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Abundance of enterovirus C in RD-L20B cell culture-negative stool samples from acute flaccid paralysis cases in Nigeria is geographically defined

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Abstract

Purpose. We recently showed that enteroviruses (EVs) andenterovirus species C (EV-C) in particular were abundant in faecal samples from children who had been diagnosed with acute flaccid paralysis (AFP) in Nigeria but declared to be EV-free by the RD-L20B cell culture-based algorithm. In this study, we investigated whether this observed preponderance of EVs (and EV-Cs) in such samples varies by geographical region.

Methodology. One hundred and eight samples (i.e. 54 paired stool suspensions from 54 AFP cases) that had previously been confirmed to be negative for EVs by the WHO-recommended RD-L20B cell culture-based algorithm were analysed. The 108 samples were made into 54 pools (27 each from North-West and South-South Nigeria). All were subjected to RNA extraction, cDNA synthesis and the WHO-recommended semi-nested PCR assay and its modifications. All of the amplicons were sequenced, and the enteroviruses identified, using the enterovirus genotyping tool and phylogenetic analysis.

Results. EVs were detected in 16 (29.63 %) of the 54 samples that were screened and successfully identified in 14 (25.93 %). Of these, 10 were from North-West and 4 were from South-South Nigeria. One (7.14 %), 2 (14.29 %) and 11 (78.57 %) of the strains detected were EV-A, EV-B and EV-C, respectively. The 10 strains from North-West Nigeria included 7 EV types, namely CV-A10, E29, CV-A13, CV-A17, CV-A19, CV-A24 and EV-C99. The four EV types recovered from South-South Nigeria were E31, CV-A1, EV-C99 and EV-C116.

Conclusion. The results of this study showed that the presence of EVs and consequently EV-Cs in AFP samples declared to be EV-free by the RD-L20B cell culture-based algorithm varies by geographical region in Nigeria.

INTRODUCTION

Enteroviruses are members of the genus *Enterovirus* in the family *Picornaviridae*, order *Picornavirales*. There are 13 species in the genus and the best studied member of the genus, poliovirus, belongs to species C (EV-C). Courtesy of the resolution of the World Health Assembly (WHA) to eradicate poliovirus [1], the Global Polio Eradication Initiative (GPEI) has eliminated indigenous strains of the virus globally, except in three countries: Afghanistan, Nigeria and Pakistan [2]. This eradication effort is driven by a two-pronged system comprising vaccination and surveillance. Vaccination uses both an inactivated polio vaccine (IPV) and the attenuated live virus vaccine [oral polio vaccine (OPV)] to inhibit virus transmission, while surveillance looks for both the street (wild-type) virus and the attenuated live (vaccine) virus in both children with acute flaccid paralysis (AFP) and the environment [3].

Ordinarily, subsequent to large scale co-ordinated immunization activities, the attenuated vaccine virus should stop circulating in the population in about 8 weeks [4]. It has,

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Abbreviations: AFP, acute flaccid paralysis; cDNA, complementary deoxyribonucleic acid; CPE, cytopathic effect; CV, Coxsackievirus; E, echovirus; ES, environmental surveillance; EV, enterovirus; F, forward; GPEI, Global Polio Eradication Initiative; GPLN, Global Polio Laboratory Network; IPV, inactivated polio vaccine; L20B, genetically engineered mouse cell line expressing the human cell surface receptor for poliovirus; NPEV, nonpolio enterovirus; OPV, oral polio vaccine; PCR, polymerase chain reaction; PE, panenterovirus; R, reverse; RD, human rhabdomyosarcoma; RNA, ribonucleic acid; UTR, untranslated region; VP1, virus protein 1; WHA, World Health Assembly; WHO, World Health Organization.

One supplementary table and one supplementary figure are available with the online version of this article.

however, been observed that many times this does not happen because the virus easily regains its transmissibility and pathogenicity by reverting to a wild-type genotype and, consequently, phenotype [5]. Such wild-type poliovirus strains of vaccine origin are referred to as vaccine-derived polioviruses (VDPV) [6]. Many VDPVs (as exemplified in the strains that caused the outbreak that started in Nigeria in 2005 and lasted for approximately a decade [6–8]), are recombinant in nature. These virus strains have a structural region of OPV origin and a non-structural region of non-polio enterovirus species C (NPEV-C) origin (OPV/NPEV-C).

This role of NPEV-Cs in VDPV emergence exemplifies their importance, and the need to carefully catalogue their diversity and geographical distribution. It has, however, been shown [9–12] that the RD-L20B cell culture-based algorithm [13, 14] recommended for and frequently used by the Global Polio Laboratory Network (GPLN) under-reports the preponderance of EV-Cs. This is because the L20B cell line component of the algorithm is largely specific for poliovirus detection due to its expression of the human poliovirus receptor [15], while the RD cell line component that is supposed to be more promiscuous for EV detection is EV-B biased [9, 11, 12, 16, 17].

We recently showed [12] that EVs were present in about 46.7 % of faecal samples from children <15 years old diagnosed with AFP but declared to be EV-free by the RD-L20B cell culture-based algorithm [13, 14]. In addition, we showed that majority of the EVs recovered in these samples were EV-Cs. This suggests that the EV-B bias of the RD component of the RD-L20B cell culture-based algorithm [13, 14] selectively accumulated samples that lacked EV-Bs but possibly had EV-Cs as 'negatives'.

Nigeria is divided into six geo-political zones: North-Central, North-West, North-East, South-West, South-East and South-South. These geo-political zones have a history of varying peculiarities that significantly influence the preponderance and dynamics of polioviruses [17–21]. For example, of the 23 lineages of circulating VDPV2s (cVDPV2) that emerged in Nigeria between 2005 and 2011 [7], 12 (52 %) emerged in North-West Nigeria, while none (0 %) emerged in South-South Nigeria. This observed and documented peculiarity of North-West and South-South Nigeria [7] informed this study. Hence, we selected AFP samples declared to be EV-free by the RD-L20B cell culture-based algorithm [12] and investigated whether the observed preponderance of EVs (and EV-Cs) in these samples [12] varied by region.

