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Viral gastroenteritis among children of 0-5 years in Nigeria: Characterization of the first Nigerian aichivirus, recombinant noroviruses and detection of a zoonotic astrovirus

M.O Japhet[a,b,c], O. Famurewa[b,d], O.A. Adesina[a], O.O. Opaleye[e], B. Wang[c], M. Höhne[c], C.T. Bock[c,f], Andreas Mas Marques[c], S. Niendorf[c,*]

[a] Department of Microbiology, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria
[b] Department of Microbiology, Faculty of Science, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria
[c] Department of Infectious Diseases, Unit of Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses, Robert Koch Institute, Berlin, Germany
[d] Department of Biological Sciences, Microbiology Unit, Kings University, Odeomu, Osun State, Nigeria
[e] Department of Medical Microbiology and Parasitology, Ladoke Akintola University, Osogbo, Osun state, Nigeria
[f] Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany

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ABSTRACT

Background: Viruses are the leading cause of acute gastroenteritis in children worldwide. Understanding of the occurrence and genetic diversity of these viruses can help to prevent infections.
Objectives: The present study describes the presence, genetic diversity and possible recombination of five enteric viruses in children with gastroenteritis in Southwestern Nigeria.
Study design: From August 2012 to December 2013, stool samples and sociodemographic data of 103 diarrheic children < 5 years were collected to detect and characterize rotavirus A, norovirus, human astrovirus, aichivirus and sapovirus using PCR techniques followed by sequencing and phylogenetic analyses.
Results: At least one virus was identified in 58.3% (60/103) of the stool samples. Rotavirus, norovirus and astrovirus were detected in 39.8% (41/103), 10.7% (11/103), and 6.8% (7/103) respectively. Notably, aichivirus was detected for the first time in Nigeria (1/103; 0.97%). Sapovirus was not detected in the study. Co-infections with rotavirus were observed in eight samples either with norovirus or astrovirus or aichivirus. Phylogenetic analyses of different genome regions of norovirus positive samples provided indication for recombinant norovirus strains. A novel astrovirus strain closely related to canine astrovirus was identified and further characterized for the first time.
Conclusions: Viruses are the common cause of acute gastroenteritis in Nigerian infants with rotavirus as most frequently detected pathogen. New norovirus recombinants and a not yet detected zoonotic astrovirus were circulating in Southwestern Nigeria, providing new information about emerging and unusual strains of viruses causing diarrhea.

1. Background

In 2015, 526,000 infants and young children died from acute gastroenteritis (AGE) worldwide [1] with viruses being the major etiological agent [2,3]. Several gastroenteritis viruses have been identified [4–7]. Predominantly, group A rotaviruses (RVA), norovirus (NoV), human astrovirus (HAstV), sapovirus (SaV) and adenovirus have emerged as etiologic causes of AGE in children (0–5 years) [4,5,7–9]. Before implementation of the RVA vaccination, recommended by Word Health Organization about 453,000 children died in 2008 from RVA infection worldwide [10]. NoV is the second most common cause of viral diarrhea in children less than 5 years. In developing countries, infection with NoV causes up to 1.1 million hospitalizations and 218,000 deaths per year in children [11]. The overall detection rates of other viruses that cause AGE like SaV, HAstV and aichivirus (AiV) are typically much lower than that of NoV and RVA [4,7,12]. NoVs are non-enveloped single stranded positive sense RNA viruses and constitute a genus within the family of *Caliciviridae* with an approximately 7.5 to 7.7 kb genome containing three open reading frames (ORFs). ORF1 encodes for the non-structural proteins including e.g. the RNA-

* Corresponding author at: Robert Koch Institute; Seestr. 10; 13353, Berlin, Germany.
E-mail address: NiendorfS@rki.de (S. Niendorf).

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dependent RNA polymerase (RdRp), ORF2 codes for the major capsid protein (VP1) with the highest degree of sequence variability in the viral genome, while ORF3 codes for the minor capsid protein (VP2). According to the ORF2 sequence NoVs are classified into seven genogroups (GI-GVII) with more than 40 genotypes. The genogroups GI and GII NoV are the leading cause of AGE in humans [13]. Human SaV, another member of the *Caliciviridae* family, is classified into four genogroups (GI, GII, GIV, and GV) and further subdivided into 17 genotypes [14]. Astroviruses cause AGE in many mammalian and avian hosts. HAstVs have been classified in the classical HAstVs species, comprising 8 genotypes, reviewed in [15] and two new clades of HAstV which have been discovered recently in human stool samples in several countries (MLB and VA). Both exhibit high genetic divergence from the classical strains [16–19]. Little is known about the epidemiology of AiV, which was first detected in a stool sample collected during an oyster-associated gastroenteritis outbreak in Japan [20]. However, recent studies on AiV have demonstrated a global distribution of this virus [20–24].

