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ISOLATION AND MOLECULAR IDENTIFICATION OF ENTEROVIRUSES IN APPARENTLY HEALTHY SCHOOL CHILDREN IN IBADAN, OYO STATE, NIGERIA.

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ABSTRACT

In this study, early morning feacal samples were collected from 212 randomly selected apparently healthy school children (age 5-10) from seven public primary schools in Ibadan during rainy and dry seasons. One gram of stool samples were processed in 10 ml mixture of 9 ml Phosphate Buffered Saline (PBS) of pH 7.2, 1ml chloroform and 1g of glass beads. The mixture was vortexed for 20 mins and centrifuged at 3000 rpm for 20 min at 4^{o}C.Two hundred microlitre of Supernatant was inoculated in Rhabdomyosarcoma (RD) cell line, incubated at 36^{o}C for 5 days and later passaged into another fresh RD cell line. The isolates that showed Cytopathic Effect (CPE) in RD cell line were subsequently passed into L20B cell line for detection of likely Poliovirus. Infected tubes showing Cytopathic Effect (CPE) were harvested and stored at -20^{0}C.Molecular identification of enterovirus isolates was done using Reverse Transcriptase-Semi nested Polymerase Chain Reaction (RT-snPCR).Sequencing of the positive viral amplicons and phylogenetic analysis of the exploitable sequenced was carried out. Out of the 18 isolates, only 14 were screened positive with RT-snPCR (12 from wet and 2 from dry seasons). Out of the 12 amplicons sequenced, only seven had exploitable sequence data which were identified as Echovirus 11 (E11) (4 isolates), E29 (1 isolate) and E33 (2 isolates). Results of this study confirmed the presence of enteroviruses recovered from apparently healthy children in Ibadan, Nigeria.

INTRODUCTION

Enteroviruses belong to the Family Picornaviridae which are small, approximately ~30 nm in size, single stranded and positive sense RNA viruses. Enteroviruses are highly prevalent enteric pathogens traditionally associated with viable symptoms (Dagan, 1996).Most of these viral infections are asymptomatic or result in only mild symptoms ranging from mild to non-specific febrile illness, hand-foot-and-mouth diseases(De W *et al*., 2011) and mild upper respiratory infection symptoms like in EV-D68virus case(Lu *et al*.,2014).Diverse group of enteroviruses can either infect and replicate in the gastrointestinal tract causing gastroenteritis, or replicate in the intestinal tract and then spread to extra-intestinal target organs where they cause more complicated and life threatening manifestations such as hepatitis,

myocarditis, meningitis, and neurological paralysis (Fong *et al*.,2005).Enteroviruses infection are mostly transmitted via the faecal oral route and they replicate in the gastrointestinal tract. Infected humans excrete faeces containing viral particles, which remain infectious for several days in environmental water (Wait and Sobsey, 2001). Infected individuals shed large amounts of virus in faeces for several weeks irrespective of whether the infection is symptomatic or not (Pallansch, 2013).Enteroviruses can be spread by close contact with an infected person who may cough or sneeze on you,by contaminating hands with stool, and by touching objects or surfaces that have the virus on them and then touching the eyes, nose or mouth(Disease Facts Sheet,2015).Infants and children are most likely to be infected with these viruses and become sick. Adults can be infected as well depending on the serotypes. (Osterback, 2015).The viruses may cause severe worldwide epidemics and they are the most common viruses causing absences from school and work, thus encumbering healthcare system and causing extensive economic effects (Osterback,2015).The aim of this study was isolation and molecular identification of enteroviruses in apparently healthy school children in Ibadan, Oyo State, Nigeria.

MATERIALS AND METHODS

Study Area and site: This study was conducted among apparently healthy children (age 5-10 years) from seven public primary schools within three Local governments in Ibadan, Oyo State (Ibadan North, Ibadan North West and Ibadan South West) with sub-optimal sanitation and low level of hygiene.

Study population: The target populations for this study were school children of age 5-10years attending public primary schools within selected Local Government Areas in Ibadan, Oyo State.

Ethical Approval: Ethical approval was obtained from Oyo State Ethical Review Board Committee, Ministry of Health, prior to the commencement of the study. Approval and consent was secured from the school administration, the guardian and parents of the children. Participant's decision to take part in the study was voluntary, and participants had the right to withdraw from the study at any time and at will.

Faecal Sample Collection: Faecal samples were collected from apparently healthy children aged 5 to 10 years attending public primary schools in Ibadan during rainy and dry season respectively. A total of 120 faecal samples were collected during rainy season and 92 faecal samples were collected during dry season. Samples were collected from each of the children into appropriately labelled sterile collection bottles. Samples were then transported to the laboratory in the Department of Virology, College of Medicine, University College Hospital, Ibadan, Nigeria, in a giostyle filled with ice packs to maintain a temperature of about 4^{o}C.On arrival at the laboratory, the faecal samples were stored at -20^{o}C until processing (WHO, 2004).

