Article

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Posted Date: 22 May 2024

doi: 10.20944/preprints202405.1456.v1

Keywords: Rotavirus, Children, diarrhoea, viral infections, Molecular characterisation.

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Abstract: Introduction: Rotavirus-induced acute diarrhoea continues to pose a significant health threat to children under 5 years old worldwide, despite the availability of effective vaccines. This study aimed to assess the prevalence, associated risk factors, and circulating genotypes of rotavirus among children under 5 admitted to Ikom Medical Centre due to diarrhea. **Methodology:** Stool samples were collected from 294 children under 5 with diarrhoea at Ikom Medical Centre. Rotavirus antigen detection was performed using rapid chromatographic immunoassay. Molecular techniques such as polyacrylamide gel electrophoresis (PAGE), RT-PCR, and semi-nested multiplex RT-PCR were used to analyze rotavirus strains. Data were obtained through questionnaires, and statistical significance was assessed using the chi-square test ($P < 0.05$). **Results:** Among the 294 samples, 20.4% (60/294) tested positive for rotavirus. Significant risk factors influencing rotavirus prevalence included feeding status, toilet system, and duration of diarrhea. The most prevalent genotype for VP7 was G4 (26.7%), and for VP4, it was genotype P[4] (23.3%). Predominant genotype combinations were G4P [4] (11.6%) and G12P[4] (6.7%), while non-typeable P[NT] was observed in 41.7% of cases. **Conclusion:** This study highlights the circulation of rotavirus in Ikom, Cross River State, emphasizing its neglected status despite its significant global impact on child mortality. Urgent attention is needed to address rotavirus as a serious public health concern, particularly in low-resource settings.

Keywords: rotavirus; children; diarrhoea; viral infections; molecular characterisation.

 doi:10.20944/preprints202405.1456.v1

1. Introduction

Human rotavirus remains a major cause of gastroenteritis worldwide despite the availability of effective vaccines (Houda et al., 2020). Rotavirus has icosahedral symmetry and a triple-layered capsid lacking an envelope and measures 70–75 nm in diameter (Meylin et al., 2020). Nine rotavirus species (A to I) have been identified. Among the predominant four species (A, B, C, H), species A is the main cause of human infections or gastroenteritis. The Rotavirus genome is composed of 11 double-stranded RNA (dsRNA) segments which encode 11 to 12 proteins, six viral structural proteins (VP1-VP4, VP6, VP7) and six non-structural proteins (NSP1-NSP5, or NSP6). Structural proteins VP7 glycoprotein (G antigen; G genotypes) and VP4 protease-sensitive protein (P antigen; P genotypes) located at the outmost layer of rotavirus virion are the host cell-attachment proteins and induce neutralizing antibodies (Heysuk et al., 2020).

Rotavirus transmission is through faecal-oral route, and it is usually spread by children or their caregivers who fail to wash their hands. Rotavirus transmission can also occur through contact with contaminated environmental surfaces or fomites where transmission and outbreaks usually occur in childcare centres or long-term health facilities (Nokes et al., 2008). Rotavirus may survive days to weeks on surfaces, four hours on hands, and weeks in recreational and drinking water (Dennehy, 2000). The faeces of an infected person can contain more than 10 trillion infectious particles per gram; only 10–100 infectious particles are required to transmit infection to another person (Shamshul et al., 2013). Some studies speculate that transmission also occurs through respiratory route (Cook et al., 1990; Estes and Kapikian, 2007; Gordon, 1982; Gurwith et al., 1981). This theory is supported by the fact that there is rapid acquisition of rotavirus antibodies in the first few years of life in all settings regardless of hygiene standards (Parashar et al., 1998). Transmission through air droplets, however, is still a theory (CDC, 2009). This follows an incubation period of 1 – 3 days before the rotavirus illness begins abruptly (Bernstein, 2009). They come in the gastrointestinal tract, survive the acidity of the stomach, and initiate their infectious cycle (Rihab et al., 2020). While in their gastrointestinal habitat, these pathogens, through a variety of pathological mechanisms trigger the over secretion of fluid in the lumen of the small intestine associated with electrolyte imbalance, and eventual diarrhoea (Humphries and Linscott, 2015; Crawford et al., 2017). In addition to diarrhoea, other major symptoms include fever, vomiting, nausea, weight loss, abdominal pain and dehydration (Stuempfig, 2018; WHO, 2019).