2. Objectives

This molecular epidemiological study describes the occurrence and genetic diversity of gastroenteritis viruses in fecal samples of pediatric patients suffering from AGE in Ile-Ife, Osun state, in the Southwestern part of Nigeria.

3. Study design

The study was conducted from August 2012 to December 2013 at the OAUTH Comprehensive Health complex, Ile-Ife, as well as the two State Hospitals of Ile-Ife (State Hospital, Oke-Ogbo and Health Centre, Enuowa). Stool samples were collected from 103 children (51 female, 52 male), 0–5 years enrolled at the outpatient clinics presenting with diarrhea (three or more watery stool over a 24 h period, with or without fever, vomiting or dehydration), some of the children were admitted to the pediatric wards of these hospitals.

3.1. Ethics approval

Ethical approval to conduct this study was obtained from the Obafemi Awolowo University Complex Teaching Hospital (OAUTHC) Research Ethics Committee (OAUTHC REC) of the Obafemi Awolowo University Ile-Ife, Nigeria (ERC/2012/10/2). The parents' signature of the informed consent forms was necessary to be included in this study. Demographic data (age, sex) were collected through the use of questionnaire completed by the parent or guardian of the children.

3.2. Viral RNA extraction

Viral RNA was extracted as previously described [25].

3.3. Viral detection by RT-PCR

For detection of NoV, reverse transcription nested PCR (RT-nested PCR) targeting NoV ORF1 region A (RdRp) was performed as previously described [26]. The P2 region within the ORF2 of NoV positive samples was analyzed as previously described [27]. For HAstV detection a RT-semi-nested PCR with the primers AV91, AV92a and AV93 (Table 1), targeting the ORF1b was used by Super-Script III One-Step RT-PCR System (Invitrogen, Carlsbad, USA). In brief, 2.5 µl RNA was used in a final reaction volume of 12.5 µl. For the first round, RT-PCR, primers AV92a and AV91 were used under the following cycling condition: 5 min 55 °C, 55 min at 45 °C, 2 min at 94 °C followed by 40 cycles of 15 s 94 °C, 30 s 42 °C, 90 s 68 °C and a final extension for 5 min at 68 °C while primers AV93 and AV91 were used for the second round PCR under the following condition: 2 min 94 °C, followed by 35 cycles of 15 s 94 °C, 30 s 42 °C, 90 s 68 °C and a final extension for 5 min at 68 °C.

To characterize canine AstV a 660bp fragment spanning ORF1b and ORF2 region was amplified using primers dAV2, dAV3 and dAV4 (Table 1) under the following condition: 5 min 55 °C, 55 min 45 °C, 2 min 94 °C, followed by 40 cycles of 15 s 94 °C, 30 s 45 °C, 60 s 68 °C and a final extension for 5 min at 68 °C for the first round of PCR with the primers dAV2 and dAV3. For the second round of PCR the primers dAV2 and dAV4 were used with following cycling conditions 2 min 94 °C, followed by 35 cycles of 15 s 94 °C, 30 s 45 °C, 60 s 68 °C and a final extension for 5 min at 68 °C. The detection of AiV was done as previously described [28]. Detection of RVA and SaV was performed by real-time PCR assays as previously described [25,29].

3.4. Nucleotide sequencing and phylogenetic analyses

All positive PCR amplicons were applied for direct sequencing using appropriate PCR primers, the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3500 xL Dx Genetic Analyzer (Applied Biosystems, USA). The software Sequencher 5.4.6 (GeneCodes, Ann Arbor, MI, USA) was used for sequence assembly and editing. Sequence alignments were performed using Geneious 9.1.6 (Biomatters, Auckland, New Zealand) with the MUSCLE algorithm [30]. Neighbor-Joining phylogenetic tree was produced using the MEGA7 software [31] with bootstrap test (1000 replicates). Genetic variation was estimated with p-distances using MEGA7 software. The NoV typing tool from NoroNet available www.rivm.nl was used to confirm the NoV genotypes of the study samples [32]. The nucleotide sequences of the viral isolates of this study are available under the following GenBank accession numbers: NoV ORF1 (KU 173780-173790), NoV P2 (KU 860550-860558), HAstV ORF1b (KU 212905-212911 and KU 860560-860562) canine AstV (KU 860559), and AiV (KU 860563).

