 3 amplified VP4 genes were sequenced and were found to be of type P [11] Strain N115 (Figures 2A and 2B). This strain showed 88.08% nucleotide identity to human rotavirus A strain G12 P [11] with accession number MH559158.1 in the GenBank. The nucleotide sequence of the N155 strain detected in this study has been deposited in the GeneBank under the accession number: EU200796.1 (Figures 3 and 4).

This strain of Rotavirus detected in this present study has not been reported in Nigeria. In contrast however, other genotype combinations have been reported in Nigeria and other African countries. For instance, genotype G12 was detected in Asaba, Delta State [44], Ibadan, Oyo State, Nigeria [45], Nairobi, Kenya [46], Dakar, Senegal [47], Democratic Republic of Congo [48] and in Tunisia [49].

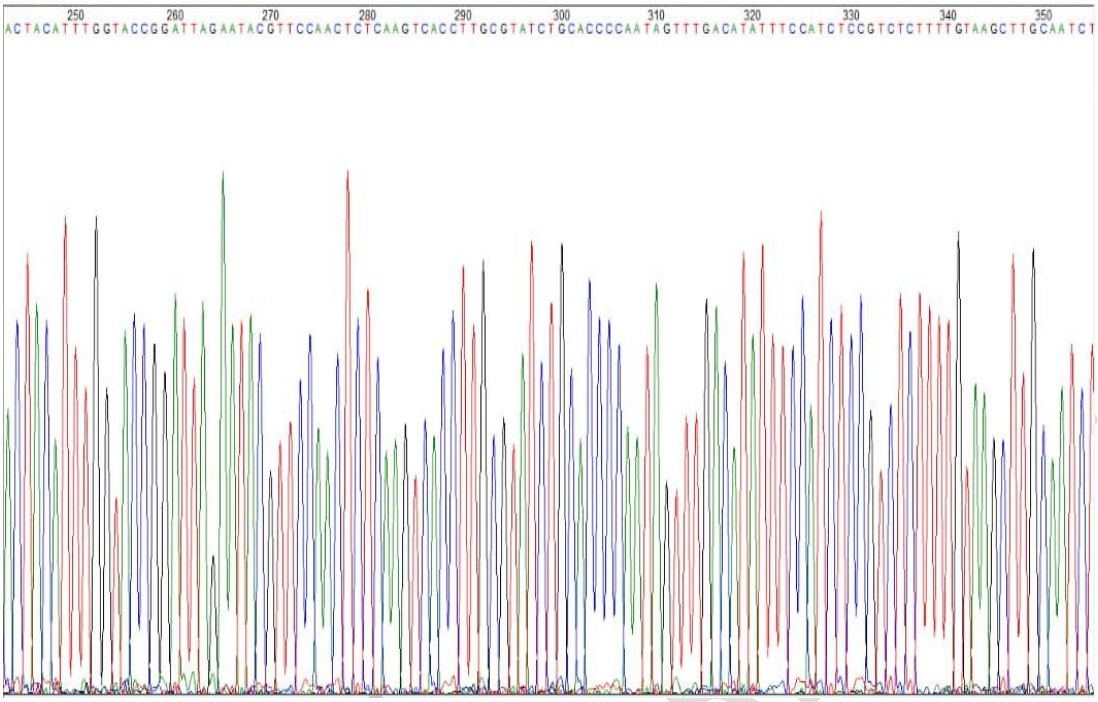

Figure 2A: Chromatograph of the Sequenced RotavirusVP4 Genes (Forward Primer)

