Research Article

Nonpolio Enterovirus-C (NPEV-C) Strains Circulating in South-Western Nigeria and their Contribution to the Emergence of Recombinant cVDPV2 Lineages

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Abstract | Recently, we showed that the nonstructural region of some of the cVDPV2 lineages described in Nigeria between 2005 and 2011 originated from NPEV-Cs circulating in Northern Nigeria. Here, we further investigate whether NPEV-Cs circulating in south-western Nigeria (SWN) contributed to the emergence of these lineages. In 2013 sewage contaminated water samples were collected from two sites in Lagos, Nigeria. Samples were concentrated and inoculated into MCF-7 and RD cell lines. Isolates from MCF-7 were passaged in RD and L20B cell lines while isolates from RD cell line were passaged in L20B cell line. Subsequently, all isolates were subjected to panenterovirus 5′-UTR, partial VP1 and EV-C-3Dpol/3'-UTR PCR assays, amplicons sequenced and subjected to phylogenetic analysis. Both sites yielded two isolates each on MCF-7 cell line while on RD cell line one site yielded one and the other seven isolates. None of the twelve isolates replicated on L20B cell line. All were positive for both the panenterovirus 5′-UTR and partial VP1 PCR reactions while only the four isolates recovered on MCF-7 where positive for the EV-C-3Dpol/3'-UTR-PCR assay. The eleven isolates with exploitable VP1 sequence data were identified as CVA13 (4 isolates), E3 (1 isolate), E7 (3 isolates) and E19 (3 isolates). Recombination analysis showed evidence of recombination events. Results of this study showed that the NPEV-Cs circulating in south-western Nigeria also contributed to the emergence of cVDPV2 previously described between 2005 and 2011. Hence, the risk of recombination between OPV and NPEV-C members to generate recombinant VDPVs in the region exist.

Editor | Muhammad Munir, The Pirbright Institute, UK.
Received | May 28, 2015; **Accepted** | July 28, 2015; **Published** | November 22, 2015
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DOI | http://dx.doi.org/10.17582/journal.bjv/2015.2.5.68.73
Citation | Faleye, T. O. C. and J. A. Adeniji. 2015. Nonpolio enterovirus-C (NPEV-C) strains circulating in South-Western Nigeria and their contribution to the emergence of recombinant cVDPV2 lineages. *British Journal of Virology*, 2(5): 68-73.

Introduction

Poliovirus (PV) is the etiologic agent of poliomyelitis and the type member of species *Enterovirus C* (EV-C) in genus *Enterovirus*, family *Picornaviridae*. Besides polioviruses, there are about 20 other non-polio enterovirus species C (NPEV-C) members including coxsackievirus (CV) CVA11, CVA13 and CVA20 (www.picornaviridae.com). There are three distinct serological types of polioviruses (PV1, PV2 and PV3), attenuated versions of which have been developed as oral polio vaccines (OPV), and are used for immunization campaigns. Occasionally, especially in sub-optimally immunized populations, the vaccine

virus reverts to wild-type virulence and transmissibility. Genomic characterization of such isolates, referred to as circulating vaccine-derived polioviruses (cVDPVs), revealed them to be mostly recombinants with OPV/NPEV-C structural and nonstructural regions respectively (Yang et al., 2003; Blomqvist et al., 2004; Arita et al., 2005; Adu et al., 2007; Rakoto-Andrianarivelo et al., 2007; 2008; Combelas et al., 2011; Burns et al., 2013).

Between 2005 and 2011, four hundred and three (403) confirmed cases of cVDPV 2 were reported in Nigeria. All but seven of which were OPV2/NPEV-C recombinants resolved into 23 independent emergences most of which occurred in Northern Nigeria (Burns et al., 2013). We recently showed that the nonstructural region of some of the recombinant cVDPV2 lineages originated from NPEV-Cs circulating in Northern Nigeria (Adeniji and Faleye, 2015). However, the origin of the nonstructural genomic region of some of the cVDPV2 lineages remained undetermined.

