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Molecular Detection of Astrovirus in Diarrhoeic Stools of Children in North East Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author OSO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AM and EEE supervised the analyses of the study. Authors NL, AM and EEE corrected the literatures searched. All authors read and approved the final manuscript.

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ABSTRACT

Background: Human astroviruses are a leading cause of severe viral gastroenteritis and are responsible for at least 95% of nonbacterial gastroenteritis outbreaks throughout the world. **Methods:** Six hundred (600) diarrhoeic stools of children under 5 years were collected between May 2013 – April 2014 and screened for astrovirus using a 3rd generation Ridascreen ELISA kit (R-Biopharm AG, Germany). Demographic data were collected via a questionnaire. Analysis of the data was done using online Easy-Chi-square ($p<0.05$) statistical package. The ELISA positive astrovirus samples were further analysed by reverse transcription polymerase chain reaction (RT-PCR) and amplicons generated were sequenced. Phylogenetic tree of sequences was constructed using the Neighbour-Joining Model with 1000 replicate bootstrap value in MEGA 6.0.
Results: Astrovirus prevalence of 5.0% (30/600) was obtained. The prevalence of astrovirus in Taraba, Bauchi and Borno states was 5.5% (11/200), 4.5% (9/200) and 5.0% (10/200), respectively. Of the 30 astrovirus positive samples, 63.3% (19/30) were male and 36.7% (11/30) female. Female

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children were more likely to be infected with astrovirus (OR= 1.38; 95% CI) compared to male children. The highest astrovirus prevalence (8%: 9/112) and lowest (1.9%: 1/54) prevalence were in children 1-2 years and 0-6 months respectively. Most children were infected before 2 years. Of the 30 astrovirus, ELISA positive samples analysed by RT-PCR, 5 (16.5%) amplicons of ORF genes with 400bp were seen and subsequently sequenced.

Conclusion: Sequence analysis showed that all the strains were HAstV-5 indicating the strain prevalent in the study area. The results of the present study suggest that astrovirus contribute significantly to the disease burden of childhood diarrhoea in parts of North Eastern Nigeria.

Keywords: Astrovirus; diarrhoea; RT-PCR; Phylogenetic tree; North East-Nigeria.

1. INTRODUCTION

Human astroviruses (HAstVs) are a leading cause of severe viral gastroenteritis and are responsible for at least 95% of nonbacterial gastroenteritis outbreaks, and 50% of all gastroenteritis outbreaks throughout the world [1,2]. HAstVs have been associated with diarrhoea in other mammals as well as birds [3-5]. Though, less pathogenic in adults, gastroenteritis due to the virus also represents an economic burden in developing countries. Worldwide, over a billion diarrhoeal cases occur each year among children below five years resulting in approximately 2.5 million deaths [6-8].

Human astroviruses are non-enveloped and positive-sense single-stranded RNA viruses, which belong to the genus Mamastrovirus, family *Astroviridae* [9,10]. The astrovirus taxonomy is mainly based on the species of origin and the serotypes within each species are defined by twenty-fold or greater cross-neutralisation titers [11]. Based mainly on the host of the virus and the genome structure, the family Astroviridae is divided into two genera. Members of the genus Avastrovirus are found in avian hosts, whereas members of the genus Mamastrovirus are found in mammalian hosts [12]. The knowledge and literature on astrovirus diversity is very limited, with only three astrovirus species from avian hosts recognized by the International Committee on Taxonomy of Viruses (ICTV) and six identified astrovirus species from mammalian hosts (bovine astrovirus, feline astrovirus, human astrovirus (serotypes 1–8), mink astrovirus, ovine astrovirus and porcine astrovirus) [12].