METHODS

Sample collection and processing

In August 2015, 86.26 % (747/866) of the AFP cases received by the WHO-accredited polio laboratory in Ibadan, Nigeria (subsequently referred to as the polio lab) were declared negative for enteroviruses using the WHO-recommended RD-L20B cell culture-based algorithm [14]. In a previous study [12], we sampled (without replacement) 30 of these 747 samples and found an abundance of EV-Cs in them. To determine whether the abundance of EV-Cs varied by region, the remaining 717 samples were sorted by region. In this study, we randomly selected 54 [27 each from North-West and South-South Nigeria (Fig. 1)] of these cases [i.e 7.53 % (54/717)] for further analysis. All the pairs of stool suspensions made from these cases were collected from the archives of the polio lab and pooled. That is, the 108 stool suspensions from the 54 cases were pooled into 54 stool suspensions and subsequently analysed in this study.

RNA extraction, cDNA synthesis and semi-nested polymerase chain reaction (snPCR)

RNA extraction, cDNA synthesis and snPCR were performed as recently described [13, 22]. Briefly, RNA extraction and cDNA synthesis were performed using Jena Bioscience kits for RNA extraction and cDNA synthesis kits (Jena Bioscience, Jena, Germany), respectively, according to the manufacturer's recommendations. However, in place of random hexamers, primers AN32– AN35 [3, 23] were used for cDNA synthesis.

For the snPCR assay, there was one first-round PCR assay and there were three [panenterovirus (PE), enterovirus species A or C (EV-A/C) and enterovirus species B (EV-B)] second-round PCR assays. All of the assays were performed in 30 µl volumes. Each contained 6 µl of Red Load *Taq* and 0.3 µl each of the forward and reverse primers. For the first-round PCR assay, 13.4 µl of RNase-free water and 10 µl of

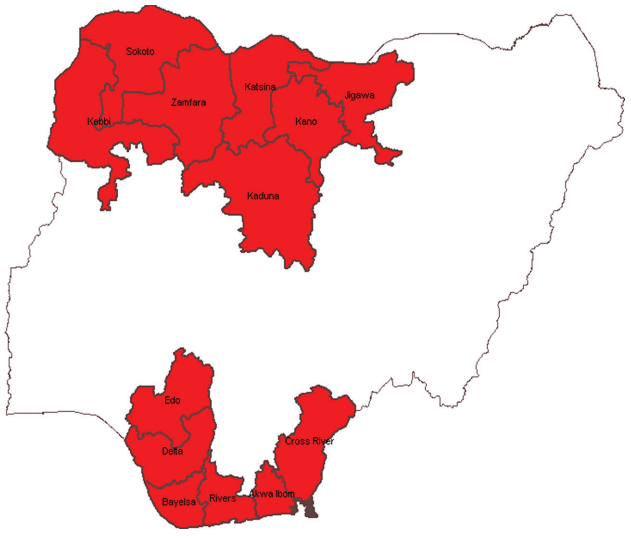

Fig. 1. Map of Nigeria highlighting North-West (seven states in the top left corner) and South-South (six states in the bottom of the image) Nigeria in red. Nigeria is divided into six geo-political zones: North-Central, North-West, North-East, South-West, South-East and South-South. In line with the justification for this study (see the final paragraph of the introduction) only samples from North-West and South-South Nigeria were analysed.

cDNA were also added to the mix. For the second-round PCR assay, 20.4 µl of RNase-free water and 3 µl of first-round PCR product were added to the mix. The primers for the first-round PCR were 224 and 222 [23]. For the second-round PCR assay, all three assays used the reverse primer AN88 [23], while the forward primers were AN89, 189 and 187 [3] for the PE, EV-A/C and EV-B PCR assays, respectively. The cycling conditions for the assays were similar [12], except for the extension times (60 s and 30 s for the first- and second-round PCR assays, respectively). All PCR products were resolved on 2 % agarose gels stained with ethidium bromide and viewed using a UV transilluminator. Samples in which the second-round PCR assay successfully amplified the expected ~350 bp amplicon were considered positive.

Amplicon sequencing and enterovirus identification

All of the appropriately sized amplicons (~350 bp) generated from the second-round PCR assays were shipped to Macrogen Inc, Seoul, Republic of Korea, for purification and nucleotide sequencing. Subsequently, the enterovirus species and genotypes were determined using the enterovirus genotyping tool (EGT) [24].

Phylogenetic analysis

Only the EV-Cs were subjected to phylogenetic analysis, because they were the most detected species. The CLUSTAL W program in MEGA 5 software [25] was used for multiple sequence alignments with default settings. Afterwards, neighbour-joining trees were constructed using the Kimura-2 parameter model [26] and 1000 bootstrap replicates in the same MEGA 5 software.

Nucleotide sequence accession numbers

The sequences generated in this study have been deposited in GenBank under accession numbers MG252505–MG252518.

RESULTS

North-West Nigeria PCR result

Eleven (40.74 %) of the 27 samples from North-West Nigeria were positive (i.e. with the expected ~350 bp successfully amplified) for the PE PCR assay. Ten (37.04 %) of the 27 samples were positive (i.e. with the expected ~350 bp successfully amplified) for the EV-A/C PCR assay. Nine of the 10 samples that were positive for the EV-A/C PCR assay were also positive for the PE PCR assay. Specifically, 1 (sample 13) of the 11 samples that were positive for the PE PCR assay was negative for the EV-A/C PCR assay and 1 (sample 23) of the 10 samples that were positive for the EV-A/C PCR assay was negative for the PE PCR assay.

