Advances in Research

16(6): 1-9, 2018; Article no.AIR.44423
ISSN: 2348-0394, NLM ID: 101666096

High Serotype Diversity of Non-polio Enteroviruses Isolated in Ghana during Acute Flaccid Paralysis Surveillance, 2010-2014

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Authors' contributions

This work was carried out in collaboration between all authors. Author JKO designed the study design, wrote the protocol, processed samples, analyzed results, wrote the first draft of manuscript, managed the literature searches, wrote and edited the manuscript. Author IA processed samples, analyzed results and edited the manuscript. Author EQ processed samples, performed the statistical analysis, data interpretation and edited the manuscript. Author KA processed samples and analyzed results. Author JAQ analyzed results, data interpretation and edited the manuscript. Author ME analyzed results, data interpretation and edited the manuscript. Author NAAN analyzed results, data interpretation and edited the manuscript. Author EO processed samples, analyzed results, managed the literature searches, wrote and edited manuscript. Author JSB supervised the study protocol, analyzed results, data interpretation and edited the manuscript. All authors have read and approved the final manuscript.

Article Information

DOI: 10.9734/AIR/2018/44423
Editor(s):
(1) Dr. Giovanni Messina, Department of Clinical and Experimental Medicine, University of Foggia, Italy.
Reviewers:
(1) Godstime Isi Irabor, Saba University School of Medicine, Netherlands.
(2) Nitesh Mohan, Bareilly International University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/26729

Original Research Article

Received 19 July 2018
Accepted 06 October 2018
Published 22 October 2018

ABSTRACT

Aim: Sabin-like polioviruses and non-polio enteroviruses (NPEVs) isolated from acute flaccid paralysis cases have continued to circulate in the country. However, no wild poliovirus has been detected in Ghana since the last case of poliomyelitis in 2008. This decline has been attributed to active surveillance and intensive oral polio vaccine immunization. As we approach polio-free world,

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surveillance of NPEVs implicated in acute flaccid paralysis (AFP) is useful for establishing temporal and geographical patterns of circulation and diversity.
Study Design: This was a retrospective study using stool samples store at -20°C.
Place and Duration: The investigation was carried out at the WHO-accredited Regional Reference Polio Laboratory, Noguchi Memorial Institute for Medical Research, Legon, Ghana from January 2010 to December 2014.
Methods: We investigated stool samples collected from 1422 patients with AFP from 2010-2014 across the country. We tested the samples for human enterovirus infection using standard cell culture methods. Serological identification of NPEV was done using RIVM-specific antisera pools A-G and H-R (The Netherlands). Untyped (UT)-NPEVs were sequenced directly using reverse transcription–polymerase chain reaction (RT-PCR). The pan-enterovirus (Pan-EV) primer (CDC, Atlanta, GA) used in the PCR assay targeted a highly conserved VP1 region of the enterovirus.
Results: Two hundred and thirty-five cases were confirmed as positive on RD cells indicating a NPEV isolation rate of 16.5%. Of these RD positive isolates, 110 (46.8%) were further analyzed using sero-neutralization and 28 different NPEVs serotypes were identified. Two additional sero-types E71 and EV A76 were identified by sequencing. All the 30 serotypes belong to four species group: 5 belong to HEV-A, 23 HEV-B, 1 HEV-C and 1 HEV-D. The mean age of the children was 3 years with a range of 8 months to 21 years and standard deviation of 3. Most infections occurred in children within the age group of 2-6 years with no statistical difference $p>0.975$. The NPEVs were found to circulate throughout the 5-year period and across the country, with the highest prevalence (24%) in the Brong Ahafo region.
Conclusion: The study provided definitive evidence on the circulation of NPEV serotypes implicated in AFP in a polio-free country, and highlights the importance of monitoring NPEVs that mimic polio as we approach polio-free world and continuous vaccination for interruption of transmission.

Keywords: Poliovirus; non-polio enterovirus; surveillance; acute flaccid paralysis; Ghana.