The astrovirus genome of approximately 6,800 nucleotides consists of three open-reading frames (ORFs): ORF1a, ORF1b, and ORF2 [13]. ORF1a encodes the non-structural polyprotein 1a; ORF1b, encodes the polyprotein 1ab, including the RNA-dependent RNA polymerase (RdRp) that is expressed by a ribosomal frameshift at the ORF1a/1b junction and ORF2, encodes a viral capsid structural polyprotein [14,15]. In humans, eight classic serotypes of astroviruses are known (HAstV1 to HAstV8). Out of these, HAstV-1 has been recognised as the most frequent genotype throughout the world [5, 11]. Transmission occurs through food and water routes, as well as incidental contact with contaminated surfaces or fomites and through person-to-person contact. It is primarily faecal-oral contamination that drives the spread of astrovirus [16]. In Nigeria, prevalence studies have been conducted at different locations [17-19], however, information on molecular studies in north-eastern Nigeria is scanty if existent at all. This study was aimed at molecular detection of astrovirus in diarrhoeic stools of children and to determine the circulating strain in the north-eastern region of Nigeria.

2. METHODS

2.1 Study Area

The study was conducted in the North Eastern region of Nigeria which comprises six states namely, Adamawa, Bauchi, Borno, Gombe, Taraba and Yobe. However, the research was carried out in three of the six states, namely, Bauchi, Borno and Taraba.

Bauchi State: Bauchi State has a population of 4,653,066 with the coordinates 10° 18’ 57”N, 09° 50’ 39”E. It is made up of twenty local government areas. Based on senatorial districts stratification into north, south and central, approximately two hundred samples were collected from the selected hospitals: General Hospital, Bauchi and General Hospital Azare.

***Borno State*:** Borno State capital is Maiduguri. The state was formed in 1976 from the split of the North Eastern State. Until 1991 it contained

what is now Yobe. Borno State has a population of 4,171,104 with the coordinates 11° 30'N, 13° 00'E. It covers a total land mass of 70,898 km^2 (27,374 sq mi). It is made up of twenty-seven local government areas. Based on senatorial districts stratification into north, south and central, approximately two hundred samples were collected from these selected hospitals: State Specialist Hospital Maiduguri, Nursing Home Maiduguri and General Hospital, Biu.

***Taraba State*:** Taraba State is a Northeastern state of Nigeria, named after the Taraba river which traverses the southern part of the state. Taraba's capital is Jalingo. Taraba State has a population of 2,294,800 with the coordinates 8°00′N 10°30. It covers a total land mass of 54,473 km^2 (21,032 sq mi). It is made up of fifteen local government areas. Based on senatorial districts stratification into north, south and central, two hundred samples were collected from the selected hospitals: Specialist Hospital Jalingo, General Wukari and General Hospital Takum.

2.2 Study Design

In this research, a hospital-based cross-sectional design was employed to allow for stool sample collection from every other child presenting at any of the selected hospitals in the study area.

2.3 Study Population

A total of 600 stool samples (200 from each representative State) were collected from children less than five years old presenting with diarrhoea at the In and Out Patient Departments and the Pediatric wards of the selected primary, secondary or tertiary hospitals as listed under the study area.

2.4 Inclusion and Exclusion Criteria

Inclusion criteria**:** Diarrhoeic children who were less than five years old whose parents/guardians consented to participate in the study were included in the study.

Non-Inclusion criteria**:** Non-diarrhoeic children were excluded from the study. Also, diarrhoeic children above the age of 5 years or those less than five years whose parents/guardian did not consent to participate in the study were excluded from the study.

2.5 Ethical Approval and Consent

Ethical approvals were sought from the Ethical Committees of the respective State Hospital Management Boards where samples were collected. A consent form was issued to all parent and guardian to explain the aim of the study and to obtain their approval for sample collection.

2.6 Data Collection using Questionnaire

Data were collected with the use of a self-structured questionnaire administered to consenting parents/guardians of children with diarrhoea attending the selected hospitals. Data collected, among others, included data on demography, clinical information and data on risk factors.

2.7 Sample Size Determination

The sample size for astrovirus was determined using the formula by [20]. The prevalence of 16% obtained in a study on astrovirus in Nassarawa State [19] was used.

$$n = \frac{Z^2Pq}{L^2}$$

Where,

n= number of samples
Z= Standard normal deviate at 95% CI = 1.96
P= 16% (Kuta *et al.,* 2014) =0.16
q= 1- 0.16 = 0.84
L= Allowable error of 5% (0.05)

$$n = \frac{Z^2Pq}{L^2}$$

$$= \frac{(1.96)^2 \times 0.16 \times 0.84}{(0.05)^2}$$

$$= 207$$

The sample size calculated for the entire study area was 207. However, to make for a sample size that will give a fair representation of the study area, 600 samples were collected from the study area/population.