Only 2 (7.4 %) of the 27 samples were positive for the EV-B PCR assay. These two samples were also positive for the PE PCR assay and one of them (sample 2) was also positive for the EV-A/C PCR assay. Thus, 9 (81.82 %) of the 11 samples that were positive for the PE PCR assay were negative for the EV-B PCR assay. Ultimately, 12 (44.44 %) of the 27 samples were positive for at least 1 of the 3 assays, but only 1 sample (sample 2) was positive for all 3 assays (Table 1).

South-South Nigeria PCR result

Four (14.82 %) of the 27 samples from South-South Nigeria were positive for the PE PCR assay. Two (7.4 %) of the 27 samples were positive for the EV-A/C PCR assay. These two samples were also positive for the PE PCR assay. Thus, two

Table 1. Results for the RT-snPCR screen of cell culture-negative AFP stool samples from North-West Nigeria

S/N	Lab ID	RT-snPCR result and enterovirus ID			Summary	Species
		PE	EV-A/C	EV-B		
1	2	+	+	+	EV-C99	EV-C
2	7	+	+	–	CV-A19	EV-C
3	9	+	+	–	CV-A24	EV-C
4	11	+	–	+	E-29	EV-B
5	13*	+*	–	–	–	–
6	14	+	+	–	EV-C99	EV-C
7	15	+	+	–	CV-A17	EV-C
8	16	+	+	–	CV-A17	EV-C
9	19	+	+	–	EV-C99	EV-C
10	21	+	+	–	CV-A10	EV-A
11	23*	–	+*	–	–	–
12	24	+	+	–	CV-A13	EV-C
	Total	11	10	2	10	

*Sequence data not usable due to multiple peaks.

+, Positive for the assay.

–, Negative for the assay.

(50.0 %) of the four samples that were positive for the PE PCR assay were negative for the EV-A/C PCR assay. Only 1 (3.7 %) of the 27 samples was positive for the EV-B PCR assay. This sample was also positive for the PE PCR assay. Hence, three (75.0 %) of the four samples that were positive for the PE PCR assay were negative for the EV-B PCR assay. Ultimately, 4 (14.82 %) of the 27 samples from South-South Nigeria were positive for at least 1 of the 3 assays, but none of the samples were positive for all 3 assays (Table 2).

North-West Nigeria sequencing and genotyping results

All 11 amplicons generated by the PE PCR assay were successfully sequenced. However, the sequence data for one of the amplicons (sample 13) was not usable due to multiple peaks. The identified strains were enterovirus (EV) C99 (three isolates), echovirus-29 (E29) (one isolate), Coxsackievirus (CV) A10 (one isolate), CV-A13 (one isolate), CV-A17 (two isolates), CV-A19 (one isolate) and CV-A24 (one isolate). Of the 10 strains successfully identified via the PE PCR assay, 1 (CV-A10) belongs to EV-A, 1 belongs to EV-B (E29) and 8 belong to , EV-C (Table 1).

For the EV-A/C assay, all of the 10 amplicons generated were successfully sequenced. However, the sequence data for one of the amplicons (sample 23) were not usable due to multiple peaks. The strains identified were EV-C99 (three isolates), CV-A10 (one isolate), CV-A13 (one isolate), CV-A17 (two isolates), CV-A19 (one isolate) and CV-A24 (one isolate). Of the nine strains successfully identified via the EV-A/C assay, one (CV-A10) belongs to EV-A and eight belong to EV-C (Table 1).

Only one (sample 2) of the two amplicons generated by the EV-B assay was sequenced and identified as EV-C99. The second amplicon (sample 11) was not sequenced because the intensity of the band was too weak (Table 1). In all, EVs were detected in 12 (44.44 %) of the 27 samples screened, and successfully identified in 10 (37.04 %) of the samples.

South-South Nigeria sequencing and genotyping results

All four amplicons generated by the PE PCR assay were successfully sequenced and three of the strains were identified as CV-A1 (one isolate), E31 (one isolate) and EV-C99 (one isolate). The fourth strain was identified as a member of EV-C, but the type was unassigned by the EGT. However, the EGT phylogenetic tree showed that it clustered with EV-C116, but with a bootstrap support of 67 (Fig. S1, available in the online version of this article). Hence, it will be referred to as EV-C116. Of the four strains successfully identified via the PE PCR assay, one (E31) belongs to EV-B and three belong to EV-C (Table 2). Both amplicons generated by the EV-A/C PCR assay were successfully sequenced and the strains were identified as CV-A1 (one isolate) and EV-C116 (one isolate). The EV-C116 was also typed using the EGT phylogenetic tree. Both strains successfully identified via the EV-A/C assay belong to EV-C (Table 2). The only amplicons generated by the EV-B PCR assay were sequenced and identified as E31. This strain belongs to EV-B (Table 2). In all, EVs were detected and successfully identified in 4 (14.81 %) of the 27 samples screened.

North-West and South-South Nigeria sequencing and genotyping result summary

Altogether, EVs were detected in 16 (29.63 %) of the 54 samples screened, and these EVs were successfully identified in 14 (25.93 %) samples. One (7.14 %), 2 (14.29 %) and 11 (78.57 %) of the strains detected were EV-A, EV-B and EV-C, respectively.

Comparison of EV types detected in the two geo-political regions

The seven EV types recovered from North-West Nigeria were spread over three EV species (EV-A, B and C) and included CV-A10 (EV-A), E29 (EV-B) and CV-A13, CV-A17, CV-A19, CV-A24 and EV-C99 (EV-Cs). On the other hand, the four EV types recovered from South-South Nigeria were spread over two EV species (EV-B and C) and included E31 (EV-B) and CV-A1, EV-C99 and EV-C116 (EV-Cs). EV-C99 (an EV-C) was the only EV type that was detected in both North-West and South-South Nigeria (Table 3).

Phylogenetic analysis

Only the seven EV-C types found in this study were subjected to further phylogenetic analysis, because they were the most detected. The CV-A1 strains found in GenBank

Table 2. Results for the RT-snPCR screen of cell culture-negative AFP stool samples from South-South Nigeria

S/N	Lab ID	RT-snPCR result and enterovirus ID			Summary	Species
		Pan-Entero	EV-A/C	EV-B		
1	9	+	+	–	EV-C116	EV-C
2	16	+	–	+	E-31	EV-B
3	20	+	–	–	EV-C99	EV-C
4	26	+	+	–	CV-A1	EV-C
	Total	4	2	1	4	

+, Positive for the assay.

