Research Article

Isolation and Complete Capsid Sequence of Enterovirus D111 from Faeces of a Child with Acute Flaccid Paralysis in Nigeria

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Abstract | The WHO recommended cell-culture-based algorithm requires enterovirus (EV) isolates to produce reproducible cytopathic effect (R-CPE) in RD and/or L20b cell lines. Samples with non-reproducible CPE (NR-CPE) are considered negative for EVs. We investigated whether there could be EVs lurking in samples with NR-CPE. Fifty-nine cell culture supernatants (CCS) (collected between 2016 and 2017) from RD and L20b cell-culture-tubes with NR-CPE, were analyzed. The tubes had been previously inoculated with stool suspension from children (<15 years) in Nigeria with acute flaccid paralysis (AFP). All CCS were screened for Enteric Adenoviruses (EAVs) and group A Rotavirus (GARV) using a rapid immunochromatographic test kit. Subsequently, they were passaged in HEp-2 cell line. All isolates were subjected to RNA extraction, cDNA synthesis, three (5′-UTR, VP1 and EV Species C [EV-C]) different PCR assays and sequencing of amplicons. EVs were further subjected to [']Illumina sequencing. All CCS were negative for EAVs and GARVs. Four CCS produced R-CPE in HEp-2 cell line, three of which were positive for the 5′-UTR assay. Of the three isolates, two and none were positive for the VP1 and EV-C assays, respectively. One of the two VP1 amplicons was successfully sequenced and identified as Echovirus 1. Illumina sequencing of the three 5′-UTR positive isolates confirmed the E1 isolate and typed the remaining two as EV-Ds (94 and 111). We describe the first EV-D isolates of Nigerian origin and the first complete capsid sequence of the virus globally. We also show that NR-CPE could sometimes be caused by EVs that do not produce R-CPE in specific cell lines.

Received | July 08, 2019; **Accepted** | August 20, 2019; **Published** | August 29, 2019
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DOI | http://dx.doi.org/10.17582/journal.hv/2019/6.4.85.92
Citation | Faleye,T.O.C., Adewumi, M. O., Olayinka.O. T. and Adeniji, J. A. 2019. Isolation and complete capsid sequence of enterovirus D111 from faeces of a child with acute flaccid paralysis in Nigeria. *Hosts and Viruses*, 6(4): 85-92.
Keywords: Acute flaccid paralysis, HEp-2, EV-D111, Enteroviruses, Nigeria

Introduction

There are 15 Species in the genus *Enterovirus*, family *Picornaviridae*, order *Picornavirales* (http://www.picornaviridae.com/enterovirus/enterovirus.htm). Enterovirus Species C (EV-C) remains the type Species and Poliovirus (PV), the prototype member of the genus. Enteroviruses (EVs) have a naked capsid with icosahedral symmetry and a diameter of 28-30nM. The capsid encapsulates a positive sense, single-stranded RNA genome that is 7,500 nucleotides in length. The genome has one open reading frame (ORF) that is flanked by untranslated regions on both ends. The ORF encodes one polyprotein that is auto catalytically cleaved to make 11 proteins; four structural proteins

(VP1 – VP4) and seven non-structural proteins (2A-2C and 3A-3D). Classically, EV identification was done by neutralization assays. However, since a correlation was shown to exist between serological types and VP1 sequence (Oberste et al., 1999), VP1 amplification and sequencing became the standard for EV identification. Recently, the European Non-polio Enterovirus Network (ENPEN) recommended (Harvala et al., 2018) that in cases where VP1 data (or the full genome) is not available, VP2 and VP4 can be utilized for EV identification.

Most of the EV data available globally is courtesy the Global Polio Laboratory Network (GPLN); the laboratory arm of the Global Polio Eradication Initiative (GPEI). GPEI was established to eradicate PV globally and have so far reduced the incidence of poliomyelitis by >99.9% (Kew and Pallansch, 2108). Their approach has so far included a blend of surveillance for PV (in acute flaccid paralysis [AFP] cases as well as in the environment) and vaccination (using both inactivated and live attenuated poliovirus vaccines) to interrupt PV transmission chains (Kew and Pallansch, 2108).