2.8 Sample Collection and Analysis

A total of 600 stool samples were collected from children under 5 years of age presenting with diarrhoea in the selected hospitals in the representative states between May 2013–April

2014. The samples were collected in clean/clear universal containers and transported to the University of Maiduguri Teaching Hospital and stored at -20°C until analysed. All the samples collected were screened for astrovirus using a 3rd generation Ridascreen ELISA kit (R-Biopharm AG, Germany) as instructed by the manufacturer. The ELISA positive astrovirus samples were further subjected to RT-PCR. The 400bp amplicons generated from the RT-PCR were subsequently sequenced.

2.9 Astrovirus RNA Detection

2.9.1 RT-PCR

All the thirty astroviral genomes extracted from the ELISA positive samples were further subjected to QIAGEN one-step RT-PCR procedure with 400bp human astrovirus-specific forward and reverse primers (SF0073 5'-GATTGGACTCGATTTGATGG-3'; SF0076 5'-CTGGCTTAACCCACATTCC-3') serving as templates for amplification.

Extracted RNA samples were then reversed transcribed. Briefly, 0.5µl of hexanucleotide random primers (20mU; PdN6; Parmacia Biotech) was added to 5µl ssRNA template. A reaction mixture (19.5µl) consisting of (4µl 5x buffer; 0.5µl avian myeloblastosis reverse transcriptase; 1µl each of 10 mM dATP, 10mM act, 10 mM dGTP, 10 mM dTTP; 11 µ1 of RNase free water) was used upon addition of 5µl of extracted sample RNA. A reverse transcription reaction at 50°C for 30min was performed.

The cDNA generated was then amplified by PCR in a 45µ1 reaction mixture containing (0.25µl each of 10mM dATP, 10mM dCTP, 10mM dGTP, 10mM dTTP; 10µl 5x Green Go Taq Buffer; 0.25µl Taq Polymerase; 30.75µl RNase free water; 5µl cDNA template). One (1) and 2µl 20 pmol of specific primers SF0073 and SF0076 (Finkbeiner *et al.,* 2009), respectively, were used in a PCR analysis using the QIAGEN One-Step RT-PCR kit with the following conditions: 94°C for 10 min (Initial PCR activation step), followed by 40 cycles of 94°C for 30secs, 56°C for 30secs, and 72°C for 50secs was performed using Primus 25 system cycler, Germany. The PCR products were loaded onto 2% agarose gel with 0.5µg/ml ethidium bromide and electrophoresed in Tris acetic EDTA (TAE) buffer at 100V for 1 hr. The amplicons were visualised on UV Transilluminator (BioRad, USA) and photographed using Polaroid camera.

2.9.2 Sequencing

The amplicons generated by RT-PCR technique were subsequently sequenced.

2.10 Data Analysis

The data obtained from the questionnaires were analysed according to demography, clinical information and risk factors. Tables and frequencies were also generated. Categorised variables were assessed using the Chi-square test. Data were entered into Easy-Chi-square ($p<0.05$) statistical package. A p-value of ≤0.05 was considered significant at 95% confident interval.

3. RESULTS

The ELISA screening for astrovirus antigen in 600 diarrhoeic samples of children in northeast Nigeria showed a statistically insignificant (χ^2= 0.3288, p=0.848) prevalence of 5.0% (Table 1). The state-based prevalence of astrovirus in Taraba, Bauchi and Borno states was 5.5%, 4.5%, 5% respectively.

Astrovirus infection among children based on sex is presented in Table 2. Out of the 600 participants enrolled for the study, male predominated with the frequency of 336 (56%) compared to female 264 (44%) (p=0.0005). However, further analysis revealed that the prevalence of astrovirus observed in female children (3.2%: 19/600) was higher compared to male children (1.8%: 11/600).