Preparation of Faecal Samples for Virus Isolation.

Centrifuge tubes were labelled with sample numbers. 10 ml PBS, 1g of glass beads and 1 ml chloroform was added to each tube. Working in a BSC, approximately 2g of each faecal sample was transfer to a labelled centrifuge tube. The remaining original sample was retained, in its original container, for storage at -20^{o}C. Centrifuge tubes were close securely and shake vigorously for 20 minutes using a mechanical shaker. The mixture was spin for 20 minutes at 1500g (3000rpm) in refrigerated centrifuge at 4 degree (because enterovirus is heat labile). Working in a Bio-Safety Cabinet, supernatant from each sample was transfer into storage vials. The faecal suspension was stored at -20^{o}C freezer for cell line to be available for inoculation.(WHO, 2004).

Cell Culture Preparation: The cells were examined for quality and absence of contamination as determined by visual examination. Growth medium was decanted from the cell culture flask and the confluent

cell layer was washed gently twice with Ca and Mg free PBS. 0.25% trypsin solution (or equal parts of 0.25% trypsin and 1:5000 Versene solution) in PBS was added to the monolayer, and disperse evenly. (A volume of 1.0 ml is adequate for a T75cm^2 flask). The flask was placed in a 36.5^{o}C incubator until the cells detach from the surface: this was assisted by tapping the side of the flask a few times. Complete detachment of cells was checked by examining under an inverted microscope. The cells were re-suspended in growth medium (4.5ml to a 25cm^2 flask), which halts the action of the trypsin. The suspension was aspirated gently a few times through a fine Pasteur pipette to break up cell clumps. Cell counting procedure was carried out and the seeding concentration was determined. By diluting with growth medium to the desired concentration, fresh culture flasks and tubes were seeded, cap tightly, and placed in a 36^{o}C incubator. The tubes were changed to maintenance medium when the monolayer was nearly confluent after 2-3 days (WHO, 2004).

Virus Isolation

Inoculation: Already prepared stool specimens aliquot was inoculated into tubes containing a labelled monolayer of cells previously prepared. Stored samples were removed from freezer and allowed to thaw for about 3 minutes, and 0.2ml (200µl) of each specimen was inoculated in duplicate on RD and other cell lines using sterile 1ml pipettes. The tubes were arranged in a rack and immediately transferred to the incubator maintained at 37^{o}C. The tubes were observed daily for cytopathic effect (CPE), toxicity, degeneration or contamination and observations recorded accordingly for 5 days.(WHO, 2004).

Harvesting of tubes showing CPE: The tubes showing CPE was closely monitored and harvested when CPE has progresses to between 75% and 100%. The cell culture fluid from such tubes was stored at -20^{o}C in aliquot of 0.5ml for subsequent passage. Other tubes with no visible CPE were also retrieved for blind passage. Samples not showing CPE after the second passage were regarded as negative. Tubes with CPE after the second passage were passed further into another fresh cell to increase viral titre. The complete CPE tubes were harvested and frozen at 20^{o}C.(WHO, 2004).

Molecular Identification of enteroviruses.

Extraction of Total RNA : Total RNA was extracted from the 18 virus suspension using total RNA purification kit by Jena Bioscience, Jena, Germany according to the manufacturer's instruction. (Silicagel membrane adsorption).

Extraction Protocol

Reagent Preparation was calculated for 30 preps: Five centrifuge tubes were properly labelled each for lysis buffer, activation buffer, primary washing buffer, secondary washing buffer and elution buffer respectively. To the first tube, 156µl of 2-Mercaptoethanol was added to 15.6ml lysis buffer preparation according to manufacturer's instruction while 3.6ml of activation buffer was aliquoted into the second centrifuge tube. Then 4.8ml of 99% ethanol was added to the primary washing buffer (19.2ml) to obtain a final volume of 24ml in the third tube. Subsequently, 19.2ml 99% ethanol was added to secondary washing buffer (4.8ml) to obtain a final volume of 24ml in the fourth tube. Lastly, 3mL of Elution Buffer was aliquoted into the fifth centrifuge tube. All the preparation was done in a class II Biosafety cabinet.

Semi-Nested Polymerase Chain Reaction (snPCR) Assays: In this study, a Semi-nested PCR assay was used. The snPCR protocol used in this study was a modification of the recently recommended WHO protocol for enterovirus surveillance (WHO, 2015). Here, the product of the first round PCR was used as template for the two (2) different second round PCR.

