

Genetic Survey of Human Rotavirus Strains Involved in Gastroenteritis among Children in Four North-Central States of Nigeria

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Abstract

A total of six hundred stool samples collected from children and infants (0-5 years) presenting diarrhea at the hospital located in the four north central states of Nigeria (Niger, Kwara, Nasarawa and Federal Capital Territory) were examined for possible detection of rotavirus antigens using enzyme linked immunosorbent assay. 27 cases (5%) were found to be positive. The result of the reverse transcription (RT) of the viral RNA to cDNA revealed that, the vp7 gene appeared large and bold while the vp4 gene had small and faint bands. Further identification of the rotavirus isolated using Reverse transcription polymerase chain reaction (RT-PCR) and Agarose Gel electrophoresis, indicated strains G1 (11%), G2 (15%), G8 (15%) and P4 (8%), P6 (11%), P8 (11%) as the strains identified. An indication that, the above mentioned strains may be the dominant strains in circulation in the study areas. Further investigation on the remaining parts of north central states is conducted in order to arrive at better conclusion on the strains of the virus present in the entire north central region.

Keywords: Gastroenteritis; Rotavirus; Strains; Diarrhea Enzyme linked immunosorbent assay.

Introduction

Diarrhea among children was conceived in the past to be a problem in the developing world where poor hygiene and sanitation, lack of clean water and proper food storage all favor the transmission of a host of different enteric pathogens [1]. None the less, in the developed world, despite all the improvements made in sanitation and the provision of clean food and water to the population, childhood diarrhea still remains one of the most common illnesses leading to hospital visits and hospitalization [2]. Why then does diarrhea continue to be a problem in both developing and industrialized nations of the world and what can we do to prevent the scourge?

The answer to this seeming paradox has only been elucidated in the past 30 years, with improvements in our understanding of the microbiology of gastrointestinal infections [1]. In 1970, only a few organisms such as Salmonella and Giardia lamblia, were recognized to cause diarrhea and with the diagnostic available, fewer than 10% diarrheal diseases could be linked to known infectious agents [1]. The remaining were attributed to conditions such as weaning, malnutrition or food allergy and the largest single diagnostic group was called "Idiopathic" a term indicating that no cause could be identified. Since 1970, a scientific explosion occurred with the discovery of more than 25 different pathogens that can cause diarrhea in children – novel viruses and others [3]. This development made the

field rich with new agents and became complicated that outside research setting, few laboratories or groups were capable of adequately establishing an etiologic diagnosis. Consequently, physicians and even research laboratories have continued to remain with an incomplete ability to detect the cause of diarrhea in their patients [1]. Despite these diagnostic shortcomings, some major advances have been made in our understanding of the causes of childhood diarrhea, their treatment and prevention [4].

First with economic development, improvements in water sanitation and hygiene, the incidence of diarrheal illness has declined so that while children in the developing world may have 3-7 episodes of diarrhea per year for the first few years of life, children in the developed world count on 1-2 episodes per year [5]. Furthermore, the agents responsible for children diarrhea have changed. In the developing world, children succumb to infections with a wide range of bacteria, parasites and viruses but with economic development, bacteria and parasites have become rare except in small outbreak settings such as daycare centres. This evolution in the profile of enteric pathogens reflect the observation that bacteria and parasites are infections spread by sewage, contaminated food or water and their transmission is aided by lapses in hygienic practices such as lack of refrigeration, closeness with domestic animals, absence of toilet facilities and limited access to clean water [6].

Unlike bacteria and parasites, most of the viruses seem to have a mode of transmission that is independent of these lapses of sanitation and hygiene. The viral agents of gastroenteritis have been termed “democratic” because all children are infected in their first few years of life and are therefore not likely to spread by poor hygiene and sanitation [1]. For instance children in the United States of America and Britain today will have 5-8 episodes of diarrhea in their first few years of life and for the most part, this will be caused by viruses. The “rogues gallery” of viral pathogens include rotaviruses and others [1]. Yet, even today, with all of our diagnostic advances, it would be difficult for even research laboratories to adequately detect the enormous agents involved in childhood diarrhea.