–, Negative for the assay.

Table 3. Diversity of enteroviruses recovered from the two geo-political regions analysed in this study

S/N	North-West Nigeria	No. of enterovirus types	South-South Nigeria	Number of enterovirus types	Total no. of enterovirus types
EV-A	CV-A10	1	Nil	0	1
EV-B	E-29	1	E-31	1	2
EV-C	CV-A13	5	CV-A1	3	7*
	CV-A17	–	EV-C99*	–	–
	CV-A19	–	EV-C116	–	–
	CV-A24	–			
	EV-C99*	–			
Total		7		4	10*

*Although EV-C99 was recovered from both regions, it is reckoned to be the same EV type. This is why the total number of EV types is 10 and not 11.

alongside the one described in this study grouped into five genotypes based on bootstrap values (Fig. 2). The CV-A1 detected in this study grouped with genotype B and is most similar to the CV-A1 recently recovered from a child with AFP in Nigeria in 2015. It is, however, different from the strain recovered from a healthy child in 2014 in Nigeria. The 2014 strain belongs to genotype E (Fig. 2).

The Nigerian strains of CV-A19 and EV-C116 are being described for the first time in this study and, based on the topology of the tree (Fig. 2), they seem to be distantly related to the strains available in GenBank. The EV strain identified as EV-C116 using the EGT phylogenetic tree (Fig. S1) did not cluster with the EV-C116 strains recovered from GenBank, instead it clustered with reference CV-A22 strain Chulman and two other CV-A22 strains (Fig. 2). To resolve this inconsistency, a BLASTN search of the sequence data of this EV strain was performed. The BLASTN results suggested that it was only similar to the CV-A22 strain recovered in Hong Kong in 2010, and this was consistent with the results of our similarity analysis (Table S1).

The CV-A13 strain recovered in this study was from North-West Nigeria (Table 3). It was most similar to a CV-A13 strain that was also recovered from a child with AFP in Nigeria in 2015 (Fig. 3). Both strains belong to the cluster sub-Saharan Africa 3, alongside strains previously described in Cameroon and the Central African Republic (Fig. 3).

The two CV-A17 strains described in this study were from North-West Nigeria (Table 3). Both strains belong to a cluster that mainly contains strains from sub-Saharan Africa. They do, however, share a common ancestor with a strain recovered in the Philippines in 2009 (Fig. 4). The two CV-A17 strains described in this study are different from the CV-A17 strains recovered in 2003 and 2015 from children with AFP in Nigeria (Fig. 4).

The CV-A24 strains described in this study belong to a cluster of CV-A24 that has repeatedly been recovered from sub-Saharan Africa (Fig. 5). It was, however, different from the strain recovered in 2010 from sewage-contaminated water in Nigeria. Rather, the CV-A24 strain recovered in this study was most similar to a strain recovered in Cameroon (Fig. 5).

Four (one from South-South and three from North-West Nigeria) EV-C99 strains were recovered in this study. The strain recovered from South-South Nigeria (Fig. 5; cluster EV-C99 d) clustered with two strains. One of these was also recovered in 2015 from a child with AFP in Nigeria. The other strain was recovered in 2014 from a healthy child in Cote d'Ivore (Fig. 5). The three strains from North-West Nigeria fell into two clusters (EV-C99 b and c) (Fig. 5). Two of the EV-C99 strains recovered in this study belong to cluster EV-C99 b, and more particularly to a sub-cluster that contains strains from Nigeria alongside one strain from the Philippines (Fig. 5). These two EV-C99 strains seem to be related to other strains recovered in 2015 from two children with AFP in Nigeria and another strain recovered in 2014 from a healthy child in the same country (Fig. 5). The third North-West Nigeria EV-C99 strain recovered in this study belong to cluster EV-C99 c and is closely related to a strain recovered from children in Cameroon and Nigeria (Fig. 5).

DISCUSSION

In this study, we investigated whether the preponderance of EVs in AFP samples declared to be EV-free by the RD-L20B cell culture-based algorithm varies by region in Nigeria. Our results suggest that this might be the case. For example, while EVs were detected and unambiguously identified in 37.04 % of the samples from North-West Nigeria, they were only detected in 14.81 % of the samples from South-South Nigeria (Tables 2 and 3). Hence, for every 10 EVs found in such samples from South-South Nigeria, 25 were found in an equivalent number of samples from North-West Nigeria. The findings of this study are therefore in agreement with the history of varying regional preponderance and dynamics of enteroviruses [17] and polioviruses [18–21] in Nigeria. Put another way, the findings of this study suggest that the figures of 52 and 0 % [for the 23 lineages of circulating VDPV2s (cVDPV2) that emerged in Nigeria between 2005 and 2011] found for North-West and South-South Nigeria, respectively, might be a reflection of the varying abundance and circulation of EV-Cs in these regions. In this study, we