Gel electrophoresis: Two grams of agarose was dissolved in 100mL of 1X Tris Boric EDTA (TBE) buffer to prepare a 2% agarose gel. The mixture was further melted in a microwave oven for three (3) minutes and allowed to cool to 45^{o}C. Five microliter (5µL) of ethidium bromide was then added carefully and the bottle containing the agarose and ethidium bromide was gently swirled for even distribution of the ethidium bromide. The mixture was then poured into the gel chamber with already fixed combs

and allowed to set for 20 to 30 minutes before the comb was carefully removed. Five microlitre of each PCR product was loaded into appropriate well and 5µL of standard DNA marker was also loaded to the middle well of each gel. On attachment of electrical leads to the electrophoresis unit, 140 volts and 400 milli- amperes was applied for 35 minutes to enable the migration of DNA to the anode. The gel was then viewed under a UV trans-illuminator. Positive samples showed the expected DNA band of 348–393 base pairs for enterovirus VP1 capsid sequence. Samples that showed no bands were considered negative. (WHO, 2015)

Amplicon Sequencing: The amplicons of positive PCR reactions for the 2nd PCR was shipped to Macrogen Inc, Seoul, South Korea, where amplicon purification and sequencing were done. Sequencing was done using the respective forward and reverse primers for each of the two assays. Subsequently, the sequence data was sent for further analysis (Kroneman *et al*., 2011).

Sequence Editing and Enterovirus Identification: Electropherograms of Sequence results were edited and subsequently, the regions of interest from the sequence data were selected using APE. This was done for both the forward and reverse complement sequence. CAP3, an online bioinformatics tool was used to generate the contig sequence from forward and reverse sequences. The contigs generated were used in Enterovirus Genotyping tool (EGT) for species and serotype identification. After identification using the EGT, known species same as the enterovirus serotype described in this study were then searched for on GenBank. Fasta format of the sequence obtained from GenBank in conjunction with the contigs, were pasted on Enterovirus genotyping tool for visual screening of the VP1 region amplified. Afterwards, unalignable sequences were deleted. Then accession numbers were searched for in GenBank for each serotype to determine their source. All data were saved for multiple sequence alignment.

Sequencing Analysis

Multiple Sequencing Analysis (MSA): Contigs obtained from this study were properly named, and then added to the strains found on GenBank. MSA was run using MEGA 5 software using CLUSTER *W* for alignment. Aligned data was then saved and prepared for Phylogenetic analysis (Tamura *et al*., 2011)

Phylogenetic Analysis: A neighbour-joining tree was constructed using the same MEGA 5 software with the Kimura-2 parameter model (Kimura, 1980) and 1000 bootstrap replicates. The accession numbers of sequences retrieved from GenBank for this analysis were indicated in the sequences name on the phylograms. A different serotype was used as outgroup and phylogenetic trees were generated.

Isolation Results: Of the 120 faecal samples collected during the rainy season and inoculated into RD cell line, only 14 (11.7 %) showed Cytopathic effect (CPE), while 92 faecal samples collected during the dry season,only 4 (4.3%) showed CPE. All 18 RD cell line isolates were inoculated into L20B cell line but none produced CPE, Therefore none of the 18 isolates are likely to be Poliovirus. Rather, all might be Non Polio enteroviruses (NPEVs). Only 18 (8.5%) of 212 samples collected from healthy children yielded isolates in RD cell line.

Molecular Identification results: Out of 18 isolates, 11(61.1%) and 7 (38.9%) were from male and female respectively). Similarly, of the 18 isolates, 11(61.1%) and 7 (38.9%) were from Ibadan southwest and Ibadan northwest local governments (LGAs), respectively. No isolate was recovered from samples collected in Ibadan north local government.

Molecular Identification Result

RT-snPCR Result*:* Of the 18 (8.5%) isolates subjected to RT-snPCR, 14 (77.8%) yielded the expected ~350bp band size. Of the fourteen (12 and 2 from rainy and dry seasons, respectively), two had very weak bands. These two were among the isolates recovered from samples collected during the rainy season. Hence, only 12 of the samples

yielded bands with sufficient intensity for sequencing.

Enterovirus Identification: Of the 12 amplicons sequenced, only seven had exploitable sequence data. The remaining five were not exploitable due to multiple peaks. The seven exploitable sequence data were identified as Echovirus 11 (E11) (four isolates), E29 (one isolate) and E33 (two isolates).