Rotavirus have become the flagship virus for all the groups of viruses involved in childhood diarrhea because it is the easiest to detect by simple diagnostic assays, it is the agent most likely to bring a child to the hospital for severe diarrhea and is now closest to control through the use of vaccines [6].

Rotavirus infection remains the most common cause of severe dehydrating gastroenteritis among children worldwide. Globally over 500,000 – 870,000 children between 0 – 5 years die every year from rotavirus gastroenteritis, with vast majority of these deaths occurring in the developing nations. In developed countries, rotavirus infection rarely results in death but remain the most common cause of hospitalization for acute gastroenteritis in children and leads to major medical and societal costs [7].

In 1998, the first vaccine against rotavirus, tetravalent rhesus – based rotavirus vaccine (RRV- TV), was approved by the United States Food and Drug Administration and recommended for inclusion in the 1999 U.S schedule for routine childhood immunization and other parts of the developing countries. In July 1999, this vaccine was withdrawn from circulation worldwide following reports of cases of intussusceptions among recently vaccinated children [4]. Since 1999, several important developments have improved the understanding of the natural history of rotavirus infection and intussusceptions, as well as the disease burden of rotavirus associated gastroenteritis. Efforts to develop a more potent and safe vaccine of intussusceptions gave birth to withdrawal of RRV – TV [4].

Epidemiological studies of rotavirus infection conducted in Africa and Nigeria in particular revealed the greatest degree of genetic diversity of strains in circulation [7]. Efforts are on-going by the World Health Organization and Africa regional network for rotavirus studies to reduce the disease burden through vaccine development, but this has been hampered due to continuous emergence of new strains (possible genetic re-assortment) of the virus in different regions of Africa. This ugly situation has continued to hinder the development of potent vaccine that could be used to stem the scourge of rotavirus gastroenteritis in Nigeria, and indeed other parts of Africa. Therefore, this study is an attempt to determine the genetic diversity of rotavirus strains in circulation within the north central states of Nigeria.

Material and Method

Sample Collection

A total of six hundred (600) stool samples was collected from children (0-5years) presented with diarrhea in the paediatric Department of the hospitals located in the capital of the four States under study. The states include Niger, Kwara, Nasarawa and Federal Capital Territory, Abuja. The samples

collection started in November, 2008 to November, 2009. The samples were collected after a written consent/questionnaire by the parent/guardians of the patient/target group. The stool samples was collected in sterile sample bottles and transported to the department of microbiology, faculty of science, Ahmadu Bello University (ABU), Zaria and stored at -200C in the refrigerator for further analysis.

Enzyme Linked Immunosorbent Assay (ELISA)

One hundred microlitre (100µl) of positive control reagent was introduced into well two and three. Also same quantity of Negative control reagent was introduced into well four and five. From well six to the 96th well, 100µl of the supernatant phase of the stool samples was introduced in each case separately and was incubated at room temperature for 30 minutes. The wells were washed with diluted buffer solution (wash solution). Two (2) drops of reagent one (blue solution) was introduced into all the wells except well one (blank). It was incubated at room temperature for 5 minutes and was rinsed with wash buffer. Two drops (2) of reagent two (red solution) was introduced to each well as specified above and was incubated at room temperature for 5 minutes and was washed again. Two drops of chromagen was added to each well according to manufacturers specifications and was mixed thoroughly using tapping strip holder. Finally two drops of stop solution was added to each well and was mixed. The results were recorded visually, and after setting the filters at 450nm the results were also recorded with the use of spectrophotometric and bichromatic device.

Extraction of Rotavirus RNA from Stool Samples

Three hundred micro liter (300µl) of lysis buffer was added to one hundred microliter (100µl) of stool samples. The mixture was transferred to collection tubes and centrifuge at 12,000 rpm for 2 minutes. The flow through from the collection tubes was discarded. 300µl RNA wash buffer was added to the column and was centrifuged at 12,000 rpm for 30 seconds. The flow through was again discarded and the zymo spin Column tubes was placed into the collection tubes. The above was repeated once. The zymo spin column tubes were spinned at 12,000 rpm for one minute in an empty collection tube in order to ensure complete removal of the wash buffer.