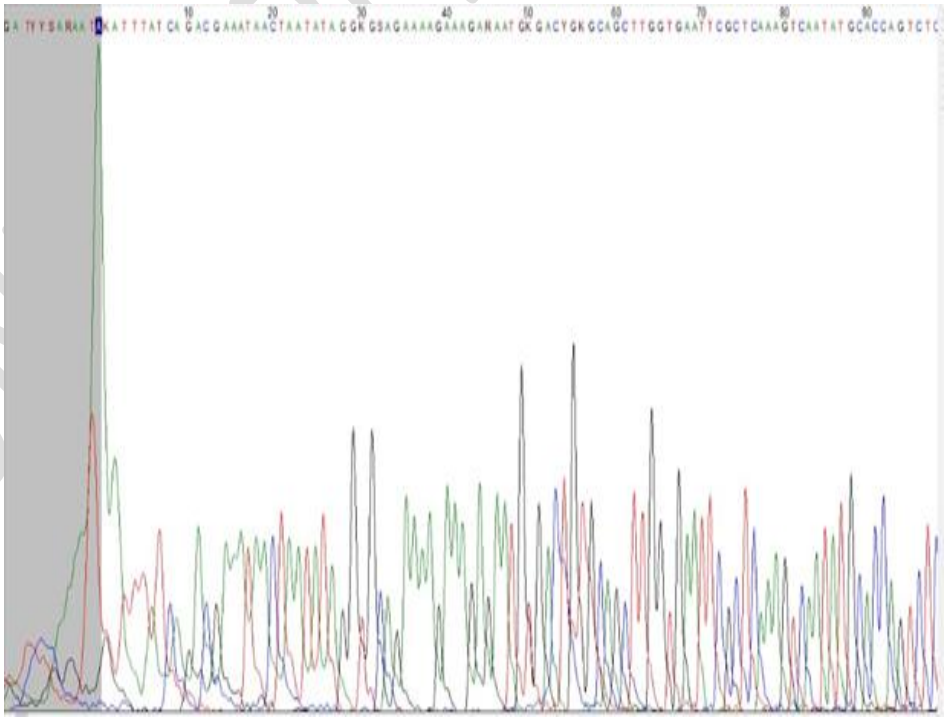

Figure 2B: Chromatograph of the Sequenced Rotavirus VP4 Gene (Reverse Primer)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Human rotavirus A isolate N155 VP4 gene, complete cds	882	882	97%	0.0	88.08%	EU200796.1
Human rotavirus A strain RVA/Hu-wt/IND/168710/2016/G12P[11] VP4 (VP4) gene, partial cds	689	689	95%	0.0	85.39%	MH559158.1
Human rotavirus A strain RVA/Hu-wt/IND/1731557/2017/G12P[11] VP4 (VP4) gene, partial cds	673	673	95%	0.0	85.14%	MH559165.1
Human rotavirus A strain RVA/Hu-wt/IND/1682239/2016/G12P[11] VP4 (VP4) gene, partial cds	673	673	95%	0.0	85.14%	MH559161.1
Human rotavirus A strain RVA/Hu-wt/IND/165460/2016/G12P[11] VP4 (VP4) gene, partial cds	665	665	95%	0.0	85.02%	MH559160.1
Human rotavirus A strain RVA/Hu-wt/IND/1617178/2016/G12P[11] VP4 (VP4) gene, partial cds	665	665	95%	0.0	85.02%	MH559159.1
Human rotavirus A strain RVA/Hu-wt/IND/169046/2016/G12P[11] VP4 (VP4) gene, partial cds	665	665	95%	0.0	85.02%	MH559153.1
Human rotavirus A strain RVA/Hu-wt/IND/1617421/2016/G12P[11] VP4 (VP4) gene, partial cds	657	657	95%	0.0	84.89%	MH559154.1
Human rotavirus A strain RVA/Hu-wt/IND/1720697/2017/G12P[11] VP4 (VP4) gene, partial cds	641	641	95%	0.0	84.64%	MH559156.1
Human rotavirus A strain RVA/Hu-wt/IND/1615595/2016/G12P[11] VP4 (VP4) gene, partial cds	641	641	95%	0.0	84.64%	MH559155.1
Human rotavirus A strain RVA/Hu-wt/IND/1842605/2018/G12P[11] VP4 (VP4) gene, partial cds	633	633	95%	0.0	84.52%	MH559171.1
Human rotavirus A strain RVA/Hu-wt/IND/1842602/2018/G12P[11] VP4 (VP4) gene, partial cds	633	633	95%	0.0	84.52%	MH559170.1
Human rotavirus A strain RVA/Hu-wt/IND/1725114/2017/G12P[11] VP4 (VP4) gene, partial cds	633	633	95%	0.0	84.52%	MH559157.1
Human rotavirus A strain RVA/Hu-wt/IND/164567/2016/G12P[11] VP4 (VP4) gene, partial cds	633	633	95%	0.0	84.52%	MH559152.1
Human rotavirus A strain RVA/Hu-wt/IND/173950/2017/G12P[11] VP4 (VP4) gene, partial cds	625	625	95%	1e-178	84.39%	MH559169.1
Human rotavirus A strain RVA/Hu-wt/IND/1722032/2017/G12P[11] VP4 (VP4) gene, partial cds	625	625	95%	1e-178	84.39%	MH559164.1
Human rotavirus A strain RVA/Hu-wt/IND/1720706/2017/G12P[11] VP4 (VP4) gene, partial cds	625	625	95%	1e-178	84.39%	MH559163.1
Human rotavirus A strain RVA/Hu-wt/IND/1720702/2017/G12P[11] VP4 (VP4) gene, partial cds	625	625	95%	1e-178	84.39%	MH559162.1
Human rotavirus A strain RVA/Hu-wt/IND/1739626/2017/G12P[11] VP4 (VP4) gene, partial cds	601	601	95%	2e-171	84.02%	MH559168.1
Human rotavirus A strain RVA/Hu-wt/IND/1739394/2017/G12P[11] VP4 (VP4) gene, partial cds	601	601	95%	2e-171	84.02%	MH559167.1

Figure 3: Image of BLAST of sample showing sequence with significant alignment with that in the GenBank.

