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Impact of cell lines included in enterovirus isolation protocol on perception of nonpolio enterovirus species C diversity

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A B S T R A C T

There has been under-reporting of nonpolio enterovirus species Cs (NPESCs) in Nigeria despite the fact that most isolates recovered from the Nigerian vaccine derived poliovirus serotype 2 (VDPV2) outbreak were recombinants with nonstructural region of NPESC origin. It has been suggested that cell lines included in enterovirus isolation protocols might account for this phenomenon and this study examined this suggestion.

Fifteen environmental samples concentrated previously and analysed using L20B and RD cell lines as part of the poliovirus environmental surveillance (ES) program in Nigeria were randomly selected and inoculated into two cell lines (MCF-7 and LLC-MK2). Isolates were identified as enteroviruses and species C members using different RT-PCR assays, culture in L20B cell line and sequencing of partial VP1.

Forty-eight (48) isolates were recovered from the 15 samples, 47 (97.9%) of which were enteroviruses. Of the enteroviruses, 32 (68.1%) belonged to enterovirus species C (EC) of which 19 (40.4%) were polioviruses and 13 (27.7%) were NPESC members. All 13 NPESC isolates were recovered on MCF-7.

Results of the study show that NPESCs are circulating in Nigeria and their under-reporting was due to the combination of cell lines used for enterovirus isolation in previous reports.

1. Introduction

Enterovirus is a genus with 12 species in the family *Picornaviridae*. Species A–D and Rhinovirus species A–C are the only species in the genus that have been documented to infect humans. Within each species are several serotypes and the genus, as a whole, has over 200 serotypes. Poliovirus is the prototype member of the genus and a member of species C alongside Coxsackievirus A11 (CVA11), CVA13, CVA17, CVA20 and others (www.picornaviridae.com). Poliovirus is the etiologic agent of poliomyelitis, a disease that results in paralysis of varying degrees in susceptible populations (Nathanson and Kew, 2010).

The enterovirus virion is naked with icosahedral symmetry and a diameter of 27–30 nm. Within the viron is an ~7.5 kb, protein-linked, single-stranded, positive-sense RNA genome. The genome has a single open reading frame (ORF) flanked by an untranslated region (UTR) at the 5^{I}-end and both a UTR and a poly A tail at the 3^{I}-end. A single polyprotein is translated from the ORF and subsequently post-translationally cleaved initially into 3 proteins (P1, P2 and P3) that are further cleaved to make 11 proteins. Five of the final subunits (VP1, VP2, VP3, VP4 and VPg) are present in the virion with VP1–VP4 forming the capsid and VPg linked to the genome. The remaining 6 proteins are nonstructural proteins. A correlation has been established between VP1 nucleotide sequence and distinct serotypes (Oberste et al., 1999; Oberste and Pallansch, 2005). Consequently, VP1 nucleotide sequences are being used to type enterovirus isolates into serotypes (Oberste et al., 1999; Oyero and Adu, 2010; Adeniji and Faleye, 2014). Also, it has been established that all regions of the enterovirus genome except the 5^{I}-UTR can be used to type enteroviruses into species (Oberste and Pallansch, 2005; Oberste et al., 2006).

The World Health Assembly in 1988 resolved to eradicate poliomyelitis by the year 2000 (WHO, 1988) and the Global Polio Eradication Initiative (GPEI) was established with this mandate. As a result of GPEI's activities, annual cases of poliomyelitis has decreased from 350,000 in 1988 to 416 in 2013 (www.polioeradication.org) and poliomyelitis has been eliminated globally except in three countries; Pakistan, Afghanistan and Nigeria (WHO, 2012). Nigeria being the only country in Africa from

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which indigenous poliovirus transmission has never been interrupted (WHO, 2012).

One of the primary instruments used by the GPEI for poliovirus eradication campaign has been the oral poliovirus vaccine (OPV). OPV is a live attenuated vaccine containing Sabin polioviruses and on administration, OPV remains in circulation within the population for up to 8 weeks (Nathanson and Kew, 2010). Several reports have documented the circulation of OPV-like viruses in suboptimally immunized or unimmunized populations for over a year (Yang et al., 2003; Blomqvist et al., 2004; Arita et al., 2005; Rakoto-Andrianarivelo et al., 2007, 2008; Burns et al., 2013). Such OPV-like viruses revert to wild type phenotype for transmissibility and pathogenicity, thus regain the ability to cause poliomyelits and have been implicated in several outbreaks of poliomyelitis globally (Kew et al., 2002; Yang et al., 2003; Arita et al., 2005; Rakoto-Andrianarivelo et al., 2007, 2008; Burns et al., 2013). These disease causing OPV-like viruses are referred to as circulating vaccine derived polioviruses (cVDPVs) and majority have been characterised at the genomic level to be recombinant viruses with capsid region of OPV origin and nonstructural region of non-polio enterovirus species C (NPESC) origin (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). Several studies have implicated CV-A13, CVA11, CVA17 and CVA20 (Arita et al., 2005; Rakoto-Andrianarivelo et al., 2007, 2008) as members of NPESC recombining with OPV to make cVDPVs. This indicates a high rate of circulation of NPESC members in regions of the world where OPV is still administered and coinfection of OPV with NPESC members in many vaccinees (Arita et al., 2005; Rakoto-Andrianarivelo et al., 2007; Combelas et al., 2011; Sadeuh-Mba et al., 2013).