3.5. Statistical analyses

Statistical analyses were done by Fischer's exact tests with SPSS version 20.0.1 for Windows. P-value < 0.05 was considered to be statistically significant.

4. Results

4.1. Detection rate of viruses among children with diarrhea in Nigeria

Of the 103 tested samples a total of 60 (58.3%) samples were positive for at least one of the tested viral gastroenteritis pathogens. The overall detection rate of RVA, NoV, HAstV, AiV was 39.8% (41/103), 10.7% (11/103), 6.8% (7/103), and 0.97% (1/103) respectively. SaV was not detected in the study population. Mono-infection was detected in 52 (86.7%) virus-positive children while eight (13.3%) of the children shed more than one type of gastroenteritis virus. Co-infection with RVA and NoV (NoV GI and GII) was the most common multiple infection observed in this study. The viral positive rate and co-infections observed is presented in Table 2.

4.2. Viral infection common in children less than one year

The majority (80%; 48/60) of the viral infections occurred in children less than one year. Specifically, RVA, NoV and AiV infections were more frequent in these children (Table 2) while HAstV detection rate was higher in children older than one year. However, there was no statistical significance between NoV, AiV, HAstV infection and age ($P = 0.94$, 0.61 and 0.82 respectively) while a significant correlation between RVA infection and age was observed ($P = 0.026$). No statistical significance between viral infection rates and gender was detected (data not shown). RVA infections were detected in 49 out of 103 samples. G12P[8] strains were predominant other, found genotypes were G9, G3, G2, P[4], P[6] and P[8]. A detailed description of the

Table 1
Modified and designed primers and probes used for amplification and sequencing.

region	name*	sequence (5'–3')	position (nt)	reference
NoV ORF1	NV1a (s)	ATGAATATGAATGAAGATGG	4226-4246[a]	[26]
	NV1b (s)	ATGAACACAATAGARGATGG	4226-4246[a]	[26]
	NV7 (as)	ATTGGTCCTTCTGTTTTGTC	4688-4707[a]	[26]
	NV7a (as)	GGYCCYTCAGTYTTGTC	4688-4704[a]	[26]
	NV6 (s)	TACCACTATGATGCAGATTA	4280-4299[a]	[26]
	NV6a(s)	TATCACTATGATGCTGACTA	4280-4299[a]	[26]
	NV4 (as)	GTTGACACAATCTCATCATC	4598-4617[a]	[26]
	NV4a (as)	ACAATYTCATCATCICCAT	4593-4611[a]	[26]
	NV4c (as)	GTGCTGACGATCTCGTCATC	4588-4611[a]	[26]
NoV ORF2 (P2 region)	NV347a (s)	GAIGATGTCTTCACAGTYTCTT	5661–5682[a]	[27]
	NV347b (s)	GATGATGTKTTCACWGTITCTT	5661–5682[a]	[27]
	NV347c (s)	GATGAYGTITTCACIGTITCMT	5661–5682[a]	[27]
	NV348a (as)	GGTTRACCCARGAATCAAA	6636–6618[a]	[27]
	NV348b (as)	GRTTMACCCAAGAITCAAA	6636–6618[a]	[27]
	NV348c (as)	GRTTRACCCAIACTTCAAA	6636–6618[a]	[27]
	NV351a (s)	CCICATGTIATTGCTGATGT	5793–5812[b]	[27]
	NV351b (s)	CCICACGTIATMGCAGATGT	5793–5812[b]	[27]
	NV352a (as)	TTCCCACAGGCTTIAGTYG	6891–6909[b]	[27]
	NV352b (as)	TTCCCACAGGCTTIAGTYG	6891–6909[b]	[27]
HAstV (ORF1b)	AV92a (s)	GGTCARTGYGGGTGGTCACC	3493-3512[c]	this study
	AV91 (as)	TTTGGWCCICCCCTCCA	4303-4319[c]	this study
	AV93 (s)	GAYTGGACICGMTWTGATGG	3583-3602[c]	this study
canine AstV (ORF1b-ORF2)	dAV2(s)	TGGAATGTGGGTYAAGCC	3845-3862[d]	this study
	dAV3(as)	GAGTTGGAAGCTGTTGATTCT	4533-4553[d]	this study
	dAV4(as)	GCAAGTTCCAGCTCTACTGC	4486-4505[d]	this study
AiV (3CD Region)	AI 1(as)	AGGATGGGGTGGATRGGGGCAGAG	6515-6538[e]	[28]
	AI 2(s)	ACACTCCCACCTCCCGCCAGTA	6226-6247[e]	[28]
	AI 3(as)	CCTTCGAAGGTCGCGGCRCGGTA	6402-6424[e]	[28]
	AI 4(s)	GTACAAGGACATGCGGCG	6245-6260[e]	[28]
SaV	SV56a (s)	GAYCAGGCTGCCACCTA	5078-5097[f]	[29]
	SV56b (s)	GATTTGGCCCTCGCCACCTA	763-780[g]	[29]
	SaV5 F (s)	TTTGAACAAGCTGTGGCATGCTAC	5112-5135[h]	[29]
	SaV1245 R (as)	CCCTCCATYTCAAACACTA	5183-5163[f]	[29]
	SV TM1	VIC-ACCACCTATRAACCA-MGB	5119-5105[f]	[29]
	SaV5 TP	VIC-TGCCACCAATGTACCA-MGB	5157-5142[h]	[29]

