Epidemiology and Molecular Identification of Rotavirus Strains Associated With Gastroenteritis in Children in Niger State.

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Abstract

The study was conducted to determine the genetic diversity of the rotaviruses associated with gastroenteritis in children in Niger State. A total of 150 stool samples were collected from diarrheic children (0 – 5 yrs) in four hospitals (Minna, Bida, Suleja and Kontagora) in Niger State. The stool samples were screened for rotavirus, using Enzyme linked Immunosorbent assay (ELISA). Eight stool samples were positive (5.33%). The prevalence of the disease according to sex of the children was as follows; male (4%) and female (1.33%). The prevalence of gastroenteritis according to water source was distributed as follows; pipe borne (2%), bore hole (1.33%), and river/stream (2%). The prevalence of rotavirus gastroenteritis among children according to the breast feeding was as follows; exclusively breastfed children had (2%) and those breastfed only recorded (3.33%). Molecular identification of the virus revealed the presence of the following genotypes: P6 (22%), G1 (22%), G2 (33%), and G8 (22%) in the study area.

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Introduction.

The epidemiology and natural history of rotavirus gastroenteritis seem quite straight forward. All children may be infected in their few years of life. The first infections are usually symptomatic and subsequent infections are silent, rarely causing symptoms and are associated with very little if any shedding (Glass *et. al.,* 2006; Wales, 2008).

Humans appear to be the main reservoir of infection but rare or novel strains can creep into humans, often representing strains from animals. In temperate climates, rotavirus has a winter peak, whereas in the tropics and the developing nations, the disease can occur year round and at a slightly earlier age (Glass *et. al.,* 2006). While outbreaks do occur in day care settings and in hospital, they probably reflect the density of immunologically naïve groups of children who are highly susceptible, the short incubation period of infection 18 – 36 hours and the low inoculums required to cause infection making spread rapid and efficient (Glass *et. al.,* 2006). In fact, children not exposed to these settings will be infected as well, although perhaps not as early or as part of an identifiable outbreak. The rationale for global programs to develop rotavirus vaccines has rested upon the enormous burden of the virus diarrhea in children.

In Nigeria, an estimated 33,000 children die each year from rotavirus gastroenteritis. Efforts to develop a candidate vaccine that could be used to bring down the disease burden, has been hampered due to continuous emergence of new rotavirus strains particularly in Africa (Aminu *et. al.,* 2008). Therefore, this study is aimed at identifying the strains of rotavirus in circulation in Niger State, in order to enrich the national data bank for rotavirus vaccine development.

Materials and Methods.

Description of the study area

Niger State

It is in the North Central part of Nigeria and the largest in terms of land mass in the country. The State Capital is Minna. It was created on 3rd February, 1976 from the defunct North Western State. It has a land area of 76,363 square kilometers and human population of 4,082,558 people. The State is made up of 25 Local Government Areas (2006 Census). Two of Nigeria's major hydroelectric power stations – the Kainji and the Shiroro Dams – are located in Niger State.

Sample Collection.

One hundred and fifty (150) stool samples were collected from diarrheic children (0-5years) at the paediatric departments of four hospitals located in Niger state. The samples were transported to the Center for Biotechnology Research and Training, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. They were stored in the temperature regulated refrigerator at -20^{0}C for further analysis.

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Preparation of Frozen Stool Samples for Enzyme linked Immunosorbent Assay.

150 frozen stool samples were thawed and each diluted to a ratio of 1:5 by adding 1 gram of stool sample to 4ml of dilute wash buffer in each case. These were mixed thoroughly and allowed to settle. The supernatants was collected and used for Enzyme linked immunosorbent assay (Aminu *et. al.*, 2009).

