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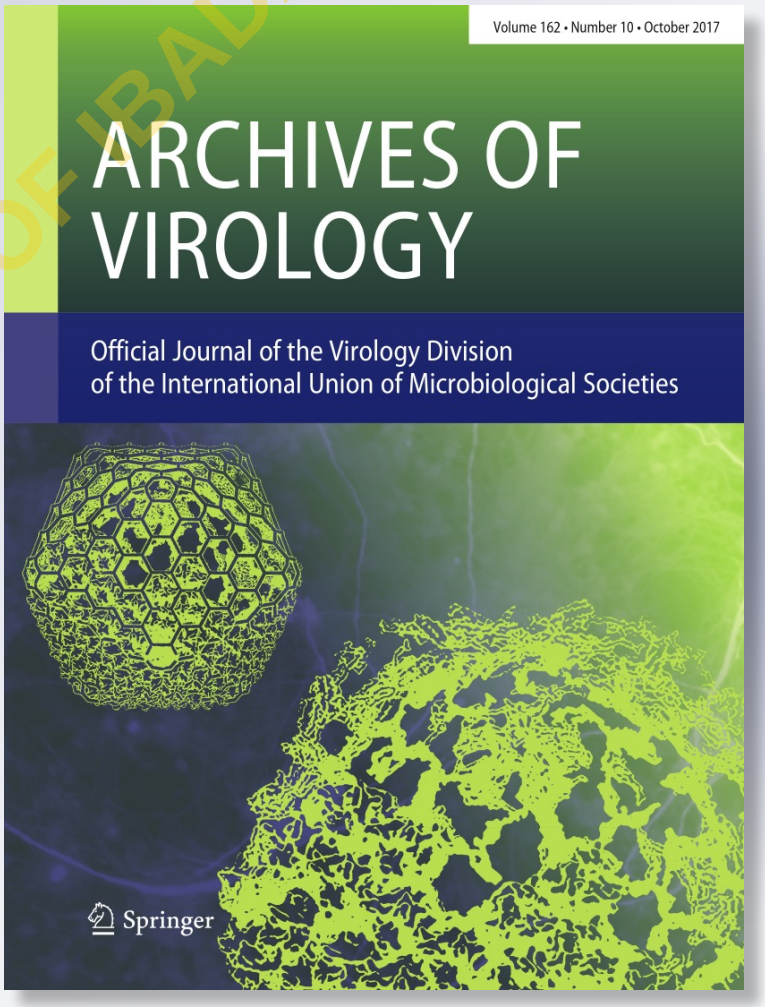

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ORIGINAL ARTICLE

Preponderance of enterovirus C in RD-L20B-cell-culture-negative stool samples from children diagnosed with acute flaccid paralysis in Nigeria

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Abstract Recently, a reverse transcriptase semi-nested polymerase chain reaction (RT-snPCR) assay was recommended by the WHO for direct detection of enteroviruses in clinical specimens. In this study, we use this assay and a modification thereof to screen acute flaccid paralysis (AFP) samples that had previously tested negative for enteroviruses by the RD-L20B algorithm. Thirty paired stool suspensions collected in 2015 as part of the national AFP surveillance program in different states of Nigeria were analyzed in this study. The samples had previously tested negative for enteroviruses in the polio laboratory in accordance with the WHO-recommended RD-L20B-cell-culture-based algorithm. Two samples that had previously been found to contain enteroviruses were included as positive controls. All samples were subjected to RNA extraction, the RT-snPCR assay and a modified version of the RT-snPCR. All amplicons were sequenced, and enteroviruses were identified using the enterovirus genotyping tool and phylogenetic analysis. Amplicons were recovered from the two controls and 50% (15/30) of the samples screened. Fourteen were successfully typed, of which, 7.1% (1/14), 21.4% (3/14), 64.3% (9/14) and 7.1% (1/14) were enterovirus (EV) -A, EV-B, EV-C and a mixture of EV-B and C (EV-C99 and E25), respectively. The two controls were identified as EV-C99 and coxsackievirus (CV) -A1, both of which belong to the species *Enterovirus C*. In one sample, poliovirus serotype 2 was detected and found to have the VP1 $_{ILE}$143 variation and was therefore identified as a vaccine strain. The results of this study showed that significant proportion of enterovirus infections (including some with Sabin PV2) are being missed by the RD-L20B-cell-culture-based algorithm, thus highlighting the value of the RT-snPCR assay and its modifications. The circulation and preponderance of EV-C in Nigeria was also confirmed.

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Introduction

Enteroviruses are members of the genus *Enterovirus* in the family *Picornaviridae*, order *Picornavirales*. The type member of the genus is poliovirus, the etiologic agent of poliomyelitis and a member of the species *Enterovirus C,*

one of the 13 species in the genus (http://www.picornaviridae.com). Many of the enterovirus serotypes (>60) described to date belong to the species *Enterovirus B*. (http://www.picornaviridae.com). Classically, serotypes have been defined using a neutralization assay [1, 2]. More recently, a correlation has been found between the nucleotide sequence of the VP1 gene and the serotype [1, 2]. Consequently, the nucleotide sequence of the VP1 gene is being used for enterovirus serotype/genotype designation, and this has allowed the discovery of several new types (http://www.picornaviridae.com).

The majority of the nonpolio enteroviruses (NPEVs) isolated until now were isolated in association with the poliovirus eradication effort (Global Polio Eradication Initiative [GPEI]). The GPEI was inaugurated in 1988 via a World Health Assembly (WHA) resolution to eradicate poliovirus [3]. The effort has centered around intensive vaccination (with both oral and inactivated polio vaccines [OPV and IPV]), and surveillance (for poliovirus in children <15 years old with acute flaccid paralysis [AFP] and sewage-contaminated water) [4–6].

As part of the surveillance effort, there is a team of about 140 laboratories (Global Polio Laboratory Network [GPLN]) that are WHO accredited and responsible for poliovirus laboratory diagnosis globally in accordance with the WHO guidelines [5, 6]. These guidelines have isolation of poliovirus in cell lines (RD and L20B) as the core on which the rest of the algorithm is built.

Although it is very sensitive for poliovirus detection and identification, studies have shown that the RD-L20B algorithm also selectively supports enterovirus B (EV-B) detection while not significantly supporting nonpolio enterovirus C (NPEV-C) isolation [7–9]. This suggests that some NPEVs are missed by the RD-L20B algorithm. Furthermore, we have previously shown [8] that by including a cell line such as MCF 7 in the isolation algorithm, enteroviruses that had been missed by the RD-L20B algorithm can be subsequently recovered from sewage-contaminated water (environmental) samples.