GenBank numbers of reference sequences are as follows:
[a] NoV GII.1 (U07611).
[b] NoV GI.1 (M87661).
[c] HAstV1 (L23513).
[d] canine AstV (KP404149).
[e] AiV-B (GQ927712).
[f] SaV-GII.1 (AJ249939).
[g] SaV-GI.2 (AF294739).
[h] SaV-GV (AY646856).
* s = sense; as = antisense.

Table 2
distribution of single and multiple infections according to age.

single infection

virus	< 6 months	6-11 months	1-2 years	3-4 years	total
RVA	20	14	6	1	41
NoV GI	0	0	0	0	0
NoV GII	1	6	0	0	7
HAstV	0	1	3	0	4
AiV	0	0	0	0	0
total	**21**	**21**	**9**	**1**	**52**

multiple infection

virus	< 6 months	6-11 months	1-2 years	3-4 years	total
RVA + NoV GI	1	0	0	0	1
RVA + NoV GII	0	2	0	1	3
RVA + HAstV	1	1	1	0	3
RVA + AiV	0	1	0	0	1
total	**2**	**4**	**1**	**1**	**8**

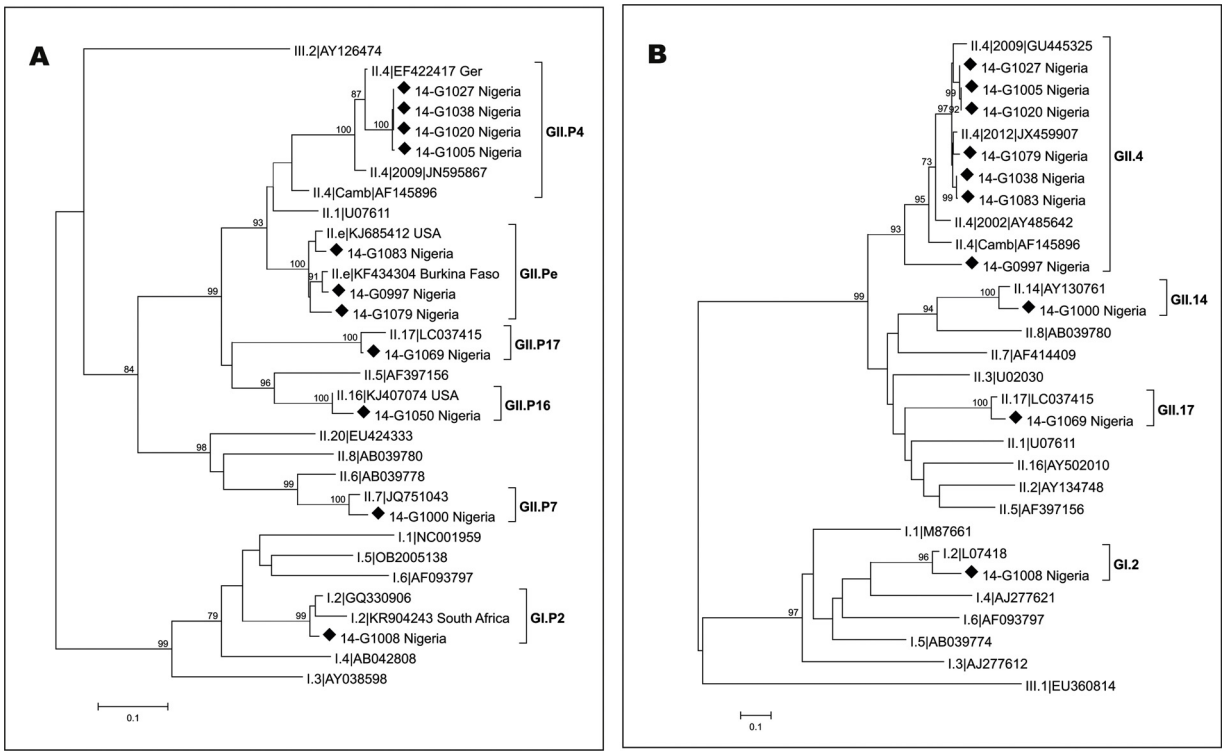

Fig. 1. Phylogenetic tree of (a) a 261 bp alignment of ORF1 region and (b) a 648 bp alignment of P2-region (ORF2) of the GI and GII NoV strains from Nigeria (denoted by rhombus) and NoV GI and GII reference strains obtained from the GenBank database (accession No. as indicated). The bar indicates the variation scale. The tree was constructed using the Neighbor-Joining method and the Kimura 2-parameter method available in MEGA 7 with Bootstrap test (1000 replicates). Bootstrap values above 70 are shown.

detected RVA G- and P types has been published previously [25].

4.3. Sequence and phylogenetic analyses of NoV

In order to determine NoV genotypes the sequences of the RdRp (ORF1) and the capsid genes (ORF2) were phylogenetically analyzed (Fig. 1a and 1b). For 10 NoV positive samples RdRp and capsid sequences and for one sample only RdRp sequences could be determined. In total, 10 of the 11 NoV positive samples belonged to genogroup II (GII) and one to genogroup I (GI). Based on the capsid and RdRp sequences six different genotypes were identified, including the intertypic recombinant strains GII.P7/GII.14 and GII.Pe/GII.4 (Table 3). For sample 14-G1050 only the RdRp sequence was available.

4.4. Sequence and phylogenetic analyses of HAstV and AstV