Rotavirus seasonality has been shown to differ widely across the world (Katarina et al., 2019). Rotavirus diarrhoea is known to follow a seasonal pattern in temperate climates, the cooler winter to early spring months is associated with the typical peak season for rotavirus infections (Bos et al., 1992; Percival et al., 2004; Page 2006; Perez-Vargas et al., 2006; Heymann, 2008; Atchison et al., 2009; McCormack and Keam 2009; Atchison et al., 2010; Kargar et al., 2011). Reports have shown that changes in environmental conditions, such as humidity, temperature cycles, rain patterns and winds, are associated with the seasonality of infectious diseases (Rohayem et al., 2006).

Rotavirus (RV) is the main etiological agent of diarrhoea in childhood; its laboratory diagnosis is crucial to guide the clinical management and prevention of its spread (Lucianne et al., 2011). It is not quite possible to diagnose rotavirus diarrhoea solely by clinical examinations, however suggestive features such as fever, dehydration and the regions' seasonal patterns may be conclusive enough (Bernstein, 2009). The most common specimen used in diagnosis is the stool, although the virus has also been found in sera, cerebrospinal fluid and throat swabs (Ushijima et al., 1994). Accurate diagnosis is necessary for surveillance, prevention, and control of diarrhoea (Ranjbar et al., 2014; Tarr et al., 2018) because it will decrease the unnecessary use of antibiotics (Arwa et al., 2016). Among different techniques used to explore new viruses; conventional microscopy, next-generation sequencing, and metagenomics has been a promising approach to study the unrevealed viral genomes since more than a decade (Garza and Dutilh, 2015; Martinez-Hernandez et al., 2017). For conducting epidemiological studies, molecular approaches such as polymerase chain reaction (PCR) and quantitative real time PCR (qPCR) have the highest sensitivity and specificity to investigate virus in clinical samples and have increasingly brought to light significant viral enteric pathogens and their virulent traits (Zhou et al., 2016). The major advantages of molecular diagnostic methods are lower

 doi:10.20944/preprints202405.1456.v1

detection limits and therefore higher analytical sensitivity for common viruses (Simpson et al., 2003; Wolffs et al., 2011; Logan et al., 2006).

Improvements in hygiene and sanitation have not reduced rotavirus diarrhoea. However, interventions to improve water quality and sanitation together with the use of oral rehydration therapy, administration of zinc supplements and breast-feeding have led to an impressive reduction in the number of diarrheal deaths from 4.6 million deaths among children in 1982 to an estimated 2.5 million deaths among children in 2003 (Bresee et al., 2005; Posfay-Barbe et al., 2008). Despite these achievements, rotavirus diarrhoea infection continues to be the major cause of severe, dehydrating gastroenteritis worldwide (Glass et al., 2006). Preventive measures for all enteric pathogens include isolating infected children from others, thoroughly cleaning and disinfecting environmental surfaces with effective agents and strictly following hand washing procedures before and after contact with infected persons and/or potentially contaminated surfaces (Penelope and Dennehy, 2000). To prevent rotavirus infections and decrease mortality, WHO recommends that vaccines should be included in all national immunization programs and considered a priority particularly in countries with high rotavirus gastroenteritis-associated fatality rates, such as in south and south-east Asia and sub-Saharan Africa (WHO, 2013). Rotarix and Rotateq vaccines present very different immunization strategies and seem to provide cross-protection against rotavirus diarrhoea caused by multiple serotypes (Mouna et al., 2011).

2. Materials and Methods

This cross-sectional investigation spanned from February 2019 to January 2020 and took place at the medical center within the Ikom local government area of Cross River State. The study targeted children aged between 0 and 5 who were hospitalized at this medical facility due to a history of diarrhea. Inclusive criteria encompassed all children within this age range who presented with diarrhea, irrespective of gender, and who provided stool samples, requiring parental or guardian consent. Questionnaire administration coincided with stool sample collection. A total of 294 stool specimens were gathered under aseptic conditions and promptly dispatched to the laboratory for analysis. Upon arrival at the laboratory, if analysis could not be immediately conducted, stool samples were preserved in a refrigerator (maintained at 2-8 ℃) for up to 20 minutes. Analysis occurred daily. Exclusion criteria comprised children above five years of age and parents who did not provide consent.