Currently, it is unclear whether the risk of emergence of cVDPVs exist in southern Nigeria considering there is paucity of data on NPEV-Cs circulating in the region. In fact most of the studies from the region mainly report the preponderance of species B enteroviruses (Oyero and Adu, 2010; Baba et al., 2012; Oyero et al., 2014; Adeniji and Faleye, 2014a). This study was therefore designed to answer two questions. Firstly, is the species B bias of previous reports (which mainly use RD cell line for nonpolio enterovirus isolation) from the region an artifact of the isolation protocol (combination of cell lines) used and can the inclusion of a cell line like MCF-7 with documented NPEV-C bias (Adeniji and Faleye, 2014b; 2015) change our perception of the enterovirus species diversity and preponderance in the region. Secondly, should NPEV-Cs be present and circulating in the region, could they have contributed in any way to the emergence of the cVDPV2 lineages described in Nigeria between 2005 and 2011 (Burns et al., 2013).

Here, we show that the species B bias of previous reports from the region is an artifact of the isolation protocol used and the inclusion of a cell line with documented NPEV-C bias changes our perception of the enterovirus diversity landscape of the region. We also show that NPEV-Cs are present and circulating in the region, and might have contributed to the emergence of the cVDPV2 lineages described in Nigeria between 2005 and 2011 (Burns et al., 2013). Finally, we show that the risk of cVDPV emergence exist in the region.

Methodology

Sewage contaminated water (SCW) samples were collected in this study as part of an annual enterovirus environmental surveillance programme carried out by our group since 2010. In 2013, samples were collected from two sites that repeatedly yielded isolates since 2010.

Samples were concentrated using a variant of the "pellet method" described in Adeniji and Faleye (2014a). Concentrates were subsequently inoculated into eight (8) tubes each containing monolayered cultures of MCF-7 and RD cell lines and incubated at 37^{0}C. The tubes were then monitored daily for the development of classical enterovirus cytopathic effect (CPE) over the course of five (5) days. Cell culture tubes that did not develop CPE after five days were blind passaged in the same cell line and monitored for an additional five days. Tubes were consequently only declared negative after they had been monitored for a total of 10 days.

Once CPE was fully developed in any cell culture tube, suspected isolates were passaged in the same cell line in which they were detected. If confirmed, isolates from MCF-7 were passaged in RD and L20B cell line while isolates from RD cell line were passaged in L20B cell line.

From all confirmed isolates, RNA was extracted using Jenabioscience RNA extraction kit (Jena Bioscience, Jena, Germany) and cDNA was synthesized using the SCRIPT cDNA synthesis kit (Jena Bioscience, Jena, Germany) as previously described (Adeniji and Faleye, 2014b). The cDNA was used as template in both the panenterovirus 5^1-UTR and partial VP1 PCR reactions as previously described (Adeniji and Faleye, 2014b). All the 12 isolates were also subjected to the EV-C-3Dpol/3'-UTR-PCR assay as previously described (Adeniji and Faleye, 2015). Only the partial VP1 and EV-C-3Dpol/3'-UTR-PCR reaction products were sequenced. Amplicons were shipped to Macrogen Inc., South Korea where sequencing was performed using the same primers used for the PCR reactions. Sequences generated in this study have been submitted to GenBank under the accession numbers KR872297-KR872307.

RESULTS

Both sites yielded isolates on both cell lines (Table 1). Both sites yielded two isolates each on MCF-7 cell line while sites 1 and 2 yielded one (1) and seven (7) isolates respectively on RD cell line. All four isolates recovered on MCF-7 cell line did not replicate in RD and L20B cell lines. None of the eight (8) isolates recovered on RD cell line replicated on L20B cell line.