Recently, a reverse transcriptase semi-nested polymerase chain reaction (RT-snPCR) assay (first described by Nix et al. [10]) was recommended by WHO for direct detection of enteroviruses in clinical specimens [11]. The sensitivity of this assay has been confirmed by several studies [12–15], and we have further improved the assay by enhancing its enterovirus co-infection resolution capacity [15].

In this study, we use the WHO-recommended RT-snPCR assay [11] and a modification thereof [15] to screen AFP samples that had been screened previously and declared negative for enteroviruses using the RD-L20B algorithm [6]. We show that 46.7% of the samples had enteroviruses in them and that enterovirus C (EV-C) was the predominant type recovered.

Materials and methods

Sample collection and processing

The samples analyzed in this study were obtained from the WHO-accredited national polio laboratory at the Department of Virology, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria (subsequently referred to as the polio lab). These stool suspensions were collected in August 2015 (the peak of the rainy season) as part of the national acute flaccid paralysis (AFP) surveillance program in different states of Nigeria. In August 2015, samples from 866 AFP cases were received by the laboratory. Enteroviruses, and specifically polioviruses, were detected in 13.74% (119/866) and 9.35% (81/866), of the cases, respectively. Hence, 86.26% (747/866) of the cases whose samples were received by the polio lab that month were declared negative for enteroviruses using the cell-culture-based algorithm [5, 6].

Thirty of these 747 (4.02%) cases whose samples showed no cytopathology in RD and L20B cell lines [6] and were consequently declared negative for enteroviruses were selected at random and analyzed in this study. The 30 cases consisted of 60 samples (two samples for each case collected within 24 hours). Each of the 30 paired samples was pooled (i.e., to make 30 samples) and analyzed. To make the pools, paired stool suspensions (previously prepared by the polio lab as described in the guidelines for poliovirus surveillance [6] and stored at -20 °C) were recovered from the sample archive maintained by the polio lab. The suspensions were thawed and vortexed for 15 seconds. Subsequently, aliquots of 100 µL were recovered from each of the two suspensions per case and mixed in an appropriately labelled, sterile 1.5-mL centrifuge tube. Each pool therefore contained 200 µL of stool suspension. In addition, two samples that had previously been confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR) amplification and sequencing of the VP1 gene to contain nonpolio enteroviruses were processed similarly and included to serve as positive controls. Figure 1 summarizes the algorithm followed in this study.

RNA extraction and cDNA synthesis

RNA was extracted from the 32 samples using an RNA extraction kit (Jena Bioscience, Jena, Germany) according to manufacturer's recommendations. Similarly, cDNA was synthesized from the RNA extract using a SCRIPT cDNA

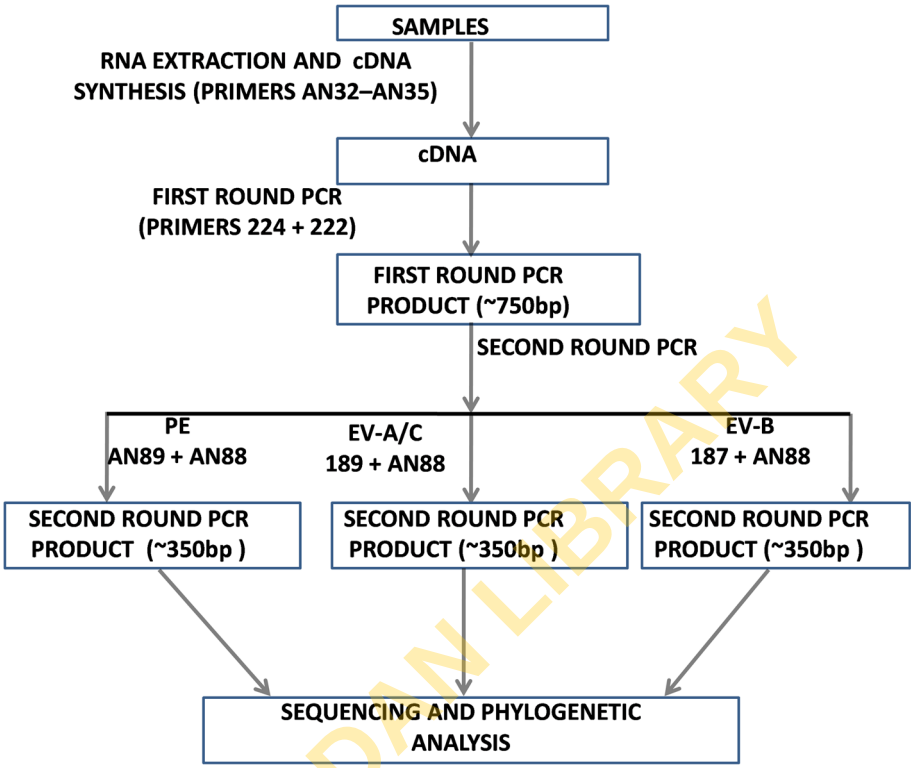


Fig. 1 Schematic representation of the algorithm followed in this study

Synthesis Kit (Jena Bioscience, Jena, Germany) according to manufacturer's recommendations. Firstly, 4.75 µL of cDNA synthesis mix was prepared per reaction, and this contained 2 µL of Script RT buffer, 0.5 µL of dNTP mix, 0.5 µL of DTT stock solution, 0.5 µL of RNase inhibitor, 0.25 µL of SCRIPT RT and 0.25 µL of each of the oligonucleotide primers AN32, AN33, AN34, and AN35 [10, 11]. Then, 5.25 µL of RNA extract was added to make 10 µL. This mixture was incubated at 42 °C for 10 minutes, followed by 50 °C for 60 minutes in a Veriti thermal cycler (Applied Biosystems, California, USA).

Semi-nested polymerase chain reaction (snPCR) screening

One first-round and three different second-round (semi-nested) PCR assays (panenterovirus [PE], enterovirus A or C [EV-A/C] and enterovirus B [EV-B]) were done (Fig. 1). For the first-round PCR assay, the reactions were done in 30 µL volumes. Each contained 6 µL of Red Load Taq, 13.4 µL of RNase-free water, 0.3 µL each of the forward (224) and reverse (222) primers, and 10 µL of cDNA. A Veriti thermal cycler (Applied Biosystems, California, USA) was used for thermal cycling with the following conditions; 94 °C for 3 minutes, followed by 45 cycles of 94 °C for 30 seconds, 42 °C for 30 seconds, and 60 °C for 60 seconds, with ramp of 40% from 42 °C to 60 °C. This was then followed by 72 °C for 7 minutes, and the temperature was held at 4 °C until the reaction was terminated.