Molecular identification of Enterovirus isolates: The Agarose gel electrophoresis image showing amplification product of the snPCR assay for isolates recovered from wet and dry seasons are shown in Plates 1 and Plate 2. Out of the 18 isolates of Enterovirus from the study respondents, only 14 were screened positive with RT-snPCR (12 from wet and 2 from dry seasons). Similarly, out of the 12 amplicons sequenced, only seven had exploitable sequence data. The seven exploitable sequence data were identified as Echovirus 11 (E11) (four isolates), E29 (one isolate) and E33 (two isolates) Three different enterovirus types (E11, E29 & E33) were detected in this study. Four isolates of E11 were recovered in this study. All belong to two of the three sub-Saharan Africa (SSA) clades that have been previously detected in the country(Palacio, 2002, Oyero *et al* 2014, Faleye *et al,* 2016). Three of the four isolates belong to SSA 1 and particularly belong to a lineage that had been detected and shown to be circulating in Nigeria since 2012. The fourth isolate (E11) belongs to SSA 3 and is ancestral to a cluster that circulated in Ghana, Central Africa Republic and Nigeria in the first decade of the 20th century. Two different clusters (SSA 1 & 2) of E29 have been described to be present and circulating in Nigeria till date(Faleye *et al,* 2016). The E29 strains that circulated in Nigeria in the first decade of the 20th century belong to SSA 1 while those detected in the second decade of the century belong to SSA 2. The E29 strain described in this study, as expected, belong to SSA 2. Only one cluster of E33 has been detected in SSA since 2003. The two E33 strains described in this study are closely related to the E33 strains previously described in SSA. The topology of the tree however suggests there might be some yet to be characterised lineages of E33 circulating in SSA. The phylogenetic tree is base domain alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. Strains previously detected in Nigeria are indicated with black triangle and diamond. The two strains indicated with black diamond is for ease of reference only. For the Nigerian strains, the GenBank accession numbers and year of sample collection are indicated in the tree. Bootstrap values are indicated if >50%. The labelled vertical bars are for ease of reference alone. The phylogenetic tree is base domain alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. Strains previously detected in Nigeria are indicated with black triangle, square and diamond. The two strains indicated with black square and diamond is for ease of reference only. For the Nigerian strains, the GenBank accession numbers and year of sample collection are indicated in the tree. Bootstrap values are indicated if >50%. The labelled vertical bars are for ease of reference alone. The phylogenetic tree is base domain alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. Strains previously detected in Nigeria are indicated with black triangle. For the Nigerian strains, the GenBank accession numbers and year of sample collection are indicated in the tree. Bootstrap values are indicated if >50%. The labelled vertical bars are for ease of reference alone.

DISCUSSION

The results obtained from these findings confirmed that enteroviruses can indeed be recovered from apparently healthy children as previously reported by Baba *et al.*,(2012) and Faleye *et al.*,(2016). Enteroviruses were recovered from 8.5% (18/212) of the apparently healthy children sampled. This is higher than the 5.5% reported by Oyero *et al.*,(2014) using same cell culture method which is the gold standard for virus isolation (WHO, 2004) Faleye *et al.,* (2016), on the other hand, reported a higher (18.3%) prevalence but did not use a cell culture based method. Rather,

the cell culture independent RT-snPCR based protocol, recently recommended by the WHO (2015) was used. The difference in prevalence found might therefore be a reflection of the impact of using different detection protocols. Processed faecal samples suspension were cultured in L20B cell and RD cell using cell culture method of virus isolation as this confirmed the earlier report of Johnston and Siegel, (1990), that isolation of enterovirus group was based on the growth of the viruses in different cell lines such as, RD , L20B, HEP-2C, LLCMK cell line. Cytopathic effect observed from culture of L20B and RD cell line used was characterized by visible rounding, shrinking, nuclear pyknosis, refractile and cell degeneration. This finding is in agreement with that of Reissig *et al*., (1956) who reported the same effect in epithelia cells. The RD cell line used which is specific for the isolation of enteroviruses, derived from a human rhabdomyosarcoma (McAllister *et al.*, 1969), yielded total of eighteen isolates. This human Cancerous cell has been known to support the replication of enteroviruses (Crowell and Goldberg, 1974; Schmidt *et al*., 1975).Out of 18 isolates obtained from this study from cell culture method, only 15 (83.3%) were positive using RT sn-PCR and 3(16.7%) were negative. This is in agreement with several studies which have established that PCR as an effective and specific method for virus isolation (Reynolds *et al.*, 2001, Greening *et al.*, 2002). The negative results obtained from PCR used confirmed that they are not entroviruses but other types of viruses that can grow in RD cell line such as Adenovirus. Thus, cell culture method is not specific since other classes of virus can be isolated from it. Polymerase Chain Reaction (PCR) result obtained was based on the specificity of the RT sn-PCR method and PCR results are obtained within a few hours compared to traditional cell culture that take about about) days. This is in agreement with the previous work of (Nijhuis *et al.*, 2002).The results of this study suggest that lifestyle and standard of living might be a major contributor to exposure of apparently healthy children to enteroviruses. For example, in this study, all the children from which isolates where recovered lived in slums and have well as one of their major (and sometimes the only) source of water. Considering that enteroviruses are transmitted fecal-orally, through contaminated hands, food and drinking water, and as was observed during sample collection in the field, overflowing sewages often enter and contaminate sources of drinking water in slums and several other low-income neighborhoods (Melnick *et al.*, 1978; Pallansch and Roos, 2007). In fact, there was a preponderance of lack of quality drinking water, open gutters, filthy and overflowing sewages, shallow wells and streams where human wastes were discharged around study areas. It is therefore likely that these children got exposed to the virus via this route.