All 12 isolates were positive for both the panenterovirus 5′-UTR and partial VP1 PCR reactions. However, VP1 sequence data from only eleven of the isolates were exploitable. The 11 (eleven) isolates were identified by the enterovirus genotyping tool (Kroneman et al., 2011) as CVA13 (4 isolates), E3 (1 isolates), E7 (3 isolates) and E19 (3 isolates). The four (4) CVA13 isolates were the ones recovered on MCF-7. Furthermore, only these four (4) isolates where positive for the EV-C-3Dpol/3'-UTR-PCR assay confirming their NPEV-C status. The ~429-nt fragment was successfully amplified and sequenced for these four isolates. Furthermore, using the 3Dpol/3'-UTR data, the enterovirus genotyping tool further confirmed all four (4) isolates to be EV-Cs (data not shown).

Table 1: *Enterovirus isolation results for Lagos 2013 using the MCF-7 – RD protocol*

Sites	Site number	Number of isolates		Identity of isolates	
		MCF-7	RD	MCF-7	RD
Makoko	1	2	1	CV-A13 (2)	E3 (1)
Ijora	2	2	7	CV-A13 (2)	E7 (3), E19 (3), UNT (1)
Total		4	8		

CV-A= *Coxsackievirus A;* **E=** *Echovirus;* **UNT=***Untypable*

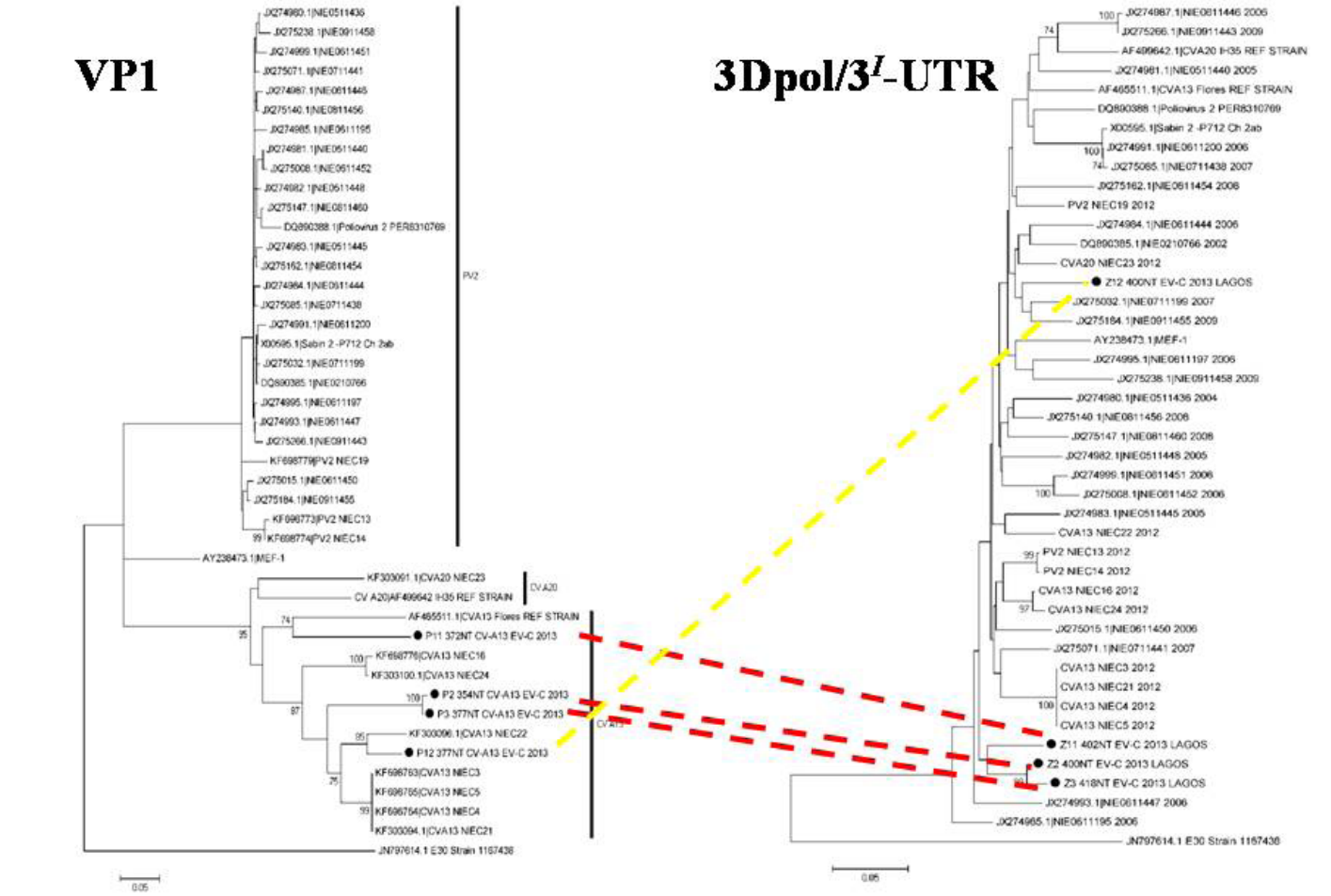

Figure 1: *Phylogenetic relationship of selected isolates at VP1 and 3Dpol/3′-UTR region*

The phylogram is based on an alignment of the VP1 (to the left) and 3Dpol/3′-UTR (to the right) sequences. The Lagos 2013 Isolates and/or genomic regions are highlighted with black circles. Connecting lines are used to trace the locations of isolates of interest on both phylograms (RED for CVA13 with similar 3Dpol/3′-UTR region and YELLOW for the CVA13 isolate that might have recombined with some of the cVDPV 2s previously reported). The GenBank accession number, strain ID and year of isolation are indicated in the 3Dpol/3′-UTR region tree only, if known. Bootstrap values are indicated if ≥70%.

