

1 **EXTENDING THE UTILITY OF THE WHO RECOMMENDED ASSAY FOR DIRECT**  
2 **DETECTION OF ENTEROVIRUSES FROM CLINICAL SPECIMEN FOR RESOLVING**  
3 **POLIOVIRUS CO-INFECTION**

4 Temitope Oluwasegun Cephas **FALEYE**<sup>1,2,\*</sup>, Moses Olubusuyi **ADEWUMI**,<sup>1</sup> Naomi Princess  
5 **OZEGBE**,<sup>2</sup> Oluwaseun Elijah **OGUNSAKIN**,<sup>2</sup> Grace **ARIYO**,<sup>2</sup> Faith Wuraola **ADESHINA**,<sup>2</sup>  
6 Oluwaseun Sarah **OGUNGA**,<sup>2</sup> Similoluwa Deborah **OLUWADARE**,<sup>2</sup> and Johnson Adekunle  
7 **ADENIJI**<sup>1,3</sup>

8  
9 Department of Virology, College of Medicine, University of Ibadan, Ibadan, Oyo State,  
10 Nigeria.<sup>1</sup> Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti,  
11 Ekiti, State, Nigeria.<sup>2</sup> WHO National Polio Laboratory, University of Ibadan, Ibadan, Oyo State,  
12 Nigeria.<sup>3</sup>

13  
14 Temitope Oluwasegun Cephas **FALEYE\***  
15 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
16 State, Nigeria. [faleyetemitope@gmail.com](mailto:faleyetemitope@gmail.com), [faleyetope@yahoo.com](mailto:faleyetope@yahoo.com); +234802 384 0394  
17

18 Moses Olubusuyi **ADEWUMI**  
19 Department of Virology, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria.  
20 [adewumi1@hotmail.com](mailto:adewumi1@hotmail.com); +2348060226655  
21

22 Naomi Princess **OZEGBE**  
23 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
24 State, Nigeria. [ozegbepressince@yahoo.com](mailto:ozegbepressince@yahoo.com)  
25

26 Oluwaseun Elijah **OGUNSAKIN**  
27 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
28 State, Nigeria [seajohndablaxczer@yahoo.co.uk](mailto:seajohndablaxczer@yahoo.co.uk)  
29

30 Grace **ARIYO**  
31 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
32 State, Nigeria [ariyograceoluwatoyin318@gmail.com](mailto:ariyograceoluwatoyin318@gmail.com)  
33

34 Faith Wuraola **ADESHINA**  
35 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
36 State, Nigeria [Skaxiety@gmail.com](mailto:Skaxiety@gmail.com)  
37

38 Oluwaseun Sarah **OGUNGA**  
39 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
40 State, Nigeria [ogungaoluwaseun@gmail.com](mailto:ogungaoluwaseun@gmail.com)  
41

42 Similoluwa Deborah **OLUWADARE**  
43 Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti,  
44 State, Nigeria [oluwadaresimiloluwa28@gmail.com](mailto:oluwadaresimiloluwa28@gmail.com)  
45

46 Johnson Adekunle **ADENIJI**  
47 WHO National Polio Laboratory, Department of Virology, College of Medicine, University of  
48 Ibadan, Ibadan, Oyo State, Nigeria. [adek1808@yahoo.com](mailto:adek1808@yahoo.com);

49 **ABSTRACT**

50 In a polio-free world there might be reduced funding for poliovirus surveillance. There is therefore  
51 the need to ensure that enterovirologist globally, especially those outside the global polio laboratory  
52 network (GPLN), can participate in poliovirus surveillance without neglecting their enterovirus  
53 type of interest. To accomplish this, assays are needed that allow such active participation.

54 In this light, we used 15 previously identified enterovirus isolates as reference samples for assay  
55 development. The first eight were enterovirus species B (EV-B). The remaining seven were EV-Cs;  
56 three of which were poliovirus (PV) 1, 2 and 3, respectively. A 16th sample was compounded; a  
57 mixture of two EV-Bs, three PVs and one nonPV EV-Cs (all part of the 15). In all, four samples  
58 contained PVs with the 16th consisting of mixture of the three PV types. All were subjected to the  
59 WHO recommended RT-snPCR assay, and five other modified (with substitution of the second  
60 round PCR forward primer) assays. The new primers included the previously described Species  
61 Resolution Primers (SRPs; 187and 189) and the Poliovirus Resolution Primers (PRPs: Sab 1, 2 and  
62 3). All amplicons were sequenced and isolate identity confirmed using the Enterovirus Genotyping  
63 Tool.

64 The PRPs detected PV types in only the four samples that contained PVs. In addition, it was able to  
65 show that the sample 16 (mixture) contained all the three PV types. On the other hand, though the  
66 SRPs and the WHO assay also detected the three singleton PVs, in sample 16, they both detected  
67 only one of the three PV types present.

68 This study describes a sensitive and specific utility extension of the recently recommended WHO  
69 RT-snPCR assay that enables independent detection of the three poliovirus types especially in cases  
70 of co-infection. More importantly, it piggy-backs on the first round PCR product of the WHO  
71 recommended assay and consequently ensures that enterovirologists interested in nonpolio  
72 enteroviruses can continue their investigations, and contribute significantly and specifically to  
73 poliovirus surveillance, by using the excess of their first round PCR product.