All the stool samples were screened as follows: One hundred microliter of positive control reagent was introduced into well two and three of the 96 titre plates. 100 microliter of negative control reagent was introduced into well four and five. 100 microliter of supernatant phase of the 150 stool samples were introduced into well six to the 96th well separately. The plates were incubated at room temperature for 30 minutes. The wells were washed with dilution buffer solution. Two drops of reagent one (blue solution) was introduced into each of the wells except well one (blank).The plates were then incubated at room temperature for 5 minutes and was rinsed with wash buffer. Two drops of reagent two (red solution) was introduced into each well and incubated at room temperature for 5 minutes and washed again. Two drops of chromogen was added to each well and was mixed thoroughly. Finally, two drops of stop solution was added into each well and was mixed. Visual observation and readings from spectrophotometric and bichromatic device was carried out and recorded.

Extraction of RNA

Enzyme linked immunosorbent assay positive stool samples were subjected to rotavirus RNA extraction as follows:

Three hundred (300) microliter of lysis buffer was added to 100 microliter of each diluted positive stool sample. The mixture was transferred to collection tube and centrifuged at 12,000 rpm for 2 minutes. The flow through from the collection tubes was discarded. 300 microliter 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 zymospin column tubes were placed into the collection tubes. 300 microliter of RNA wash buffer was added to the column tubes, spinned at 12,000 rpm for 30 seconds, and the flow through from the collection tubes was also discarded. The zymospin column tubes were further spinned at 12,000 rpm for one minute in an empty collection tube in order to ensure complete removal of the wash buffer.

The zymospin column tubes were placed into RNase free tubes. 10 microliter of the RNase free water was introduced into the column tubes and the mixture was allowed to stand at room temperature for one minute. The mixture was then centrifuged at 12,000 rpm for one minute to elute the viral RNA from stool samples and was used immediately for the generation of cDNA for VP7 and VP4 genes of the virus.

Generation of VP7 cDNA.

Eight (8) microliter of the RNA was introduced into 500 microliter PCR tubes. A One microliter primer pair (Beg 9 and End 9) was introduced into PCR tubes. The mixture was heated to 95^{0}C for 5 minutes to denature the dsRNA and was cooled on ice immediately for 2 minutes. One microliter reverse transcriptase (RT) was introduced into the mixture. Finally the mixture was centrifuged at 10,000 rpm for one minute and was incubated at 42^{0}C for 30 minutes for the generation of cDNA.

Generation of VP4 cDNA

Eight(8) microliter of the extracted RNA was introduced into 500 microliter PCR tubes. One microliter each of the primer pairs (con2 and con3) were introduced into PCR tubes. The mixture of the RNA and primer was heated to 95^{0}C for 5 minutes to denature the dsRNA and was cooled on ice immediately for 2 minutes. Reverse transcriptase (RT) was introduced into the mixture. Finally the mixture was centrifuged at 10,000 rpm for one minute and was incubated at 42^{0}C for 30 minutes for the generation of cDNA.

Preparation and Casting of gel.

Twenty milliliter (20ml) tris acetase buffer (TAE) was mixed with 80 ml of distilled water to make 100ml. Two grammes of agarose was weighed and dissolved into the solution. The mixture was heated at 85^{0}C for 2 minutes. The gel was allowed to cool to 45^{0}C, after which 10 microliter ethidium bromide was added and allowed to diffuse into the gel. The gel was poured onto the gel cassette tray with a comb in place. The gel was allowed to set at room temperature for 45 minutes. The tray was

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submerged beneath TAE running buffer in an electrophoresis apparatus and the comb removed.

Loading of the Gel.

Ten (10) microliter of each cDNA product was mixed with loading dye and loaded into separate wells on the gel. The first and the last well of the gel were loaded with 1 kilobase 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 using gel documentation machine.

Amplification of VP7 cDNA.

Eight (8) microliter of the VP7 cDNA was introduced into 500 microliter PCR tube. Two microliter RNA water was added and mixed. In addition to the mixture, 15 microliter of the PCR master mix was introduced to the PCR tubes. Finally one microliter of each of the G-genotypes specific primers and 2 microliter of consensus primer such as Beg 9 and End 9 were added to the mixture. The samples were amplified in PCR machine programmed to run 30 cycles.

Amplification of VP4 cDNA.