After the above, the zymo-spin column tubes were placed into the RNase free tubes. 10µl of the RNase free water was introduced into the column tubes and the mixture was allowed to stand at room temperature for one minute. Then the mixture was centrifuged at 12,000 rpm for one minute to elute the viral RNA from the stool samples and was used immediately for the generation of cDNA for VP7 and VP4 genes of the rotaviruses.

Generation of VP7 and VP4 cDNA

The cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) using extracted rotavirus dsRNA, as described below.

Generation of VP7 complimentary DNA (cDNA)

In order to generate full length VP7 cDNA from the dsRNA extracted from the stool samples, 8 micro liter (8µl) of the extracted dsRNA was introduced into 500µl PCR tube. Then the primer pair sBeg9 and End9 were used at concentration of 1µl each. The mixture was heated to 95°C for 5 minutes to denature dsRNA and was cooled on ice immediately for 2 minutes. Reverse transcriptase master mix (RT – MM) was introduced into the mixture above. Finally the mixture was centrifuged at 10,000rpm for one minute and was incubated at 42°C for 30 minutes for the generation of cDNA.

Generation of VP4 cDNA

The procedure for the generation of VP4 cDNA was similar to that of VP7 cDNA except that different primer pair (Such as con2 and con3) was used, at concentration 1µl [8,9].

Preparation of Agarose Gel

Twenty milliter (20ml) Tris acetate buffer (TAE buffer) was mixed with 80ml of distilled water to make it 100ml. 2gramm of agarose was weigh and was dissolved in the solution. The mixture was heated at 85°C for 2 minutes to ensure complete dissolution of the agarose. The gel was allowed to cool at 45°C, after which 10µl ethidium bromide was added to the gel and was allowed to diffuse into the gel.

Casting of the Gel

The gel was poured into the gel cassette tray with a comb in place. The gel was allowed to set at room temperature for 45 minutes. The tray was submerged beneath TAE running buffer in an electrophoresis apparatus and the comb was removed.

Loading of the Gel

Ten microliter (10µl) of each cDNA product was mixed with loading dye and loaded into separate wells of the gel. The first and the last wells of the gel were loaded with 1kb DNA ladder. The gel was electrophoresed at 100v for 45 minutes at room temperature. The electrophoretic apparatus was switched off and the gel was removed, it was viewed first under uv-light and the image captured (gel documentation machine). The bands for the cDNA of vp7 genes were identified as thick or bright bands and faint bands.

Amplification of VP7 cDNA

The amplification of vp7 cDNA was conducted using nested multiplex primers, to determine the specific genotypes (i.e. G-types) on the basis of the migration pattern on agarose gel. Eight microliter (8µl) of the vp7 cDNA was introduced into 500µl PCR tube, also 2µl of RNA water was mixed with the cDNA in PCR tube. In addition to the mixture above 15µl of the PCR-master mix was introduced to the PCR tube. Finally 1µl of each of the six G-type specific primers and 2µl of consensus primer such as Beg9 was added to the mixture. The samples were amplified in PCR (Tech gene....UK) machine programmed to run 30cycles as indicated below: Denaturing at 94°C for 1 minute, annealing at 42°C for 2 minutes extension at 72°C for 3 minutes and final extension at 72°C for 7 minutes.

Amplification of vp4 cDNA

The procedures for the PCR of vp4 cDNA was similar to that of vp7 cDNA except that, the primers used specifically for determination of P-types were different from the ones used for NP7 cDNA (G-groups). In the same vein the loading procedure was same as conducted in the case of vp7 cDNA [9] and the results obtained was also documented.

Results

The result of the reverse transcription (RT PCR) of the viral RNA to cDNA revealed that, the G and P genotypes of the rotaviruses identified was G1 (11%), G2 (15%), and G8 (15%). The P4 (8%), P6 (11%) and P8 (8%) (Table 1 & 2).