74 **KEYWORDS:** Enteroviruses, Polioviruses, WHO, Surveillance, Nigeria

75 **WORD COUNT:** 336 Words

76 **INTRODUCTION**

77 In 1988, the World Health Assembly resolved to eradicate poliovirus (WHO, 1988), and as at today  
78 circulation of indigenous wild poliovirus has remained uninterrupted in only three countries  
79 (Afghanistan, Nigeria and Pakistan) globally ([www.polioeradication.org](http://www.polioeradication.org)). The Global Polio  
80 Eradication Initiative (GPEI) has been the major vehicle driving this effort using both  
81 immunization and active surveillance. The surveillance programme has been centred on finding  
82 poliovirus in the stool specimen of AFP cases and sewage contaminated water. The detection and  
83 confirmation of poliovirus is however, done globally in about 150 WHO accredited laboratories  
84 referred to as the Global Polio Laboratory Network (GPLN).

85 Detection and identification of polioviruses in specimen submitted to the GPLN is done by firstly  
86 isolating the virus in cell culture. Subsequently, the isolate is identified as a poliovirus using an  
87 array of assays ranging from serology using monoclonal virus specific antibodies to nucleic acid  
88 based tests as stipulated in the protocol (WHO, 2003; 2004). Ultimately, definitive identification of  
89 any isolate is done by nucleotide sequencing and phylogenetic analysis of the VP1 gene (WHO,  
90 2003; 2004).

91 The strength of this cell culture based algorithm is the ability to detect poliovirus even at very low  
92 titre. However, a major draw-back is its dependence on infectious particles. This can constitute an  
93 avenue for short-changing the surveillance effort if the specimens are not appropriately preserved to  
94 ensure that infectious particles are viable on arrival in the laboratory. The impact of this might not  
95 be evident in developed economies where refrigeration is readily available. In resource-limited  
96 economies, where the reverse is the case, this is accommodated by moving specimen in ice chest  
97 from sampling site to laboratories. This system has challenges that range from getting ice for the

98 chest to ensuring that refrigeration temperature is maintained in the chest from sampling site to the  
99 laboratory which may be up to hundreds of kilometres apart in a tropical climate like Nigeria. In  
100 such situation, poliovirus particles in specimens that were not well handled in transit might no  
101 longer be infectious on arrival in the laboratory. Consequently, the laboratory might not be able to  
102 detect poliovirus in such specimen. This (alongside the ongoing unrest in the three countries  
103 [Afghanistan, Nigeria and Pakistan] where indigenous circulation of wild poliovirus has remained  
104 uninterrupted) can partly account for the sporadic detection of poliovirus strains referred to as  
105 orphan polioviruses (poliovirus strains that have been previously deemed eliminated due of lack of  
106 detection for over one year)

107 In this light, the WHO recently recommended the Reverse Transcriptase–seminested Polymerase  
108 Chain Reaction (RT–snPCR) assay described by Nix and colleagues (Nix et al., 2006) for direct  
109 (cell culture independent) detection of enteroviruses from clinical specimen (WHO, 2015). The Nix  
110 et al., (2006) assay is an upgrade (seminested and Consensus Degenerate Hybrid Oligonucleotide  
111 Primers [CODEHOP]) version of the Oberste et al., (2003) assay. It has been shown that this  
112 algorithm is very sensitive for cell culture independent enterovirus detection and identification  
113 (Faleye et al., 2016a, Rahimi et al., 2009, Sadeuh-Mba et al., 2014). However, we (Faleye et al.,  
114 2016b) have recently shown that the assay lacks the capacity to resolve enterovirus types present in  
115 cases of co-infection. Significantly, the prevalence of enteroviruses co-infections in Nigeria is  
116 underscored by the independent emergence of 29 lineages of circulating Vaccine Derived  
117 Polioviruses (cVDPVs) between 2004 and 2014 (Pons-Salort et al., 2016), most of which are of  
118 recombinant origin (Burns et al., 2013). This necessitates the need for assays that can detect and  
119 resolve enterovirus co-infections.

120 Further, we had also shown (Faleye et al., 2016b) that by including primers 189 and 187  
121 (subsequently referred to in this study as Species Resolution Primers) in the second round PCR of  
122 the WHO recommended RT–snPCR assay the resolving power of the assay could be improved.

123 However, in a polio-free world, that there might be reduced funding for enterovirus surveillance,  
124 assays are needed that can in one swoop detect and resolve enterovirus co-infections, including  
125 different poliovirus types. Therefore, in this study, we extend the utility of the WHO recommended  
126 RT-snPCR assay by inclusion of three poliovirus-specific forward primers in the second round  
127 assay, thus, making it possible to independently detect the three poliovirus types even in cases of  
128 co-infection.

129

130

131

132

133

134

135

136

137

138

139

140

141

142

143

144

145

146

147

148

## 149 **METHODOLOGY**

### 150 *Samples*

151 Sixteen enterovirus isolates (Table 1) previously recovered from sewage contaminated water were  
152 used as reference samples in this study and analyzed as depicted in the study algorithm (Figure 1).  
153 Isolation and characterization of samples 1 through 12 have been previously described (Faleye and  
154 Adeniji 2015). Briefly, samples 1 through 8 are enterovirus species B (EV-B), and were isolated on  
155 RD cell line (Faleye and Adeniji 2015). Samples 9 to 12 are enterovirus species C (EV-C) and were  
156 isolated on MCF-7 cell line (Faleye and Adeniji 2015). Samples 13 to 15 are Sabin poliovirus 1-3  
157 respectively. Samples 13 and 15 were isolated and characterized by the WHO Environmental  
158 Surveillance (ES) laboratory in Ibadan, Nigeria and provided to us as references for Sabin 1 and 3  
159 polioviruses, respectively. Sample 14 (a poliovirus 2) was isolated as part of a previous study  
160 (Adeniji and Faleye, 2014) but identified subsequent to the study. Sample 16 is a mixture of six  
161 isolates, two species B (samples 1 and 3) and four species C (samples 11, 13, 14 and 15) (Table 1).