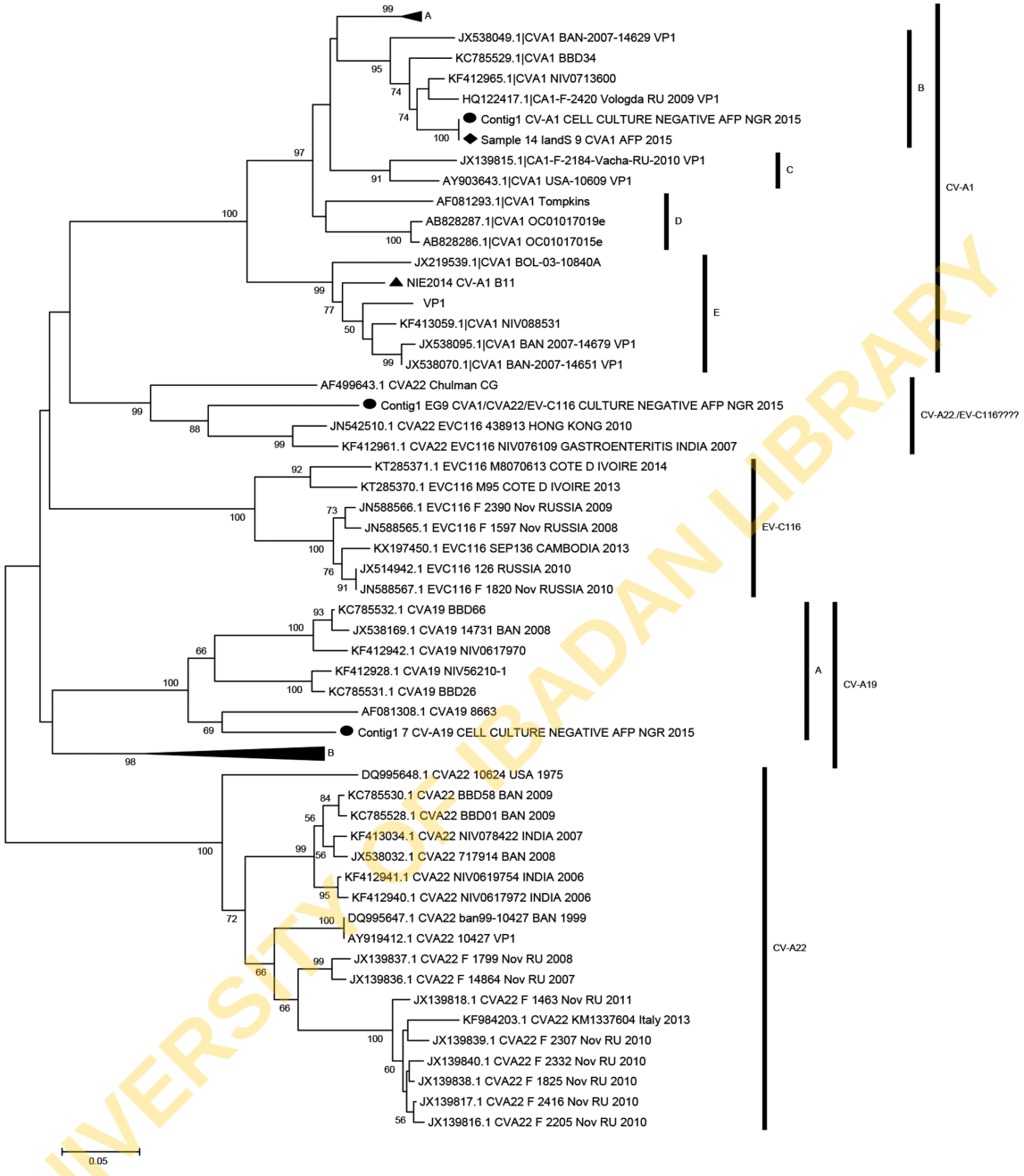

Fig. 2. Phylogram of the genetic relationship between the VP1 nucleotide sequences of the CV-A1, CV-A19, CV-A22 and EV-C116 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circles. Strains previously detected in Nigeria are indicated with black diamonds (2015) and triangles (2014). Bootstrap values are indicated if >50 %. *CV-A22/EV-C116???, ambiguous serotype (not clustering with major groups of either CV-A22 or EV-C116).

also found that 78.57 % (11/14) of the EVs detected were EV-Cs. This further confirms the findings of previous reports [12, 27, 28] on the EV-B bias of the RD component of the RD-L20B cell culture-based algorithm [13, 14]. This allows the selective accumulation of AFP samples that lack EV-Bs but possibly have EV-Cs as 'negatives'. Furthermore, our results suggest that the preponderance of EV-Cs in AFP samples declared to be EV-free by the RD-L20B cell culture-based algorithm varies by region in Nigeria. For instance, while EV-Cs were found in 29.6 % (8/27) of the samples from North-West Nigeria, they were only detected in 11.1 % (3/27) of the samples from South-South Nigeria (Tables 2 and 3). Consequently, for every 10 EV-Cs found in AFP samples from South-South Nigeria that have been declared