Seven AstV infections were detected in this study. Sequence and phylogenetic analysis of sequences from ORF1b (Fig. 2) demonstrated that 4 samples (14-G1014, 14-G1017, 14-G1051, 14-G1090) belong to the classical HAstV and two samples belong to the newly described HAstV-VA clade, including one HAstV-VA2 (14-G1010) and one HAstV-VA3 (14-G1045). HAstV strains belonging to the MLB clade were not detected in the study population.

Interestingly, one of the AstV strains detected in this study (14-G1060) was completely different from the other ORF1b sequences and the genetic distance to the other HAstV sequences from this study ranged between p = 0.461 and p = 1.955. The most similar astrovirus reference sequences available in the GenBank were KR349489 from Brazil (p = 0.033) and HQ623148 from China (p = 0.035) which represents canine AstV. In order to characterize this canine AstV in more detail, a canine AstV specific RT-semi-nested PCR was performed and the resulting 660 bp fragment was sequenced and phylogenetically analyzed (Fig. 3).

Table 3
Genotype distribution of identified NoV strains ([a]recombinant strain).

genogroup	sample ID	genotype	n (%)
GI	14-G1008	GI.P2/GI.2	1 (9%)
GII	14-G1005; 14-G1020; 14-G1027; 14-G1038	GII.P4/GII.4	4 (36%)
	14-G0997; 14-G1079; 14-G1083	GII.Pe/GII.4[a]	3 (27%)
	14-G1069	GII.P17/GII.17	1 (9%)
	14-G1000	GII.P7/GII.14[a]	1 (9%)
	14-G1050	GII.P16	1 (9%)
total			**11**

4.5. Sequence and phylogenetic analyses of human AiV

A single AiV infection was detected in this study. Phylogenetic analysis of a 172 bp fragment of the 3CD gene region of the AiV revealed that the Nigerian AiV strain belonged to the AiV-1 genotype B (Fig. 4).

5. Discussion

In Nigeria AGE is responsible for about 77,000 deaths in 2015 [33]. However, little is known about the circulating gastroenteritis viruses in this region. Therefore, a study was conducted to determine the distribution and genetic diversity of viruses that cause AGE among children < 5 years in Nigeria using molecular epidemiology. The overall detection rate of viral gastroenteritis infections (58.3%) in infants with AGE in this study is not unexpectedly high and comparable with previously published studies [7,9].