### 162 *RNA Extraction and cDNA synthesis*

163 In accordance with the manufacturer's instructions, all samples were subjected to RNA extraction  
164 and cDNA synthesis using JenaBioscience RNA extraction kit and Script cDNA synthesis kit (Jena  
165 Bioscience, Jena, Germany) respectively. Primers AN32, AN33, AN34 and AN35 (Nix et al.,  
166 2006) were used in combination for cDNA synthesis.

### 167 *Seminested Polymerase Chain Reaction (snPCR) Assay for Enterovirus VP1 gene*

168 All primers were re-constituted in concentrations of 100 $\mu$ M and first round PCR was done in  
169 50 $\mu$ L reactions. The first round PCR contained 0.5 $\mu$ L each of primers 224 and 222 (Nix et al.,  
170 2006), 10 $\mu$ L of Red load Taq, 10 $\mu$ L of cDNA and 29 $\mu$ L of RNase free water. A Veriti thermal  
171 cycler (Applied Biosystems, California, USA) was used for thermal cycling as follows; 94°C for 3  
172 minutes followed by 45 cycles of 94°C for 30 seconds, 42°C for 30 seconds and 60°C for 60

173 seconds with ramp of 40% from 42°C to 60°C. This was then followed by 72°C for 7 minutes and  
174 held at 4°C till terminated.

175 The second round PCR was done in 30µL reactions. It contained 0.3µL each of forward and  
176 reverse primers (Figure 2), 6µL of Red load Taq, 5µL of first round PCR product and 18.4µL of  
177 RNase free water. As shown in the algorithm (Figure 1), six different second round PCR assays  
178 were done using the first round PCR product as template. Hence, all six second round PCR assays  
179 used different forward primers (Table 2) but the same reverse primer (AN88) (Figure 2). Based on  
180 the expected amplicon size (Figure 2), the PCR extension time for the second round assays were 30  
181 seconds for primers AN89, 189 and 187 (WHO, 2015; Oberste et al., 2003; Nix et al., 2006) and 60  
182 seconds for primers Sab-1, Sab-2 and Sab-3 (Sadeuh-Mba et al., 2013). The extension temperature  
183 was however retained at 60°C. Subsequently, PCR products were resolved on 2% agarose gel  
184 stained with ethidium bromide, and viewed using a UV transilluminator.

#### 185 *Nucleotide sequencing and enterovirus identification*

186 All amplicons generated from the six second round PCR assays were shipped to MacroGen Inc,  
187 Seoul, South Korea, for purification and sequencing. The primers used for each of the second round  
188 PCR assays (Figure 2) were also used for sequencing, respectively. The identities of the sequenced  
189 isolates were determined using the enterovirus genotyping tool (Kroneman et al., 2011).

#### 190 *Nucleic acid sequences described in this study*

191 The accession numbers for the newly described sequence data generated in this study are  
192 **KX856914-KX856921.**

193

194

195

196 **RESULT**

197 For isolates one to eight which were previously known to be EV-Bs, primer AN89, amplified only  
198 six of the eight samples (Table 1). It is noteworthy that the two isolates not amplified by primer  
199 AN89 were previously identified as E19. Also, the only previously identified E19 (sample 2)  
200 subsequently amplified by primer AN89 in this study was identified as E7 (Table 1). Further, the  
201 identity of the previously unidentified (sample 8) was shown to be E7, and all the other previously  
202 identified E7s and the E3 were confirmed (Table 1).

203 The Species Resolving Primers (SRPs [189 and 187]) identified the isolates to a large extent in  
204 accordance with their previously stated predilections (WHO, 2015). Hence, as expected primer 187  
205 confirmed all but one (sample 4) of the eight (8) EV-B isolates as such. All its type identities were  
206 in accordance with that previously determined prior this study. It also identified the previously  
207 unidentified sample 8 as E7. Primer 189 however showed the presence of E20 in an isolate (sample  
208 4) previously shown to be E19. The Poliovirus Resolving Primers (PRPs [Sab 1, 2 and 3]) however,  
209 did not amplify any of the isolates in samples 1 to 8 (Table 1).

210 For isolates nine to twelve which were all previously identified as CV-A13 (EV-C) (Faleye and  
211 Adeniji, 2015), Primer AN89 (Nix et al., 2006; WHO, 2015) detected and identified all as such.  
212 The SRPs also confirmed all as such. However, it is worth mentioning that primers 189 and 187  
213 could not confirm samples 9 and 11 respectively because the sequence data were unexploitable.  
214 The PRPs did not amplify any of the isolates in samples 9 to 12 (Table 1).