All three second-round PCR assays were carried out with the first-round PCR product as template, with similar thermal cycling conditions except for the extension time, which was reduced to 30 seconds. The same reverse primer (AN88) was used for all three second-round PCR assays. The forward primers were AN89, 189 and 187 for the PE, EV-A/C and EV-B PCR assays, respectively (Fig. 1). Subsequently, all PCR products were resolved on 2% agarose gels stained with ethidium bromide and viewed using a UV transilluminator.

Amplicon sequencing and enterovirus identification

Amplicons of all the second-round PCR assays with the expected band size (i.e., ~350 bp) were shipped to Macrogen Inc., Seoul, South Korea, for purification and nucleotide sequencing. Afterwards, the enterovirus genotyping tool (EGT) [16] was used for enterovirus species and genotype determination.

Phylogenetic analysis

Considering EV-C was the most commonly detected virus, the EV-C sequences were subjected to phylogenetic analysis. Multiple sequence alignments were done using

reference sequences downloaded from the GenBank database and aligned using the CLUSTAL W program in MEGA 5 software with default settings [17]. Subsequently, neighbor-joining trees were constructed using the same MEGA 5 software with the Kimura 2-parameter model [18] and 1,000 bootstrap replicates. The accession numbers of sequences retrieved from GenBank are indicated in the sequence names on the phylogenetic trees.

Nucleotide sequence accession numbers

All of the sequences determined in this study have been deposited in GenBank under accession numbers KY748282-KY748295.

Results

PCR results

The PE assay successfully amplified the expected ~350 bp from 15 of the 30 (50%) samples screened in this study and the two controls. The EV-A/C assay successfully amplified the expected ~350 bp from 11 of the 30 (36.7%) samples screened as well as the two controls. The EV-B assay successfully amplified the expected ~350 bp from nine (9) of the 30 (30%) samples screened as well as the two controls. Altogether, amplicons were recovered from 15 of the 30 (50%) samples screened as well as the two controls (Table 1).

Sequencing and genotyping results

For the PE assay, of the 15 amplicons generated, 13 were successfully sequenced alongside the two controls, while two were not sequenced due to very weak bands. The sequenced amplicons were identified as enterovirus (EV) A119 (one strain), echovirus 21 (E21) (one strain), EV-B97 (one strain), EV-B111 (one strain), poliovirus serotype 2 (PV-2) (one strain), coxsackievirus (CV) -A13 (two strains), CV-A17 (one strain), CV-A20 (one strain), and EV-C99 (four strains), Specifically, of the 13 strains detected by the PE assay, one, three and nine belonged to EV-A, EV-B and EV-C, respectively. The two controls were identified as EV-C99 and CV-A1, both EV-Cs (Table 1).

For the EV-A/C assay, all 11 amplicons were successfully sequenced alongside the two controls and identified as EV-A119 (one strain), PV-2 (one strain), CV-A13 (two strains), CV-A17 (one strain), CV-A20 (two strains), and EV- C99 (four strains). Of the eleven strains detected in this study, 10 belonged to EV-C, while one was EV-A. The two controls were identified as EV-C99 and CV-A1, both of which belong to the species *Enterovirus C* (Table 1).

For the EV-B assay, all nine amplicons and the two controls were successfully sequenced. However, sequence data from four of the amplicons and the two controls were not usable due to the presence of multiple peaks. The remaining five amplicons were successfully typed and identified as echovirus 21 (E21) (one strain), E25 (one

Table 1 Enterovirus types detected and identified in this study. Only results for samples that were positive in at least one test are shown

S/N	Sample ID	PE		EV-A/C		EV-B		Summary	Species
		PCR	ID	PCR	ID	PCR	ID		
1	1	+	EV-C99	+	EV-C99	+	E-25	EV-C99/E-25	EV-B/C
2	4	+	EV-C99	+	EV-C99	+	EV-C99	EV-C99	EV-C
3	5	+	E-21			+	E-21	E-21	EV-B
4	7	+	PV-2	+	PV-2			PV-2	EV-C
5	13	+	EV-B111			+	EV-B111	EV-B111	EV-B
6	14	+	CV-A17	+	CV-A17			CV-A17	EV-C
7	15	+	EV-A119	+	EV-A119			EV-A119	EV-A
8	17	+	EV-B97			+	EV-B97	EV-B97	EV-B
9	18	+	CV-A13	+	CV-A13	+	NU	CV-A13	EV-C
10	19	+	EV-C99	+	EV-C99			EV-C99	EV-C
11	23	+*	NS	+	CV-A20	+	NU	CV-A20	EV-C
12	24	+*	NS						
13	27	+	CV-A13	+	CV-A13	+	NU	CV-A13	EV-C
14	28	+	EV-C99	+	EV-C99	+	NU	EV-C99	EV-C
15	30	+	CV-A20	+	CV-A20			CV-A20	EV-C
C1	31	+	EV-C99	+	EV-C99	+	NU	EV-C99	EV-C
C2	32	+	CV-A1	+	CV-A1	+	NU	CV-A1	EV-C

NU, not usable; *, very weak band; NS, not sequenced; C1, control 1; C2, control 2

strain), EV-B97 (one strain), EV-B111 (one strain) and EV-C99 (one strain) (Table 1).

Coinfection resolution

Only sample number one contained a mixture of viruses (EV-C99 and E25), and this was resolved (Table 1). It is, however, important to note that, had we relied on only the PE assay as recommended by WHO [11], the presence of E25 in this sample would have been missed. Similarly, we might have missed CV-A20 in sample 23 (Table 1) had we relied on the PE assay alone.

Sequence-based determination of the type of the detected PV-2 strain

Multiple sequence alignment showed that the PV-2strain that was detected had the amino acid isoleucine at the 143rd position of the VP1 protein, suggesting it to be of vaccine strain origin (Fig. 2a). However, alignment of nucleotides corresponding to residues 45 to 159 of the amino acid sequence alignment in Figure 2a showed that, within the 345 nucleotides sequenced (Fig. 2b), four changes (C138A, A147G, T270C and T477C) are evident that differentiate the PV2 detected in this study from the reference Sabin strains (AY184220.1 and X00595.1).