2.1. Ethics Approval

Ethical clearance was obtained from the Cross Rivers State Ministry of Health with REC No: CRSMOH/RP/REC/2018/100. A consent form was given to all participants (mother, father, or guardian) to seek their opinion on their participation in this research and those willing indicated by signing the form. A questionnaire containing socio-demographic, economic, environmental, and clinical factors about the project was administered to all participants after they had read and signed the consent form. They were instructed to fill in the form and aseptically collect the sample.

2.2. Laboratory Diagnosis

We performed a quick screening for rotavirus in children's stool samples using a rapid chromatographic immunoassay method with the Combo cassette from BIOZEK Laboratory, Netherlands. Rotavirus viral RNA was then extracted from the samples using the Quick-RNA™ Viral Kit from ZYMO RESEARCH CORP, India, following the manufacturer's instructions. A 400 µl Viral RNA Buffer was added to each 200 µl stool sample and mixed very well. The mixture was transferred into a Zymo-Spin™ IC Column 2 in a collection tube and centrifuged for 2 minutes, and the column was transferred into a new collection tube. A 500 µl Viral Wash Buffer 3 was added to the column, centrifuged for 30 seconds and the flow-through was discarded and the step was repeated. A 500 µl ethanol (95-100 %) was added to the column and centrifuged for 1 minute to ensure complete removal of the wash buffer. The column was carefully transferred into a nuclease-free tube. A 15 µl

 doi:10.20944/preprints202405.1456.v1

DNase/RNase-Free Water was directly added to the column matrix and centrifuged for 30 seconds, then the eluted RNA was used for PCR as described previously (Fujii et al., 2014).

2.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The presence of viruses in stool samples were determined using the Polymerase Chain Reaction (PCR) technique. Reverse transcription was done using One taq RT-PCR kit (Biolabs New England) following the manufacturer's protocol. The extracted dsRNA was used as a template for reverse transcription. Briefly, a 5 µl of dsRNA was added with 2 µl of mix of d(T) VN (50 µM) (random primers (60 µM) and 1 µl of nuclease free water added to make a volume of 8 µl and placed in the PCR machine for 5 min at 65 ^{o}C for denaturation to take place, then followed by addition of 10 µl of M-MuLV Reaction Mix (2X) and 2 µl of M-MuLV Reaction Mix (10X) making a total volume of 20 µl. It was then incubated in the PCR machine at 5 min for 25 ^{o}C, 42 ^{o}C for 1h and inactivated for 5 min at 80 ^{o}C to produce the complementary (cDNA) to be used for PCR amplification of rotavirus. All the cDNA samples were stored at -20 ^{o}C until ready for use (Jain et al., 2016).

2.4. Semi-Nested Multiplex RT-PCR for G and P Genotyping

Genotyping of positive samples was performed according to the international standard of genotyping method with specific primers for regions of the genes encoding the VP7 (G-type) and VP4 (P-type) as developed by Iturriza-Gomara et al., (2001); Gouvea et al., (1990); Simmond et al., (2008) and Gentsch et al., (1992) and approved by WHO.

2.5. VP7 Amplification

The first round of PCR amplification of the VP7 gene was performed by adding 2.5 µl of cDNA into a master mix of 12.5 µl (10 mM dNTP's, 50 mM MgCL2, x10 Buffer ll, Taq Polymerase 5U/ µl) in a clean Eppendorf tube, 0.2 µl each of the forward primer VP7-F (5'- GGC TTT AAA AGA GAG AAT TTC CGT CTG G- 3') and the reverse primer VP7-R (5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3') were added, followed by 9.6 µl of Rnase free H20 making a total volume of 25 µl. PCR was performed on a thermocycler under the following conditions: 2 minutes at 94 °C; 30 cycles of 60 seconds at 94 °C, 30 cycles of 60 seconds at 52 °C, 30 cycles of 60 seconds at 72 °C, and a final step of 7 minutes at 72 °C followed by holding at 15 °C according to the protocol described (Iturriza-Gomara et al., 2001; Gouvea et al., 1990).

The second round VP7 multiplex was performed in 25 µl total volume containing a 0.2 µl of the first PCR product as a template (VP7-R) to 5 µl of master mix (10mM dNTP's, 50 mM MgCL2, X10 Buffer ll, Taq Polymerase 5U/ µl), 0.2 µl of each G-type-specific primer (G1-G4, G8-G12), 1 of the templates and 17.2 of Rnase- free H2O. The thermal cycling conditions were as follows: 5 minutes at 94 °C, followed by 30 cycles at 94 °C for 1 minute, 30 cycles at 42 °C for 2 minutes, 30 cycles at 72 °C for 1 minute, then a final extension step of 7 minutes at 72 °C followed by holding at 15 °C. Both the positive and negative controls were run parallel along with the test samples. The amplified products were analysed on 1% agarose gel and the genotype determined based on the size of the resultant amplicon (Iturriza-Gomara et al., 2001; Gouvea et al., 1990).