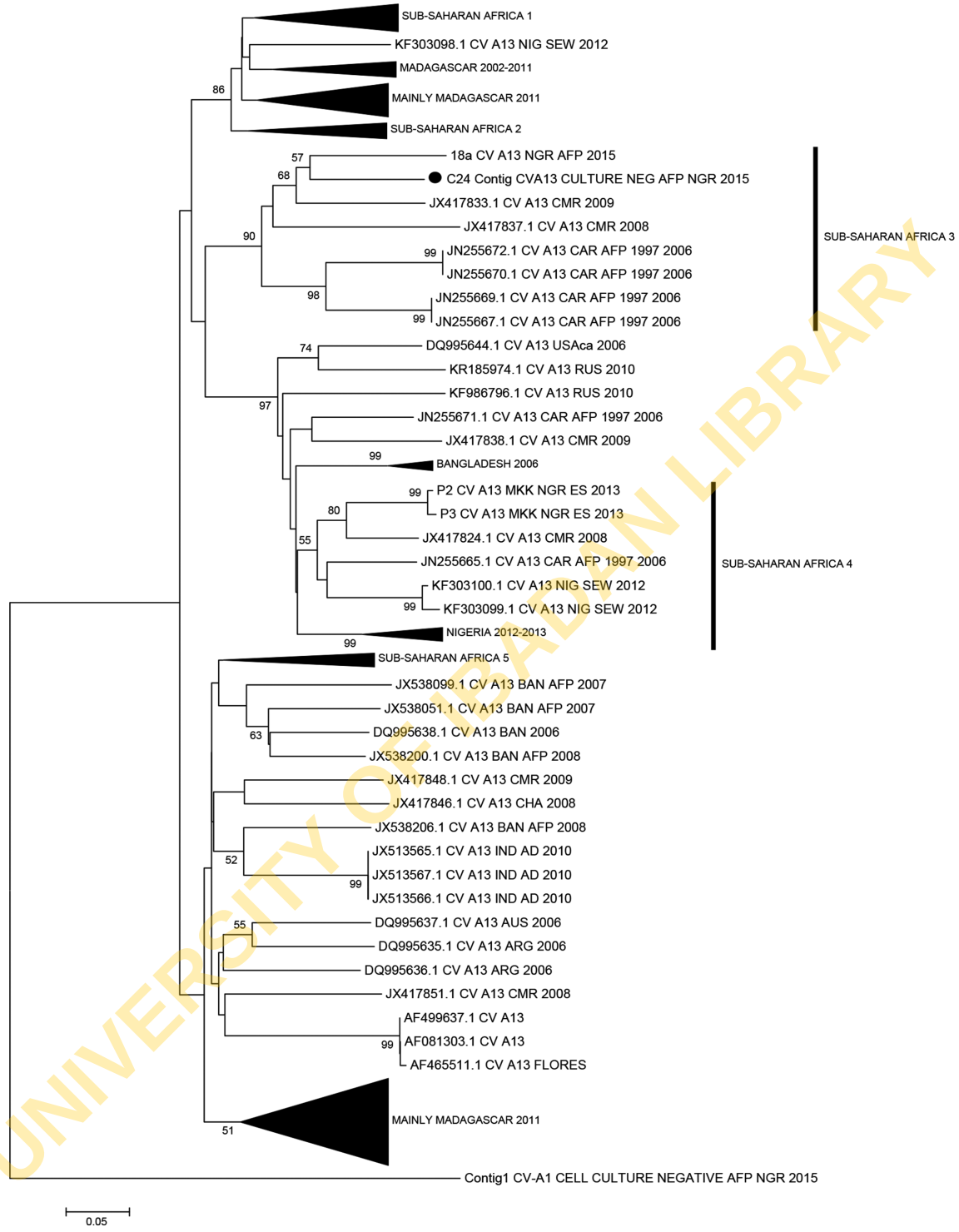

Fig. 3. Phylogram of the genetic relationships between the VP1 nucleotide sequences of the CV-A13 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strain is indicated with a black circle. Bootstrap values are indicated if >50 %. The labelled vertical bars are for ease of reference only.

to be EV-free by the RD-L20B cell culture-based algorithm, we find 27 in an equivalent number and type of samples from North-West Nigeria.

It is known that a population's gut (mucosal) immunity to PVs determines the proportion of the population that can participate in PV transmission (and, consequently, the