Phylogenetic analysis

In this study, we detected five EV-Cs, but phylogenetic trees were constructed for only four of them. No phylogenetic tree was prepared for the PV-2 strain that was detected because the majority of global PV sequences are not available in GenBank but are in specialized databases managed by the Global Polio Eradication Initiative. Therefore, any phylogenetic tree of PV2 that is based on the sequences in GenBank would not be representative.

Two strains of CV-A13 were recovered in this study (Table 1), and both (18a and 27a) belong to two different lineages of CV-A13 (sub-Saharan Africa 1 and 2, respectively; Fig. 3) that have only been described in sub-Saharan Africa. In sub-Saharan Africa 1 (SSA1) 18a shares a common ancestor with a strain recovered from a child in Cameroon in 2009 [7]. In sub-Saharan Africa 2 (SSA2), 27a shares a common ancestor with an isolate recovered from sewage-contaminated water in 2012 in Nigeria [19].

The coxsackievirus A17 (CV-A17) strain recovered in this study (Table 1) is very different from that detected in Nigeria in 2003 (Fig. 4). In fact, they belong to two different lineages (sub-Saharan Africa and Madagascar and Nigeria 2015; Fig. 4). The CV-A17 strain detected in this study shares a common ancestor with a lineage found in Madagascar in 2002 [20, 21] (Fig. 4).

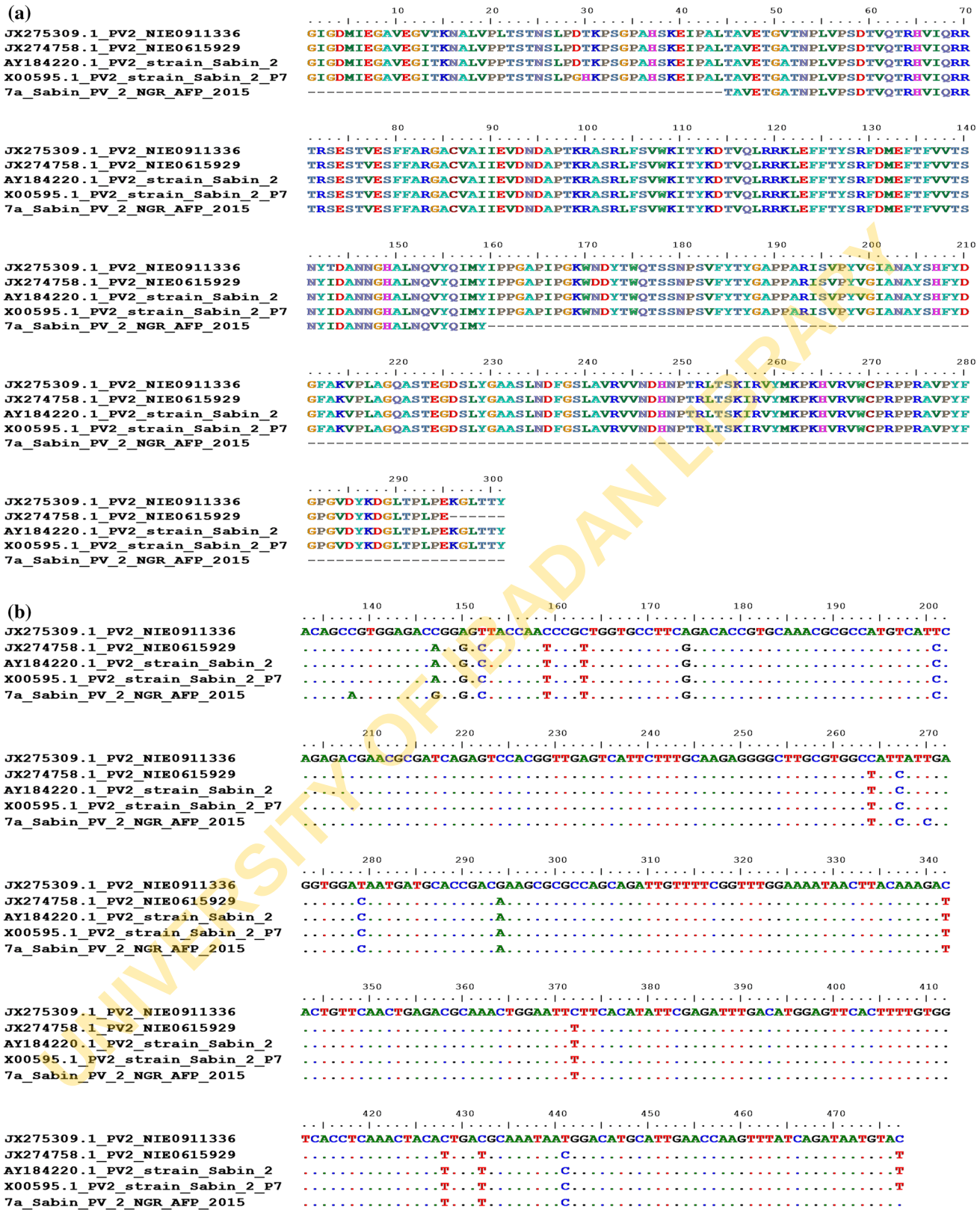
The two CV-A20 strains (23b and 30a) recovered in this study (Table 1) belong to a lineage that has been circulating in sub-Saharan Africa for at least a decade (Fig. 5). In this lineage, designated sub-Saharan Africa (SSA), 23b shares a common ancestor with a strain recovered from a child in Cameroon in 2008 [7]. On the other hand, 30a shares a common ancestor with a strain recovered from a child in Nigeria in 2014 [14].

