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Short communication

The fallacy of identification by neutralization in the light of cytopathic effect non-producing enterovirus strains.

ABSTRACT:

We describe the characterization of an enterovirus isolate recovered from untreated raw sewage in Ibadan, southwest Nigeria in 2010. The isolate was neutralized by specific antisera and consequently identified as Echovirus 7 (E7). Subsequent molecular characterization showed the isolate to be a mixture of E7 and Coxsackievirus A24 (CV-A24) thereby suggesting the CV-A24 strain to be non-cytopathic effect producing. It is therefore crucial that identities of enterovirus strains determined by neutralization assay be verified by molecular characterization to ensure they do not have any non-cytopathic effect producing strain(s) lurking unnoticed.

Keywords: Nigeria, Enterovirus, Neutralization, CV-A24, Cytopathic effect.

Word Count: 90

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Enteroviruses (EVs) are members of the family *Picornaviridae*, order *Picornavirales*. There are 13 species in the genus and while Enterovirus Species B (EV-B) contain the most serotypes (>60), EV-C contains the prototype member of the genus (Poliovirus). EVs have a 27 – 30nm diameter naked icosahedral capsid which encapsidates a 5^{l} protein-linked, positive-sense, single-stranded, ~7.5kb, RNA genome. The genome is flanked on both ends by 5^{l} and 3^{l} untranslated regions (UTRs) and has a single open reading frame (ORF) between the UTRs. The ORF is translated into a polyprotein that is self-cleaved into three smaller proteins (P1, P2 and P3) and subsequently into 11 protein products. VP1-VP4 (from P1) are the structural proteins which form the capsid. While 2A-3D (from P2 & P3) are nonstructural proteins crucial for virus replication.

Classically enterovirus detection is by isolation in cell (usually RD and L20B) culture [1, 2] although this is fast being replaced by cell-culture independent strategies [3]. Also, identification was classically by neutralization using polyclonal antisera. However with an association shown between VP1 sequence data and enterovirus serotypes VP1 amplification and sequencing is now being used for enterovirus identification [4, 5].

In June 2010 we isolated an enterovirus in RD cell culture. The isolate was from a sample of untreated raw sewage collected in Ibadan, southwest Nigeria. The sample was collected as part of an environmental surveillance study conducted by our group (unpublished). It was subsequently concentrated and enterovirus recovered from it by culture in RD cell line as previously detailed [6]. The RD cell culture isolate was subjected to neutralization [2, 6] using a panel of antisera provided by RIVM, Netherland and identified as Echovirus 7 (E7). On passage in L20B cell line, the isolate did not show cytopathic effect (CPE). Interestingly, on subjection to both the Panenterovirus and Panpoliovirus realtime reverse transcriptase polymerase chain reaction (rRT-PCR) assay in use by the Global Polio Laboratory Network (GPLN) and previously detailed [6], the isolate was positive for both but with variations.

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While the Panenterovirus assay was clearly positive with an early (≤ 30 cycles) Ct value, the Panpoliovirus assay was controversially positive with a late (≥ 32 cycles) Ct value. The result of the Panpoliovirus rRT-PCR assay did not improve despite repeated cycles of passage in RD cell line and subsequent Panpoliovirus rRT-PCR screen.

In, this study we attempt to better characterize this E7 isolate in a bid to determine the reason an RD cell culture positive but L20B negative isolate is showing late positivity with very high Ct value on subjection to the Panpoliovirus rRT-PCR assay. The algorithm followed in this study is depicted by Figure 1.

RNA was extracted using the JenaBioscience RNA extraction kit (Jena Bioscience, Jena, Germany). Using the Script cDNA synthesis kit (Jena Bioscience, Jena, Germany) two different cDNAs were made (Figure 1). The basic difference was that the first cDNA (cDNA 1) was done using random hexamers while the second cDNA (cDNA 2), was done using primers AN32, AN33, AN34 and AN35 [3]. A Veriti thermal cycler (Applied Biosystems, California, USA) was used for thermal cycling.