In the GPLN, PV detection is done using a cell culture-based algorithm that requires PV isolates to produce reproducible cytopathic effect (R-CPE) in RD and L20b cell lines (WHO, 2003, 2004, 2015). The algorithm also results in the isolation of non-polio enteroviruses (NPEVs) which are also required to produce R-CPE in RD and/or L20b cell lines (WHO, 2003, 2004, 2015). Samples with non-reproducible CPE (NR-CPE) are considered negative for EVs. This phenomenon of NR-CPE has been suggested to be caused by nonspecific cytotoxicity, Adenovirus or Reoviruses (to which the Rotaviruses belong) (WHO, 2011). However, we (Adeniji et al., 2018) recently showed that EVs could also contribute to NR-CPE. In this study, we go a step further to find out how often the three (Adenoviruses, group A Rotaviruses and EVs) virus types are present in cell culture supernatants (CCS) recovered from L20b cell culture tubes with NR-CPE.

Materials and Methods

Samples

Fifty-nine (59) cell culture supernatants (CCS) were analyzed in this study following the algorithm depicted in Figure 1. All 59 CCS were recovered from L20b cell culture tubes with NR-CPE. The tubes had been previously inoculated with stool suspension from children (≤15 years old) in Nigeria with acute flaccid paralysis (AFP). Prior this study, the samples were accumulated over the course of one (1) year (2016-2017) during which they were stored at -20°C.

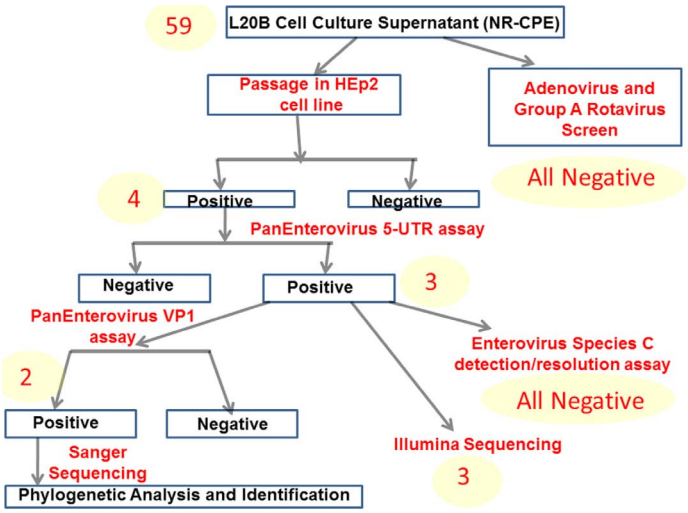


Figure 1: *A schematic representation of the algorithm followed in this study.*

Adenovirus and group a rotavirus screen

A rapid immunochromatographic test kit (Rotavirus Group A antigen/Enteric Adenovirus Antigen Rapid test kit, Accumed Technology Co. Ltd, Beijing) was used to screen the samples for Enteric Adenoviruses and Group a Rotavirus. As controls, a fecal suspension from a child with Rotavirus diarrhea that we had confirmed by molecular testing (unpublished data) and reference Adenovirus 1 and 6 from the BBSRC (a kind gift from Oladipo K.E.) were used as positive controls.

Passage in Hep2 cell line

The HEp-2 cell line used in this study was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% Fetal Calf Serum, 100IU/ml penicillin and 100mg/ml streptomycin. All CCS were passaged in HEp-2 cell line and incubated for 14 days at 37°C. For each CCS, 200µL was inoculated into two tissue culture tubes each containing HEp-2 cells. The tubes were then incubated at 37°C and monitored for 14 days. Daily the tubes where visually screened (using an inverted microscope) for EV characteristic cytopathic effect (CPE). Tubes in which CPE was observed were further passaged to confirm such. Samples were only declared negative for CPE if none was observed after 14 days of observation.

