Research Article

Detection of Non-Cytopathic Enteroviruses in Supernatant of RD and L20B Cell Cultures

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Abstract | We investigated the likely presence of enteroviruses in supernatants of cytopathic effect (CPE) negative RD and L20B cell culture tubes in which faecal suspension from acute flaccid paralysis (AFP) cases were cultured. Samples analyzed were collected in 2017 as part of the AFP surveillance program in Nigeria and declared negative for enteroviruses because they did not produce CPE in RD and L20B cell lines. In all, 120 cell culture supernatants (60 each on RD and L20B cell lines) that emanated from 30 stool suspensions (2 samples per case) were analyzed as 60 pools (pooled per case by cell line). Pools were subjected to RNA extraction, RT-snPCR, amplicon sequencing and phylogenetic analysis. Eleven and one of the 30 pools of RD and L20B cell culture supernatants, respectively, were positive for the RT-snPCR. Nine of the 11 amplicons from RD and the only from L20B were sequenced and identified as seven EV types; Coxsackievirus A4 (CV-A4), CV-A6, CV-A13, CV-A17 (both RD and L20B), CV-B2, Echovirus 9 (E9) and Enterovirus A76 (EV-A76). Our findings suggest some enteroviruses are present in and might be replicating in RD cell line without producing CPE. We also report the existence of CV-A6 genotype E (possibly sub-Saharan Africa restricted).

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Introduction

The largest source of information on enteroviruses globally (Global Polio Eradication Initiative [GPEI]) defines the presence or otherwise of enterovirus in any faecal suspension or concentrate of sewage or sewage contaminated water (SCW) by the development of classic enterovirus cytopathic effect (CPE) on inoculation into any cell line of interest (WHO, 2003, 2004). This classic enterovirus CPE includes cells becoming rounded, refractive and detaching from culture surface (WHO, 2003, 2004).

Recently, studies in our laboratory suggested that some enteroviruses might be replicating in RD and L20B cell lines (which are the main stay of the poliovirus detection for eradication programme) without producing CPE. For example, in 2010, we (Adewumi et al., 2017) isolated an enterovirus from SCW that both neutralization by polyclonal antisera (Oberste et al., 2000) and Panenterovirus molecular identification (Oberste et al., 2003) identified as Echovirus 7 (E7). Enterovirus Species specific molecular characterization further showed the presence of a Coxsackievirus A24 (CV-A24) in the same isolate (Adewumi et al.,

2017). Ultimately, our investigations suggested that the CV-A24 was missed by both the neutralization and Panenterovirus molecular identification assays because it was not producing CPE in RD cell culture. The observed absence of CPE might be due to the EV-B bias of the cell line (Faleye and Adeniji, 2015) or low virus titre (Adewumi et al., 2017). We have subsequently made similar observation with a CV-B6/CV-A24 (unpublished) and CV-A1/CV-B4 (Adeniji et al., 2017) co-infection of children with acute flaccid paralysis (AFP) in Nigeria.

These findings led us to investigate the likelihood that enteroviruses are present in the supernatants of CPE negative RD and L20B cell culture tubes into which faecal suspension from AFP cases were inoculated and incubated for the specified 10 days at 37°C.

Materials and Methods

Samples

Routinely, the WHO polio laboratory in Ibadan Nigeria receives faecal samples from children <15years old diagnosed with AFP in the country as part of the national AFP surveillance programme. The samples are screened for the presence of polioviruses (and other non-polio enteroviruses) following the cell culture-based algorithm stipulated in the WHO polio manual (WHO, 2004). In accordance with the algorithm, two faecal samples are collected (at least 24 hours apart) from the suspected case of AFP and transported in a cold box to the laboratory. On arrival in the laboratory, the samples are re-suspended in phosphate buffered saline (PBS) and simultaneously inoculated into cell culture tubes independently containing RD and L20B cell lines. Samples from which isolates (defined by the production and confirmation of classic enterovirus CPE) were recovered, are processed further as defined by the algorithm (WHO, 2004). On the other hand, samples that showed no CPE on the cell lines throughout the stipulated period of incubation and observation are considered negative and discarded.