In accordance with previous reports [34–36] on the distribution of viruses causing diarrhea, our findings showed that RVA was predominant, followed by NoV, HAstV, and AiV. The detection rate of

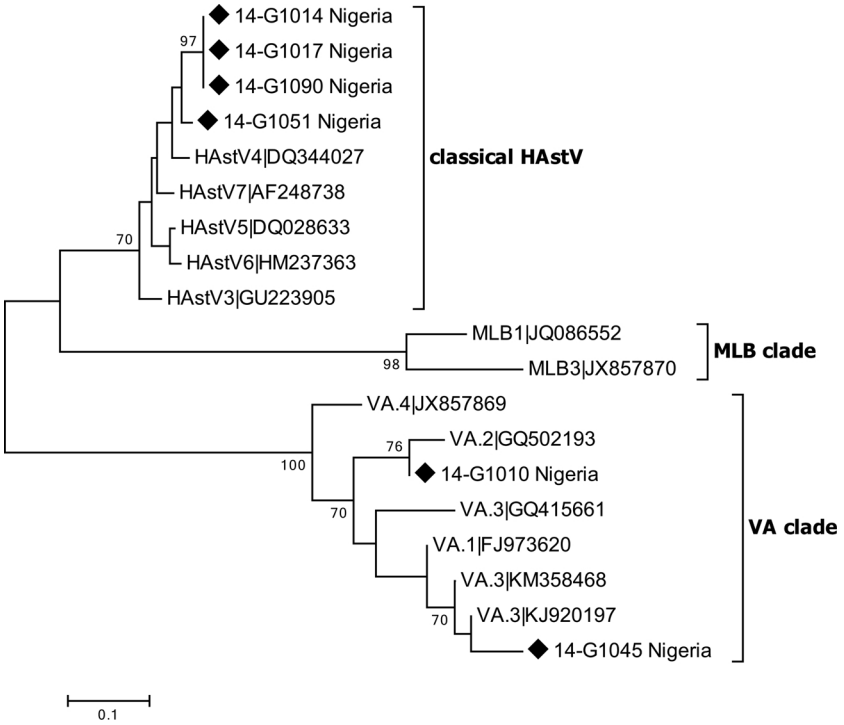

Fig. 2. Phylogenetic tree of a 178 bp alignment of ORF1b region of the HAstV strains from Nigeria (denoted by rhombus) and HAstV reference sequences (accession No. are indicated). The tree was constructed using the Neighbor-Joining method and the Kimura 2-parameter method with Bootstrap test (1000 replicates). Bootstrap values above 70 are shown. The Bar indicates the variation scale.