2.6. VP4 Amplification

The first-round of PCR amplification of the VP4 gene was performed by adding 2.5 µl of cDNA into a master mix of 12.5 µl (10 mM dNTP's, 50 mM MgCl2, x10 Buffer ll, Taq Polymerase 5 U/ µl) in a clean eppendorf tube, 0.2 µl of the forward primer VP4-F (5'-TAT GCT CCA GTN AAT TGG - 3') and the reverse primer VP4-R (5'- ATT GCA TTT CTT TCC ATA ATG -3') were added, follow by 9.6 µl of Rnase free H20 making a total volume of 25 µl. PCR was performed on a thermocycler under the following conditions: 2 minutes at 94 °C; 30 cycles of 60 seconds at 94 °C, 30 cycles of 60 seconds at 50 °C and the last 30 cycles of 60 seconds at 72 °C, and a final step of 7 minutes at 72°C followed by holding at 15 °C (Simmond et al., 2008; Gentsch et al., 1992)

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The second round VP4 multiplex was performed in 25 µl total volume containing 0.2 µl of the first PCR product as a template (VPR-R) to 5 µl of master mix (10 mM dNTP's, 50 mM MgCl2, X 10 Buffer ll, Taq Polymerase 5 U/µl), 0.2 µl of each P-type-specific primer (P4, P6, P8-P11), 1 µl of the template and 17.2 µl of Rnase- free H2O. The thermal cycling conditions were as follows: 4 minutes at 94 °C, followed by 30 cycles at 94 °C for 1 minute, 30 cycles at 45 °C for 2 minutes, 30 cycles at 72 °C for 1 minute, then a final extension step of 7 minutes at 72 °C followed by holding at 15 °C. Both the positive and negative controls were run parallel along with the test samples. The amplified products were analysed on 1% agarose gel and the genotype determined based on the size of the resultant amplicon (Simmond et al., 2008; Gentsch et al., 1992)

2.7. Agarose Gel Electrophoresis

One gram (1 g) of agarose was measured and poured into 30 ml of 1xTBE in a conical flask and mixed very well, 70 ml of 1xTBE was added to make 100 ml, it was microwave for 1-3 min until the agarose was completely dissolved and allowed to cool to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins, 1 µl of ethidium bromide was added and swirl to mix very well and pour into a gel tray with the well comb in place and allow to solidify at room temperature for 20-30 mins (Chudzio et al., 1989).

2.8. Loading of Samples and Running of Agarose Gel

Once solidified, the agarose gel was placed into the gel box (electrophoresis unit) and fill the gel box with 1xTBE until the gel is covered, about 3 µl of gel loading dye was added to each amplicon and gently mix, ladder was carefully loaded into the centre lane of the gel and samples into the additional wells of the gel; the gel was run at 80-150 V for 30 min; The power was turned off, the electrodes disconnected from the power source, and the gel carefully removed from the gel box. UVP Photodoc-It Imaging System (Fisher Scientific) was used to visualize the DNA fragments referred as 'bands' due to their appearance on the gel alongside 100 base pair ladders (Dubai et al. 2015).

2.9. Sequencing and Phylogenetic Analysis of Rotavirus Outer Proteins

Representative amplicons for G and P genotype whose primary amplification showed intense brightness were randomly selected across all sentinel sites for sequencing which was performed at Inqaba Biotechnical Industry (Pretoria, South Africa). Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10 uM PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96 °C for 10s, 55 °C for 5s and 60 °C for 4min. Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT) (Juliana et al., 2019).