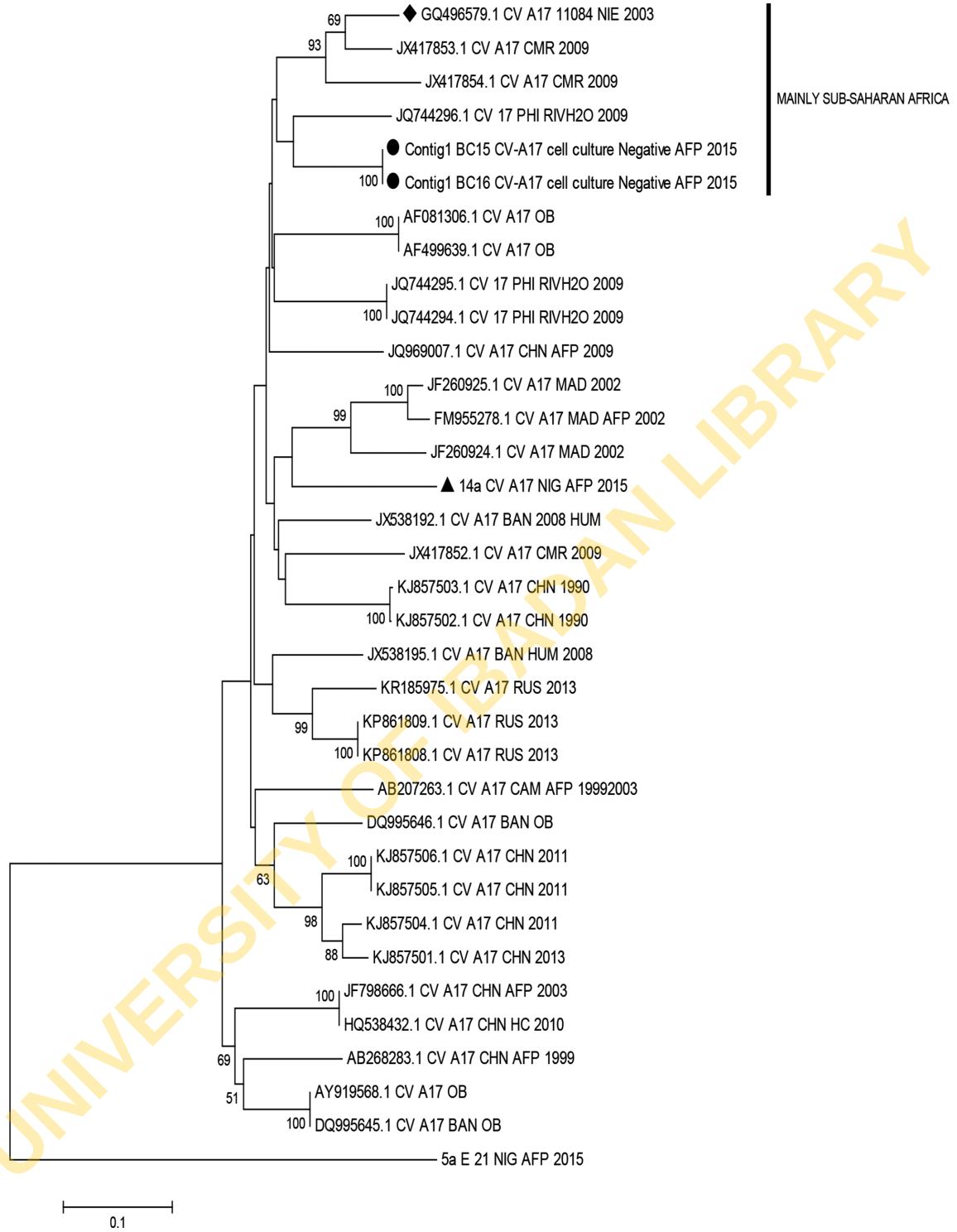

Fig. 4. Phylogram of the genetic relationships between the VP1 nucleotide sequences of the CV-A17 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strain is indicated with a black circle. Strains recovered in Nigeria in 2003 and 2015 are indicated with a black diamond and a triangle, respectively. Bootstrap values are indicated if >50 %. The labelled vertical bars are for ease of reference only.

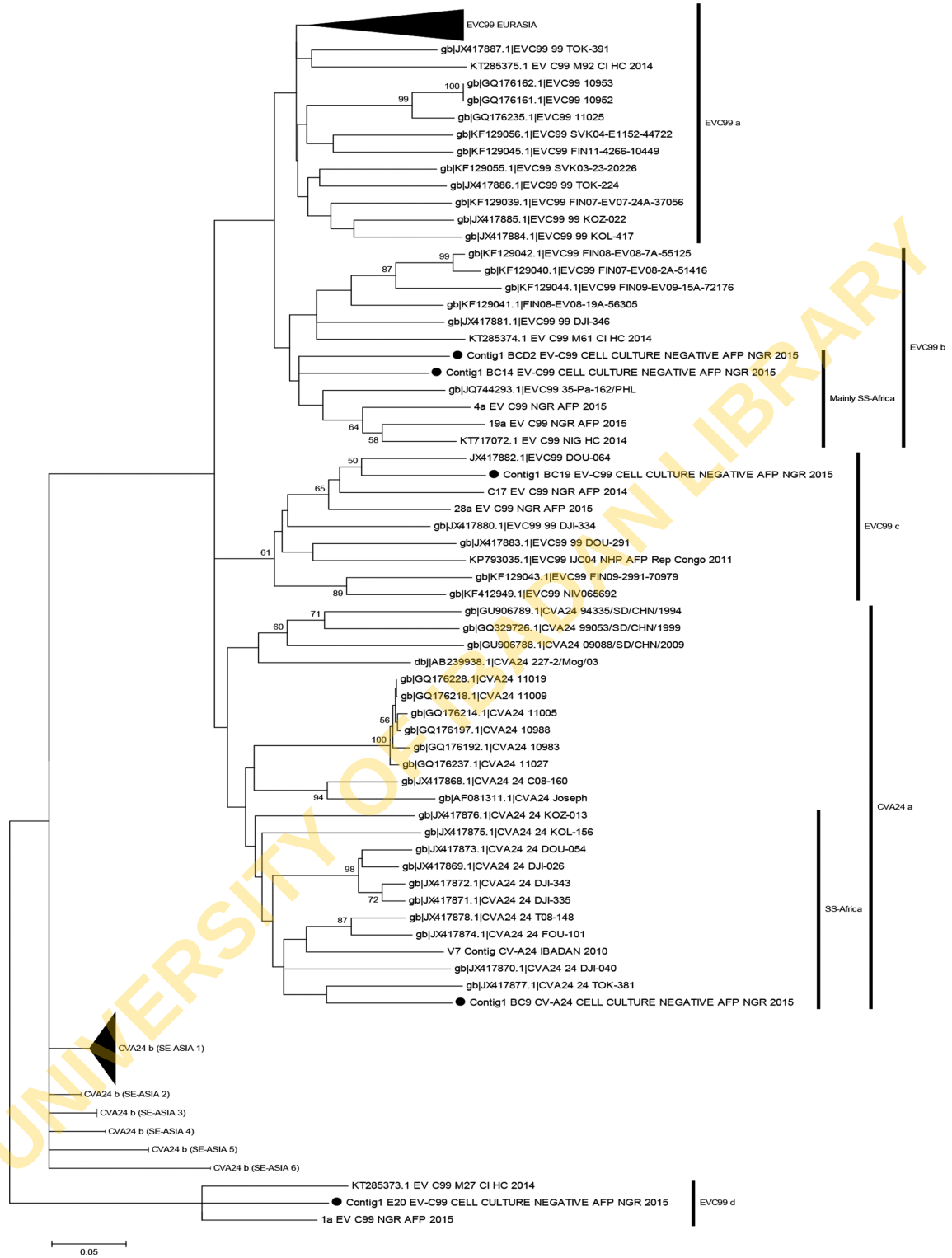

Fig. 5. Phylogram of the genetic relationships between the VP1 nucleotide sequences of the CV-A24 and EV-C99 strains. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circles. Bootstrap values are indicated if >50 %. The labelled vertical bars are for ease of reference only.

duration of transmission) to a significant degree [4, 8]. However, it appears that the higher preponderance of EV-Cs in North-West Nigeria compared to South-South Nigeria might have also contributed to the emergence in North-West Nigeria of over 50 % (12/23) of the lineages of cVDPV2 (most of which were recombinants with NPEV-C non-structural region) that circulated in Nigeria between 2005 and 2011 [7]. The results of this study therefore further highlight the importance of NPEV-Cs and the need to carefully and exhaustively catalogue their diversity and geographical distribution in Nigeria, given their role in VDPV emergence [7].