Table 1: *Details of PCR assays.*

S/N	Assay name	Primer name	Region amplified	Amplicon size (bp)	Extension time (Seconds)	cDNA used	Reference
1	PE-5-UTR	PanEnt-5^{I}-UTR-F	5^{I}-UTR	~114	30	1	Adeniji and Faleye, 2014
		PanEnt-5^{I}-UTR-R					
2	EC 3Dpol/3^{I}-UTR PCR	HEV-C-3D	3Dpol-3^{I}-UTR	~429	30	1	Adeniji and Faleye, 2014
		HEV-C-3^{I}-UTR					
3	*PE-VP1a	224	VP3-VP1	~750	60	2	Nix et al., 2006
		222					
4	*PE-VP1b	AN89	VP1	~350	30	NA	Nix et al., 2006
		AN88					

*NA: Not Applicable; *Both PE-VP1a and PE-VP1b are both components of the Nix et al., 2006 semi-nested PCR protocol. PE-VP1a is the first round while PE-VP1b is the second round PCR; cDNA 1= synthesized using random hexamers (Faleye et al., 2017); cDNA 2= synthesized using Oligos AN32-AN35 (Nix et al., 2006).*

Total RNA extraction and cDNA synthesis

Total RNA was extracted from all samples that produced CPE in HEp-2 cell line using the Total RNA extraction kit (Jena Bioscience, Jena, Germany). RNA was extracted following the guidelines detailed by the manufacturer. The SCRIPT cDNA synthesis kit (Jena Bioscience, Jena, Germany) was used for cDNA synthesis following manufacturer's instructions. Two different cDNAs were prepared; cDNA 1 (using random hexamers, (Faleye et al., 2018)) and cDNA 2 (using primers AN32 to AN35, (Nix et al., 2006)).

Polymerase Chain Reaction (PCR) assays

Three (PE-5-UTR, EC 3Dpol/3^{I}-UTR PCR and PE-VP1) different PCR assays that target three different (5^{I}-UTR, 3Dpol-3^{I}-UTR and VP1) EV genomic regions were done in this study (Table 1). It is however crucial to mention that the VP1 assay is a semi-nested PCR assay with PE-VP1a and PE-VP1b being the first and second round assays, respectively.

All primers used were made in 100µM concentrations. All PCR assays were done in 30µL volumes containing 6µL of Red Load PCR mix (Jena Bioscience, Jena, Germany), 0.3 µL of each of the forward and reverse primers, 5µL of cDNA or first round PCR product and 18.4µL of PCR grade water. Amplification was done using the Veriti thermal cycler as follows; 94°C for 3 min followed by 45 cycles of 94°C for 30s, 42°C for 30s and 60°C for 30s with ramp from 42°C to 60°C adjusted to 40%. This was followed by 72°C for 7 min and held at 4°C till terminated. It should however be noted that the extension temperature varied based on the expected amplicon size as detailed in Table 1. All PCR products were resolved on 2% agarose gels stained with ethidium bromide and viewed using a transilluminator.

Sanger sequencing

Only the amplicons generated by the PE-VP1b assay (Table 1) were subjected to Sanger sequencing. The amplicons were shipped to Macrogen Inc, Seoul, South-Korea, where gel extraction and Sanger sequencing were done. Sanger sequencing was done using the primers AN89 and AN88.

Illumina sequencing

For Illumina Sequencing, cDNA 1 was used. The genomes were amplified in overlapping fragments of 2 – 3kb as previously described (Faleye et al., 2018). Irrespective of the intensity of amplicon or whether or not there were amplicons, for each isolate, the overlapping genomic fragments were pooled and shipped to a commercial facility (MR DNA, Texas, USA) where library preparation (Nextera DNA sample preparation kit), Illumina sequencing (paired end, 300 cycles, HiSeq) were done. The Illumina sequencing data was assembled using the Kiki assembler v0.0.9. Subsequently, detection of EV contigs was done using the Enterovirus Genotyping Tool (EGT) v1.0 (Kroneman et al., 2011).

Enterovirus identification and Nucleotide accession numbers

Enterovirus types were determined using the EGT v1.0 (Kroneman et al., 2011). Nucleotide sequences generated in this study have been submitted to GenBank under the accession numbers MK190417-MK190420.