In this study, cell culture supernatants from 30 CPE negative cases of children <15years old with AFP were analyzed. Both the RD and the L20B cell culture supernatants were analysed. In all 120 cell culture supernatants (60 each for the RD and corresponding L20B supernatants) were analyzed. The 60 tubes for each cell line consisted of 30 pairs of samples. Thus, the 60 tubes of each cell line were pooled into 30 samples. The faecal samples inoculated into the cell lines analyzed in this study were collected in 2017 as part of the national AFP surveillance programme in Nigeria. The cell culture tubes analyzed were set to be autoclaved and subsequently discarded in accordance with the WHO algorithm (WHO, 2004). They were however, anonymized by the WHO polio laboratory in Ibadan, Nigeria before they were handed over to us for further analysis.

Molecular detection and strain identification

All samples were subjected to RNA extraction and the RT-snPCR assay first described by Nix et al. (2006) and recommended by the WHO (2015). Briefly, RNA extraction and cDNA synthesis were done using the Jena Bioscience Total RNA extraction and cDNA synthesis kits (Jena Bioscience, Jena, Germany) as recently described (Adeniji et al., 2017). The mixture was incubated at 42 °C for 10min followed by 50 °C for 60 minutes in a Veriti thermal cycler (Applied Biosystems, California, USA).

The PCR assay was a seminested assay as recommended by the WHO (2015). Primers 224 and 222 were used for the first round assay while primers AN89 and AN88 were used for the second round assay. The first round PCR reaction (Figure 1) contained 6µL of Red Load Taq, 13.4µL of RNase free water, 0.3µL each of the forward and reverse primers and 10µL of cDNA. Thermal cycling was done as follows; 94°C for 3 minutes, then, 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 held at 4°C until the reaction was terminated. The second round PCR reaction was similar to the first but with slight modifications. Instead of 10µL of cDNA, 3µL of first round PCR product was used. Also, the volume of RNase free water was increased to 20.4µL. Thermal cycling conditions were also the same except for the extension time that was reduced to 30 seconds. The second round amplicons were resolved on 2% agarose gels stained with ethidium bromide and viewed using a UV transilluminator.

All amplicons of the required size (~350bp) were sequenced at Macrogen Inc using the second round primers. The enterovirus strain and species determination was done using the Enterovirus Genotyping Tool (Kroneman et al., 2011). Phylogenetic analysis and pairwise distance enumeration was done

using MEGA 5 software as previously described (Adeniji et al., 2017). The sequences obtained from this study have been deposited in GenBank with accession numbers MG761735-MG761743. The algorithm followed in this study is depicted in Figure 1.

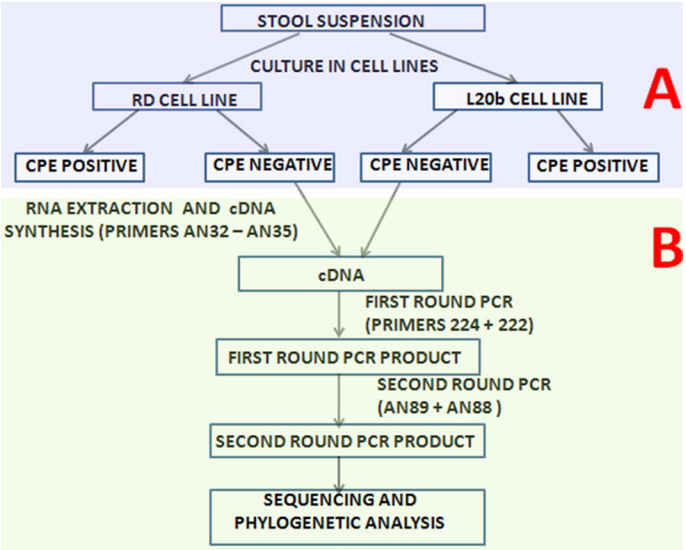

Figure 1: *The algorithm followed in this study. The section depicted 'A' was executed by the WHO polio laboratory in Ibadan, Nigeria while the section depicted 'B' was executed in this study. The cell culture supernatants from RD and L20B cell culture tubes that showed no CPE were collected from the WHO polio laboratory and analyzed in this study.*

Results and Discussion

Eleven of the 30 RD cell culture suspensions analyzed in this study were positive for the Panenterovirus RT-snPCR assay. On the other hand, only one of the 30 L20B cell culture suspensions were positive for the Panenterovirus RT-snPCR assay (Table 1).