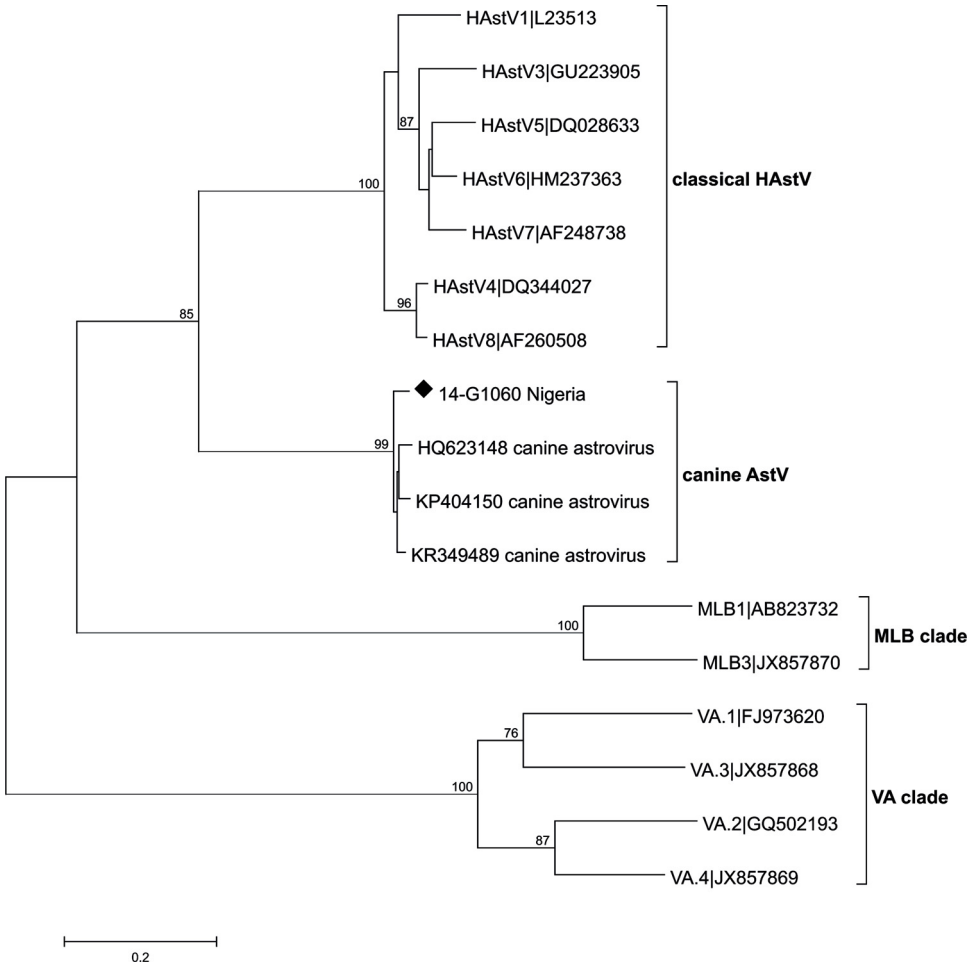

Fig. 3. Phylogenetic tree of a 660 bp alignment of a region spanning the ORF1 and ORF2 genes of the canine AstV strain from a Nigerian child (denoted by rhombus) and canine AstV reference sequences (accession No. are indicated). The tree was constructed using the Neighbor-Joining method and the Tamura-Nei parameter method with Bootstrap test (1000 replicates). Bootstrap values above 70 are shown. The bar indicates the variation scale.

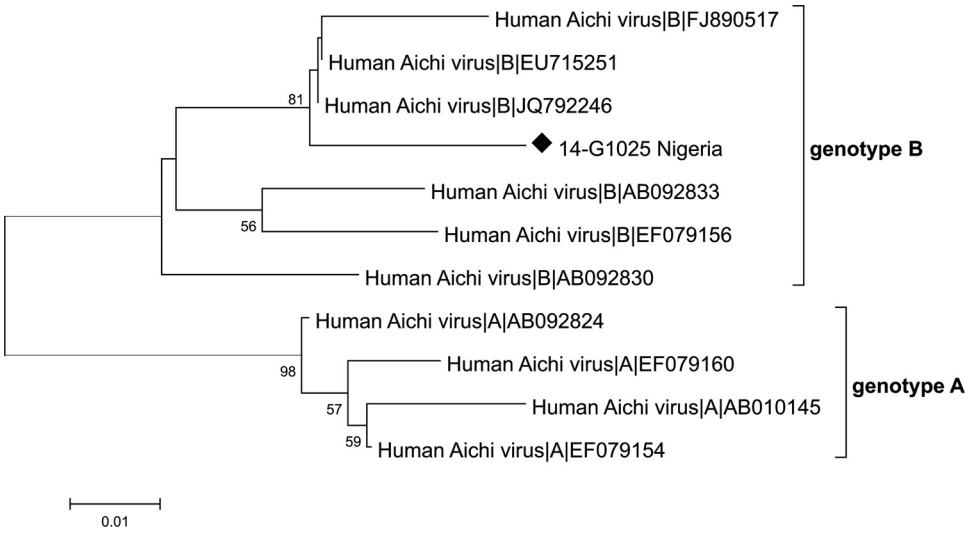

Fig. 4. Phylogenetic analysis of a 172 bp alignment of the 3CD region of the AiV positive sample from Nigeria (denoted by rhombus) and its relation to AiV reference strains (accession No. are indicated). Reference sequences were obtained from the GenBank database. The tree was constructed using the Neighbor-Joining method and the Kimura 2-parameter method with Bootstrap test (1000 replicates). Bootstrap values above 50 are shown. The bar indicates the variation scale.

HAstV is comparable with other studies [4,8,9] but relatively low compared to a previous study conducted in Nigeria with a reportedly unexpected high detection rate of 40.4% [37], which might be caused by using different diagnostic methods. AiV is a less prevalent gastroenteritis virus, not detected in previous studies in Nigeria; however, the low detection rate in this study is consistent with previous AiV studies from France, and China where comparable detection rates were observed [22,38]. The published detection rate of NoV infections varies strongly worldwide; depending on the country and time of sampling, the setting of testing, and age of patients, and ranged from 7.7% to 39% [4,5,7–9,39]. The detection rate in this study falls within this range of previous reports.