Phylogenetic analysis

All the sequences of the EV types of interest in GenBank were downloaded. Using the EGT v1.0 (Kroneman et al., 2011), sequences that did not cover the VP1 region of interest were removed. The sequences were then aligned using the Clustal W program in MEGA 5 software with default settings (Tamura et al., 2011). Subsequently, neighbor-joining trees were constructed using 1000 bootstrap replicates (Tamura et al., 2011). The accession numbers of sequences retrieved from GenBank for analysis are shown in the phylograms.

Results and Discussion

Adenovirus and group a rotavirus screen

All the samples screened in this study were negative for the Enteric Adenovirus and Group a Rotavirus Screen (Figure 1). However, the positive controls (a fecal suspension from a child with Rotavirus diarrhea and reference Adenovirus 1 and 6) were repeatedly and consistently detected by the rapid detection kit.

Passage in HEp2 cell line

On passage in HEp2 cell line, only 4 (6.8%) of the 59 samples yielded isolates that were reproducible on passage in HEp2 cell line (Figure 1).

PCR assays

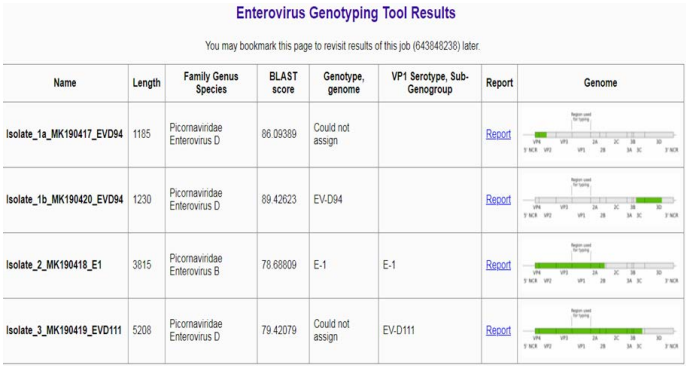
Three (75%) of the four isolates were positive for the Pan Enterovirus 5-UTR (PE-5-UTR) assay. None of the three (3) PE-5-UTR positive isolates was positive for the Enterovirus Species C detection/resolution (EC 3Dpol/3[1]-UTR PCR) assay. Two (2) of the three (66.7%) PE-5-UTR positive isolates were positive for the Pan Enterovirus VP1 (PE-VP1) assay (Figure 1).

Sanger sequencing

Of the two (2) samples positive for the PE-VP1 and shipped to the sequencing facility, only one was successfully sequenced. The other could not be sequenced due to insufficient volume. The sequenced isolate was typed as Echovirus 1 using the EGT (Table 2).

Illumina sequencing

This showed that isolates 1, 2 and 3 contained EV-D94, E1 and EV-D111 (Table 2). One contig spanning the entire capsid region was assembled for each of isolates 2 and 3 (Figure 2). On the other hand, two contigs were assembled for isolate 1. One each within the capsid (P1) and nonstructural protein (P3) encoding genomic regions (Figure 2).



Enterovirus Genotyping Tool Results

You may bookmark this page to revisit results of this job (643848238) later.

Name	Length	Family Genus Species	BLAST score	Genotype, genome	VP1 Serotype, Sub-Genogroup	Report	Genome
Isolate_1a_MK190417_EVD94	1185	Picornaviridae Enterovirus D	86.09389	Could not assign		Report	
Isolate_1b_MK190420_EVD94	1230	Picornaviridae Enterovirus D	89.42623	EV-D94		Report	
Isolate_2_MK190418_E1	3815	Picornaviridae Enterovirus B	78.68809	E-1	E-1	Report	
Isolate_3_MK190419_EVD111	5208	Picornaviridae Enterovirus D	79.42079	Could not assign	EV-D111	Report	

Figure 2: *The Contigs assembled from the Illumina sequencing data generated for the three PE-5UTR positive isolates. The contigs and the genomic regions covered are displayed using the EGT.*

A glitch in the enterovirus genotyping tool

It was observed that for isolate 3 (Table 2), there was disparity in the genotype and serotype as determined by the EGT (Figure 2). To resolve this aberration and confirm the identity of isolate 3, nucleotide similarity between the VP1 sequence of isolate 3 (and other EV-D111 from GenBank) and a reference EV-D70 was estimated (Table 3). This confirmed that isolate 3 was indeed EV-D111. Furthermore, using the EGT to type the six (6) EV-D111 VP1 sequences available in GenBank, prior this study, showed that the aberration was not unique to isolate 3 (Figure 3).