2.10. Statistical Analysis

All the data obtained from each patient were entered into a spreadsheet created in MS Excel. Data were analyzed using the Statistical Package for Social Sciences (SPSS for IBM software version 21.0). The Chi-square test was used to test the statistical significance of differences observed between different proportions. A P value < 0.05 was considered as an indicator of statistical significance. Descriptive summaries were presented in Tables and Figures.

 doi:10.20944/preprints202405.1456.v1

3. Results

3.1. Rotavirus Infection and Socio-Demographic Factors

From February 2019 to January 2020, 294 children with acute diarrhoea were admitted in Ikom Medical Center and were tested for rotavirus infection. Out of 294 stool samples collected for this study, 20.4 % (60/294) were found to be positive for rotaviruses. The socio-demographic factors of 294 children in relation to rotavirus infection is presented on table 1. Patients were divided in six age groups from 1-60 months, high-rate infection of rotavirus 36.4% was recorded among patients 21-30 months. Males and females' participation was relatively equal 50.3% (148/294) and 49.7% (146/294) respectively but infection was higher in male 22.3% (33/148). Nigerian children representation was very high but Cameroonian kids had the highest infection rate 46.7% (21/45). There was a statistical difference among nationality group ($P < 0.05$).

Table 1. Socio-demographic factors of children infected with diarrhoea.

Gender	**No. Tested**	**No. of positive**	**No of Negative**	**p-value**
Male	148(50.3)	33(22.3)	115(77.7)	p=0.41
Female	146(49.7)	27(18.5)	119(81.5)	
Total	294	60	234	
Nationality				
Nigerian	249(84.7)	39(15.7)	210(84.3)	p=0.000
Non-Nigerian	45(15.3)	21(46.7)	24(53.3)	
Total	**294**	**60**	**234**	
Age group				
01-10	57 (19.4)	15(26.3)	42(73.6)	p=0.1
11-20	39(13.3)	6(15.4)	33(84.6)	
21-30	33(11.2)	12(36.4)	21(63.6)	
31-40	42(14.3)	6(14.3)	36(85.7)	
41-50	30(10.2)	6(20)	24(80)	
51-60	93(31.6)	15(16.1)	78(83.9)	
Total	294	60	234	

The socio-demographic factors of 294 children in relation to rotavirus infection among Nigerians and Non-Nigerian children admitted at Ikom Medical Center.

The G type (VP7) produced seven (7) different strains of VP7 detected were G1, G2, G4, G8, G9, G10 and G12. The most predominant G genotype observed was G4 with a prevalence of 26.7 % (16/60), followed by G9 20 % (12/60). Fifteen (15) children had infections of dual nature 25% (15/60) with G2G8 group the most prevalent with 6.7% (4/60). Two (2) samples had mixed infection of triple nature G2, G9G12 with prevalence rate of 3.3 % (2/60).

The P types (VP4) were successfully determined in 30 samples out of 60 positive rotaviruses by combo 50 % (30/60). Five (5) samples were found negative with conventional polymerase chain reaction 8.3 % (5/60) and 25 samples 41.7 % (25/60) were untypeable. The four (4) P different strains of VP4 detected were P [4], P [8], P[10] and P[11. The most predominant P genotype observed was P [4] with a prevalence of 23.3 % (14/60), followed by P [8] strain 10 % (6/60), P [10] and P[11] prevalence's rate were 6.7 % and 3.3% respectively. The mixed infections found in this study population were P [8]P[10]. The P [6] and P [9] genotypes were not found in circulation during this study period.

For each isolate the G typing and P typing results were combined to get the G-P combination genotype. Thirty (30) G-P combinations were successful 50 % (30/60) in which 19 were normal combinations 63.3 % (19/30) and 11 were mixed combinations 36.7 % (11/30). G4P [4] was found to be the prominent combination at 11.7 % (7/60) followed by G12P[4] with a prevalence rate of 6.7 % (4/60). Other combinations such as G4P [11] and G9[10] were represented at a low frequency of 1.7 % (1/60) each. The most prevalent mixed infections for the G-P combination were G2G8P [10] and G4G10P [8]

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at a frequency of 3.3 % (2/60). The presence of P[NT] (non-typeable) strain of P in combination with G typing produced G4P[NT] and G12P[NT] at a frequency of 10 % (6/60), followed by G9 P[NT] with the frequency of 8.3 % (5/60). Mixed infections of dual nature of G type with P[NT] were reported in G1G4P[NT] and G2G8P[NT] with prevalence of 5 % (3/60) and 3.3% (2/600 respectively. Mixed infection of triple nature of G type in combination with P[NT] was recorded in 2 samples G2G9G12 P[NT] at a prevalence rate of 3.3 % (2/60). (Table 2).

Table 2. Distribution of rotavirus genotypes among children with diarrhoea.