Table 3 shows the state-based astrovirus infection according to age. Astrovirus infection was significantly associated with age (χ^2=19.367, p=0.01302). The distribution of astrovirus prevalence was various: the highest in Taraba state (13.3%) was among the 25 – 36 month age, while the highest in Bauchi (9.1%) and Borno (10.2%) states, were among children between 13 – 24 month ages. Overall, astrovirus positivity rate was observed to be even (n=15) for children 1-2 years and those who are > 2 but ≤ 5 years.

A total of 30 ELISA-positive samples were analysed by using the RT-PCR and the numbers of amplicons generated according to representative states. Of these, HAstVs amplicons were generated from 1(11.1%) of 9 samples from Bauchi State, 2(20.0%) of 6 samples from Borno State, and 2(18.2%) of 7

samples from Taraba State (Table 4). In Borno state, the positive samples were mostly in the cold season (November, December and January). However, in Taraba state, the positive samples did not show specific seasonal distribution (as it was detected in May and December). RT-PCR products were subjected to sequencing. A total of 5 amplicons of ORF2 regions of the HAstV (Plate 1) were sequenced. The gel photo of the electrophoresis of the 400bp PCR products is presented on Plate 1. The isolates designated NIBOR 007 and 021; NITAR 041 and 089; NIBAU 032 are presented in duplicate.

The phylogenetic tree constructed using the sequences obtained in this study and comparing them with reference sequences from the GenBank is presented in Figure 1. Amplicons size of 400bp from ORF2 region was used. Significant bootstrapping values (>70%) are shown at relevant nodes. The viruses identified in this study were designated as NITAR 041; NITAR089; NIBOR 021 and NIBOR 007. NIBAU 032 did not reflect in the phylogenetic tree due to the incompatibility of sequence length with software used for construction. Scale bar represents the number of nucleic acid difference.

Table 1. Distribution of astrovirus in stool of diarrhoeic children 0-5 years in North Eastern Nigeria

State	Total no. of sample tested	Number of astrovirus positive (%)	P value	X^2
Bauchi	200	9(4.5)	0.848	0.3288
Borno	200	10(5.0)		
Taraba	200	11(5.5)		
Total	600	30(5.0)		

Table 2. Association of astrovirus with diarrhoea in children in North Eastern Nigeria

Sex	Total number of sample tested	Astrovirus positive (%)	O.R (95% CI)
Male	336	19(5.7)	1.38
Female	264	11(4.2)	
Total	600	30(5.0)	

Key: O.R : Odd Ratio

Table 3. Distribution of astrovirus according to age of diarrhoeic children in North Eastern Nigerian States

Age group (month)	Taraba		Bauchi		Borno		p-value
	Total number of sample	Astrovirus positive (%)	Total number of sample	Astrovirus positive (%)	Total number of sample	Astrovirus positive (%)	
0 – 6	18	0(0)	23	0(0)	13	1(7.7)	0.013
7 – 12	58	3(5.2)	25	1(4.0)	44	1(2.3)	
13 – 24	41	2(4.9)	22	2(9.1)	49	5(10.2)	
25 – 36	15	2(13.3)	57	1(1.8)	35	2(5.7)	
37 – 48	35	1(2.9)	43	1(2.3)	36	1(2.8)	
49 – 60	33	3(9.1)	40	4(10)	23	0(0)	

Table 4. One Step RT-PCR analysis of ELISA Astrovirus-positive diarrhoeic stools of children 0-5 years in a part of North Eastern Nigeria

State	No. of ELISA positive	400bp ORF gene generated by RT-PCR (%)
Bauchi	09	01(11.1)
Borno	10	02(20.0)
Taraba	11	02(18.2)
Total	30	05(16.7)

Fig. 1. Phylogenetic analysis of human astrovirus ORF gene as compared with data from the GenBank