	Name	Length	Family Genus Species	BLAST score	Genotype, genome	VP1 Serotype, Sub-Genogroup	Report	Genome
1	JN255685_EVD111_CAF-OUP-05-059	295	Picornaviridae Enterovirus D	73.037544	EV-D70	EV-D111	Report	
2	JN255684_EVD111_CAF-NGB-06-107	295	Picornaviridae Enterovirus D	73.22034	EV-D70	EV-D111	Report	
3	JN255683_EVD111_CAF-BAN-06-150	295	Picornaviridae Enterovirus D	73.037544	EV-D70	EV-D111	Report	
4	EVD111_NGR_2017	295	Picornaviridae Enterovirus D	73.46939	EV-D70	EV-D111	Report	
5	JX431302_EVD111_111_TOK-230_HC	295	Picornaviridae Enterovirus D	75.17007	EV-D70	EV-D111	Report	
6	JF416935_EVD111_KK2640_Chimpan	295	Picornaviridae Enterovirus D	73.12926	EV-D70	EV-D111	Report	
7	EF127249_EVD111_17-04_DRC_2000	295	Picornaviridae Enterovirus D	73.61111	EV-D70	EV-D111	Report	
8	DM0820_EVD70_J670_71	295	Picornaviridae Enterovirus D	100.0	EV-D70	EV-D70	Report	

Figure 3: *All the EV-D111 sequences in GenBank described till date alongside a reference EV-D70 and the EGT identification glitch.*

Phylogenetic analysis

Considering EVDs are being described for the first time in the country, it was subjected to phylogenetic analysis and the data showed that two (1 and 2) lineages of EV-D111 have been documented globally (Figure 4). The EV-D111 lineage described in this study belongs to lineage 1 alongside those recovered

in Cameroon and Central Africa Republic (CAF) (Figure 4). It is crucial to mention that, prior this study, only six (6) EV-D111 sequences have been described till date and all were detected in sub-Saharan Africa (Figure 5).

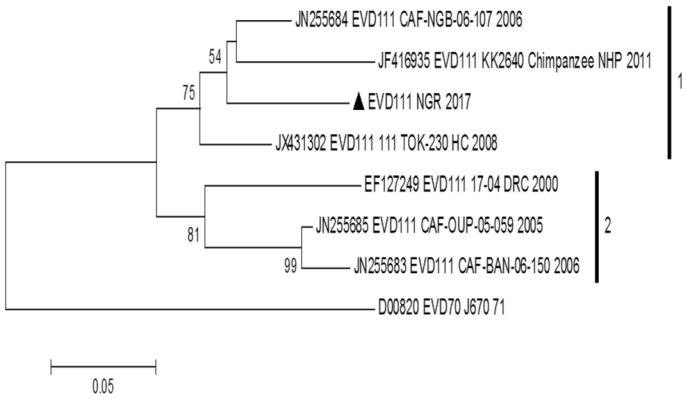


Figure 4: *Phylogram of partial VP1 sequences of EV-D111. The phylogenetic tree is based on an alignment of partial EV-D111 VP1 sequences available in GenBank and the one described in this study. The EV-D111 described in this study is indicated with a black triangle. Bootstrap values are indicated if >50%.*