All the 11 amplicons from the RD cell culture suspensions were successfully sequenced but only nine could be identified. The identifiable nine were typed by the EGT as Coxsackievirus A4 (CV-A4) (1 strain), CV-A6 (1 strain), CV-A13 (1 strain), CV-A17 (1 strain), CV-B2 (1 strain), Echovirus 9 (E9) (2 strains) and Enterovirus A76 (EV-A76) (2 strains). Hence, four EV-As (CV-A4, CV-A6 and two EV-A76), three EV-Bs (CV-B2 and two E9) and two EV-Cs (CV-A13 and CV-A17) were recovered from the RD cell culture supernatant. The only amplicon from the L20B cell culture suspensions was successfully sequenced and identified as CV-A17 (1 strain), an EV-C and exactly what was found in its corresponding RD supernatant (Table 1).

The CV-A17 strain recovered in this study was subjected to phylogenetic analysis because CV-A17 was found in both RD and L20B cell culture suspensions of the same sample. Phylogenetic analysis showed that both CV-A17 strains found were very similar (Figure 2). They were however different from other CV-A17 strains that had been previously detected in Nigeria from 2003 till date (Figure 2).

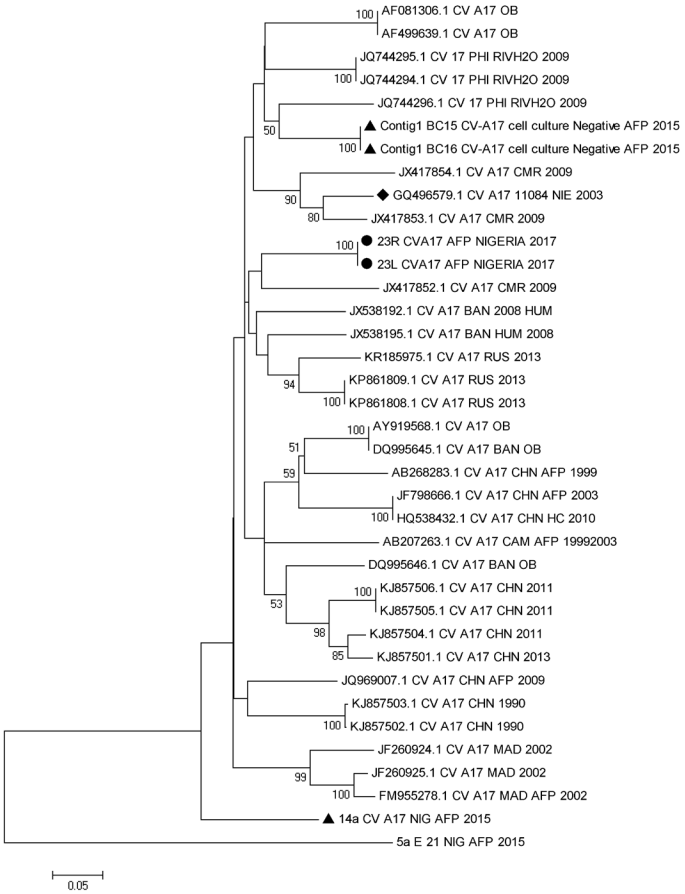

Figure 2: *Phylogenetic tree of genetic relationship between VP1 nucleotide sequences of CV-A17. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. Strains detected in Nigeria in 2003 and 2015 are indicated with black diamond and triangle, respectively. Bootstrap values are indicated if >50%.*