Four (two each for EV-B [7, 8] & EV-C [9, 10]) different polymerase chain reaction (PCR) assays were done. For each species one of the PCR assays targeted the VP1 (structural region) and the other the nonstructural (3C/D for EV-B and 3D/3^{I}-UTR for EV-C) region (Figure 1). All the four assays (EV-B-3C/D-PCR, EV-B-VP1-PCR, EV-C-3D/3^{I}-UTR-PCR and EV-C-VP1-PCR) were consequently two-step RT-PCR assays except for EV-C-VP1-PCR which an RT-seminested PCR (RT-snPCR) assay [3]. All amplicons generated were sequenced and isolate identity determined using the enterovirus genotyping tool [12]. Multiple sequence alignments and phylogenetic trees were done using the default settings of the CLUSTAL ***W*** program in MEGA 5 software [13] as previously described [11]. The

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sequences obtained from this study have been deposited in GenBank with accession numbers KM264408, KM264416, MF535106 & MF535107.

The isolate was positive for all four assays. All four amplicons were successfully sequenced and exploited for virus identification. The, EV-B-3C/D-PCR result confirmed that the isolate contained a Species B enterovirus. The EV-B-VP1-PCR result confirmed that the EV-B in the isolate is E7, thereby confirming the results of the neutralization assay. The EV-C-3D/3^{I}-UTR-PCR showed that the isolate also contained a Species C enterovirus and the EV-C-VP1-PCR showed that the EV-C contained in the isolate is Coxsackievirus (CV) A24 (CV-A24).

Considering the EV-C was missed by the neutralization assay, it was further subjected to phylogenetic analysis. Figure 2 shows that, with respect to the VP1 sequence, the CV-A24 strain detected in this study belongs to a lineage that has only been described in sub-Saharan Africa till date.

In this study we attempted to determine the reason an already serotyped E7 that showed CPE on RD but not in L20B cell culture, is showing late positivity with very high Ct value (>32 cycles) on subjection to the Panpoliovirus rRT-PCR assay. We found that the isolate contained both E7 and a CV-A24. The Panpoliovirus rRT-PCR assay was specifically designed to screen for the presence of poliovirus in isolates that had shown CPE in both RD and L20B cell lines in tandem (which is not the case with this isolate). The results of this study however show that the assay might also be valuable for detecting non-polio enterovirus C (NPEV-C) members when used outside of its prescribed algorithm.

Though the isolate was a mixture of E7 and CV-A24, the fact that it was neutralized by antisera specific for E7 suggest that either the CV-A24 was very slow growing or was not replicating in RD cell line with evident CPE. In the light of Jiang et al., [14], this is not totally strange. In fact, we have also recently isolated some EV-Cs that replicate in RD cell line

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without cytopathic effect (Adeniji et al., unpublished) and thus, further buttress the likelihood of such find. If indeed the CV-A24 does not produce CPE in RD cell line and was replicating alongside the CPE producing E7, it is crucial that the logic underlying enterovirus neutralization assays be revisited.

The neutralization assay done in cell culture is predicated on the assumption that any enterovirus added to the cell line of interest will produce CPE unless it is neutralized by the specific antibodies the virus was previously mixed and incubated with. The findings of this study question this assumption and shows it can be false if the isolate is a mixture of CPE producing and non-producing enterovirus strains. As shown by the result of this study, with such mixtures, the antibodies specific for the CPE producing strain will neutralize it and prevent CPE development. The fact that the other strain in the mixture does not produce CPE in the cell line of choice gives the impression that the CPE producing strain is the only one present in the mixture. This error can only be corrected by confirming the results of neutralization assays with other assays like the molecular ones used in this study.

Although enterovirology is moving away from the classic technique of enterovirus identification by neutralization and depending more on molecular identification, a lot of studies are still dependent on the use of antibodies raised against enterovirus isolates. It is therefore essential to ensure that pure cultures intended for such purpose are truly pure and do not have any non-CPE producing strain(s) lurking unnoticed.