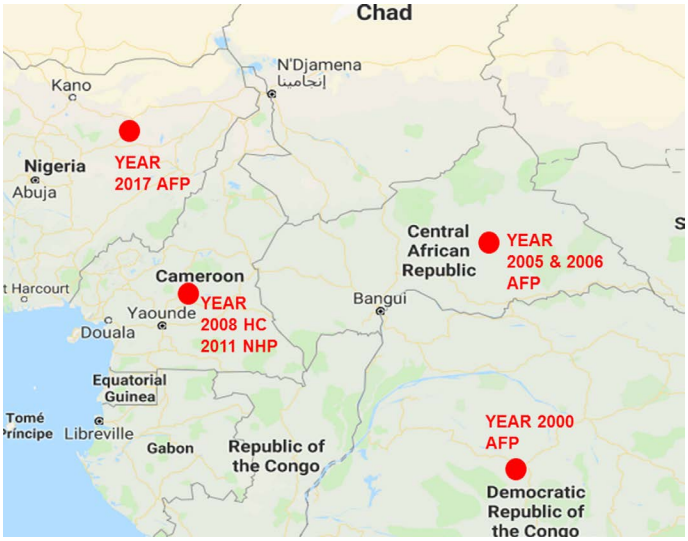


Figure 5: *Global distribution of EV-D111 detection sources. It is crucial to note that, till date, all have been detected in sub-Saharan Africa.*

In this study we screened 59 CCSs with NR-CPE for Enteric Adenovirus or Group a Rotavirus and found them to be negative. Though, it is currently impossible to assay for nonspecific cytotoxicity and several other non-Enteric Adenoviruses and Reoviruses (asides the Group a Rotaviruses) remain unaccounted for in this study, the results suggest that cases of NR-CPE might not always be accounted for by Enteric Adenoviruses or Group A Rotaviruses.

We (Adeniji et al., 2018) had previously shown that EVs could be recovered from CCSs of NR-CPE by direct RT-snPCR (Nix et al., 2006; WHO, 2015). We (Adeniji et al., 2018) also showed that EVs were detectable by direct RT-snPCR in 30.8% (4/13; 2 each of EV-Cs and EV-Bs) of the CCSs. We have taken a step further to recover the EV-B and EV-C isolates on RD (Adeniji et al., 2018) and Hep-2 (Faleye et al., 2019) cell lines, respectively. Considering, Hep-2 cell line supports replication of EV-Bs and has been documented to be better than RD in supporting the replication EV-Cs (Sadeuh-Mba et al., 2013), we selected it for use in this study. Using Hep-2 cell line, we recovered four (4) isolates from the samples screened and showed unequivocally, that three of them were EVs. Of the EVs detected in this study, one (E1) belongs to EV-B while the remaining two are members of EV-D (EV-D111 and EV-D94). To our surprise, none of the isolates recovered were EV-C members despite the documented predilection of Hep-2 cell lines for EV-Cs (Sadeuh-Mba et al., 2013) and our previous findings of EV diversity in NR-CPE (Adeniji et al., 2018).

Specifically, 5% (3/59) of the NR-CPE CCSs screened in this study had EVs that were missed by the RD-L20b (R-L) cell-culture-based algorithm (WHO, 2003, 2004, 2015). We therefore confirm in this study that NR-CPE could sometimes be caused by EVs that possibly do not produce R-CPE in RD and L20b cell lines but do so in other cell lines like HEp-2. How often these EVs cause NR-CPE in R-L cell lines need further investigation. However, pending that, it seems 5% (this study) to 30% (Adeniji et al., 2018) of CCSs from NR-CPE on R-L cell lines might contain EVs.

Three EVs (E1, EV-D111 and EV-D94) were identified in this study. While VP1 data was available to identify E1 and EV-D111, the EV-D94 isolate had no VP1 data available (Figure 2). It has been recommended (Harvala et al., 2018) and we have found it to be true (Adewumi et al., 2018) that in cases where VP1 data is not available, VP2 and VP4 can be utilized for EV identification. Hence, identification of the EV-D94 was done using a combination of the EGT and BLASTn result (data not shown). While the BLASTn result (data not shown) identified the isolate as EV-D94, the EGT could not assign it to any EV type using the VP2 and VP4 data. It however identified the isolate as EV-D94 using sequence data from the P3 genomic region, confirming the BLASTn result for the same region.