Based on the VP1 sequence data, the identities of the four EV-C isolates according to the phylogram (Figure 1) was in accordance with the results of the enterovirus genotyping tool. These CVA13s clustered with sequences of other CVA13s from Nigeria obtained from GenBank and previously described (Adeniji and Faleye, 2015).

Phylogenetic analysis of 3Dpol/3'-UTR sequences alongside VP1 for the four (4) CVA13 isolates from Lagos 2013 alongside other Nigerian CVA13 and PV2 sequences previously described (Adu et al., 2004; 2007; Burns et al., 2013; Adeniji and Faleye, 2015) showed a previously undescribed cluster of 3Dpol/3'-UTR sequences (Figure 1). Furthermore, the 3Dpol/3'-UTR region of one of the CVA13 isolates (Z12 EV-C 2013) did not cluster with the other three CVA13 isolates recovered in this study (Figure 1). Rather, it clustered with 3Dpol/3'-UTR sequences of two PV2 isolates (phylogeny violation) (Figure 1). These PV2 isolates (NIE0711199 and NIE0911455) had been previously shown to be recombinants with nonstructural genomic region of unknown origin (Burns et al., 2013).

Discussion

The results of this study showed that NPEV-Cs, particularly CVA13s, are also present and circulating in South-Western Nigeria. It further shows that the nonstructural genomic region of cVDPV2s NIE0711199 and NIE0911455whose origin was previously "unknown" (Burns et al., 2013) and remained unidentified despite our previous effort (Adeniji and Faleye, 2015), share a common ancestor with that of a CVA13 recovered from south-western Nigeria. In fact, the 3Dpol/3'-UTR sequences of the CVA13 isolate (Z12 EV-C 2013) is less than eight (8) percent divergent from those of NIE0711199 and NIE0911455 (data not shown). This suggests that these cVDPV2 nonstructural regions of previously "unknown" origin might have originated from EC lineages circulating in south-western Nigeria. This therefore implies that EV-C lineages circulating in south-western Nigeria might have contributed to the emergence of some of the Nigerian cVDPV2 lineages previously described (Burns et al., 2013).