NoV GI strains are less frequently detected than NoV GII genogroups strains in this study population. A comparable result has also been observed in previous studies [7,39–41]. The most frequently detected NoV genotype in this study was GII.P4/GII.4 with 36.4% of the detected NoV strains. This finding is in agreement with previous studies, which reported that GII.4 strains are more frequently detected than any other GII genotype [9,39–41]. It will be of interest whether NoV will become the predominant diarrhea virus among children after RVA vaccine introduction into the Nigerian national immunization program as observed in previous studies from other countries [2,42]. Genetic recombination has been described as a widespread phenomenon in NoV, which has a major impact on evolution of this virus. NoV recombinants could be associated with the emergence of new pandemic NoV variants [43,44]. Only few studies from South Africa have reported combined RdRp/capsid typing data [45,46]. Two different recombinant norovirus strains were detected in the study. GII.Pe appears to be an obligatory recombinant strain that has been linked to a number of ORF 2 genotypes including GII.3, GII.4 and GII.12 [47–49]. In this study the only ORF2 genotype associated with the ORF1 GII.Pe genotype was GII.4. An additionally detected recombinant NoV of this study was the intertypic recombinant GII.P7/GII.14. This recombinant was first detected in a study between 1982 and 1986 in Brazil [50].

The results of the present study suggest that HAstV is less common in children with AGE compared to RVA and NoV in Nigeria. There is a dearth of data by molecular studies on HAstV to establish the virus as a causative agent of gastroenteritis among children in Nigeria. Our study demonstrated the circulation of different HAstV genotypes in Nigeria (HAstV-VA2, HAstV-VA3 and classical HAstV) for the first time in Southwestern Nigeria among diarrheic children. Additionally a novel AstV strain which was found to be highly homologous to a canine-related AstV was detected in this study, suggesting a zoonotic transmission. AstVs were initially considered to be species specific [51]; however, zoonotic transmission of astroviruses to humans has been suggested recently [52].

AiV has been proposed as novel causative agent of AGE with lower detection rates compared to other gastroenteritis viruses [4,21,22,38,53] and it is more frequently detected in mixed infections with other gastroenteritis viruses [20–22]. The latter is supported by findings in the present study; the only AiV infection was detected in a co-infection with RVA. However, we cannot conclude whether AiV was the etiological agent causing the symptoms of AGE. There is need for further studies with larger sample size and an additional healthy control group to analyze the role of AiV as a possible cause of AGE. Genotyping revealed that the detected AiV strain belongs to the genotype B; however, genotypes A, B, and C have been reported in previous African studies [8,53].

The most common mixed infection in this study was RVA and NoV with a mixed infection rate of 6.7%. This result is consistent with findings from other studies showing dual RVA and NoV infections in 4.4–9.5% of children with gastroenteritis [5,7,54].

The limitation of the present study is the relatively small sample size and the local restriction to Osun State in the South Western area of Nigeria; however, it will add some new insights into the circulation and occurrence of different gastroenteritis viruses in children in Sub-Saharan Africa.

In summary, this study shows high viral detection rate (58.3%) among children < 5 years with AGE in South-West Nigeria. To the best of our knowledge this study provides the first report on the detection of AiV in Nigeria. Additionally, we could identify and characterize for the first time a canine AstV strain in humans and here in an infant with AGE. We could also detect and characterize recombinant NoV strains (GII.Pe/GII.4 and GII.P7/GII.14) and various genotypes of HAstV not reported previously in Nigeria. Our data suggest the need for constant molecular epidemiological surveillance of viruses causing diarrhea in Nigeria as it has provided new information about unusual and emerging gastroenteritis virus strains, which may have implications not only for the efficacy of vaccine formulations but also implementations in the nearby future.

Conflict of interest

The authors declare that they have no conflict of Interest.

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Authors' contributions

JMO and FO conceived and designed the study. Input on the design was given by BCT, OOO, AOA, MMA, WB, HM and NS. JMO collected samples. JMO performed the experiments. JMO, HM, AOA, and NS analyzed the data. JMO, NS, and BCT wrote the manuscript. All authors revised the manuscript, read and approved the final draft. FO and HM supervised this study.

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