Four EV-C99 strains (1a, 4a, 19a and 28a) were detected in this study (Table 1), and they all belonged to three different lineages (Fig. 6). 1a is ancestral to a lineage that has been detected in Cote d'Ivoire [22], Cameroon [7] and Finland [23]. Strains 4a and 19a form a cluster (Nigeria 2014-2015) with an EV-C99 strain detected in a child in Nigeria in 2014 [14]. Finally, 28a belongs to a lineage that has so far only been detected in sub-Saharan Africa. This lineage also includes an EV-C99 strain detected in Nigeria in a child with AFP in 2014 (Faleye et al., unpublished) and another detected in a captive chimpanzee in Congo in 2011 (Mombo et al., unpublished, with accession number KP793035)

Discussion

In this study, enteroviruses were detected in 50% (15/30) of the samples screened. However, enteroviruses could only be accounted for unambiguously in 46.7% (14/30) of the samples screened. Hence, the findings of this study suggest that about 46-50% of the stool samples from children <15 years old diagnosed with AFP in Nigeria that tested negative for enteroviruses by the RD-L20B-cell-culture-based algorithm might actually contain enteroviruses. Considering that only ~4% (30/747) of the cell-culture-negative samples for the month in question were screened in this study, one can only imagine the number of enterovirus infections missed by the RD-L20B-cell-culture-based algorithm annually. The failure of this algorithm to detect some EVs may be due to factors like the cell lines not being susceptible and/or permissive to the EVs in question, the presence of slow-growing EVs in stool samples, poor handling of stool samples during transport to the laboratory (and consequent arrival with no viable virus) and/or very low titre of viruses in the sample. We understand, however, that the detection limit of any PCR assay and the virus genome concentration in any sample both ultimately influence the sensitivity of the PCR assay. Hence, it is crucial to state that we are aware that the 46-50% detected in this study might represent an underestimate.

Analysis of the enterovirus types found in this study revealed that 7.1% (1/14), 21.4% (3/14), 64.3% (9/14) and 7.1% (1/14) of the strains were EV-A, EV-B, EV-Cs and a mixture of EV-B and C, respectively (Table 1). This study



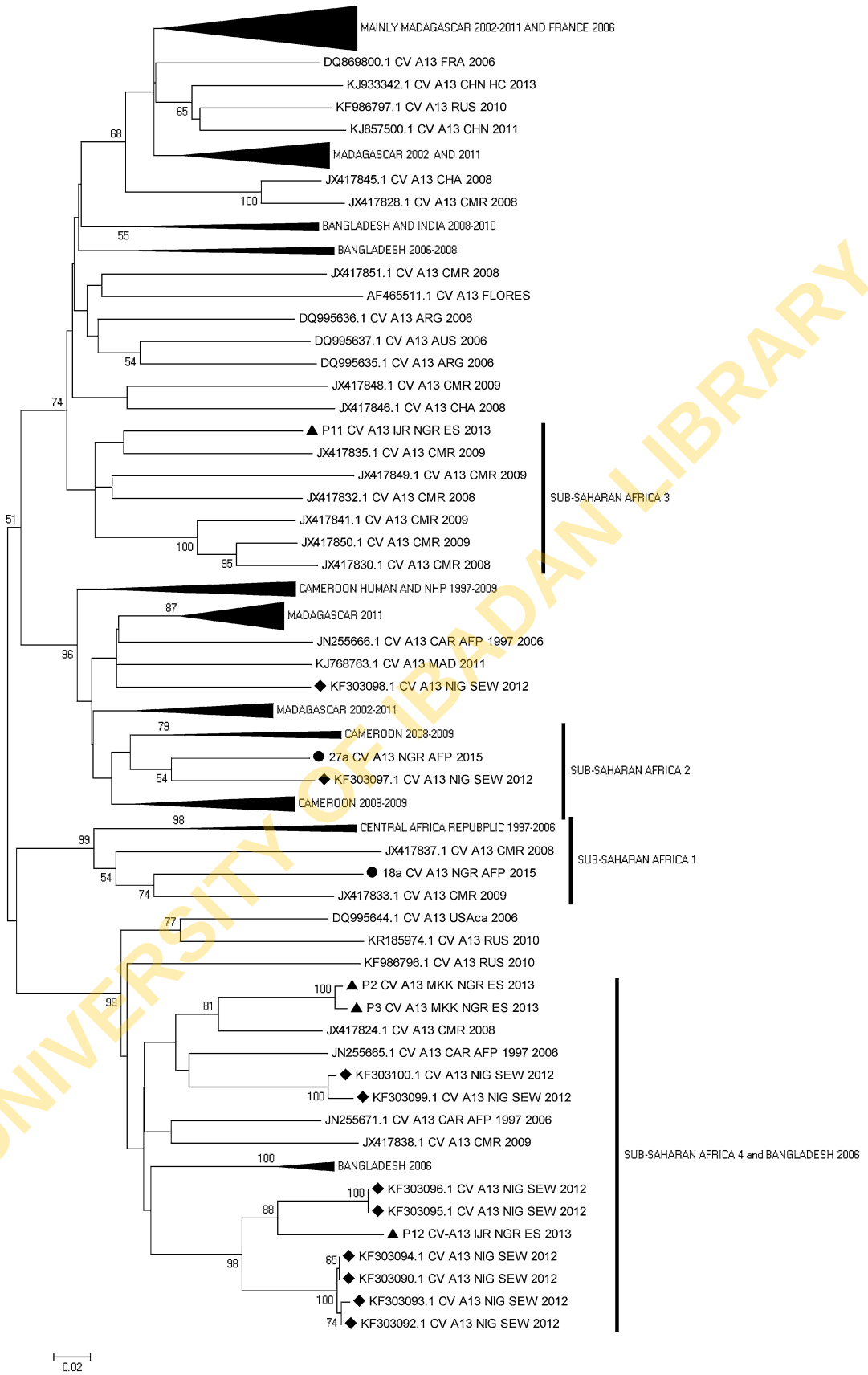
◀**Fig. 2** Alignment of the PV2 strain detected in this study (7a_Sabin_PV_2_NGR_AFP_2015) with some reference strains from GenBank. A) Alignment of VP1 amino acid sequences. Note the isoleucine at position 143 ($_{ILE}$143), which indicates that the strain is of vaccine strain origin. B) Alignment of nucleotide sequences corresponding to residues 45 to 159 of the amino acid sequence alignment in Fig. 2A. Note that, of the 345 nucleotides sequenced, there are four nucleotide changes (C138A, A147G, T270C and T477C) that differentiate the PV2 detected in this study from the reference Sabin strains (AY184220.1 and X00595.1)

therefore confirms a preponderance of EV-Cs. Other studies in which cell lines with an EV-C 'bias' (e.g., HEp-2c or MCF-7) were incorporated into the cell culture algorithm also reported a preponderance of EV-C [7, 8]. In fact, phylogenetic analysis (Figures 3, 4, 5, 6) showed that the nonpoliovirus EV-C strains recovered in this study belonged to the same lineages previously found in the region when cell lines with an EV-C 'bias' were used for enterovirus isolation [7, 8] or when viruses were detected directly in fecal specimens [14] as was done in this study. However, it is important to mention that previous studies in the region that, like the GPLN, used the RD-L20B-cell-culture-based algorithm [19, 24, 25] reported a preponderance of EV-B. The obvious dissimilarity between the results of these studies might therefore be due to the cell culture used. Evidently, the RD-L20B algorithm had selectively filtered out the majority of the EV-B-containing samples (bearing in mind the EV-B bias of the RD cell line [7, 8, 19]), leaving behind more EV-C-containing samples. Hence, it is not unexpected to recover more EV-C than EV-B in this study and for the EV-C strains recovered to be very similar to those found previously in the region when EV-C-'biased' cell lines were used for enterovirus isolation [7, 8]. The results of this study therefore add more credence to the hypothesis that the RD cell line has a bias for EV-Bs.