Phylogenetic Analysis:

Three different enterovirus types (E11, E29 & E33) were detected in this study. All the strains of the three enterovirus types detected belong to lineages that have been previously detected and described to be circulating in sub-Saharan Africa (SSA). The results of this study therefore support the *'regional confinement hypothesis'* which suggests that certain enterovirus lineages circulating in SSA are confined to the region (Sadeuh-Mba et al., 2013, Adeniji and Faleye, 2014, Faleye and Adeniji, 2015). The E11 genotype (lineage) that circulated between 2002 and 2010 in Nigeria (Adeniji and Faleye 2014) appears to have been replaced by another lineage that has been detected between 2012 (Faleye and Adeniji, 2015) and 2017. Furthermore, the isolate 8B appears to be the first member of a new circulating lineage that is yet to be described. These observations suggest that circulating E11 genotypes remain predominant for about five to eight years before being replaced by another. This therefore confirms the idea that genotype replacement is a central part of enterovirus evolutionary strategies in populations (Adeniji and Faleye, 2014).

Plate 1: Agarose gel electrophoresis image showing amplification product of the snPCR assay for isolates recovered from rainy season samples. The expected band size is approximately 350bp.

Key: Lane L: Molecular ladder. 1-4: VP1 Amplicon. PC: Positive Control- Rainy season

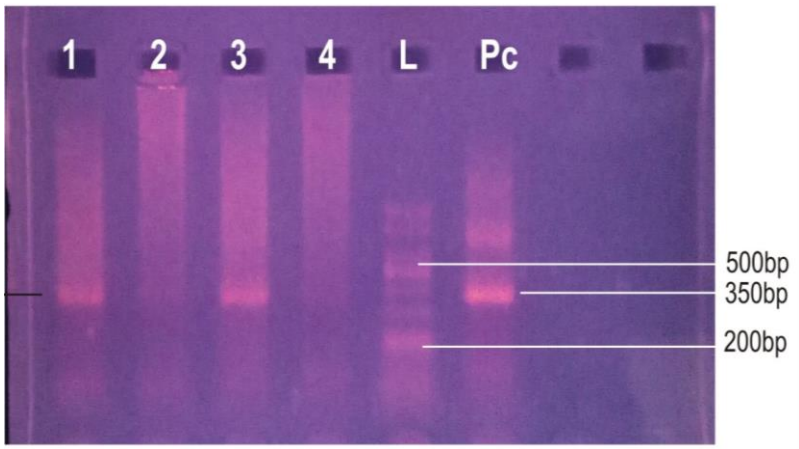

Plate 2: Agarose gel electrophoresis image showing amplification product of the snPCR assay for isolates recovered from dry season samples. The expected band size is approximately 350bp.

Key: Lane L: Molecular ladder. 1-4: VP1 Amplicon. PC: Positive Control. Dry season.

Table 1: Sequencing results of the isolated Enterovirus by serotypes.

Name	Length	Family	Genus Species	Serotype, Sub-Genogroup
2B_SW160	246	Picornaviridae	Enterovirus B	E-33
3B_SW041	245	Picornaviridae	Enterovirus B	E-29
8B_NW049	290	Picornaviridae	Enterovirus B	E-11
9B_SW079	360	Picornaviridae	Enterovirus B	E-33
10B_SW083	310	Picornaviridae	Enterovirus B	E-11
12B_SW177	367	Picornaviridae	Enterovirus B	E-11
14B_SW129	291	Picornaviridae	Enterovirus B	E-11

Table 2: Sequencing results of the Enterovirus by serotypes with Primer Label.

Name	Length	Family	Genus Species	Serotype, Sub-Genogroup
2B_AN89	246	Picornaviridae	Enterovirus B	E-33
3B_AN89	245	Picornaviridae	Enterovirus B	E-29
8B_AN89	290	Picornaviridae	Enterovirus B	E-11
9B_Contig9B	360	Picornaviridae	Enterovirus B	E-33
10B_AN89	310	Picornaviridae	Enterovirus B	E-11
12B_Contig12B	367	Picornaviridae	Enterovirus B	E-11
14B_AN89	291	Picornaviridae	Enterovirus B	E-11