	VP4 type							
VP7 type	**P[4]**	**P[8]**	**P[10]**	**P[11]**	**P[8]P[10]**	**P[NT]**	**Negative**	**Total (%)**
G4	7	0	0	1	2	6	0	**16(26.7)**
G9	2	3	1	0	1	5	0	**12(20)**
G12	4	0	0	0	0	6	0	**10(16.7)**
G1G4	0	0	0	0	0	3	0	**3(5)**
GIG9	0	0	0	0	0	1	0	**1(1.7)**
G2G8	0	0	2	0	0	2	0	**4(6.7)**
G4G10	0	2	0	1	0	0	0	**3(5)**
G4G12	1	0	0	0	1	0	0	**2(3.3)**
G9G12	0	1	1	0	0	0	0	**2(3.3)**
G2G9G12	0	0	0	0	0	2	0	**2(3.3)**
Negative	0	0	0	0	0	0	5	**5(8.3)**
Total (%)	**14(23.3)**	**6(10)**	**4(6.7)**	**2(3.3)**	**4(6.7)**	**25(41.7)**	**5(8.3)**	**60**

Distribution of rotavirus genotypes among children with diarrhoea.

3.2. Sequence Analysis of Rotavirus Compared to NCBI.

Identification of genotypes of the sequences was achieved by comparison with reference sequences available in the NCBI GenBank database using BLASTN. The G1-VP7 fragment of the isolate showed a percentage similarity of 98 % to Rotavirus A strain MD-51 (MH381891), MD 49 (MH381890) and 100% to Rotavirus A isolate NGR (MN304728). The P1-VP4 and P2-VP4 fragments showed a percentage similarity of 99 % to Rotavirus A isolated NGR, and Rotavirus A isolated RV/IND/TG/GMC/2016/1194/P8 respectively.

4. Discussion

Rotavirus gastroenteritis is responsible for substantial morbidity and mortality among children younger than 5 years of age (Negar et al., 2019). Rotavirus gastroenteritis has previously accounted for an estimated 1.9 million episodes per year of severe acute gastroenteritis requiring hospital admission among children younger than 5 years of age. In 2013 alone, an estimated 215,000 deaths related to rotavirus gastroenteritis occurred in this age group, with four countries (Nigeria, Pakistan, India, and the Democratic Republic of the Congo) accounting for 49 % of these deaths (Tate et al., 2016). Group A rotavirus has consistently been reported to be the single most common cause of diarrhoea worldwide. Better hygiene and sanitation have not been very effective in reducing rotavirus disease. This is illustrated by the fact that virtually everyone in developed and developing countries is infected by rotavirus disease during the early years of life, despite differences in sanitation between countries (Tate et al., 2012; 2016). In this study, 20.4 % recorded for rotavirus infection is following the 20.8 % reported by Sani et al., (2022) in Zaria Nigeria but lower than the 23% reported by Digwo et al., (2023) in another study conducted in Nigeria. Outside Nigeria, a lower prevalence of 14.8% was reported in Ghana by Flavia et al., (2023) while high infection rates of 24.5 % and 30.6% were recorded in Palermo and Southwestern China by Emmanuel et al., (2023) and Longyu et al., (2022) respectively.

4.1. Distribution of VP7 Genotypes

 doi:10.20944/preprints202405.1456.v1

The current study revealed the presence of the following VP7 genotypes: G1, G2, G4, G8, G9, G10 and G12. The result is in accordance with the systematic review of Babatunde et al., (2018) who reported the same genotypes in circulation in other parts of Nigeria. The genotype G4 was the most predominant 26.7% (16/60). This result is lower than the 59.3% reported by Alkali et al. (2016) in Sokoto Nigeria. Another study in Enugu State by Tagbo et al., (2019) reported a very low rate of 1.2 % of G4. Contrary to this study, Japhet et al., (2019) reported 0 % of G4 in Ile Ife a South–Western part of Nigeria. Our findings for G9 20 % (12/60) are lower than the 28% detected by Amadou et al. (2019) Ilorin Nigeria.