CONFLICT OF INTERESTS

The authors declare that no conflict of interests exist.

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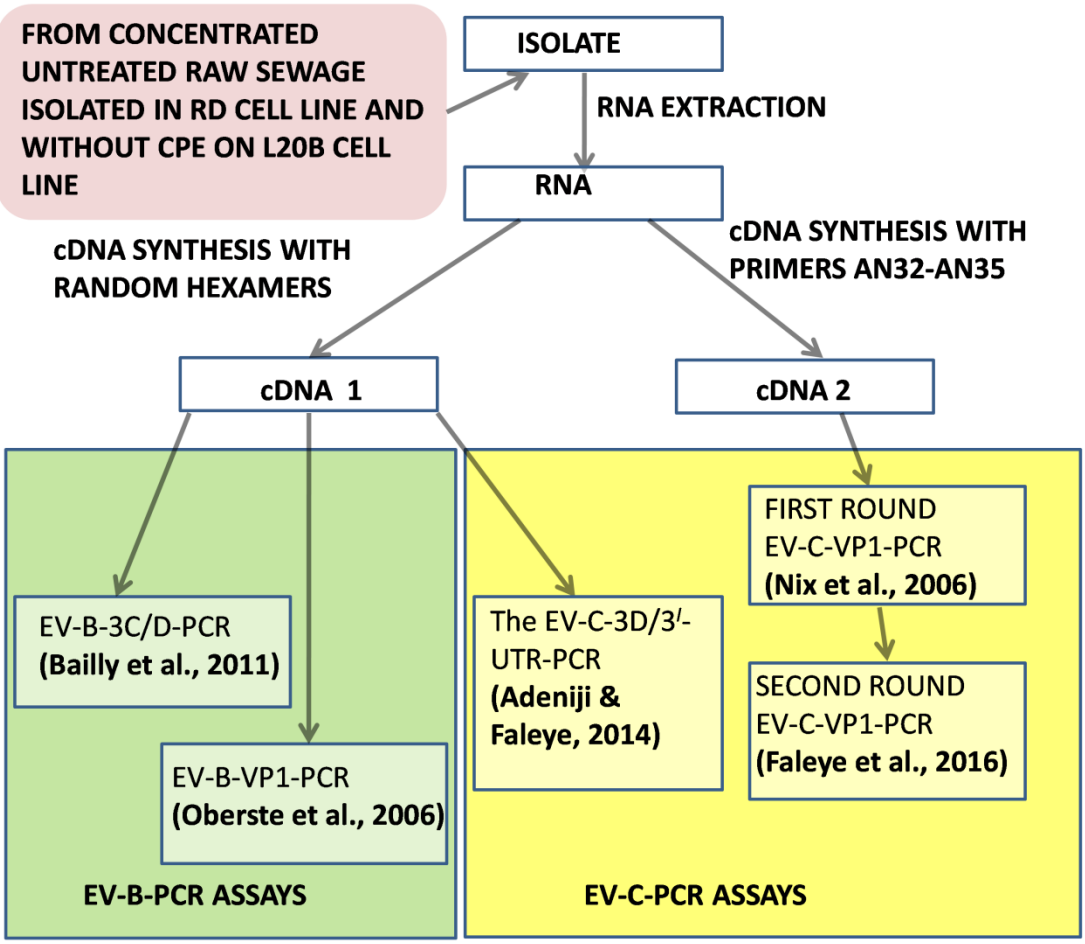

Figure 1: A schematic representation of the algorithm followed in this study.