1. INTRODUCTION

Enteroviruses (EVs), members of the family Picornaviridae are small, non-enveloped RNA viruses that continue to represent a significant global public health threat. EVs were originally classified into four groups on the basis of human disease and virulence or pathogenicity in intracranially inoculated suckling mice as polioviruses (PVs), coxsackieviruses A and B (CVA and CVB), echoviruses (E), and the numbered enteroviruses (EV) [1,2]. In the present classification scheme which takes into account the nucleotide and amino acid sequences of the viral protein genes, HEVs have been subgrouped into ten species namely HEV-A to D (over 100 serotypes), Bovine enterovirus groups A and B (10 serotypes), Pocine enterovirus B (11 serotypes), Semian enterovirus group A (1 serotypes), unclassified semian enterovirus (11 serotypes) and human rhinovirus A, B, and C [3,4].

Members of the species of HEV A-D apart from polioviruses also known as non-polio enteroviruses (NPEV) circulate in all populations and are known to infect a billion people or more annually worldwide [5]. Infection can be associated with a vast range of presentations, from asymptomatic to acute infections like common cold, febrile rash, aseptic meningitis, acute flaccid paralysis (AFP), and neonatal sepsis-like disease. NPEV infections among neonates and infants occur with varying degrees of severity [6,7]. Furthermore, several studies provide more evidence that enteroviruses are also the causative or contributory agents to chronic diseases including insulin-dependent diabetes mellitus and dilated cardiomyopathy [8,9].

According to WHO, it is not necessary to characterize NPEVs for polio eradication but can just be reported [6], however the similarities and challenging links between some of the clinical presentations of NPEV infection, polio and emerging diseases, poses a great difficulty in ensuring a total eradication of polio especially in the tropics where emerging and re-emerging infections abound particularly in the post eradication era. For instance it has been reported that Echoviruses have been implicated in multiple human disease syndromes, including paralysis [7], hence inadequate data on the characterization pattern of transmission of NPEVs may present the possibility that poliomyelitis could emerge in the post eradication era.

Virus isolation and subsequently serotyping of enteroviruses by cell culture is being replaced by genetic characterization for enterovirus strains classification. Sequence analysis of enterovirus genomes has been used to determine the current molecular-based criteria for defining enterovirus serotypes, leading to identification of an increasing number of new enteroviruses [8], however, in resource limited settings, serotyping which has long been a gold standard [9] until the introduction of characterization by sequencing is still very common and relevant. While serotyping may have little influence on the clinical management of a given patient, identification of the serotype is important to firmly establish an epidemiological link among cases during an outbreak and to recognize serotype-specific clinical illness [9].

From a public health standpoint, it is important to be able to distinguish sporadic cases from an outbreak so that intervention and prevention strategies may be targeted logically and effectively.

In Ghana wild poliovirus has been eliminated with the last indigenous case seen in 1999 and the country declared polio-free in 2015. Nevertheless, NPEV have been implicated to cause 10-20% of all AFP cases in the country [10]. This present study sought to provide data on the characteristics, diversity, seasonality and geographical pattern of circulation of NPEVs isolated in Ghana during an AFP surveillance from 2010 to 2014.

2. METHODS

The Department of Virology, Noguchi Memorial Institute for Medical Research (NMIMR) is a WHO-accredited Regional Reference Polio Laboratory (RRPL) for receiving, processing, and analyzing AFP specimens for polio and NPEVs. At least 2 specimens, collected ≈24 hr apart, from each person with AFP by the Disease Surveillance Department of the Ghana Health Service, Ministry of Health during 2010–2014 were sent to the RRPL, NMIMR. A total of 1422 specimens from AFP children under < 15 years of age were chloroform-treated and the supernatants inoculated on confluent monolayer of RD (Human rhabdomyosarcoma) and L20B (Mouse fibroblast cells expressing with poliovirus receptor) cells containing serum-free Modified Eagle's Medium (MEM) in tubes and incubated at 35^{o}C. The L20B is intended for the specific isolation of PV but recently it has been reported that it can also propagate some CVA types 4, 8, and 10. The tubes were examined for EVs by observing cytopathic effects in both cell lines characterized by rounding necrosis. The infected cells were harvested and kept frozen (-20°C).

EV serotyping was by microneutralization tests with pools of antisera specific for common EV serotypes according to WHO standard protocols [11]. Serotype-specific immune antisera for the EV serotypes EV pools from the National Institute for Public Health and the Environment, Bilthoven, the Netherlands (RIVM) were used. The EV pools contain reference horse typing polyclonal antisera against the HEV serotypes isolated most frequently combined as nine antiserum pools (A-G pool). The untypable isolates were further subjected to another microneutralization assay using a second set of polyclonal antiserum pools H-R.