4. DISCUSSION

In this hospital based-study, the prevalence of astrovirus in stools of children in North Eastern Nigeria was 5.0%. This prevalence is representative of only diarrhoeic patients who presented at hospitals during the duration of the research because many who suffer from diarrhoea resort to traditional treatment at home. Also, it is within the prevalence range of 2–16% of human astrovirus (HAstV) infection reported among children hospitalised with diarrhoea and 5–17% in community studies that used either EIA or RT-PCR analysis [21-23]. This prevalence is

also similar to those obtained in previous studies in Nigeria is similar to 5% prevalence in northwest Nigeria [17]; but less than 5.3%, 8.3% and 40.4% prevalence in Niger, Nasarawa and Lagos state, Nigeria [18,19]. However, it was more than 4.9% prevalence reported in Mexico but less than 10.8% in the United States [24]. Attributable reasons for the variations in prevalence reported in different studies may be due to the period samples were collected relative to the duration of illness, a number of samples collected, age inclusion criteria and the sensitivity of the method employed in the analysis.

However, unlike in developed countries, the literature on molecular studies on human astrovirus (HAstVs) in North-east Nigeria is scanty, and no definite investigation is routinely made at health facilities for viral etiologies of diarrhoea or gastroenteritis. Yet HAstVs is one of the important viral agents of diarrhoea. In this study, 5 amplicons (400bp) were generated from the thirty samples analysed on RT-PCR. This outcome may be attributable to the following factors: the quality and purity of the RNA template, nonspecific amplification due to assembly of amplification reactions at room temperature, reaction conditions and presence of contaminants (inhibitors).

The 400bp genomic sequence from the ORF2 region of HAstV was phylogenetically compared to some reference genomic sequences for astroviruses available in GenBank. The resulting phylogenetic tree with the bootstrapping values (>70%) at branching points between the astrovirus species indicated shows clearly that the HAstV obtained in this study is most closely related to HAstV-5 suggesting that HAstV-5 is the prevalent genotype in Borno and Taraba states and indicates that there is no simultaneous circulation of another genotype in the region. This is consistent with the report of [24] in a study conducted in Houston and Mexico City [5] on an outbreak of astrovirus in adults with acute gastroenteritis in Korea but contrary to studies in most countries e.g Spain: [25]; Brazil: [26,27]; Bangladesh: [28] where HAstV-1 is reported to be the prevailing genotype. The HAstV-5 isolates in this study showed high identity to each other and were located in one cluster, indicating the circulation of one transmission link. However, the absence of other genotypes does not mean that only HAstV-5 was prevalent in the region. The use of more primers different from ours could help detect other strain(s) which might be present in the study area.