Phylogenetic Analysis

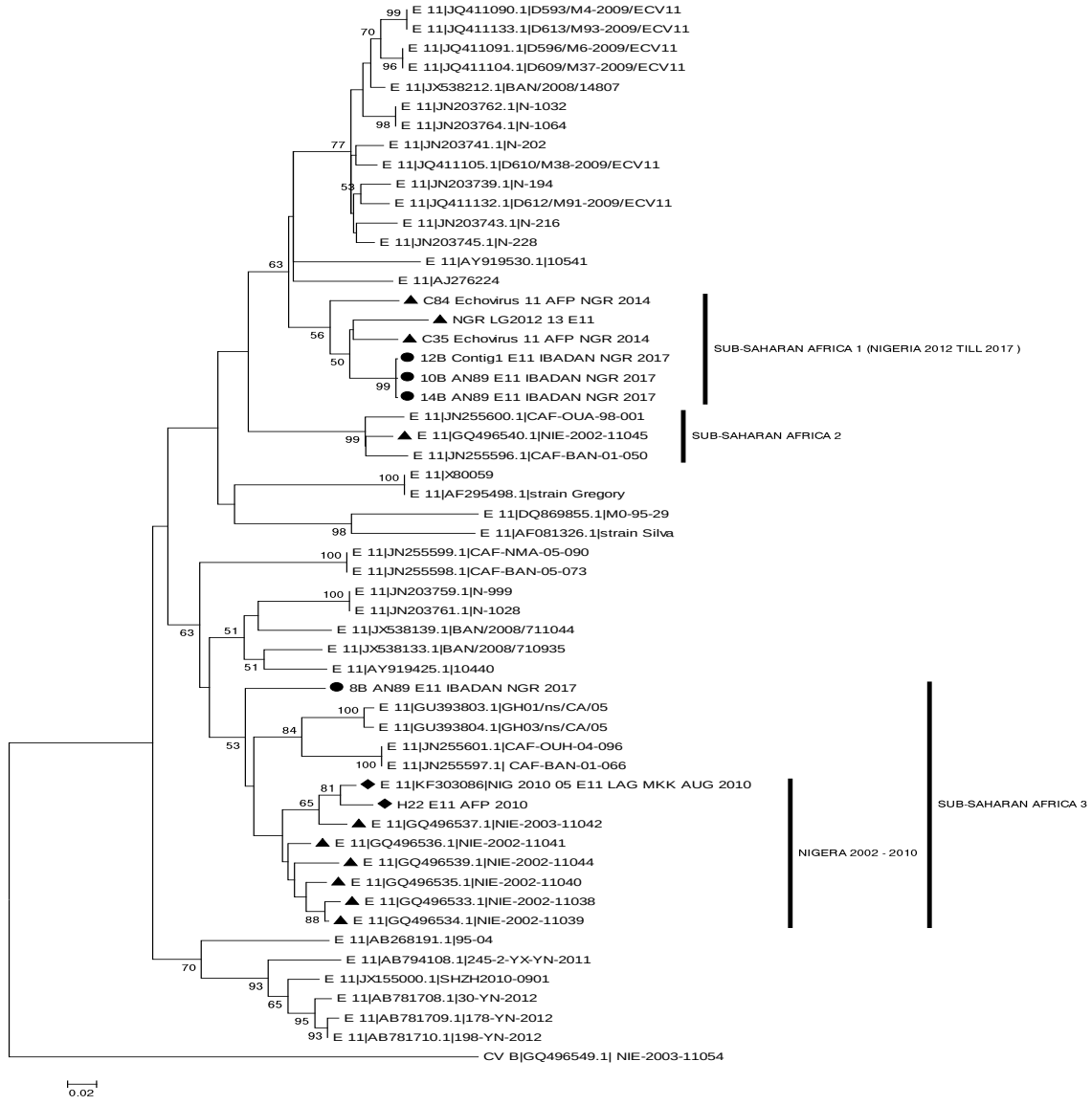

Figure 1: Phylogram of geneticrelationshipbetweenVP1 nucleotidesequences of Echovirus 11 (E11) isolates.