The CV-A6 strain recovered in this study was subjected to phylogenetic analysis because the only two CV-A6 strains from Nigeria sequenced and available in GenBank were recovered in 2003 (Oyero et al., 2014). The CV-A6 strain detected in this study therefore represents a re-sampling after 14 years. Phylogenetic analysis showed that the CV-A6 strain described in this study and those previously described in Nigeria in 2003 and Guinea in 2014 clustered together with strong bootstrap support (Figure 3). The fact that this cluster was an independent genotype that conforms with the ~15% divergence rule (Brown et al., 1999; Song et al., 2017) was confirmed by pairwise distance

Table 1: *Result of the RT-snPCR screen showing only samples positive for the assay.*

S/N	Sample ID	RD			L20B		
		PCR Result	Species	Serotype/Genotype	PCR Result	Species	Serotype/Genotype
1	1	+	EV-B	E9	--	NA	NA
2	5	+	EV-A	CV-A4	--	NA	NA
3	7	+	Bad Data	NA	--	NA	NA
4	10	+	Bad Data	NA	--	NA	NA
5	12	+	EV-A	CV-A6	--	NA	NA
6	13	+	EV-A	EV-A76	--	NA	NA
7	15	+	EV-A	EV-A76	--	NA	NA
8	16	+	EV-B	CV-B2	--	NA	NA
9	17	+	EV-C	CV-A13	--	NA	NA
10	23	+	EV-C	CV-A17	+	EV-C	CV-A17
11	26	+	EV-B	E9	--	NA	NA

+: Positive; --: Negative; NA: Not Applicable; EV: Enterovirus; CV: Coxsackievirus.

Table 2: *Pairwise distance between the CV-A6 strain identified in this study and other reference strains from GenBank.*

Strain of interest	Reference strain	Dist	Dist %	Siml %
CVA6 AFP NGR 2017	KY433795.1 CVA6 318 GUINEA 2014	0.137	13.71	86.29
CVA6 AFP NGR 2017	GQ496524.1 CVA6 11029 NGR 2003	0.152	15.20	84.80
CVA6 AFP NGR 2017	GQ496523.1 CVA6 11028 NIGERIA 2003	0.152	15.20	84.80
CVA6 AFP NGR 2017	JQ364886.1 CVA6 GENOTYPE B1 92022/SD CHINA 1992	0.206	20.58	79.42
CVA6 AFP NGR 2017	KP143073.1 CVA6 AFP024/GD CHINA 2004	0.206	20.64	79.36
CVA6 AFP NGR 2017	KC866915.1 CVA6 JB143090087 CHINA 2009	0.212	21.15	78.85
CVA6 AFP NGR 2017	KP143076.1 CVA6 AFP265 GD CHINA 2005	0.224	22.40	77.60
CVA6 AFP NGR 2017	HQ538431.1 CVA6 dl-114 CHINA 2010	0.224	22.40	77.60
CVA6 AFP NGR 2017	JF798665.1 CVA6 CA6-104-YN-2003AFP CHINA 2003	0.224	22.40	77.60
CVA6 AFP NGR 2017	KP143075.1 CVA6 GENOTYPE B2 AFP262/GD CHINA 2005	0.224	22.40	77.60
CVA6 AFP NGR 2017	KP143074.1 CVA6 GENOTYPE B2 AFP560/GD CHINA 2004	0.228	22.84	77.16
CVA6 AFP NGR 2017	KP143078.1 CVA6 GENOTYPE B2 AFP051/GD CHINA 2007	0.234	23.44	76.56
CVA6 AFP NGR 2017	KU736939.1 CVA6 GENOTYPE D3 SHAPHC5696/SH CHINA 2015	0.254	25.44	74.56
CVA6 AFP NGR 2017	KP143077.1 CVA6 GENOTYPE D2 AFP569/GD CHINA 2006	0.255	25.51	74.49
CVA6 AFP NGR 2017	AY421764.1 CVA6 GENOTYPE A Gdula USA 1949	0.265	26.55	73.45
CVA6 AFP NGR 2017	JN203517.1 CVA6 GENOTYPE C2 N-313 INDIA 2008	0.266	26.60	73.40
CVA6 AFP NGR 2017	LC126143.1 CVA6 GENOTYPE D1 Hyogo1278 JAPAN 1999	0.267	26.75	73.25
CVA6 AFP NGR 2017	JQ364887.1 CVA6 GENOTYPE C1 96188/SD CHINA 1996	0.288	28.77	71.23
CVA6 AFP NGR 2017	KM114057.1 CVA6 GENOTYPE D3 Finland 2008	0.292	29.24	70.76
CVA6 AFP NGR 2017	EVA76 NGR AFP 2017 OUTGROUP	0.768	76.85	23.15