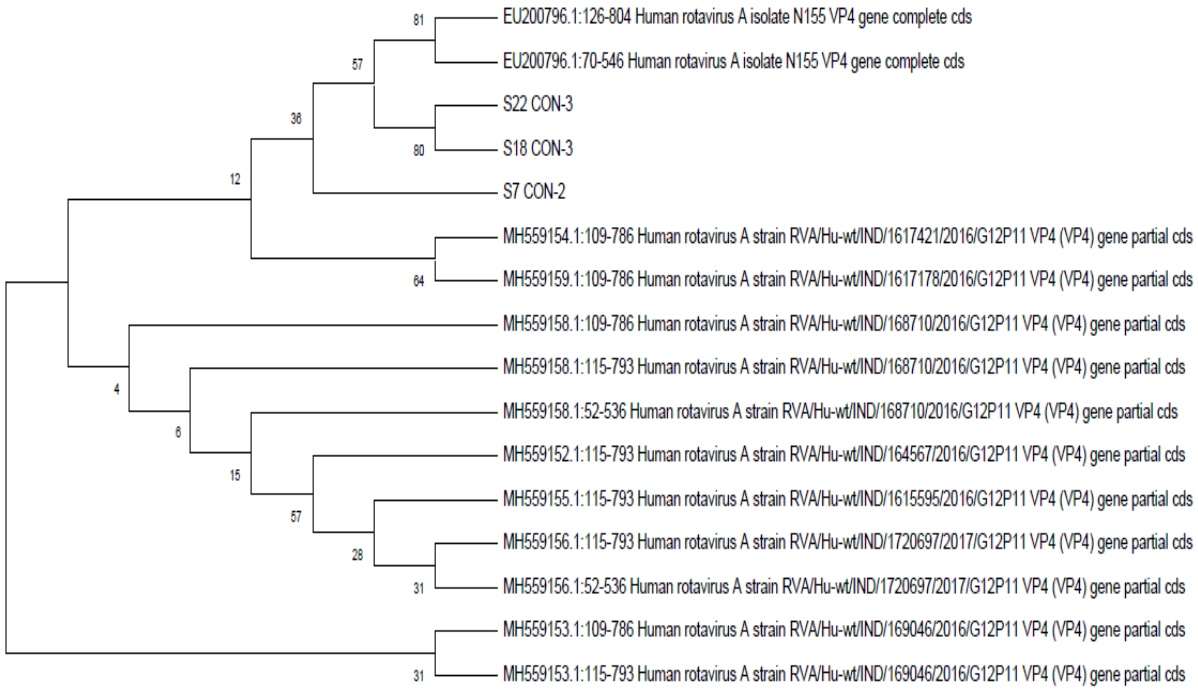

Figure 4: Phylogeny of rotavirus VP4 gene.

Furthermore, in India, Giri *et al*. [50] identified genotype G12 P [11] while Ray *et al*. [51] and Ramani *et al*. [52] reported the detection of strain N155 with a difference in the G-type (G10) among neonates with gastroenteritis in India. Additionally, Libonati *et al*. [53] also reported the detection of G10 P [11] type in India. These strains of group A rotavirus are believed to be from bovine origin as a result of bovine-human gene re-assortment [50].

4. CONCLUSION

This study recorded 17.8% prevalence of infection with type P [11] Strain N115 (5.6%) of Rotavirus among children aged 0-5 years with gastroenteritis in 2 selected healthcare centres in Keffi, Nigeria. The detection of this rare rotavirus strain in this study is a cause for concern and hence there is an urgent need for the Nigerian health authorities to implement a nationwide surveillance system for monitoring rotavirus molecular epidemiology, before considering introduction of rotavirus vaccination into the expanded program on immunization (EPI). This will help to give necessary information on current genotypes and novel introductions as well as evolution of mutant strains to help augment current rotavirus prevention and control.

CONSENT

All parents/guardians of the children included in this study completed and signed an informed consent form.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have been conducted in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki.

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