Table 2: *Identity of PE-5UTR positive samples using VP1 Sanger and Illumina data.*

EV Isolate S/N	PE-VP1 Assay	Identity		EV Species
		Sanger	Illumina	
1	Positive	Not Sequenced	EV-D94	EV-D
2	Positive	Echovirus 1	Echovirus 1	EV-B
3	Negative	Not Applicable	EV-D111	EV-D

Table 3: *Estimation of similarity between EV-D111 and EV-D70 VP1 to confirm identity of Isolate 3.*

EV-D111	Reference EV-D70	Distance (%)	Similarity (%)
JX431302 EVD111 111 TOK-230 HC 2008	D00820 EVD70 J670 71	31.03	68.97
JN255684 EVD111 CAF-NGB-06-107 2006	D00820 EVD70 J670 71	32.15	67.85
EF127249 EVD111 17-04 DRC 2000	D00820 EVD70 J670 71	33.27	66.73
EVD111 NGR 2017	D00820 EVD70 J670 71	33.89	66.11
JF416935 EVD111 KK2640 Chimpanzee NHP 2011	D00820 EVD70 J670 71	33.94	66.06
JN255683 EVD111 CAF-BAN-06-150 2006	D00820 EVD70 J670 71	34.52	65.48
JN255685 EVD111 CAF-OUP-05-059 2005	D00820 EVD70 J670 71	34.58	65.42

The EV-D94 and EV-D111 described in this study (Table 2) are being described for the first time in Nigeria. In fact, should the information in GenBank and the Picornavirus Study Group website be real-time, as at the 4th of November 2018 (http://www.picornaviridae.com/enterovirus/ev-d/ev-d_seq.htm), no complete genome of EV-D111 has been described and complete sequences exist for only the VP1 and VP4 genes. The EV-D111 sequence data we provide here, has the complete VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A and 3B genes (Figure 2). It also has partial sequences for 5-UTR and 3C (Figure 2). Hence, some of the sequence data we provide for EV-D111 are being described for the first time globally. It is however, crucial to note that this valuable data would have been missed if the CCSs from these NR-CPEs had not been re-screened. We believe, that as the EV community move more towards direct detection of EVs from samples using RT-PCR and NextGen sequencing strategies (Isaacs et al., 2018; Joffret et al., 2018; Majumdar et al., 2018), it is likely that we have less of these omissions. However, as we have previously shown (Adeniji et al., 2017), it should be noted that RT-PCR assays have limit of detection and their sensitivity can be enhanced when augmented with cell culture.

To the best of our knowledge, till date, EV-D111 has only been detected in sub-Saharan Africa (Junttila et al., 2007; Harvala et al., 2011; Bessaud et al., 2012; Sadeuh-Mba et al., 2013). Why this is the case is not clear. It therefore remains to be shown whether EV-D111 is present or circulating outside sub-Saharan Africa. Pending that, it seems EV-D111 is confined to the region (Figure 5). Though confined to sub-Saharan Africa, EV-D111 has been recovered from feaces of NHPs (Harvala et al., 2011), healthy children (Sadeuh-Mba et al., 2013) and those with AFP (Bessaud et al., 2012; Junttila et al., 2007 and this study). Hence, its zoonotic potential is clear. More importantly, the strain found in the AFP case in Nigeria (this study, Figure 4) belongs to lineage 1; the same lineage to which that found in NHP in Cameroon (a country sharing border with Nigeria) belongs. Considering the global distribution of EV-D68 (another member of EV species D) and its recurrent association with outbreaks with severe clinical manifestations (Esposito et al., 2015; Knoester et al., 2018), it is essential that the molecular epidemiology of this EV type be better investigated.

Acknowledgements

We thank the WHO National Polio Laboratory in Ibadan, Nigeria for providing the HEp-2 cell line and anonymous cell culture supernatants analyzed in this study. We thank Oladipo Kolawole Elijah for providing us reference Adenovirus 1 and 6 from BBSRC. This study was partly funded by a TET Fund grant to Adeniji J.A. PhD.

Author's Contribution

FTOC, AMO, OOT and AJA study design. OOT

sample collection. FTOC and AMO laboratory analysis. FTOC, AMO, OOT, AJA reagent contribution. FTOC wrote the first draft of the manuscript. FTOC, AMO, OOT, AJA edited the manuscript. FTOC, AMO, OOT, AJA read and approved the final draft of the manuscript.
AMO and AJA supervision. AJA funding.

Statement of conflict of interests

The authors declare that no conflict of interests exist. In addition, no information that can be used to associate the isolates analyzed in this study to any individual is included in this manuscript.

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