215 For isolates 13 to 15 (previously identified as Sabin PV-1, 2 and 3 respectively), primer AN89 (Nix  
216 et al., 2006; WHO, 2015) detected all, and were subsequently confirmed as such. The SRPs also  
217 confirmed all as such. Similarly, primer 189 detected and identified

218

219



220 Table 1: Identity of Isolates determined using the algorithm.

S/N	Previously determined ID	Species	WHO 2015	Species Resolving Modification (SRM)		Poliovirus Resolving Modification (PRM)			SUMMARY
			AN89	189	187	Sab 1	Sab 2	Sab 3	
1	E3	EV-B	E3	unx	E3				E3
2	E19	EV-B	E7	unx	E19				E7/ E19
3	E7	EV-B	E7	unx	E7				E7
4	E19	EV-B		E20					E20/E19
5	E7	EV-B	E7		E7				E7
6	E19	EV-B		unx	E19				E19
7	E7	EV-B	E7	unx	E7				E7
8	UNT	EV-B	E7	unx	E7				E7
9	CV-A13	EV-C	CV-A13	unx	CV-A13				CV-A13
10	CV-A13	EV-C	CV-A13	CV-A13	CV-A13				CV-A13
11	CV-A13	EV-C	CV-A13	CV-A13	unx				CV-A13
12	CV-A13	EV-C	CV-A13	CV-A13					CV-A13
13	PV-1	EV-C	PV-1	PV-1	PV-1	PV-1	PV-2	PV-3	PV-1, PV-2, PV-3
14	PV-2	EV-C	PV-2	PV-2	unx		PV-2*	unx	PV-2
15	PV-3	EV-C	PV-3	PV-3	unx			PV-3 <sup>§</sup>	PV-3
16	PV-1, PV-2, PV-3, CV-A13 (S/N 11), E3 (S/N 1) E7 (S/N 3),	EV-B & C	PV-2	unx	PV-1	PV-1	PV-2*	PV-3 <sup>§</sup>	PV-1, PV-2, PV-3

221

222 E = Echovirus; EV= Enterovirus; PV = Poliovirus; CV = Coxsackievirus; unx = Unexploitable due to  
 223 bad sequence data; Unt = Untypable; \* and <sup>§</sup> = isolates are genetically the same

224 Note: The Primers included in the Species and Poliovirus Resolving Modifications (SRMs and PRMs) are  
 225 referred in the text as Species and Poliovirus Resolving Primers (SRPs and PRPs), respectively.

226

227

228

229

230

231 all the three, but primer 187, though detected all three could only confirm sample 13 as PV-1. The  
232 remaining two were unexploitable due to bad sequence data. The PRPs also confirmed all as such.  
233 Precisely, the Sabin-1 primer only detected sample 13 and confirmed it to be PV-1. The Sabin-2  
234 primer produced the expected amplicon size in both samples 13 and 14. The sequence data  
235 subsequently confirmed both to contain two different Sabin 2 viruses that are 99.65% similar (data  
236 not shown). In fact, the Sabin 2 in sample 13 has an Isoleucine (I) at position 143 of VP1 while that  
237 in sample 14 has an Asparagine (N) at the same position. The Sabin-3 primer also produced the  
238 expected amplicon size in both samples 13 and 15. The sequence data also confirmed both to  
239 contain two different Sabin-3 viruses that are 99.65% similar (data not shown). Hence, primers  
240 AN89 and the SRPs (189 and 187) did very well in identifying all three polioviruses. The PRPs  
241 (Sab1-Sab3) also confirmed the identity of the three samples. The PRPs however, further showed  
242 that sample 13, which was previously identified by the GPLN algorithm (Kilpatrick et al., 2009) as  
243 PV-1 and confirmed by primers AN89 and the SRPs as such, also contained PV-2 and PV-3 (Table  
244 1).

245 For sample 16 which contained four EV-Cs (PV-1, PV-2, PV-3 and CV-A13), and two EV-Bs (E3  
246 and E7), all the six primers produced their expected band sizes. Sequence data analysis however  
247 showed that while the amplicon from primer 189 produced unexploitable sequence data, primers  
248 AN89, 187, Sab-1, Sab-2 and Sab-3 detected PV-2, PV-1, PV-1, PV-2 and PV-3, respectively  
249 (Table 1). Otherwise stated, AN89 and the SRPs (primers 189 and 187) only detected PV-2 and  
250 PV-1 respectively from the mixture while the PRPs specifically detected all the three poliovirus  
251 serotypes. Importantly, the PV-2 and 3 detected in sample 16 were exactly those found in samples  
252 14 and 15 respectively (Table 1).

253

254

255

Table 2: Sequences of the different forward primers used for second round PCR in this study.

S/N	PRIMER NAME	PRIMER SEQUENCE	PRIMER BINDING REGION	REFERENCE
1	AN89	CCAGCACTGACAGCAGYNGARAYNGG	VP1	Nix et al., 2006
2	189	CARGCIGCIGARACIGGNGC	VP1	Oberste et al., 2003
3	187	ACIGCIGYIGARACIGGNCA	VP1	Oberste et al., 2003
4	Sab 1	AGTCGTCCCTCTTTCGACA	VP3	Sadeuh-Mba et al., 2013
5	Sab 2	TAGGGTTGTTGTCCCGTTG	VP3	Sadeuh-Mba et al., 2013
6	Sab 3	TGTGGTGCCACTGTCCACC	VP3	Sadeuh-Mba et al., 2013

**Note:** Sab 1-Sab 3 was originally designed by M.L. Joffret but first detailed in Sadeuh-Mba et al., 2013 as unpublished data

256  
257

258

259

260

261

262

263

264

265

266

267 **DISCUSSION**

268 *Extending the Utility of the WHO recommended RT-snPCR assay*

269 The results of this study confirm our previous findings (Faleye and Adeniji, 2015a, Faleye et al.,  
270 2016b) that though the Oberste et al., (2003) and the Nix et al., (2006) (recently recommended by  
271 the WHO [2015]) protocols are very sensitive for enterovirus detection and identification, both lack  
272 the capacity to resolve enterovirus co-infection. For example, samples 1-8 (Table 1) were all  
273 previously identified (Faleye and Adeniji, 2015b) using the Oberste et al., (2003) protocol.  
274 However, the results of this study showed that samples 2 and 4 were mixtures; a fact that was  
275 missed when the Oberste et al., (2003) protocol was used for identification (Faleye and Adeniji,  
276 2015). Hence, in this study, the inclusion of the SRPs in the second round PCR of the WHO  
277 recommended RT-snPCR (WHO, 2015) assay enabled enterovirus co-infection detection and  
278 thereby improved the resolving capacity of the assay.