The G9 genotype was first identified in the USA in 1983 and remained undetectable for approximately a decade. It re-emerged in the mid-1990s, and due to the increased number of countries that have reported G9, it has become the fifth most prevalent genotype globally (Doro et al., 2014). This genotype G9 has been detected sporadically and in localized outbreaks in various African countries, including South Africa, Botswana, Malawi, Kenya, Cameroon, Ghana, Nigeria, Guinea-Bissau, Libya and Mauritius (Page et al., 2010). Outside the continent, high occurrence of G9 genotype 64.4 % and 76 % were reported by Tian et al., (2018) in Beijing and Zhao et al., (2021) in Shanxi Province, China. Moreover, the G9 genotype has been humanized since early 2000, it has been reported to be associated with more severe diseases (Silapong et al., 2017). Before it was a typical rotavirus porcine genotype but today this is still one major VP7 genotype of rotavirus infecting pigs (Donato et al., 2012; Theuns et al., 2015).

The existence of genotype G12 was also reported in this study with a prevalence rate of 16.7 % (10/60). This finding is above the report of Moussa et al., (2017) who reported 5.2 % of G12 in circulation in Tunisia while Boni-Cisse et al., (2018) reported high prevalence of 27 % in Ivory Coast. This G12 strain is not included in the target strains of the two vaccines recommended by WHO and should be a major concern. Its emergence has been observed globally in several studies. In Thailand, Maneekarn et al., (2014) observed the predominance of the G12 from 2007 to 2009. In Australia, Kirkwood et al., (2014) reported incidence of G12P [8] 23 % in 2012, and Wylie (2014; 2015) reported an emergence of this strain in the same year in Saint Louis, United States. This emergence was also observed in Cameroon and Nigeria during that period by Ngum Ndze et al., (2012) and Ayolabi et al., (2013) respectively. The G12 strain was also reported by Rakau et al., (2021) to be in circulation in other African countries and are closely related, irrespective of country of origin and year of detection, except for the Ethiopian strains that clustered distinctly. The reason for its rapid spread has not yet been fully understood and could be attributed to its relatively recent introduction in countries such as Nigeria. The global spread of G12 rotavirus strains from the Philippines to USA, South America, South Asia, Africa and finally to Europe appeared to be like that of G9 which became the fifth most important global genotype in the late 1990s (Iturriza Gómara et al., 2003; Santos et al., 2005). Although Malik et al., (2014) suggested that G12 rotavirus strains came from evolution of rotavirus porcine, till date the exact origin of G12 strain remains unclear. The diversity found among the G12 strains described worldwide may suggest either accidental human infection by animal rotavirus strains or reassortant rotaviruses generated in nature between animal and human RVA strains. Since animal rotaviruses and human–animal reassortant rotaviruses circulate simultaneously in the environment, more intensified investigation of animal rotavirus strains should be conducted to evaluate the origin of emerging genotypes. Indeed, such survey may identify the possible animal ancestor of each new genetic lineage (Moussa et al., 2017). In Cross river state, no previous studies are available on the characterization of rotavirus, so it is not known if the dominance of G4, G9 and G12 in this study is attributable to natural fluctuation of rotavirus genotypes or if they represent a unique situation. Their emergence indicates that no barriers can prevent emerging strains to any corners of the world.

The high rate of mixed VP7 genotype in this study 25 % (15/60) with G2G8 been the most predominant 6.8% (4/60) is concordant with others finding in Northern Nigeria (Aminu at al., 2010). Nevertheless, studies reported far back by Adah et al., (1997), Pennap et al., (2000) and Audu et al., (2002) revealed another rotavirus strains G1G3 specificity which was not detected in this study. It is important to note that the other studies have used different techniques, including antigenic analyses with monoclonal antibodies (Pennap et al., 2000) and RT-PCR typing with various primers.

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Therefore, though these mixed infections should be further studied to elucidate this phenomenon in Nigerian children (Aminu et al., 2010) and their effect on the development of new vaccine should thoroughly be investigated.

4.2. Distribution of VP4 Genotypes

The genotype P[4] by far prevailed 23.3 % (14/60) followed by P[8] with prevalence rate of and 10 % (6/60). The predominance of P[4] genotype in this study is closely in accordance with the publications of Pimmada et al., (2019) in Thailand and that of Giri et al., (2020) in India who reported 25.6 % and 25.3 % respectively of P[4] genotype in children with gastroenteritis. A previous study conducted by Wardana et al., (2015) in Indonesia reported P[4] prevalence rate of 31.8 % among children with severe diarrhoea. Others studies conducted by Gikonyoa et al., (2020) in Nairobi and Sadik et al., (2019) in Pakistan reported a close related prevalence's of 20.4 % and 22 % respectively of P[4] in circulation in their respective locations. On the other hand, Shrivastava et al., (2019) in India reported low prevalence of P[4] genotype 6.5 % while Alkali et al., (2016) in Sokoto Nigeria reported 0% among children.