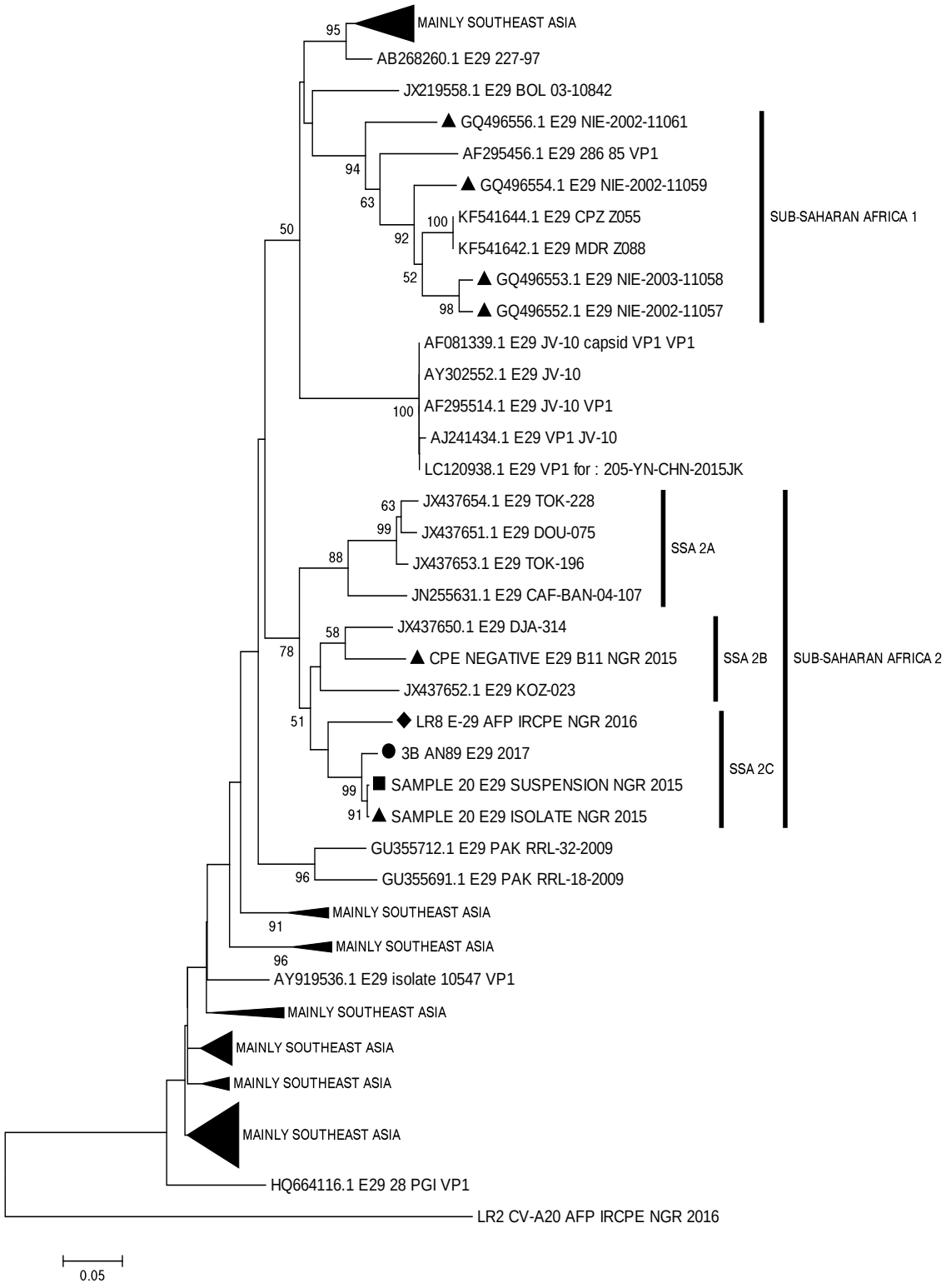

Figure 2: Phylogram of genetic relationship betweenVP1 nucleotide sequences of Echovirus 29 (E29) isolates.