analysis (Table 2). Hence the sub-Saharan Africa cluster has been designated genotype E (Figure 3).

The EV-A76 strains recovered in this study were subjected to phylogenetic analysis because this is the first description of EV-A76 in children with AFP in Nigeria. Phylogenetic analysis showed that both EV-A76 strains (Nigeria, 2017) found in this study were different from all previously described in sub-Saharan Africa (Figure 4). Particularly, they were also different from the strain we detected in a nonhuman primate (NHP) in 2016 in Jos, Nigeria (sub-Saharan Africa 3) (Figure 4).

In this study, we investigated the likelihood that enteroviruses are present in the supernatants of CPE negative RD and L20B cell culture tubes into which faecal suspension from AFP cases were inoculated and incubated for the specified 10 days at 37°C. We succeeded in identifying enteroviruses in 30% (9/30)

of such samples screened. This confirms our previous finding (Adeniji et al., 2017) that enteroviruses are present in some of the AFP samples declared 'negative' for enteroviruses on the grounds that they did not produce CPE in RD and L20B cell line as recommended (WHO, 2004). The results of this study further confirm that these samples were falsely declared as 'negative' for enteroviruses. This diagnostic error however, has its origin in the dependence on CPE (WHO, 2003, 2004) as the criteria for scoring a cell culture tube as positive for enteroviruses.

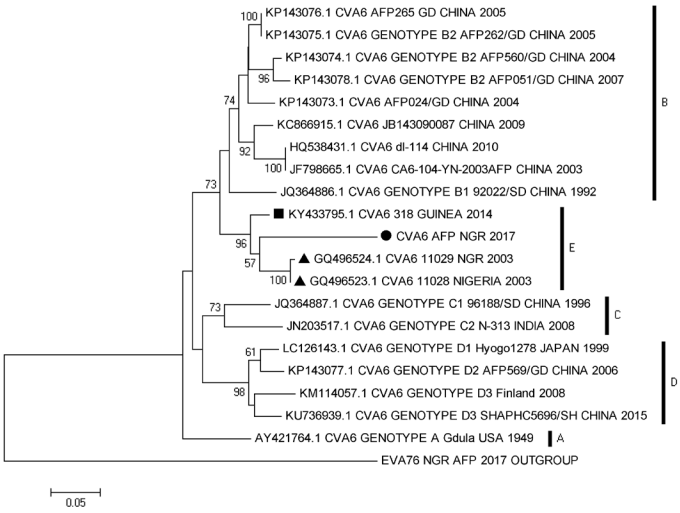

Figure 3: *Phylogenetic tree of genetic relationship between VP1 nucleotide sequences of CV-A6. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. Strains detected in Nigeria in 2003 are indicated with black triangle, while that detected in Guinea in 2014 is indicated with a black square. Bootstrap values are indicated if >50%.*