In Nigeria OPV is administered both during the routine and supplementary immunization activities and northern Nigeria has been battling an outbreak of cVDPV since 2005 with 403 cases reported between 2005 and 2011 (Wassilak et al., 2011; Burns et al., 2013). The 403 cases were resolved into 23 independent emergence of cVDPVs (Burns et al., 2013) and all but seven of the cVDPV isolates were recombinants with nonstructural region of NPESC origin (Burns et al., 2013). This implies the circulation of NPESCs in northern Nigeria and co-infection of vaccinees and/or their contacts with NPESC members. However, efforts to identify the NPESC members recombining with OPV to make cVDPVs in Nigeria have not been very successful because only the RD and L20B cell lines (R–L protocol) were used for enterovirus isolation in all such studies (Oyero and Adu, 2010; Baba et al., 2012; Adeniji and Faleye, 2014).

The R–L enterovirus isolation protocol is highly optimised to detect poliovirus and studies have shown that asides poliovirus, very few other NPESC members (e.g. CVA17 and CVA21) are detected by this protocol (Newcombe et al., 2003; Oyero and Adu, 2010; Rao et al., 2012). In fact, it has been documented that most NPESC members use intercellular adhesion molecule 1(ICAM-1) as their cellular receptor for entry into cells (Newcombe et al., 2003; Shafren et al., 1997) and it has been shown that RD and Mouse L cell lines do not express human ICAM-1 on their surface (Wawryk et al., 1989; Newcombe et al., 2003). Most studies that have documented the isolation of NPESC members, used isolation protocols slightly different from the R–L protocol. Such studies included HEp-2 cell line for enterovirus isolation as HEp-2 has documented cell surface expression of ICAM-1 (Huang et al., 2000) and has been shown to greatly enhance the recovery of NPESC members especially the Coxsackievirus As (Arita et al., 2005; Rakoto-Andrianarivelo et al., 2007; Sadeuh-Mba et al., 2013; Bessaud et al., 2012).

As the end game of poliovirus eradication is being considered (Minor, 2012), the public health significance of cVDPVs increases as do the significance of NPESC members circulating globally because of the role they play in the emergence of cVDPVs (Rakoto-Andrianarivelo et al., 2007, 2008; Combelas et al., 2011; Burns et al., 2013). Despite this, the indigenous NPESC members recombining with OPV to make cVDPV2 in Nigeria are yet to be isolated and described. Therefore, in this study, including MCF-7 cell line, with documented expression of ICAM-1 (Komi and Lassila, 2000; Rosette et al., 2005; Viola et al., 2013), and another cell line, we analysed sewage samples from northern Nigeria for NPESC members. The results of this study confirm the circulation of NPESC members in northern Nigeria. Furthermore, it demonstrates that the cell lines included in enterovirus isolation protocols greatly influence the species diversity detected.

2. Materials and methods

2.1. Sample collection and processing

A total of 15 randomly selected concentrates of sewage contaminated water samples were analysed in this study. These were concentrates of samples collected from Sokoto and Kano States between July and August 2012 as part of the environmental surveillance (ES) component of the WHO National polio surveillance programme in Nigeria. Samples were collected by grab method and concentrated using the two-phase concentration technique as described in the Guidelines for environmental surveillance for poliovirus circulation (WHO, 2003). Furthermore, the concentrates were analysed previously by the WHO National Polio Laboratory in Ibadan, Nigeria, according to the Guidelines for environmental surveillance for poliovirus circulation (WHO, 2003) and the results already used to guide mop-up and supplementary vaccination campaigns in Northern Nigeria.

2.2. Cell lines and virus isolation

Human rhabdomyosarcoma (RD) and L20B (murine transgenic L cells expressing the poliovirus receptor; CD155) cell lines were previously used by the WHO National polio surveillance programme to screen the concentrates analysed in this study as described in the Guidelines for environmental surveillance for poliovirus circulation (WHO, 2003). However, in this study, LLC-MK2 (rhesus monkey kidney), MCF-7 (human mammary gland adenocarcinoma) and L20B cell lines were used for virus isolation and poliovirus detection. Cells were grown in Medium 199 supplemented with 5% foetal calf serum.

To test the impact of cell lines included in the enterovirus isolation protocol on NPESC recovery all concentrates were inoculated into MCF-7 and LLC-MK2 cell lines (the M–L protocol). For each cell line used, each of 8 tubes of monolayered cells was inoculated with 200 μL of concentrate. Consequently, 1.6 mL of each concentrate was screened per cell line and 3.2 mL of each in 2 cell lines. Inoculated cell culture tubes were microscopically checked for 10 days to detect the appearance of characteristic enterovirus cytopathic effect (CPE). CPE was confirmed by passage in fresh cell culture tubes of the cell line in which the CPE was previously observed.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from tissue culture supernant using the QIAamp viral mini kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. From the extract, cDNA was synthesized using Script cDNA syntheses kit (Jena Bioscience, Jena, Germany). Twelve microlitres of extract was added to 8 μL cDNA synthesis mixture containing the following volumes of stock solution; 4 μL of SCRIPT RT buffer complete, 1 μL of 10 mM dNTP mix, 1 μL of 40 unit RNase inhibitor, 1 μL of 100 mM DTT, 0.5 μL of 100 μM random hexamers and 0.5 μL of 200 unit SCRIPT reverse-transcriptase. The reaction was incubated at 42 °C for 10 min