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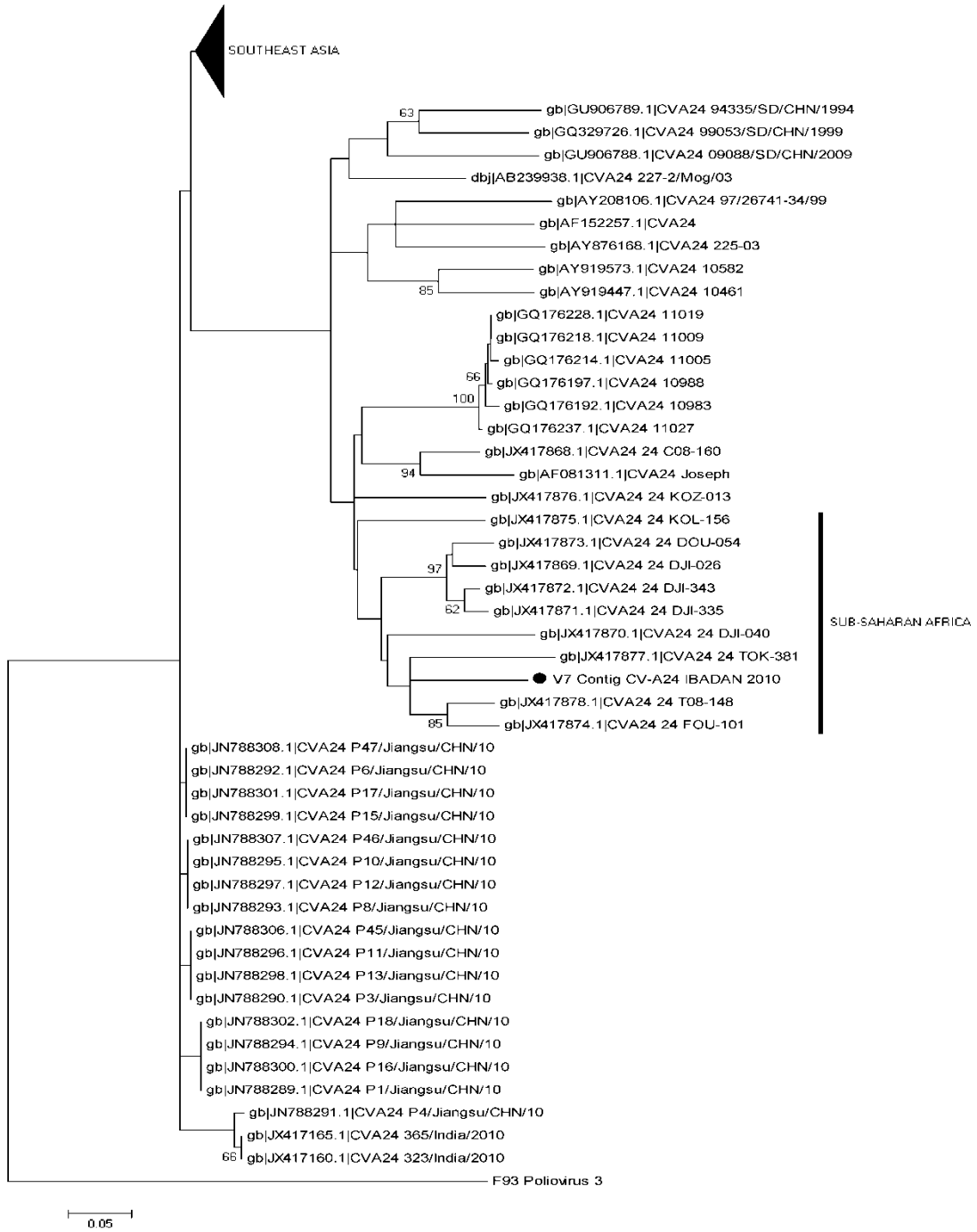

Figure 2: Phylogram of Coxsackievirus A24. The phylogram is based on an alignment of partial VP1 sequences. The newly sequenced strain is highlighted with Black circle. The GenBank accession number of the strains are indicated in the phylogram. Bootstrap values are indicated if > 50%. The labelled vertical bar is for ease of reference only.