In this study, we detected CV-A1, CV-A19 and EV-C116 (Fig. 2), enterovirus types whose prototype strains have not been grown in cell culture [29–31]. In particular, they are known not to grow in RD cell culture [31, 32]. Therefore, the selective accumulation of these EV-C types in AFP samples that showed no sign of growth in RD and L20B cell lines is in agreement with previous studies [29–32]. More recently, Sun *et al.* [33] described two CV-A1 strains that developed cytopathology in RD cell culture. In 2015, we (Adeniji *et al.*, unpublished) also recovered a CV-A1 strain that developed cytopathology in RD cell culture (Fig. 2). Particularly interesting is the fact that this CV-A1 strain is very similar to the strain recovered in this study, despite the fact that it was recovered from North-West Nigeria about 1 month before the strain recovered in this study was recovered from South-South Nigeria (Fig. 2). Why the North-West Nigerian strain replicated in cell culture, but the South-South Nigerian strain did not (despite their similarity in the VP1 region) is not clear. One possibility is the likely possession of different non-structural regions, which might be responsible for the varying phenotypes, as as been described for polioviruses [5].

One other striking observation about the North-West Nigerian CV-A1 strain (Adeniji *et al.*, unpublished) is the fact that it was growing simultaneously in RD cell culture alongside a CV-B4. Although only the CV-A1 virus was detected in the stool suspension, both CV-A1 and CV-B4 were detected in the RD cell culture isolate. This suggests that the CV-B4 strain might have been at very low titre in the faecal suspension. Furthermore, given that on the two occasions on which we have detected CV-A24 in RD cell culture in our laboratory to date, in one instance it was replicating alongside echovirus 7 (E7) and in the other it was replicating alongside CV-B6 (unpublished data), it seems likely that co-replication with a CPE-producing species B enterovirus either enables some EV-C members to replicate in RD cell culture or facilitates their detection. However, more extensive investigations might be required to determine whether these observations are just chance occurrences or have a biological basis.

To the best of our knowledge, this represents the first description of Nigerian strains of CV-A19 and EV-C116. However, while the CV-A19 strain was identified unambiguously, identifying the EV-C116 was challenging. The EV strain identified as EV-C116 using the EGT phylogenetic tree (Fig. S1) did not cluster with the other EV-C116 strains recovered from GenBank (Fig. 2). Rather it clustered with the reference CV-A22 strain Chulman (AF499643) and two other CV-A22 strains (JN542510 and KF412961) (Fig. 2). When we subjected the two non-Chulman CV-A22 strains (JN542510 and KF412961) that clustered with the EV-C116 strain identified in this study to further EGT, they both typed as EV-C116. This suggests that these CV-A22 strains (JN542510 and KF412961) were not really CV-A22, but were erroneously typed as such based on the sequence data available in GenBank at the time they were initially sequenced. What is puzzling, however, is the fact that the Chulman strain CV-A22 clustered with this group [CV-A22/EV-C116? (Fig. 2)], but not with the remaining CV-A22 sequences (Fig. 2), while the enterovirus classification criteria of Oberste *et al.* [34] (Table S1) validated the phylogenetic analysis results. Given the strong bootstrap support both clusters have (Fig. 2), the question that needs to be answered is, which of the two clusters is CV-A22 and what is the identity of the other?

The CV-A10 described in this study is the first strain of this EV type to be described in Nigeria, to the best of our knowledge. CV-A10, alongside EV-A71 and other EV-A types, has been implicated in hand, foot and mouth disease (HFMD) [35, 36], which sometimes has neurological complications. Although CV-A10 is being described for the first time in Nigeria, EV-A71 has been detected repeatedly [22, 27]. Usually, EV-A71 strains (of genotype E) that are not associated with HFMD are detected in Nigeria [22, 27]. However, as Fernandez-Garcia *et al.* [37] found in some West-African countries in 2013 and 2014, we discovered in 2014 (unpublished data), a genogroup C of EV-A71 (which is associated with HFMD) in a child with AFP but co-infected with echovirus 13 (E13). These sightings of CV-A10 and EV-A71 genogroup C in children with AFP in Nigeria might be a clarion call for a more intensive search for HFMD in the population or, better still, a call to ascertain why the disease condition is hard to find in the population in spite of the presence of these viruses. It is, however, crucial to mention that, as shown for the other EV types phylogenetically analysed in this study (Figs 2–5), it is likely that several lineages of CV-A10 and EV-A71 genogroup C are present and circulating in Nigeria. Hence, the sporadic detection of these viruses in the country should not be interpreted as evidence of their absence. Rather, this should be seen as being the tip of the iceberg and, consequently, as an incentive for the surveillance of these viruses in the population.

Finally, in this study we were able to detect EVs in 29.63 % (16/54) of the RD-L20B cell culture-negative AFP samples from children <15 years old who had been screened. We were, however, only able to identify the EVs present in 25.93 % (14/54) of the samples. Consequently, as found in this study, the preponderance of EVs in such samples is lower than the 46.7 % (14/30) previously described [12].

The samples analysed in this study and those described in Adeniji *et al.* [12] were all recovered from AFP cases in August 2015 (but independently and without replacement), and overall 33.33 % (28/84) of these samples had EVs in them. This value (33.33 %) might therefore be a more accurate description of the prevalence of EVs in RD-L20B cell culture-negative AFP samples from children <15 years old in Nigeria. It is, however, likely that, going forward, more samples of this type will be analysed. This will help us to grow better at reproducibly estimating the true prevalence of EVs in such sample types. It is important to mention that we would have liked to further examine the impact of regional dynamics on EV-C preponderance in the samples analysed in this study in combination with those analysed in Adeniji *et al.* [12]. However, this could not be done because the selection of the samples in Adeniji *et al.* [12] was performed at random from an anonymized set and not regionally based.

Of note, we found a Sabin strain PV2 in Adeniji *et al.* [12], but found none in this study. This implies that only 1.2 % (1/84) of the samples analysed so far had poliovirus present. It does, however, clearly show the downside of analysing this sample set by random sampling, especially given the significance of missing poliovirus strains that are present to the eradication programme. Against this backdrop, it is essential that all (and not a random sample) of the AFP samples declared negative for EVs by RD-L20B cell culture be further screened using cell culture-independent techniques.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
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 strains analysed in this study to any individual.

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