Our result for P[8] is lower than that of Sadik et al., (2019) who reported 39 % in Pakistan and Manar et al., (2021) who reported 33.6 % at Zagazig University Hospitals. Very high prevalence's of 75.6 % and 85.4 % were also reported by Vrdoljak et al., (2019) in Croatia and Eva et al., (2020) in Mozambique. In disagreement with our result, recent literature worldwide showed that P[8] is the most prevalent genotype (Abugalia et al., 2011; Khoury et al., 2011).

The only mixed P genotype detected in this study was P [8]P[10] at a frequency of 6.7 % (4/60). Mascarenhas et al., (2007) suggested that the comparatively low rate of mixed rotavirus P-type infections may be associated with the high (about 50%) proportion of nosocomial diarrhoea cases, which are mostly associated with one or two (largely predominant) strains circulating at hospital environment.

The genotype P[10] and P[11] detected in this study are unusual serotype and have also been reported from other African countries like Cameroon, Ghana, Libya and they are currently emerging from different part of the word (Esona et al., 2010). Very few data about P [11] are available in literature (Matthijnssens et al., 2010). In this our present study, this genotype was associated with G4 genotype and mixed G4G9 which are unusual combinations.

Non typeable P[NT] were recorded at a very high prevalence of 41.7 % (25/60) which disagrees with Sprengers et al., (2015) who reported that only 1.6 % of his cases were non-typeable. The detection of a high proportion of untyped or non-typeable samples in our study could be due to very low number of viral particles with intact RNA in stool samples, non-recognition of the viruses by the primer sets due to point mutations at the primer binding sites, or the viruses belonging to genotypes which are not included in the primer set used in the RT-PCR assays (Santos et al., 2005; Saluia et al ., 2014). Further investigations of these untyped P genotypes will be required to confirm their presence and determine their genetic character (Aminu et al., 2010).

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5. Conclusions

This study revealed an important rate of 20.4% of rotavirus in circulation in Cross river state. Risk factors that were reported to significantly influence the prevalence of rotavirus in this study were nationality, feeding status, toilet system and number of days of diarrhoea. Genotyping analysis revealed that G4 and P [4] were the major genotypes present in cross river state. The high percentage of non-typeable P[NT] in combination with one of the G strains to produce G2G9G12P[NT] is an indication that children probably acquire rotavirus infection from various sources and could serve as sources of new strains globally (Giri et al., 2020). This study to the best of our knowledge is the first comprehensive report to provide an estimation of the prevalence and genotypes of rotavirus associated with childhood diarrhoea in Cross River State and can be used as baseline for evaluation of vaccine implementation.

It is crucial to discover and introduce simple, inexpensive rapid tests for rotavirus and other enteric pathogens detection should be encouraged in local health centres and this can be beneficial on the other hand since it can be the basis of the national surveillance system. Studies on etiological agents of diarrhoea should focus on all known enteric pathogens associated with diarrhoea and not be limited to rotavirus only. This will help know the true aetiology of diarrhoea disease and allow the design of prevention and treatment strategies, availability and affordability of some sequences methods which provide alternative tools for characterization of unusual strains, all the non-typeable strains should be subjected to sequencing analysis. Since the diversity of primer sequences are needed to identify non-typeable strains, there is a need to design a cocktail of primers that use both human and animal primer sequences to identify non-typeable strains.

Authors' Contributions: Konlak D Gilbert: Conceptualizing, data analysis, writing of the first draft, Euslar Nnenna Onu; supervisor, Okorie Nnaemeka; writing review and editing, Azi Cornelius Ituma; visualisation Ubong Aniefiok Udoh; writing the original draft, Okparaoka Samuel Uchechukwu writing the original draft, Ernest Ochang; Project administration, Okparaoka Vivian Chisom Project administration, Chinedu Obasi Akpa; writing methodology, Samuel Eleojo Agada writing method and study design, Okechukwu Emmanuel Ogbonna; investigator, Ogbonnaya Ogbu; investigator.

Acknowledgments: We would like to express our gratitude to all participants enrolled in this study and the entire medical personnel of Ikom Mother Health Care Centre for their great cooperation in this research. We would also like to say thank you to the Coordinator of Nigeria Red Cross River for providing his laboratory premises for the primary analysis.

Conflicts of Interest: The authors declare that they have no conflicts of interest or funding disclosure.

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