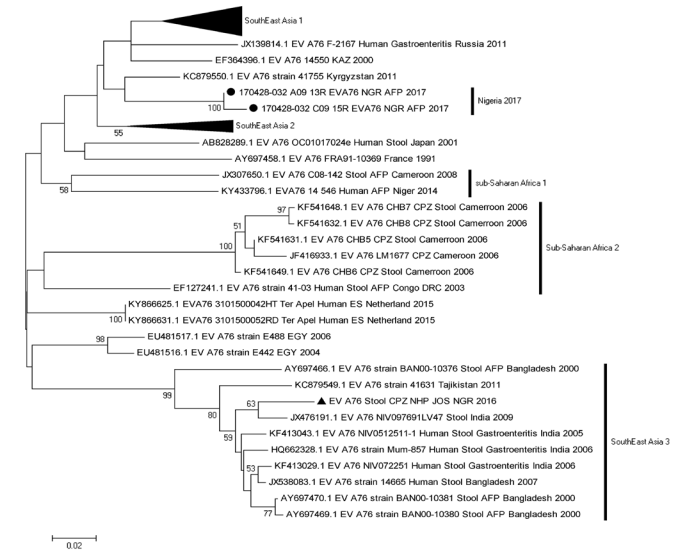

Figure 4: *Phylogenetic tree of genetic relationship between VP1 nucleotide sequences of EV-A76. The phylogenetic tree is based on an alignment of the partial VP1 sequences. The newly sequenced strains are indicated with black circle. A strain detected in a nonhuman primate in Nigeria in 2016 is indicated with black triangle. Bootstrap values are indicated if >50%.*

Of course, it can be argued that the enteroviruses found in the cell culture supernatants, in this study, might not have replicated in the different cell lines but might rather represent a relic of the inoculum introduced. That argument might only be correct for sample 23 in which both cell lines yielded the same enterovirus type; CV-A17 (Table 1 and Figure 2). For the remaining eight samples in which six different EV types were detected in RD cell line supernatant but not in the L20B equivalent, that argument might be incorrect. This is because, if the EV found were a relic of the inoculum then it should have been detected in both the RD and L20B supernatants as found in sample 23 (Table 1 and Figure 2). Rather, the fact that EVs were only found in the RD and not the L20B supernatants suggests the possibility of at least, these six different EV types might have been replicating in RD cell line without showing CPE (Table 1).

It did not escape our notice that while there is always EV-B bias of strains recovered when the RD-L20B cell culture based, CPE dependent algorithm is used (Baba et al., 2012; Sadeuh-Mba et al., 2013; Adeniji and Faleye, 2014; Oyero et al., 2014; Faleye and Adeniji, 2015; Faleye et al., 2017; Fernandez-Garcia et al., 2017), in this study, EV-Bs (3/9) did not form the most abundant species recovered (Table 1). Rather EV-As (4/9) were the most abundant supporting previous findings (Faleye et al., 2016) in which CPE was not the basis for detecting the presence or otherwise of EVs in samples of interest. Furthermore, while in other studies where the RD-L20B cell culture based, CPE dependent algorithm was used, all the other species summed up was usually less than the EV-Bs recovered (Baba et al., 2012; Sadeuh-Mba et al., 2013; Adeniji and Faleye, 2014; Oyero et al., 2014; Faleye and Adeniji, 2015; Faleye et al., 2017; Fernandez-Garcia et al., 2017), in this study, reverse is the case (Table 1). The results of this study therefore confirm that the EV-B preponderance observed in many RD-L20B cell culture based, CPE dependent studies might not be a true reflection of EV diversity in the population sampled. Specifically, the results of this study further showed that some EV infections are being missed by the RD-L20B cell culture based, CPE dependent algorithm resulting in selective accumulation as 'negatives', samples with EV strains that either do not replicate or replicate in RD cell line but without producing CPE in such. The results of this study thereby highlight the need to revise the use of CPE as the basis for detecting or determining the

presence of EVs in any sample of interest.

Two (CV-A6 and EV-A76) interesting EV types were found in this study. CV-A6 is interesting because it is associated with hand, foot and mouth disease (HFMD) in Asia (Song et al., 2017; Tao et al., 2017). Till date, besides the CV-A6 strain described in this study, two Nigerian strains of CV-A6 were previously described in 2003 (Oyero et al., 2014). Phylogenetic analysis (Figure 3 and Table 2) showed that these three Nigerian strains and the 2014 Guinea strain (Fernandez-Garcia et al., 2017) form a new genotype (genotype E) that has only been detected, till date, in sub-Saharan Africa. This thereby suggests possible confinement of CV-A6 genotype E to the sub-region. It is however crucial to state that members of CV-A6 genotype E have only been recovered from healthy children (Oyero et al., 2014) and those with AFP (Fernandez-Garcia et al., 2017). It therefore remains to be shown whether, like EV-A71 genotype E, CV-A6 genotype E might be restricted to sub-Saharan Africa and possibly not associated with HFMD.