This shows that the risk of recombination between OPV and NPEV-C members to generate recombinant VDPVs previously documented and described in Northern Nigeria (Adu et al. 2004; Burns et al., 2013; Adeniji and Faleye, 2015) might not be restricted to the region and might also exists in south-western Nigeria. Furthermore, the fact that silent circulation of WPV3 was detected in these same sites in South-Western Nigeria less than a year ago (both by our group and the WHO polio laboratory in Ibadan Nigeria (Asghar et al., 2014; Faleye and Adeniji, 2015) signals that herd immunity to PVs does not exist in the region and transmission and circulation of PVs in the region might still be ongoing silently.

The continued administration of OPV in immunization campaigns, coupled with circulation of NPEV-Cs and the lack of herd immunity to the PVs might be further indication that, just like in Northern Nigeria (Adeniji and Faleye, 2015), the conditions might also be perfect for cVDPV emergence in the South-Western Nigeria. Consequently, it is important to ensure that vaccination is optimal and population immunity closely monitored to prevent circulation should such recombination events occur.

The results of this study showed that about 33% (4/12) of the isolates recovered from this study are NPEV-Cs. This is similar to the 27.66% recently reported in Northern Nigeria (Adeniji and Faleye, 2014b; 2015). In a study done in the same region but in which only RD and L20B cell lines were used (Adeniji and Faleye, 2014a) all isolates recovered belonged to species B. This species B bias reported in most RD cell line based enterovirus isolation studies in the region (Oyero and Adu, 2010; Baba et al., 2012; Oyero et al., 2014; Adeniji and Faleye, 2014a) is a cell line dependent phenomenon. In fact, the results of this study showed that had MCF-7 been excluded from this study all the isolates recovered would have been species B members (Table 1). Thus, the increased prevalence of NPEV-C is, most likely, not due to increased circulation but a reflection of the bias contributed by the isolation protocol, thereby confirming the conclusions of Sadeuh-Mba et al. (2012). It is therefore obvious that the combination of cell lines used for enterovirus isolation determines the serotype and species diversity detected.

The value of MCF-7 as a regent for NPEV-C isolation cannot be over emphasized. However, MCF-7 also appears to have a bias for CVA13 because in our laboratory, since we incorporated MCF-7 into our enterovirus isolation protocol in 2012, only one

NPEV-C isolate (CVA20) besides CVA13 has been isolated. However, this awaits confirmation as effort is ongoing to determine the susceptibility and permissiveness profile of the cell line. On the other hand, if this predilection for CVA13 is a valid biological property of MCF-cell line, being of breast cancer origin, understanding the molecular basis of this selection might shed light on the probable development of CVA13 strains as oncotherapeutic agents to target breast cancer.

Finally, the results of this study showed that NPEV-Cs are present and circulating in South-Western Nigeria. It also shows that EC lineages circulating in south-western Nigeria might have contributed to the emergence of some of the Nigerian cVDPV2 lineages previously described (Burns et al., 2013). Furthermore, it shows that the risk of recombination between OPV and NPEV-C members to generate recombinant VDPVs previously documented and described in Northern Nigeria (Adu et al. 2004; Burns et al., 2013; Adeniji and Faleye, 2015) might not be restricted to the region and might also exists in south-western Nigeria. It also demonstrates the value of MCF-7 as a reagent for NPEV-C isolation, and confirms that the combination of cell lines used for enterovirus isolation determines the serotype and species diversity detected. It is however important to mention that all isolates detected in this study (E3, E7, E19 and CVA13) belonged to sub-Saharan Africa specific clades (data not shown) as previously described (Sadeuh-Mba et al. 2012; Adeniji and Faleye, 2014a; 2014b).

Author's Contribution

This article is emanated from FTOCs PhD research and JAA is his PhD supervisor.

Acknowledgements

We thank Dr. M. O. Adewumi for thoughtful critique of our manuscript. We thank Ayorinde James for providing us with the MCF-7 cell line. We also thank the WHO National Polio Laboratory in Ibadan, Nigeria, for providing us with the L20B and RD cell lines.

Conflict of Interest

The authors declare that there exist no conflict of interests.

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