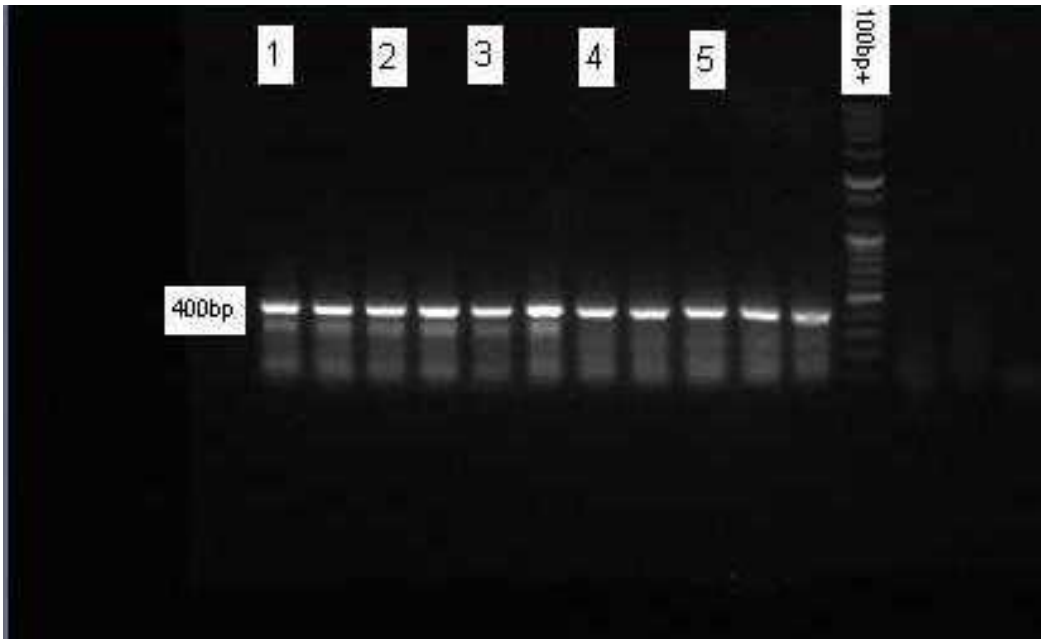

Plate I. Amplicons of 400bp of Astrovirus ORF$_2$ gene detected in the diarrhoeic stool of children. Lanes 1-5 (in pairs)

Lane 1 = NIBOR 007; Lane 2 = NIBOR 021; Lane 3 = NITAR 041; Lane 4 = NITAR 089; Lane 5 = NIBAU 032

This observed circulation of HAstV-5 in the study area is also contrary to the widely reported prevailing HAstV-1 in early studies conducted in Spain, Germany, Brazil, Vietnam, Japan, and China which indicated that HAstV-1d is the predominant type in these countries [29]. Other studies have also reported that HAstV-1 is the predominant strain in Egypt, Italy and France [30].

Phylogenetic analysis showed a Nigerian HAstV-1 isolate (GQ441176) which was closely related to an Indian isolate (KT159910) as reported by [31] but it does not possess a close relationship with the isolate in this study. The viral cluster of the isolate in this study contains sequences identical to a previously isolated Nigerian strain (GQ441183) by [31], indicating that one HAstV-5 transmission strain circulated in Borno and Taraba states concurrently and the occurrence of frequent inter-state spread during the survey period. The phylogenetic analysis also showed that a strain found in Brasil (DQ028633) had a close relationship with the viral strain cluster in this study. Therefore, it may be inferred that the strains in this study should have the same ancestor.

Also, a reference Nigerian human astrovirus isolates (GQ441171) was observed to have a close relationship with duck astrovirus strain (KJ173710) (Fig. 1) which suggests possible cross-infection from animal host (duck) to man especially domestic rearers, commercial poultry farmers/workers, and consumers of ducks and other avians. This is significant because it buttresses the report by [31] that two species of astrovirus which caused animal gastroenteritis were suspected of causing human diarrhoea due to the high recombination events in astroviruses which have been described in cattle, swine, humans [32-34] and poultry [35-38].

Therefore, the need to establish an effective and efficient HAstVs surveillance, not only in the study area but in Nigeria is apparent to avoid an outbreak of HAstV gastroenteritis of highly pathogenic astroviruses resulting from mutation or recombination events. This surveillance will also generate baseline information to be used to develop a possible vaccine against the virus.

5. CONCLUSION

The prevalence of astrovirus (5.0%) obtained in this study indicate that the virus contributes to the burden of diarrhoea among children in the study area. Also, this study has shown the presence of HAstVs in the diarrhoeic stool of under-five-years old children in northeast Nigeria. The HAstVs-5 was the circulating strain recognised during this study. Phylogenetic analysis showed that this strain had a close relationship to a reference strain from Brazil, with seasonal variability. This study has provided the latest information on the circulating HAstVs strain in North East Nigeria. This can be helpful for formulating an effective vaccine against the virus.

KEY POINTS

- This was a study aimed at the molecular detection of astrovirus in diarrhoeic children in North Eastern region of Nigeria.
- Six hundred (600) diarrhoeic stools of children under 5 years were screened for astrovirus using a 3rd generation Ridascreen Enzyme-Linked Immunosorbent Assay (ELISA) kit (R-Biopharm AG, Germany) and the ELISA positive astrovirus samples were further analysed by reverse transcription polymerase chain reaction (RT-PCR) and amplicons generated were sequenced.
- An overall Astrovirus prevalence of 5.0% (30/600) was obtained.
- Sequence analysis showed that all the strains belong to the HAstV-5 indicating the strain prevalent in the study area.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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