279 In the same light, by including poliovirus serotype specific forward primers (Sadeu-Mba et al.,  
280 2013) in the second round PCR of the WHO recommended RT-snPCR (WHO, 2015) assay, we  
281 were able to selectively and specifically detect and identify the different poliovirus serotypes. More  
282 importantly, this was accomplished in a situation where six different enterovirus types were  
283 present. The results of this study showed that in such instance, the RT-snPCR assay recommended  
284 by the WHO (WHO, 2015) detected only one of the six different enterovirus types present (Table 1;  
285 sample 16). It therefore confirmed that the WHO recommended RT-snPCR assay (WHO, 2015)  
286 might not be dependable for the resolution of enterovirus mixtures. Furthermore, the results of this  
287 study showed that the WHO recommended RT-snPCR assay (WHO, 2015) can be modified or  
288 tailored as described in this study (for detection and identification of the polioviruses) to  
289 specifically detect other enterovirus types especially in cases of co-infection. Such serotype-specific

290 modifications would be very valuable for low-income economies as it will broaden the surveillance  
291 capacity of enterovirologists in such settings with minimal increase in cost.

292

### 293 *Sensitivity of the modified assay*

294 It is pertinent to note that this modification appears to be more sensitive for poliovirus detection and  
295 identification than both the WHO RT-snPCR algorithm (Nix et al., 2006; WHO, 2015) and the  
296 current algorithm for poliovirus identification in use by the GPLN (Kilpatrick et al., 2009). For  
297 example, while the GPLN algorithm (Kilpatrick et al., 2009) and the WHO RT-snPCR algorithm  
298 (WHO, 2015) identified sample 13 as PV-1, the modification described here showed that sample 13  
299 contained PV-1, 2 and 3. More importantly, sequence comparison showed that the PV-2 and 3 in  
300 sample 13, and those in samples 14 and 15 were different (data not shown). Consequent of this  
301 discovery, the initial result of this reference sample of environmental origin was retraced. It was  
302 then discovered that the results of the five L20B and one RD flask (WHO, 2004) showed that PV-1,  
303 2 and 3 were all isolated from the parent environmental sample but in different flasks (unpublished  
304 data). However, the PV isolate in the L20B flask from which sample 13 was aliquoted was  
305 identified as PV-1 by the current GPLN poliovirus detection and identification algorithm  
306 (Kilpatrick et al., 2009). Altogether, this suggests that the isolate in sample 13, contained PV-1, 2  
307 and 3 but PV-1 had a titre that is significantly higher than others and was consequently the type  
308 detected in sample 13 both by the GPLN algorithm and primers AN89, 189 and 187 (Table 1;  
309 sample 13). Further buttressing the influence of PV-1 titre hypothesis is the fact that the PV-2 and 3  
310 in sample 13 could not be detected in the sample 16 mixture. Rather it was the PV-2 and 3 in  
311 samples 14 and 15 that were detected in sample 16 (Table 1).

312 Considering that about 25,000 genomic equivalents are required for the current GPLN algorithm to  
313 detect the presence of PV-1 (Arita et al., 2015), this finding is not unexpected. Rather it suggests

314 that while the genomic equivalents of PV-1 in sample 13 might be up to the required, those of PV-2  
315 and PV-3 are below and account for the inability of the assay to detect both. This has implications  
316 for the polio eradication and endgame strategic plan 2013-2018 (WHO, 2013) and particularly the  
317 WHO global action plan (GAP III) for poliovirus containment and sequential withdrawal of the  
318 Sabin strains (WHO, 2014). For example, in the course of Sabin PV-2 containment, isolates  
319 containing Sabin PV- 2 but with titre below the detection limit of the GPLN assay for Sabin PV-2  
320 detection might be missed. It is therefore suggested that for containment, all isolates that contain  
321 any of the poliovirus types should be handled as potentially containing the other two types.  
322 Furthermore, to reduce the misclassification of mixed isolates with low titre components and  
323 consequently enhance the containment programme, effort should be put into increasing the  
324 sensitivity of the assays in use by the GPLN and others; like that described in this study. In  
325 addition, effort should also be put into mainstreaming serotype-independent NextGen sequencing  
326 strategies recently described for the polioviruses (Montmayeur et al., 2016) and other Species C  
327 members (Bessaud et al., 2016).