The modifications [15] to the WHO-recommended RT-snPCR assays showed that 3.3% (1/30) of the culture-negative stool samples from AFP cases analyzed in this study using the WHO-recommended RT-snPCR assay had evidence of being mixed (i.e., the patient had a co-infection with two different enterovirus types). The sample (serial number 1) was shown to contain both EV-B and C (E25 and EV-C99) (Table 1). The inability of the WHO-recommended RT-snPCR VP1 assay [11] to detect and resolve mixed isolates has been discussed previously [15]. Hence, as suggested previously [15], the modification of the WHO-recommended assay [11] to include independent use of primers 187 and 189 (forward primers) alongside AN88 (reverse primer) for the second-round PCR ensured that the assay retained its sensitivity for enterovirus detection while providing a simple method to identify and resolve co-infection with different enterovirus types. It is therefore crucial that we do not allow our desire for a panenterovirus detection assay to overshadow the need to adequately catalogue all enterovirus types present in cases of co-infection. On the other hand, as also discussed previously [15], it is important to reiterate that the snPCR result without sequencing should be interpreted with caution. This is because although primers 189 and 187 mostly selectively amplify EV-A/C and EV-B, respectively, as shown previously [15] and confirmed in this study (Table 1, sample 4), they sometimes amplify members of other species. Hence, basing species designation on evidence of amplification without confirmation by sequencing may, on occasion, be misleading.

One of the important enterovirus types detected in this study is poliovirus serotype 2 (PV-2). It was found (Fig. 2a) that the amino acid at the 143rd position of the VP1 protein is an Isoleucine ($_{Ile}$143), implying that the virus was of vaccine (Sabin) strain origin [26]. However, the fact that 1.16% (4/345) of the nucleotides in the region sequenced were different from that of the vaccine strain (Fig. 2b) might indicate that this strain was circulating. Considering that the cell lines used by the polio lab during the period in question were sensitive (Table S1), it is currently not clear why this virus was missed by the WHO-recommended RD-L20B-cell-culture-based algorithm [5, 6]. One possibility could be a break in the reverse cold chain en route the laboratory and the consequent arrival of the samples with nonviable virus. Hence, the inability of the cell lines to detect it. Whatever may be the case, the finding of PV-2 in a sample declared negative for enteroviruses highlights the value of the WHO-recommended RT-snPCR assay for detection of enteroviruses, even in cases where the enterovirus that is present might not be detectable by the WHO-recommended RD-L20B-cell-culture-based algorithm [5, 6]. The significance of this to the poliovirus eradication and WHO global action plan (GAP III) for poliovirus containment and sequential withdrawal of the Sabin strains [27] cannot be overemphasized. For example, samples containing Sabin strains but with a break in reverse cold chain en route the laboratory and consequently with nonviable virus on arrival at the lab might be missed. Hence, we suggest that all samples found negative by the RD-L20B-cell-culture-based algorithm be further subjected to the WHO-recommended RT-snPCR assay (and possibly a poliovirus-specific modification of it [Faleye et al., unpublished but available on BioRxiv]) before samples are ruled as truly negative for poliovirus.

Among all of the enterovirus types detected in this study, EV-A119 and EV-B111 are being described for the first time in Nigeria. The significance of this first find of EV-A119 in a child with a clinical condition, particularly AFP, and its zoonotic potential has been described [28]. As regards EV-B111, as at the 4th of March 2017, only three



◀**Fig. 3** Phylogram showing the genetic relationship between VP1 nucleotide sequences of CV-A13 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated by a black circle. Strains detected in Nigeria in 2012 and 2013 are indicated by a black diamond and a triangle, respectively. The GenBank accession numbers and year of sample collection are indicated in the tree, if known. Bootstrap values are indicated if >50%. The labeled vertical bars are for ease of reference only

publicly available sequences of EV-B111 (KF312882, JX538160 and JX538130) existed in GenBank, and they were all from Southeast Asia. This might therefore represent its first description in Africa. The previously described EV-B111 strains were recovered in 2000 in China (KF312882) [29] and 2008 in Bangladesh (JX538160 and JX538130) [30], from humans (KF312882 and JX538160)