Eight (8) microliter of the VP4 cDNA was introduced into 500 microliter PCR tube: Two microliter RNA water was added and mixed. In addition to the mixture, 15 microliter of the PCR master mix was introduced to the PCR tubes. Finally one microliter of each of the P-genotypes specific primers and 2 microliter of consensus primer such as Con 2 and Con 3 were added to the mixture. The samples were amplified in PCR machine programmed to run 30 cycles.

Two percent (2%) agarose gel containing 10 microliter ethidium bromide was prepared and casted. Ten microliter of each of cDNA product was mixed with loading dye and loaded into separate wells on the gel. The first and the last well of the gel were loaded with 1 kilobase 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 under UV-light and the image captured using gel documentation machine.

Results.

Out of 150 stool samples screened for rotavirus, 8 were found to be positive representing (5.33%). The prevalence of infection according to sex of the children was as follows: Male (4%) and Female (1.33%) (Table 1):

Table 1: Prevalence of Infection according to Sex

Sex	No. of Sample Screened	No. of Positive Sample	Prevalence (%)
Male	70	6	4.00
Female	80	2	1.33
	150	8	5.33

The prevalence of infection according to water source was distributed as follows: children that drink water from tap recorded (2%), bore hole (1.33%) and those that drink water from streams/rivers had (2%) (Table 2).

Table 2: Prevalence of Infection according to Water Source

Water Source	No. of Sample Screened	No. of Positive Sample	Prevalence (%)
Pipe Borne	50	3	2.0
Bore Hole	80	2	1.33
Streams/Rivers	20	3	2.0
	150	8	5.33

The prevalence of the infection according to breast feeding was as follows: exclusively breastfed children recorded (2%), breastfed children had (3.33%) (Table 3).

Table 3:Prevalence of Rotavirus Infection according Breast Feeding.

Breast Milk	No. of Sample Screened	No. of Positive Sample	Prevalence (%)
Exclusively Breastfed Children	70	3	2
Breastfed Children only	80	5	3.33
	150	8	5.33

The strains of rotavirus identified in Niger State include: P6 (22%), GI (22%), G2 (33%) and G8 (22%) (Table 4).

Table 4:Strains of Rotavirus Identified in Niger State

Genotype	Frequency	Percentage (%)
P6	2	22
G1	2	22
G2	3	33
G8	2	22

Discussion.

In this study, 150 stool samples were collected from diarrheic children in Niger State and screened for rotavirus. Prevalence of 5.33% was recorded. Higher prevalence (9%) has been reported in North Western Nigeria in previous study (Aminu *et. al.*, 2009). This

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could be attributed to poor hygienic practices, coupled with the inadequacies in the sanitary laws in Nigeria in the past. Although previous studies (Steele *et. al.*, 2002; Armah *et. al.*, 2003; Glass *et. al.*, 2006) have reported that no link between the quality of environment and rotavirus infection, it is difficult to rule out the influence of environment in disease transmission particularly pediatrics diarrhea. The prevalence of rotavirus infection according to the sex of the children was Male (4%) and Female (1.33%) (Table 1). Several studies (Glass *et. al.*, 2006; Armah *et. al.*, 2003) have reported that male children are vulnerable to rotavirus gastroenteritis. The outcome of this study is in conformity with the previous reports.

The prevalence of rotavirus infection according to water source was distributed as follows; children that drink water from Tap (2%), borehole (1.33%), streams/rivers (2%) (Table 2). This could be attributed to contamination. Already United States Food and Drug Administration (2007) have reported that rotaviruses have been found in water samples at infectious level, the outcome of this study is not surprising. However, the identification of rotaviruses in tap water is an indication that there are inadequacies with the water treatment procedures or could be attributed to leakages on the pipe lines. However, chi square test revealed that the difference sources of water were not a factor in rotavirus infection in the study area. The prevalence of rotavirus infection according to the intake of breast milk was as follows: Exclusively breastfed children had (2%), breastfed children only record (3.33%) (Table 3). Previous report by Mishra *et. al.* (2010) has indicated that exclusively breast milk can confer immunity against rotavirus gastroenteritis. The outcome of this study seems to agree with the previous report. However, statistically, there was no relationship between breastfeeding and the rate of rotavirus infection in the study area.

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