328

### 329 *Field implementation of the modified assay*

330 It is imperative to mention that in this study; cell culture isolates were used and as such it might be  
331 difficult to remark on how the assay might fair for direct detection of polioviruses from clinical  
332 specimen. However, considering that the initial development of the RT-snPCR assay (Nix et al.,  
333 2006) recently recommended by the WHO (WHO, 2015) was done using isolates, we see no reason  
334 why implementing this algorithm with clinical specimen should be problematic. Furthermore, this  
335 assay does not in any way change the assay described by Nix and colleagues (Nix et al., 2006) and  
336 subsequently recommended by the WHO (WHO, 2015). Rather, considering its limitations with  
337 respect to resolving mixtures, this modification piggy-back on the first round PCR product of the

338 Nix algorithm (Nix et al., 2006) in a bid to improve its mixture resolving capacity and thereby  
339 extend its utility.

340 In a polio-free world there might be reduced funding for poliovirus surveillance and only essential  
341 facilities (WHO, 2014) with appropriate safety mechanisms in place to avoid facility associated  
342 escape of poliovirus might be cleared for poliovirus isolation in cell culture . In such strictly  
343 regulated and potentially resource limited setting, the modification described in this study might be  
344 of significance because it allows the enlistment of nonessential facilities and others without the  
345 capacity or infrastructure for cell culture to participate in poliovirus surveillance. Particularly, in  
346 such settings, enterovirologists interested in nonpolio enteroviruses can continue their  
347 investigations, and also contribute significantly and specifically to poliovirus surveillance, by using  
348 the excess of their first round PCR product.

349 The modification described in this study is also valuable in regions where maintaining the reverse-  
350 cold chain for sample transport to the laboratory might constitute a bottle-neck. In such settings,  
351 using this modification as an addendum to the current cell culture based algorithm, even if only for  
352 cell culture negative samples, will improve the detection of poliovirus in samples that might no  
353 longer contain infectious particles on arrival in the laboratory. This might help reduce the incidence  
354 of orphan polioviruses by serving as an early warning system in determining regions where samples  
355 arrive in nonviable conditions. Such early detection can result in quick intervention and the  
356 consequent forestalling of the occurrence of orphan polioviruses. Also, like the RT-snPCR assay  
357 recently recommended by the WHO (2015), this algorithm will significantly reduce the turn-around  
358 time from sample arrival in the laboratory to availability of results to less than 48 hours. This is  
359 significantly less than the minimum of 10 days for the cell culture based GPLN algorithm (WHO,  
360 2004) and is of immense value for early outbreak detection of a virus that only shows clinical  
361 manifestation in one out of 100 to 250 people infected (Nathanson and Kew, 2010).

362 ***Limitations and Conclusion***

363 The limit of detection of the modification described here is currently not known. Hence, effort is  
364 ongoing to conduct spiking experiments with plaque purified and titrated reference isolates in a bid  
365 to better define the sensitivity of the assay. Furthermore, it is crucial to mention that the sequence  
366 data this modification provides do not cover the entire VP1 region. As such, unlike the ECRA assay  
367 (Arita et al., 2015), the sequence data generated might not be sufficient for extensive molecular  
368 epidemiology. Consequently, this modification is currently proposed as addenda and not as  
369 substitute for either the current GPLN algorithm (WHO, 2004) or the other cell culture independent  
370 assays (Arita et al., 2015; Krasota et al., 2016) with the capacity to provide sequence data of the  
371 entire VP1 region. In addition, its value for early outbreak or extended vaccine virus circulation  
372 detection (currently in resource-limited settings and in a polio-free world) by increasing the number  
373 of laboratories that can contribute in a meaningful way to poliovirus surveillance is espoused.

374 **Acknowledgements:** The authors would like to thank the members of staff of the WHO  
375 Environmental Surveillance Laboratory in Ibadan, Nigeria, for providing us with Sabin strains of  
376 poliovirus types 1 and 3.

377 **Compliance with ethical guidelines:** Isolates recovered from environmental samples were  
378 analyzed in this study. Hence, this article does not contain any studies with human participants  
379 performed by any of the authors. The poliovirus 2 analysed in this study has been destroyed.

380 **Conflict of Interest:** The authors declare no conflict of interests

381 **Funding:** This study was not funded by any organization rather it was funded by contributions  
382 from authors.