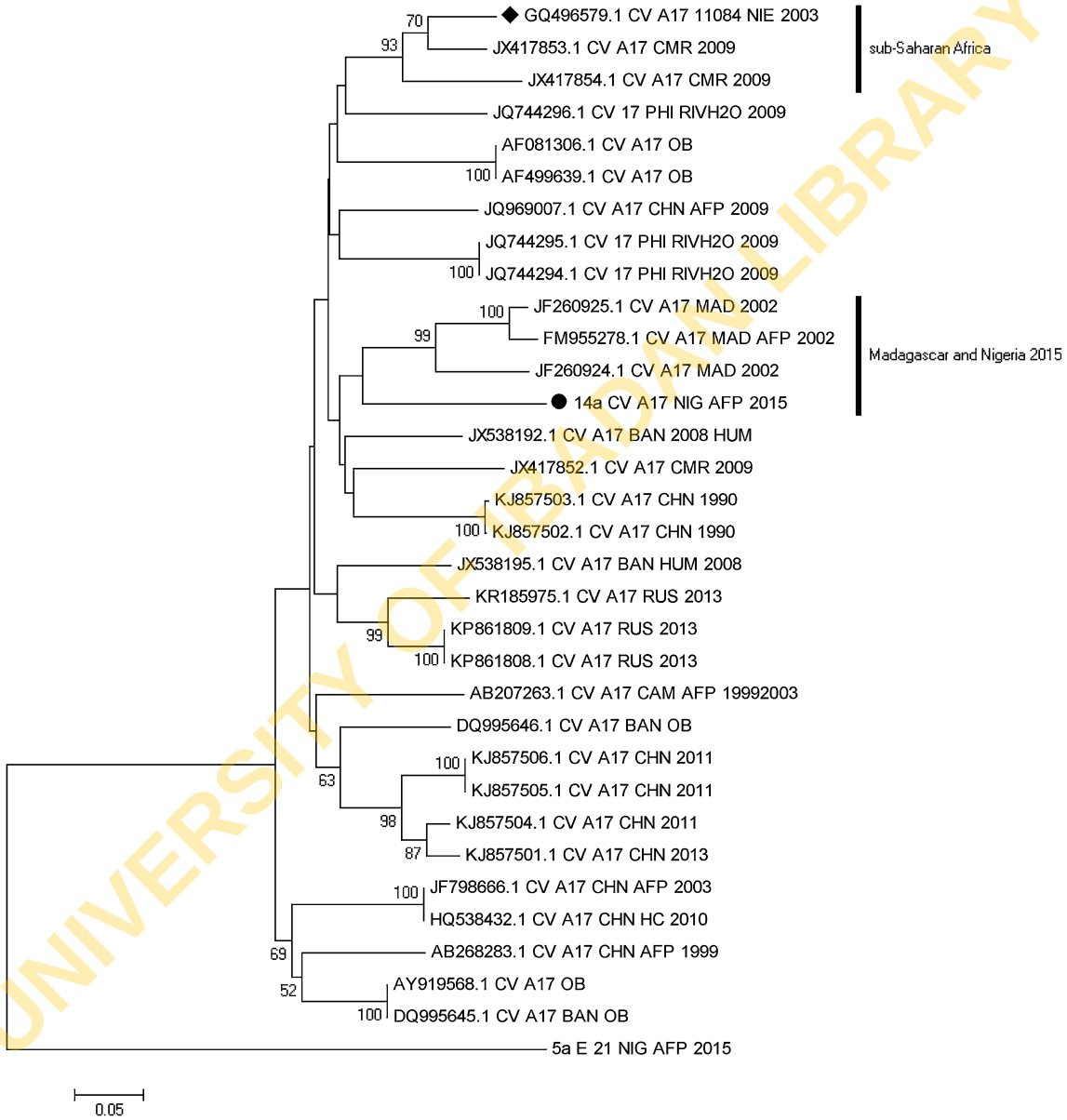


Fig. 4 Phylogram of the genetic relationship between VP1 nucleotide sequences of CV-A17 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strain is indicated by a black circle. A strain recovered in Nigeria in 2003 is indicated by a black diamond. The GenBank accession numbers and year of sample collection are indicated in the tree, if known. Bootstrap values are indicated if >50%. The labeled vertical bars are for ease of reference only

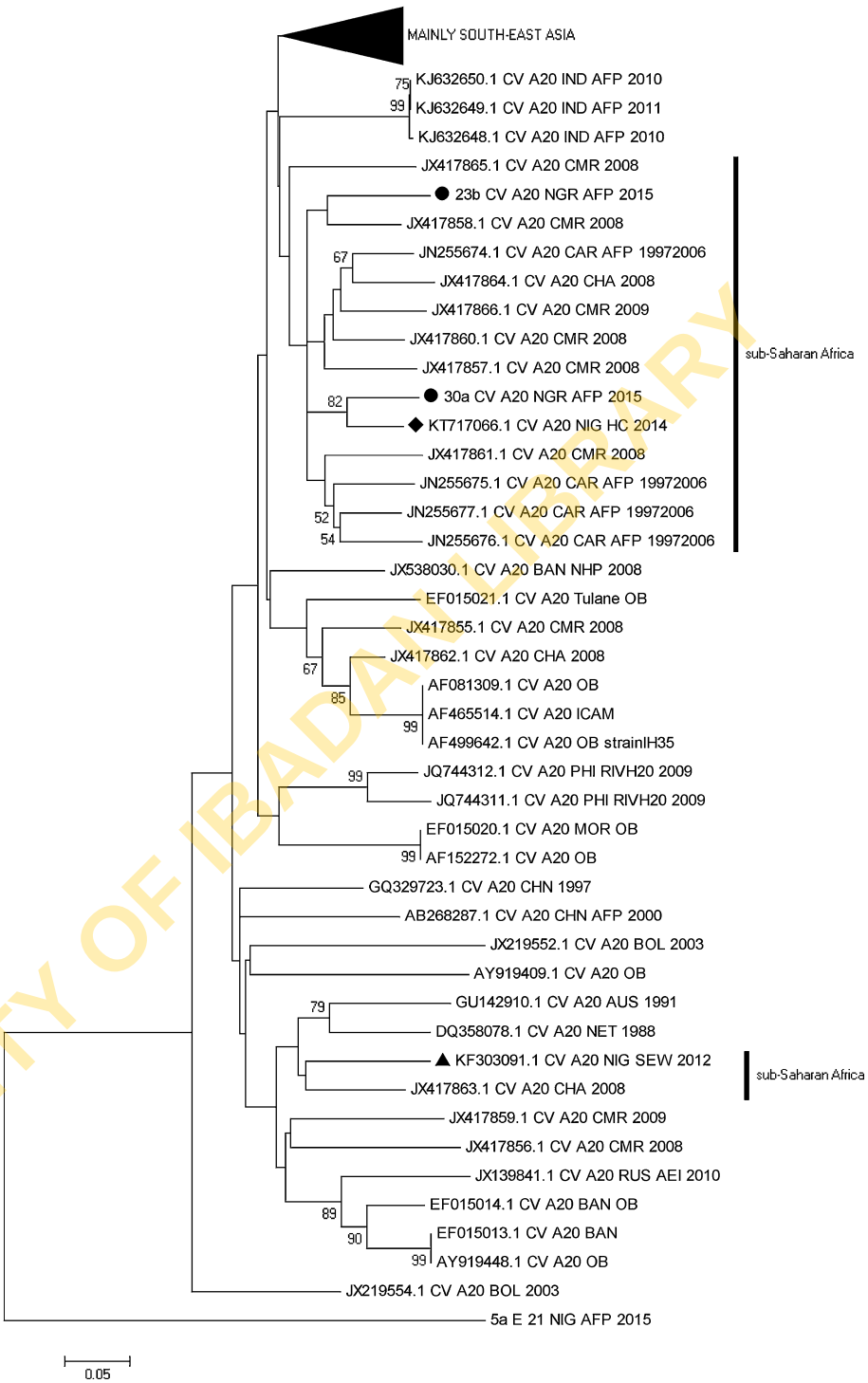


Fig. 5 Phylogram showing the genetic relationship between VP1 nucleotide sequences of CV-A20 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated by black circles. Strains detected in Nigeria in 2012 and 2014 are indicated by a black triangle and a diamond, respectively. The GenBank accession numbers and year of sample collection are indicated in the tree, if known. Bootstrap values are indicated if >50%. The labeled vertical bars are for ease of reference only