Finally, we recently found (in 2016) an EV-A76 of Asian ancestry in a NHP that was born and has been in captivity all its life in Jos, North-Central Nigeria (Oragwa et al., 2018) buttressing the importation of EV strains from Asia into the country (Faleye et al., 2019). Thus, our interest when two EV-A76 strains were detected in children with AFP in the country. Phylogenetic analysis however showed that the EV-A76 strains found in this study were unrelated to the Jos, NHP strain (Figure 4) and even the strain detected in Niger in 2014 (Fernandez-Garcia et al., 2017). This suggests that as observed for CV-A17 too (Figure 2), several lineages of EV-A76 are also circulating in Nigeria.

Conclusions and Recommendations

The findings of this study show it is necessary to revise the use of CPE as the basis for detecting enteroviruses in RD and other cell culture systems used for enterovirus isolation. We also report the existence of CV-A6 genotype E. It however remains to be shown whether, like EV-A71 genotype E, CV-A6 genotype E might be restricted to sub-Saharan Africa and possibly not associated with HFMD.

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Author's Contributions

AJA, AMO and FTOC study Design. All authors sample collection, laboratory and data analysis. All authors wrote, revised, read and approved the final draft of the manuscript.

Statement of conflict of interests

The authors declare that no conflict of interests exist.

References

Adeniji, J.A., A.O. Oragwa, U.E. George, U.I. Ibok, T.O.C. Faleye and M.O. Adewumi. 2017. Preponderance of enterovirus C in RD-L20B-cell-culture-negative stool samples from children diagnosed with acute flaccid paralysis in Nigeria. Arch. Virol. 162(10): 3089-3101. https://doi.org/10.1007/s00705-017-3466-2

Adeniji, J.A. and T.O.C. Faleye. 2014. Isolation and identification of enteroviruses from sewage and sewage contaminated water in Lagos, Nigeria. Food Environ. Virol. 6(2): 75-86. https://doi.org/10.1007/s12560-014-9137-5

Adewumi, M.O., T.O.C. Faleye and J.A. Adeniji. 2017. The fallacy of identification by neutralization in the light of cytopathic effect nonproducing enterovirus strains. Microbiol. Res. J. Int. Med. Res. J. Int. 22(4): 1-6. https://doi.org/10.9734/MRJI/2017/38280

Baba, M.M., B.S. Oderinde, P.Z. Patrick and M.M. Jarmai. 2012. Sabin and wild polioviruses from apparently healthy primary school children in northeastern Nigeria. J. Med. Virol. 84(2): 358-364. https://doi.org/10.1002/jmv.23184

Brown, B.A., M.S. Oberste, J.P. Alexander, M.L. Kennett and M.A. Pallansch. 1999. Molecular epidemiology and evolution ofenterovirus 71 strains isolated from 1970 to 1998. J. Virol. 73: 9969–9975.

Faleye, T.O.C. and J.A. Adeniji. 2015. Enterovirus species B bias of RD cell line and its influence on enterovirus diversity landscape. Food Environ. Virol. 7(4): 390-402. https://doi.org/10.1007/

s12560-015-9215-3

Faleye, T.O.C., M.O. Adewumi, M.O. Japhet, O.M. David, A.O. Oluyege, J.A. Adeniji and O. Famurewa. 2017. Non-polio enteroviruses in faeces of children diagnosed with acute flaccid paralysis in Nigeria. Virol. J. 14(1): 175. https://doi.org/10.1186/s12985-017-0846-x

Faleye, T.O.C., O.M. Adewumi and J.A. Adeniji. 2019. Genomic characterization of an echovirus 7 isolate of Southeast Asian ancestry recovered from a child in Nigeria with acute flaccid paralysis. Microbiol. Resour. Announc. 8: e00830-19. https://doi.org/10.1128/MRA.00830-19

