Research Article

Non-polio Enterovirus Species C infection in AFP Cases and its Implication for the Emergence of Recombinant Circulating Vaccine-Derived Polioviruses

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Abstract | Using 40 faecal samples from acute flaccid paralysis (AFP) cases collected in 2019, we examined how coupling Enterovirus (EV) isolation in RD and MCF-7 cell culture with direct detection from clinical sample might impact our ability to detect non-polio EV Species C (NPESC) members in stool samples. In all, 20% (8/40) of all the samples screened had NPESCs and 50% (8/16) of the EVs detected were NPESCs. However, the RD cell line missed all NPESCs. Hence, RD cell line alone should not be used for EV isolation if the desire is to also isolate NPESC.

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Introduction

Enteroviruses (EVs) are members of the genus *Enterovirus* in the family *Picornaviridae*, order *Picornavirales* [1]. Within the genus are 15 species (EV-A to EV-L and Rhinovirus A-C). EV-B has the highest number of serotypes while EV-C has the best studied member of the genus, which is the poliovirus. Other non-poliovirus members of Species C are referred to as Non-polio enterovirus species C (NPESC) members.

The GPEI was established in 1988 with the mandate of poliovirus eradication [2]. As at the time of writing, wild poliovirus circulation had been interrupted globally except in Pakistan and Afghanistan [3]. This progress has had at its core a very strong surveillance (environmental and acute flaccid paralysis [AFP]) and vaccination program. Poliovirus vaccination is being done globally using a combination of a live attenuated vaccine (oral poliovirus vaccine [OPV]) and inactivated poliovirus vaccine (IPV). While the IPV is great at protecting vaccinees from poliomyelitis, it performs poorly at inducing mucosal immunity which is essential for interrupting transmission chains. OPV on the other hand, both protects the vaccinees from developing poliomyelitis and is also great at interrupting poliovirus transmission because it is great at inducing mucosal immunity. Hence, to interrupt transmission chains during an outbreak, OPV is usually administered [4].

The downside of OPV is that it sometimes losses its attenuated phenotype and consequently reverts back to a wild type virulence and transmissibility. These revertant OPV strains are referred to as circulating Vaccine-Derived Polioviruses (cVDPVs) if the chain of transmission has lasted for over eight weeks [5]. More crucial is the observation that most cVPDVs are recombinants with structural genomic region of OPV origin and nonstructural genomic region of NPESC origin [6]. This suggests that there is baseline NPESC infection in children in most places where recombinant cVDPVs emerge.

Previously, we [7-9] had tried to learn about NPESC infection in children below the age of 15 years in Nigeria and most of our studies taught us how the algorithm of choice influences what we see [10]. Particularly, we learnt that RD cell line (which is recommended alongside L20B cell line for EV detection during surveillance and used in laboratories that are part of the GPEI) tend not to support the replication of NPESCs [11,12]. Here we attempt to determine the prevalence of NPESC infection in children diagnosed with AFP in Nigeria. However, leaning on our prior understanding of how EV detection algorithms introduce bias, we attempt to ensure most sources of bias are addressed. Our results show that about 20% of children diagnosed with AFP have NPESCs that were not detectable using RD cell line. It also shows that 50% of children diagnosed with AFP and from whom EVs were detected, had NPESCs in them which were missed using the RD cell culture-based algorithm.

Materials and Methods

Samples from 40 anonymized individuals were analyzed in this study. All samples were previously processed in one day in the WHO polio laboratory in Ibadan, Nigeria. The samples consist of 31 paired (i.e. 62 samples) faecal samples from cases of AFP and nine (9) singleton fecal samples of their contacts. The samples were collected as part of the National AFP surveillance program in Nigeria and processed (resuspended in PBS and inoculated into both RD and L20B cell lines) as recommended by the WHO [13]. Isolates were recovered in RD cell line from 11 of the 31 cases. All 11 RD cell culture isolates did not replicate with evidenced CPE in L20B cell culture. All the RD isolates were analyzed in this study.