[29, 30] and nonhuman primates (NHPs) (JX538130) [30]. Consequently, the zoonotic potential of EV-B111 cannot also be ruled out. The fact that only the year 2000 isolate recovered in China was isolated in culture might suggest that EV-B111 does not replicate readily in the cell lines that are used routinely for cell culture in most enterovirology laboratories. Consequently, it is and will mostly be detected by cell-culture-independent methods like the WHO-recommended RT-snPCR assay [11] used in this and the other study [30]. On the other hand, as evidenced by its varying serology ten years after its description in different prefectures of the same region in China, it is also likely that EV-B111 is not well established in the human population and is thus not circulating efficiently [29]. However, as

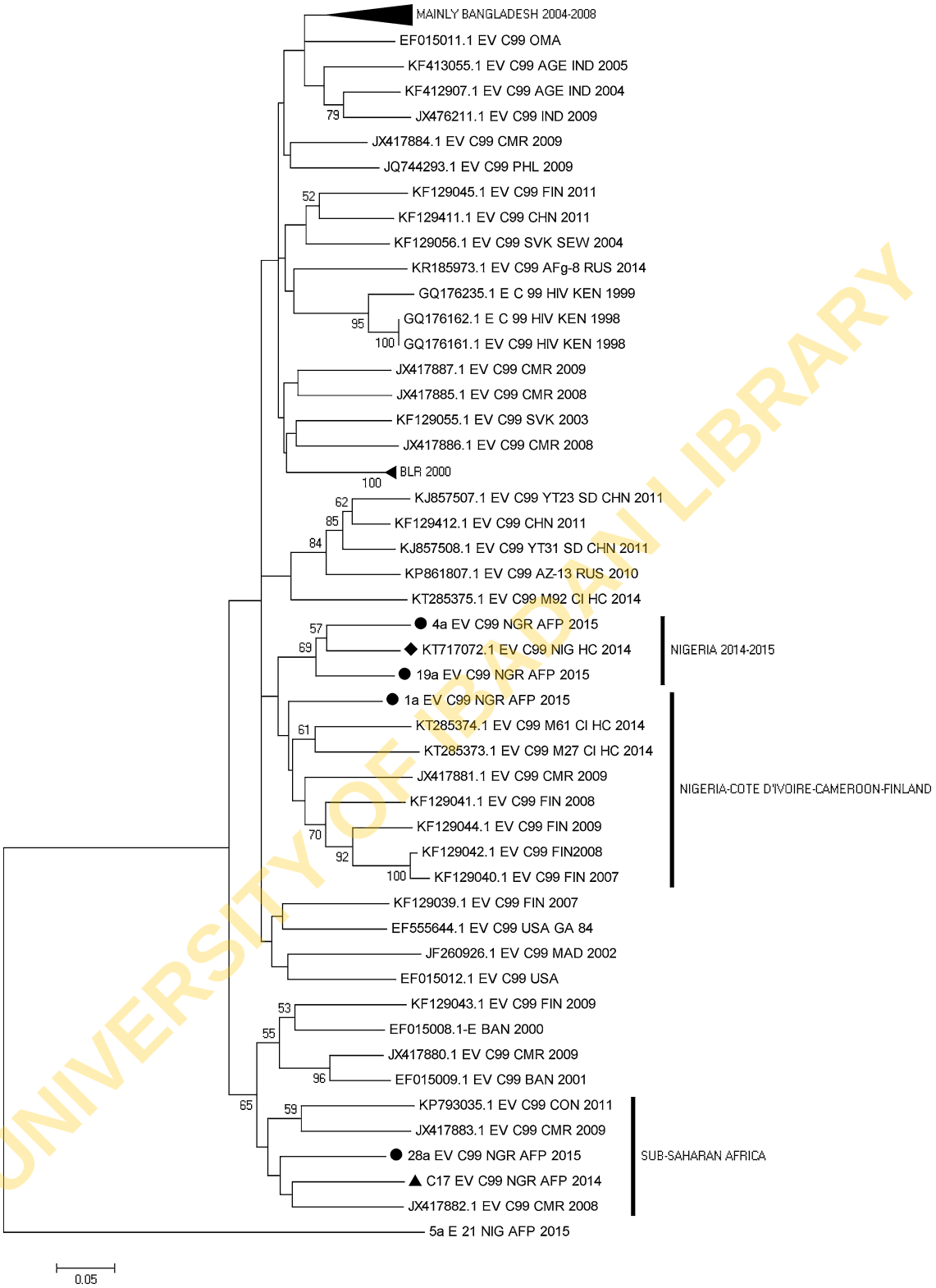


Fig. 6 Phylogram showing the genetic relationship between VP1 nucleotide sequences of EV-C99 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated by black circles. Strains detected in 2014 in Nigeria from a child with acute flaccid paralysis and a healthy child are indicated by a black triangle and a diamond, respectively. The GenBank accession numbers and year of sample collection are indicated in the tree, if known. Bootstrap values are indicated if >50%. The labeled vertical bars are for ease of reference only

tempting as this proposition is, the fact that EV-B111 has been found at least four times and on two different continents over the last 16 years is cause for concern. Furthermore, if EV-B111 is not well adapted to humans, then where has it been surviving and circulating between detections? More troubling is the fact that besides its finding in NHPs [30], the other strains detected in humans to date were found in the stool of children diagnosed with AFP [29, 30]. Of course, the evidence might not be suggestive of causality and might just be indicative of a sensitive AFP surveillance system. In spite of this, this occurrence provides a reason for further investigation.

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Author contributions 1. Study design (JAA, MOA, TOCF). 2. Sample collection, laboratory and data analysis (all authors). 3. Wrote, revised, read and approved the final draft of the manuscript (all authors).

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

Ethical approval There was no contact with human participants by any of the authors, and the article does not contain any information that can be used to associate the enterovirus types analyzed in this study to any individual.

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