Faleye, T.O.C., M.O. Adewumi, B.A. Coker, F.Y. Nudamajo and J.A. Adeniji. 2016. Direct detection and identification of enteroviruses from faeces of healthy Nigerian children using a cell-culture independent RT-seminested PCR assay. Adv. Virol. Article ID: 1412838, 12 pages. https://doi.org/10.1155/2016/1412838

Fernandez-Garcia, M.D., O. Kebe, A.D. Fall and K. Ndiaye. 2017. Identification and molecular characterization of non-polio enteroviruses from children with acute flaccid paralysis in West-Africa, 2013-2014. Sci. Rep. 7: 3808. https://doi.org/10.1038/s41598-017-03835-1

Kroneman, A., H. Vennema, K. Deforche, H.V.D. Avoort, S. Penarandac, M.S. Oberste, J. Vinjéc and M. Koopmans. 2011. An automated genotyping tool for enteroviruses and noroviruses. J. Clin. Virol. 51: 121–125. https://doi.org/10.1016/j.jcv.2011.03.006

Nix, W.A., M.S. Oberste and M.A. Pallansch. 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J. Clin. Microbiol. 44(8): 2698–2704. https://doi.org/10.1128/JCM.00542-06

Oberste, M.S., K. Maher, M.R. Flemister, G. Marchetti, D.R. Kilpatrick and M.A. Pallansch. 2000. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J. Clin. Microbiol. 38(3): 1170–1174.

Oberste, M.S., W.A. Nix, K. Maher and M.A. Pallansch. 2003. Improved molecular identification of enteroviruses by RT–PCR and amplicon sequencing. J. Clin. Virol. 26(3): 375-377. https://doi.org/10.1016/S1386-6532(03)00004-0

Oragwa, A.O., U.E. George, T.O.C. Faleye, M.O. Adewumi and J.A. Adeniji. 2018. Enterovirus-A76 of South-East Asian ancestry in a Captive Chimpanzee (*Pan troglodytes*) in Jos, Nigeria. J. Adv. Microbiol. 9(3): 1-8. https://doi.org/10.9734/JAMB/2018/40008

Oyero, O.G., F.D. Adu and J.A. Ayukekbong. 2014. Molecular characterization of diverse species enterovirus-B types from children with acute flaccid paralysis and asymptomatic children in Nigeria. Virus Res. 189: 189-193. https://doi.org/10.1016/j.virusres.2014.05.029

Sadeuh-Mba, S.A., M. Bessaud, D. Massenet, M.L. Joffret, M.C. Endegue, R. Njouom, J.M. Reynes, D. Rousset and F. Delpeyroux. 2013. High frequency and diversity of species C enteroviruses in Cameroon and neighboring countries. J. Clin. Microbiol. 51(3): 759-770. https://doi.org/10.1128/JCM.02119-12

Song, Y., Y. Zhang, T. Ji, X. Gu, Q. Yang, S. Zhu, W. Xu, Y. Xu, Y. Shi, X. Huang, Q. Li, H. Deng, X. Wang, D. Yan, W. Yu, S. Wang, D. Yu and W. Xu. 2017. Persistent circulation of Coxsackievirus A6 of genotype D3 in mainland of China between 2008 and 2015. Sci. Rep., 7: 5491. https://doi.org/10.1038/s41598-017-05618-0

Tao, J., X.Y. He, Y. Shi, G. Zhu, S. Liu, Z. Zhang, S. Tang, R. Zhang, B. Peng, Z. Liu, J. Tan, Q. Chen, W. Wang, L. Bao, L. Zou and P. Zhang. 2017. Epidemiology of 45,616 suspect cases of hand, foot and mouth disease in chongqing, China, 2011- 2015. Sci. Rep. 7: 45630. https://doi.org/10.1038/srep45630

WHO. 2003. Guidelines for environmental surveillance of poliovirus circulation. Geneva.

WHO. 2004. Polio laboratory Manual. 4th Edition, Geneva.

WHO. 2015. Enterovirus surveillance guidelines: Guidelines for enterovirus surveillance in support of the Polio Eradication Initiative, Geneva.