For this study, all the fecal suspensions and their corresponding 11 isolates on RD cell line were collected from the WHO Polio Laboratory in Ibadan, Nigeria and subsequently analyzed. The 62 (31 paired) fecal samples from AFP cases were then pooled by cases making 31 fecal suspension samples. All the 31 fecal suspension samples and the 9 fecal suspension samples from their contacts were inoculated into MCF-7 cell culture and observed for development of cytopathic effect (CPE) for 10 days in culture. All 40 fecal suspensions, isolates recovered on MCF-7 and the 11 from RD cell line collected from the WHO National Polio laboratory in Ibadan were subjected to RNA extraction, cDNA synthesis and RT-snPCR as previously described [8]. All PCR products were subsequently resolved on a 2% Agarose gel and amplicons shipped to Macrogen Inc., Netherlands where Sanger sequencing was done using primers AN89 and AN88 [14]. Sequences generated were identified using the enterovirus genotyping tool [15]. All sequences generated in the study have been submitted to GenBank with Accession Numbers MN718829-MN718840 and MN738732-MN738740.

Results and Discussion

Isolates were recovered from seven (7) of the 40 samples inoculated into MCF-7 cell culture. Hence, from the 40 faecal suspensions, isolates were recovered from 27.5% (11/40) and 17.5% (7/40) on RD and MCF-7 cell lines, respectively. Altogether, 40, 11 and 7 samples from faecal suspensions, RD and MCF-7 cell culture supernatants, respectively were analyzed as previously described [8].

Of the 40 faecal suspensions, amplicons were recovered from 13 (32.5%) but only nine (9) produced useable nucleotide sequence data. The nine amplicons sequenced include four EV-Cs (CVA13 [3 isolates] and EV-C99 [1 isolate]) and five EV-Bs (CV-B2 [1 isolate], E20 [2 isolates], E30 [1 isolate] and EV-B77 [1 isolate]) (Table 1).

Of the 11 isolates recovered on RD cell culture, amplicons were recovered from 10 (90.9%) and all ten (100%) produced useable nucleotide sequence data. All 10 were successfully identified as members of EV-B. Specifically, CV-B2 [1 isolate], CV-B3 [3 isolates], E7 [1 isolate], E12 [1 isolate], E20 [3 isolates] and E24 [1 isolate] (Table 1).

Of the seven (7) isolates recovered on MCF-7 cell culture, amplicons were recovered from four (4) and all four (100%) produced useable nucleotide sequence data. All four were successfully identified as members of EV-C. Specifically, EV-C99 [1 isolate], CV-A13 [2 isolates] and CV-A20 [1 isolate] (Table 1).

Table 1: *Enterovirus types detected in this study and the samples from which they were detected.*

	Isolate ID		
Sample code	Faecal suspension	RD cell line	MCF-7 cell Line
3	CV-A13	---	---
8	---	E-12	---
9	---	CV-B3	---
12	E-20	E-20	---
13	E-20	E-20	---
14	---	E-20	---
16	EV-C99	---	---
17	E-30	E-7	EV-C99
19	CV-B2	CV-B2	---
22	CV-A13	---	---
24	EV-B77	---	---
30	---	---	CV-A13
31	CV-A13	E-24	---
32	---	---	CV-A13
33	---	CV-B3	---
34	---	CV-B3	CV-A20

It is worth noting that of the samples that were negative for EVs by direct detection from faecal suspension using RT-snPCR [14,16], seven (7) had EVs that were only detectable after the samples had been subjected to culture on either or both RD and MCF-7 cell line. Also, of the samples that did not show CPE on RD cell line, two (2) had CV-A13 on MCF-7 cell line while another four (4) had EVs (CV-A13 [2 isolates], EV-B77 [1 isolate] and EV-C99 [1 isolate]) detected directly in their faecal suspension. It is also worth noting, that all isolates recovered on RD and MCF-7 cell lines belong to Species EV-B and EV-C, respectively. In addition, all samples from which EV-C members were detected in the faecal suspension were different from those in which EV-C members were recovered on MCF-7 cell line. Precisely, in samples 17 and 34, EV-Bs were detected on RD cell line but EV-Cs on MCF-7 cell line. Also notable, three (3) different EV types (E30, E7 and EV-C99 from faecal suspension, RD and MCF-7 cell lines) were detected in sample 17. Finally, of the 16 samples from which EVs were unambiguously identified, 50% (8/16) were NPESCs and none of these NPESCs was detected on RD cell line.