Table 1. Distribution of rotavirus G-types rotavirus

G – TYPE (Serotype)	FREQUENCY	PERCENTAGE (%)	STUDY AREA
G1	3	11	Kwara, Niger, FCT. FCT, Niger Kwara, FCT, Niger and Nasarawa
G2	4	15	
G8	4	15	
NT	16	59	

G1 = Serotype G1; G2 = Serotype G2; G8 = Serotype G8; NT = Non Typeable

Table 2. Distribution of P-types rotavirus

P –TYPE (Genotype)	FREQUENCY	PERCENTAGE (%)	STUDY AREA
P4	2	8	FCT, Kwara Niger, Kwara, FCT FCT, Nasarawa
P6	3	11	
P8	3	11	
NT	19	70	

P4 = Genotype P4; P6 = Genotype P6; P8 = Genotype P8; NT= Non typeable

Discussion

Genetic (strains) diversity of rotavirus was observed with relative prevalence of G1, G2, G8 (Table 1). The P-types identified also include P4, P6 and P8 (Table 2). This present study represents the first and elaborate attempt to identify the genetic diversity (strains) of rotaviruses in four north central states of Nigeria. Although only few G-P genotypes were identified in this study, about 64% represented the globally most common strains (G1 and G2) (Table 1). No G3 and G4 were detected. The G9 strain, which is an emerging strain worldwide, was also not identified of all the positive samples analysed. The uncommon G1 strain was identified in 11% of the samples (Table 1). Other globally uncommon genotypes that was also identified in this present study was G2 (15%) (Table 1). This makes the result of this present study comparable with previous studies [9,10]. The relatively high frequency of non typeable strains of rotavirus is quite unusual in tropical countries particularly in Africa (Table 1 & 2). However earlier report [9], have indicated that, dual infection could some times lead to non typing strains due to genetic reassortment of the rotaviruses. We therefore hypothesize that, the high rate of nontypeable strains of rotavirus in this study could also be due to methods applied including singles locus PCR.

We found no G3 strain (usually common with cats and dog) but a relatively high frequency of G2 (15%) (Table1). The probable reasons could not be established in this and earlier reports. However, we believe that a more likely explanation for the occurrence of strain G2 at a relatively high frequency and uncommon G1 and G2 types among the rotavirus specimens has been under reported in previous studies [7,10,11].

Following our experience in this present study, the use of multiplex PCR in the identification of rotavirus strains and the high frequency of non typeable rotavirus should be handled with sufficient caution. The possibility of non specific primers binding has to be considered as a critical factor that may have contributed substantially to the high frequency of non typeable strains. Further more, in non typeable strains, the rotavirus might have been present at a low concentration, resulting in an uneven amplification, making the interpretation or the visibility of the bands pattern difficult or completely absent as the case may be.

We were also aware of the risk of contamination when so many samples were handled, however adequate measures were taken (such as wearing of facial mask, conducting the molecular part of the work in an insolated and aseptic condition) in order to reduce level of contamination to the bearest level. The predominant P-types identified in this study were P6 and P8 (Table 2). It is interesting to note that P6, an usual strain formerly known to infect predominantly neonates, is now an important cause of diarrhea in children in four north central states of Nigeria as revealed in this study. A similar trend has been reported in previous studies carried out in parts of Africa [12,13]. It is also worth nothing that, P4 occurred in a low frequency compared with other P-types (P6 and P8) (Table 2) in this study. What is important is that, strain P4 was identified as one of the globally important P-type in this study. The outcomes conform with earlier reports of [10,8,12].

A general comparative analysis of the methods used in this study to detect rotavirus found the ELISA technique to be highly sensitive, more reliable and specific compared with other techniques such as latex agglutination technique. RT-PCR and Electrophoresis are known to be the most sensitive techniques for the determination of rotavirus serotypes [12]. This sensitivity was also noticed in this study.

Conclusions

The result of the investigation revealed that, strains G1, G2, G8 and P4, P6, P8 as the common strains in circulation in the study areas. Further investigation should be conducted in other parts of north central states to arrive at better conclusion on the etrains of the virus in the entire region.

Conflict of Interest

The authors declare that they have no conflict of interest.

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