383

384

385

386

387



388 **REFERENCES**

- 389 Adeniji J.A., and Faleye, T.O.C., 2014. Impact of Cell Lines Included in Enterovirus Isolation  
390 Protocol on Perception of Nonpolio Enterovirus Species C Diversity. *J.Virol.Methods.* **207**: 238-  
391 247.
- 392  
393 Arita M., Kilpatrick D.R., Nakamura T., Burns C.C., Bukbuk D., Oderinde S.B., Oberste M.S.,  
394 Kew O.M., Pallansch M.A., Shimizu H., 2015. Development of an efficient entire-capsid-coding-  
395 region amplification method for direct detection of poliovirus from stool extracts. *J. Clin.*  
396 *Microbiol.* 53:73–78.
- 397  
398 Bessaud M, Sadeuh-Mba SA, Joffret ML, Razafindratsimandresy R, Polston P, Volle R, Rakoto-  
399 Andrianarivelo M, Blondel B, Njouom R, Delpeyroux F.(2016). Whole Genome Sequencing of  
400 Enterovirus species C Isolates by High-Throughput Sequencing: Development of Generic Primers.  
401 *Front Microbiol.* 26;7:1294. doi: 10.3389/fmicb.2016.01294. eCollection 2016.
- 402  
403 Faleye, T.O.C., and Adeniji, J.A., 2015. Nonpolio enterovirus-C (NPEV-C) strains circulating in  
404 South-Western Nigeria and their contribution to the emergence of recombinant cVDPV2 lineages.  
405 *Brit. J. Virol.* 2(5): 68-73.
- 406  
407 Faleye, T.O.C., Adewumi, M.O., Coker, B.A., Nudamajo, F.Y. & Adeniji J.A. 2016a. Direct  
408 Detection and Identification of Enteroviruses from Faeces of Healthy Nigerian Children Using a  
409 Cell-Culture Independent RT-Seminested PCR Assay. *Adv. Virol.*, Article ID: 1412838, 12 pages,  
410 <http://dx.doi.org/10.1155/2016/1412838>.
- 411  
412 Faleye, TOC, Adewumi, MO, Kareem, SA, Adesuyan, YO, Fapohunda, FA, Fasanya, ST, Jimeto,  
413 T, Lawrence, OE, Obembe, AA, Adeniji, JA. 2016b. The impact of a panenterovirus VP1 assay on  
414 our perception of the enterovirus diversity landscape of a sample. *J Hum Virol Retrovirol* 4(3):
- 415  
416 Nathanson, N. and Kew, O. M. 2010. From Emergence to Eradication: The Epidemiology of  
417 Poliomyelitis Deconstructed. *American Journal of Epidemiology* **172**(11), 1213 – 1229.
- 418  
419 Nix, W. A., Oberste, M. S. & Pallansch, M. A. 2006. Sensitive, Seminested PCR Amplification of  
420 VP1 Sequences for Direct Identification of All Enterovirus Serotypes from Original Clinical  
421 Specimens. *J. Clin. Microbiol.* **44**(8): 2698–2704.
- 422  
423 Kilpatrick, D. R., Yang, C. F., Ching, K., Vincent, A., Iber, J., Campagnoli, R., Mandelbaum, M.,  
424 De, L., Yang, S. J., Nix, A. & Kew, O. M. 2009. Rapid Group-, Serotype-, and Vaccine Strain-  
425 Specific Identification of Poliovirus Isolates by Real-Time Reverse Transcription-PCR Using  
426 Degenerate Primers and Probes Containing Deoxyinosine Residues. *J. Clin. Microbiol.* **47**(6):  
1939-1941.
- 427  
428 Krasota, A., Loginovskih, N., Ivanova, O. and Lipskaya, G. 2016. Direct Identification of  
429 Enteroviruses in Cerebrospinal Fluid of Patients with Suspected Meningitis by Nested PCR  
430 Amplification. *Viruses* 8, 10.
- 431  
432 Kroneman, A., Vennema, H., Deforche, K., Van der Avoort, H., Pe narandac, S., Oberste, M.S.,  
433 Vinjéc, J., Koopmans, M. 2011. An automated genotyping tool for enteroviruses and noroviruses.  
434 *Journal of Clinical Virology.* 51(2), 121–125.

- 435 Montmayeur AM, Ng TF, Schmidt A, Zhao K, Magaña L, Iber J, Castro CJ, Chen Q, Henderson E,  
436 Ramos E, Shaw J, Tatusov RL, Dybdahl-Sissoko N, Endegue-Zanga MC, Adeniji JA, Oberste MS,  
437 Burns CC. 2016. High-Throughput Next Generation Sequencing of Polioviruses. *J Clin Microbiol.*  
438 2016 Dec 7. pii: JCM.02121-16. [Epub ahead of print]  
439
- 440 Oberste, M. S., Nix, W. A., Maher, K. & Pallansch, M. A. 2003. Improved molecular identification  
441 of enteroviruses by RT-PCR and amplicon sequencing. *J. Clin. Virol.* **26(3)**, 375-377.  
442
- 443 Rahimi, P, Tabatabaie, H, Gouya, M.M., Mahmudi, M, Musavi, T, Rad, K.S., Azad, T.M. &  
444 Nategh, R. (2009). Direct identification of non-polio enteroviruses in residual paralysis cases by  
445 analysis of VP1 sequences. *J Clin Virol.* **45(2)**:139-41.  
446
- 447 Sadeuh-Mba S.A., Bessaud M., Joffret M.L., Endegue Zanga M.C., Balanant J., Mpoudi Ngole  
448 E., Njouom R., Reynes J.M., Delpeyroux F. & Rousset D. (2014). Characterization of  
449 Enteroviruses from non-human primates in cameroon revealed virus types widespread in humans  
450 along with candidate new types and species. *PLoS Negl Trop Dis.* **31**; 8(7):e3052. doi:  
451 10.1371/journal.pntd.0003052.  
452
- 453 Sadeuh-Mba, S. A., Bessaud, M., Massenet, D., Joffret, M. L., Endegue, M. C., Njouom, R.,  
454 Reynes, J. M., Rousset, D. & Delpeyroux, F. 2013. High frequency and diversity of species C  
455 enteroviruses in Cameroon and neighbouring countries. *J. Clin. Microbiol.* **51(3)**: 759-770.  
456
- 457 World Health Organisation, 1988. Global Eradication of Poliomyelitis by the Year 2000 (World  
458 Health Assembly resolution, WHA41-28).  
459 [http://www.polioeradication.org/content/publications/19880513\\_resolution.pdf](http://www.polioeradication.org/content/publications/19880513_resolution.pdf)
- 460 World Health Organisation. 2003. Guidelines for environmental surveillance of poliovirus  
461 circulation. Geneva.
- 462 World Health Organisation. 2004. Polio laboratory Manual. 4th Edition, Geneva.  
463
- 464 World Health Organisation. 2013. Polio Eradication & Endgame Strategic Plan 2013 – 2018.  
465 Geneva.  
466
- 467 World Health Organisation. 2014. WHO global action plan to minimize poliovirus facility-  
468 associated risk.3rd Edition, Geneva.
- 469 World Health Organisation. 2015. Enterovirus surveillance guidelines: Guidelines for enterovirus  
470 surveillance in support of the Polio Eradication Initiative, Geneva.  
471
- 472
- 473
- 474
- 475