In this study we set out to determine the true prevalence of NPESCs in faecal samples of children with AFP and their contacts in Nigeria. More importantly, we tried in this study to address two of the biases in previous studies describing such data; 1) using only RD cell line alone [17] and 2) using direct detection using an RT-snPCR assay alone [8,9] to estimate the prevalence of NPESCs in such samples. With respect to RD cell line, we showed that all the NPESC members detected in this study were missed by RD cell culture (Table 1). With respect to using the direct detection with an RT-snPCR assay alone to estimate the prevalence of EV-Cs in such samples, we showed that 50% of the NPESCs in the samples were missed using this algorithm (RD cell culture isolation) (Table 1). The results of this study therefore showed that both the NPESC cell culture sensitive methods and culture independent molecular identification strategies need be pooled into a robust protocol that ensures we capture as much NPESC diversity as possible from every sample screened. Hence, result of studies [7,18,19,20] in which the samples were subjected directly to the recommended molecular identification assay [14,16] without first subjecting the samples to culture in a cell line with documented sensitivity for EV-Cs or *vice-versa* [17,21,22,23] should be correctly interpreted as documenting only a subset of NPESCs present in such samples.

This bias of RD cell line for propagating EV-Bs coupled with the fact that about 140 laboratories globally (as part of the GPEI) are using RD cell line routinely[13,16] might explain the fact that EV-Bs (with 63 types already documented to exist [www.picornaviridae.com]) are one of the best documented species in the genus Enterovirus. Hence, the EV-B bias of RD cell line might be helping to selectively filter out and document EV-B diversity at the expense of other EV species. Further, in nations where recombinant circulating vaccine-derived polioviruses (cVDPVs) emerge and are circulating, cataloguing non-polio EV isolates recovered on RD cell line [17] tends not to be very useful in finding the NPESCs recombining with OPV to make recombinant cVDPVs.

In the light of recombinant cVDPV emergence, this baseline circulation of NPESC members in Nigeria

and possibly in sub-Saharan Africa [24,25] becomes a major cause for concerns especially now when cVDPVs outbreaks are being tackled in nine (9) different countries on the continent [26]. The presence of NPESCs coupled with the lack of herd immunity (manifest in continued circulation of cVDVPs) to the polioviruses suggests that the conditions are again perfect for co-infection of host with both NPESCs and OPV, recombination between both and emergence of recombinant cVDPVs. We may therefore still be in for more cVDPV emergence and circulation in the sub-region. It is therefore essential that this endemic circulation of NPESCs in the sub-region be taken into consideration when designing the next-generation (Next-Gen) of live poliovirus vaccines. These Next-Gen vaccines should be designed to induce/provide immunity to recombinants when in the company of (co-infecting with) other wild-type NPESC members. It is crucial that this is exhaustively examined especially as clinical trials are ongoing for novel OPVs.

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Ethics approval and consent to participate

The faecal suspensions analysed in this study were collected in Nigeria in accordance with the national ethical guidelines as part of the National AFP surveillance programme and sent to the WHO National Polio Laboratory in Ibadan, Nigeria to determine whether poliovirus is the etiologic agent of the diagnosed AFP using the WHO recommended algorithm. Both the faecal suspensions and RD cell culture isolates analyzed in this study were subsequently anonymized for further studies before use in this study. Hence, this article does not report direct interaction of authors with study participants, and participants' IDs were anonymized. 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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Author's Contributions

AJA, AOM, FTOC design the study. All authors were involved in sample collection, laboratory and data analysis. All authors wrote, revised, read and approved the final draft of the manuscript.

Conflict of interest

The authors declare that no conflicts of interests exist.

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