2.1 Sequence Analysis of Untypable NPEV

Four samples that could not be typed by seroneutralization were selected for sequencing. Sequencing was carried out directly from RT–PCR products using pan-enterovirus (Pan-EV) primer (CDC, Atlanta, GA) [12]. Highly conserved VP1 region EV primers was used; EV VP1-AN88-forward primer; – 5′-CCAGCACTGACAGCAGYNGARAYNGG- 3′ and EV VP1 SO222-reverse primer; – 5′-CIC CIG GIG GIA YRW ACA T- 3′. One-step RT–PCR was used to amplify the product; 660bp-sized band of the PCR amplified product was visualized on I% agarose gel electrophoresis under UV trans-illuminator. Non-infectious RNA was used as positive control and culture supernatant from uninfected cells was used as negative reagent control.

Data Analysis: The MS excel data base was imported into SPSS version 17 and analyzed. Univariable analysis of case investigation and administrative data by person, place and time were expressed as frequency distributions, percentages and charts. NCBI BLAST-Phylogenetic Tree View was applied for data analysis of untypable NPEV.

Ethical Issues: This study was conducted on specimens collected upon conclusion of routine examinations and stored as anonymous. Patient identifiers including personal information (name, address) and hospitalization number were removed from these samples to protect patient

confidentiality and neither did they appear in any part of document in this study. The research protocol was approved by the Institutional Review Board (IRB reference number DF. 22), Noguchi Memorial Institute for Medical Research. IRB waived the need for consent because the samples were de-identified.

3. RESULTS

Ghana implemented the nationwide surveillance for AFP was in 1996. About a decade and half later, wild polioviruses were eliminated and the country attained a polio-free status in 2015. A total number of 1422 cases of AFP were investigated from 2010 and 2014, with majority of 389 cases investigated in 2014 (Table 1). Of the total cases, 235 were isolated on RD cells as NPEVs given an isolation rate of 16.5%. The highest NPEV isolation rate of 18.6% was in 2013. However, only 110 (46.8%) NPEVs were available in the lab for further analysis as at the time of this study. NPEV infections were significantly $p>0.03$ detected in males 67 (60.9%) than females 39.1% over the 5-year period. The mean age of the cases was 3 years, the median was 2 years, the mode was 1 year, the age range was from 8 months to 21 years with variance of 10 and standard deviation of 3. Several serotypes of NPEVs circulated over the study period with the highest frequency of 14 NPEVs in 2012 followed by 13 NPEVs in 2011 and 7 NPEVs recorded in 2014. Children less than 2 years were the most infected with NPEVs in 2010. Similarly, children within the age group of 2 to 6 years were mostly affected but with no significant difference ($p>0.975$). The study patients have received between 2 and 6 doses of trivalent OPV immunization except for 5 children whose statuses were unknown.

As shown in Fig. 1, more NPEV cases occurred in the Brong Ahafo region followed by Volta region, with the least number of cases in the Greater Accra region. Central and Upper West regions did not record any NPEV case in 2010. Likewise, Greater Accra region that did not record any NPEV case in 2010 and 2012. However the remaining seven regions recorded NPEV cases throughout the 5-year study period. NPEVs were found circulating throughout the year with peaks during the raining seasons of May to -July and September to October (Fig. 2). Sero-neutralization using antisera pools A-G from RIVM, The Netherlands, on 110 RD isolates identified 75 (68.2%) typable NPEV while 25 (31.8%) remained untypable. All the typable NPEVs belong to human enterovirus (HEV) group B. A second pool of antisera H-R from the same source serotyped the remaining 21 NPEV isolates while the remaining 4 were identified by sequencing. The sequences obtained in VP1 were compared with those included in GenBank database and were assigned the serotype of the strain that gave the highest identity score. The sequences revealed their serotypes when homology in VP1 sequence was at least 86% to prototype strains. Of the isolates sequenced, 2 belong to HEV-A, and 2 HEV-B.