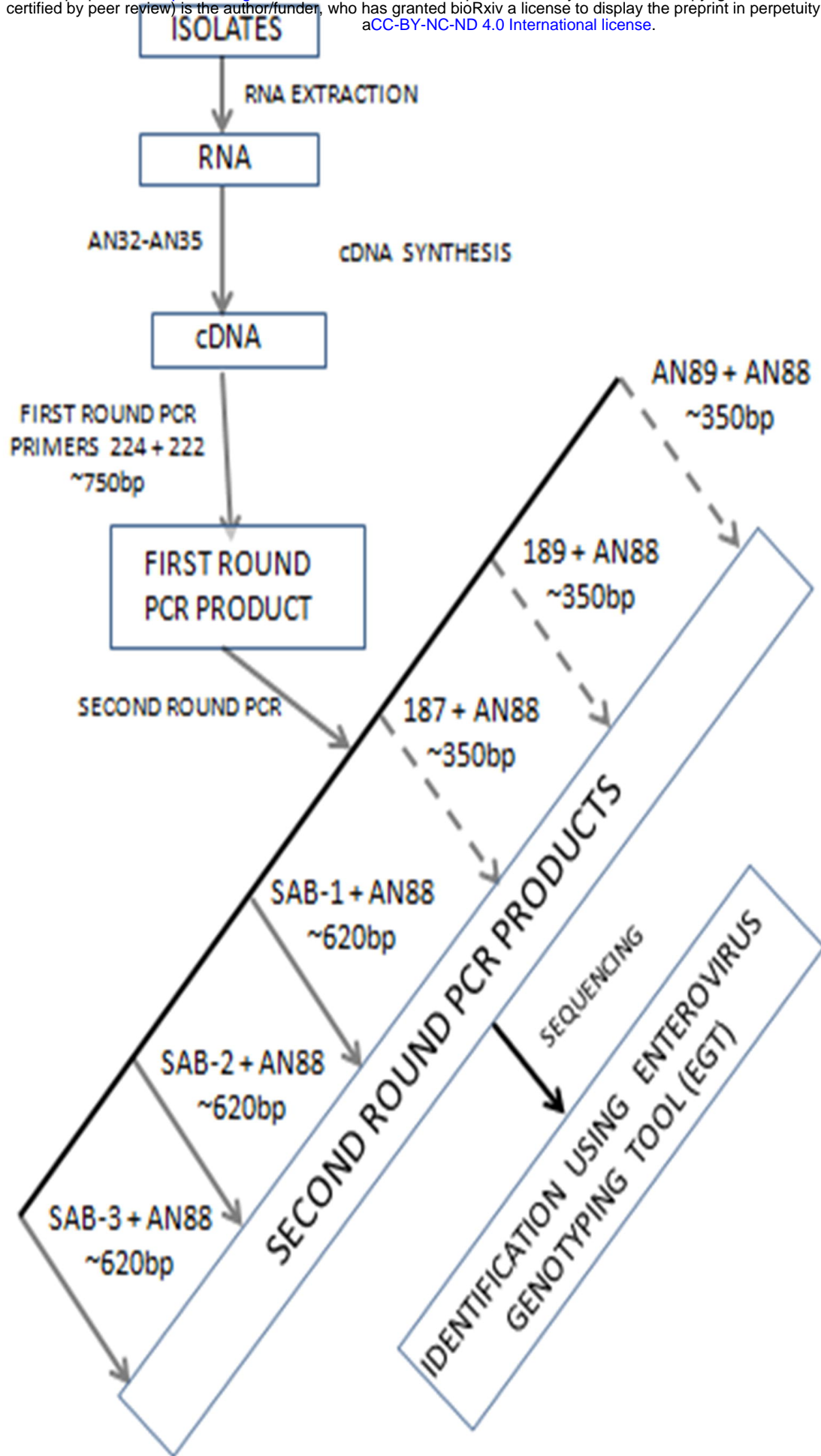
476 **Figures**

477 Figure 1: The algorithm followed in this study.

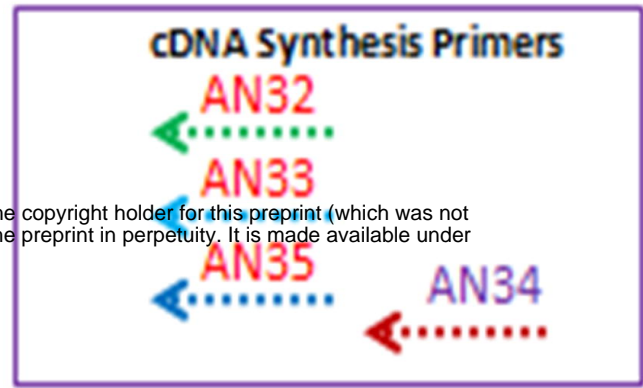
478

479 Figure 2: A schematic representation of the annealing sites of the different primers used in this study relative  
480 to the enterovirus genome and the consequent amplification product (arrows depict primers and their  
481 orientation).

482



## Enterovirus Genomic RNA



## Second Round PCR