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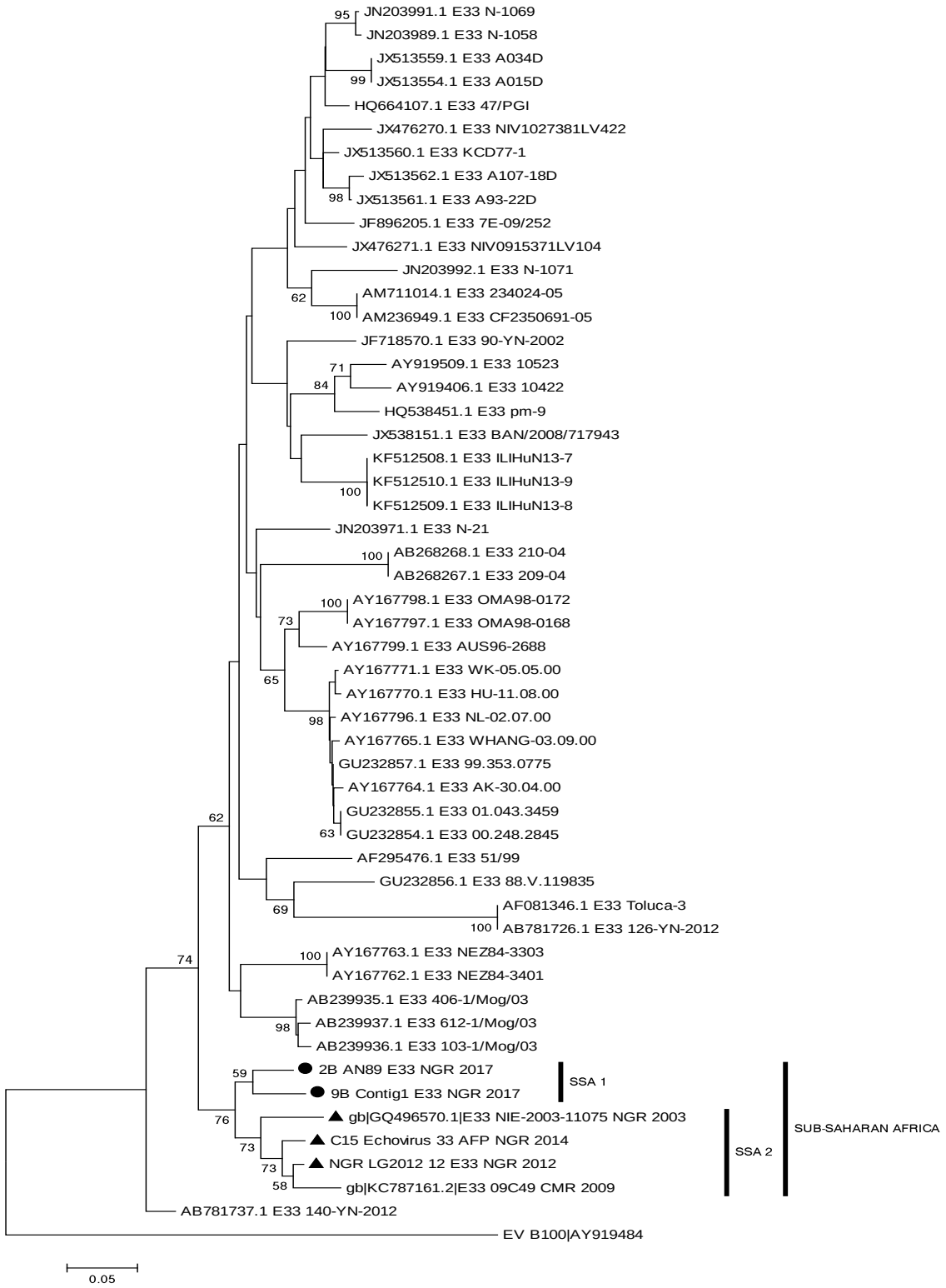

Figure 3: Phylogram of geneticrelationshipbetweenVP1 nucleotidesequences of Echovirus 33 (E33) isolates.

What defines this time interval is currently not clear. On the other hand, the E33 lineage described in this study appears to have been somewhat circulating in Nigeria since 2003 (i.e. for 14 years). Why E11 genotypes are replaced after 5-8 years but an E33 lineage can circulate sustainably for 14 years is not clear. It is however important that the

dynamics of this duo of sustained transmission versus genotype replacement be perfectly understood in a bid to help guide enterovirus detection, prevention and control strategies.Echovirus 29 (E29) strains have been described in Nigeria since 2002 (Oyero et al., 2014) and these early strains (SSA 1) were isolated on RD cell line as is the strain described in this study. However, we recently came across two LR8 (diamond) and B11 (triangle) SSA 2B and C strains of E29 that were not cytopathic on RD cell line but were only detected by a cell culture independent enterovirus detection and identification method (Nix et al., 2006, WHO, 2015). These two strains were recovered from 2015 and 2016. It is however crucial to mention that all the other strains described in the country till date showed cytopathology in RD cell culture. This led to re-assess one of the two non-cytopathology producing strains LR8 (diamond) and SSA 2C. On further passage beyond the stipulated 10 days in the WHO recommended algorithm (WHO, 2004), the virus started developing cytopathology in RD cell line. Hence, it is likely, that there are no non-cytopathology producing strains of E29. Rather, the virus might be a late cytopathology producing strains that do not produce cytopology within the stipulated 10 days culture period. In this study, three different enterovirus types (E11, E29 & E33) were identified. Particularly interesting is the fact that these enterovirus types had been previously detected in Nigeria (Oyero et al., 2014; Adeniji and Faleye, 2014) and therefore confirmed by this study to still be circulating. Further confirming their circulation is the fact that, the four E11 isolates recovered in this study were recovered from healthy children from four different schools distributed across both Ibadan Northwest and southwest LGAs. How these viruses are transmitted across the different LGAs is not known but it is likely that sewage contaminated running surface water might be the major vehicle moving the viruses across the city of Ibadan.

CONCLUSION

The results of this study confirmed that enteroviruses can indeed be recovered from apparently healthy children in Ibadan. This suggests that lifestyle and standard of living might be a major contributor to exposure of apparently healthy children to enteroviruses in Ibadan, Nigeria. It highlights that children living in slums at highest risk of exposure and infection due to a preponderance of lack of quality drinking water, open gutters, filthy and overflowing sewages, shallow wells and streams where human wastes were discharged in such communities. Therefore, environmental factors which encourage transmission of enteroviruses should be properly monitored, personal hygiene should be practised, vaccinations and routine immunization should also be encouraged.

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