Results of serological and molecular tests were summarized in Fig. 3. Thirty different NPEV serotypes were found within the 5-year period. Of these, HEV-A made up of Cox A3, Cox A10, Cox A16, EV A76 and E71 formed 16.7%; HEV-B comprising Cox A9, Cox B4, Cox B5, Cox B, E2, E3, E5, E6, E7, E11, E12-14, E17, E19-E22, E25, E29, E30 and E33 was 76.7%; HEV-C and HEV-D contributed 3.3% each of Cox A24 and E70 respectively. A greater proportion of the total NPEV population in Ghana were species under the HEV-B with Cox B group (32.7%) having the highest number of the total isolates (Fig. 3). Serotype of HEV-C group of which poliovirus belongs and HEV-D were rarely observed. Apart from 2 Cox B that were selected and typed Cox B4 and Cox B5, the remaining were not typed. Of the 30 different NPEV serotypes identified, Cox B viruses were isolated throughout the 5-year study period with E7 in four out of the 5 years. E6, E11, E13, E14 and E33 were also isolated in 3 of the 5-year period while 14 different NPEVs were identified in only one particular year.

The sequenced isolates were characterised as shown in Fig. 4. Two of the strains clustered with reference E6 strains, 1 with EV A76 while the other clustered with EV71.

4. DISCUSSION

Isolation of NPEVs from AFP cases is common worldwide [13,14,15]. In Ghana, there is no routine surveillance of NPEVs but their isolation in the laboratory is only documented to monitor the sensitivity of the national polio eradication surveillance program and the circulating pattern of the different serotypes. NPEVs rate was found to be slightly higher in males than in females, but with no statistical difference ($p>0.9$). This finding was quite similar to that described earlier from Pakistan [16] and Taiwan [17], however, differed from that of Austria [18] and Tunisia [19] reporting predominance in males. The activity of NPEVs recorded in this study was found to be

high during the raining seasons with low peaks during the dry harmattan period. This observation was contrary to that described in earlier studies from northern India [20,21] where NPEV peaks during the summer periods. The climatic factors may thus contribute to affect the transmission and infection rates of NPEVs [17].

Our study has indicated a high degree of NPEVs diversity with multiple patterns of circulation in Ghana over the 5-year period. Thirty different NPEV serotypes were identified with as high as 26 NPEVs and low as 16 NPEVs to have co-circulated each year. Four most commonly reported serotypes Cox B, E6, E7 and E33 accounted for 49.1% in the study. Circulation of individual serotypes varied from endemic, recurrence, and epochal patterns. The recurrence of enterovirus serotypes (Cox B, E6, E7, E11, E13, E14 and E33) may suggest that these serotypes have circulated probably during a period when high population immunity have waned and reintroduction occurs when a new build-up of susceptible individuals take place. The introduction of single serotype at different years and the existence of epochal strains with few isolates are patterns which can be explained by importation and epidemic transmission and not endemic serotypes of the country.

Coxsackie B viruses, commonly detected in Ghana was the predominant strain found every year. Similar studies have reported Cox B dominance in the Philippines [22], Slovakia [23] and India [24]. Cox B viruses were found to be circulating significantly more in AFP cases aged 2 to 6 years than in under 2 years and predominant in 2011 and 2014. Similarly, E7 was found circulating all year round except for 2012 when the virus was not seen.

Sequence analysis of the 4 (3.6%) untypable NPEV after neutralization revealed two E6 belonging to HEV-B, and EV 71 and EV A76 belonging to HEV-A. The two E6 could have been typed by the neutralization assay but this was not successful. The possible explanation reported elsewhere in India [25] could be that they remain untypable probably because of high titre of virus used for neutralization or presence of mixed serotypes. The two E6 further clustered with E6 lineages from Kenya in the phylogenetic analysis and underscores a possible link between different populations. The study also reports for the first time the circulation of Cox A10, Echo 22, EV71 and EV A76 in the country. Probably, these viruses were importation into the country but for lack of sustained transmission they died naturally. It is also possible these were mixed in our previous studies because no genomic sequencing was done and all such viruses were reported as untypable.

The incidence of different species of enteroviruses in different countries has been established with data from temperate countries demonstrating an isolation frequency pattern: HEV-B > HEV-A > HEV-C >HEV-D while in tropical countries studies have revealed different isolation pattern of HEV-B > HEV-C >HEV-A > HEV-D [26,27]. Our study however showed that Ghana which is a tropical country has isolation pattern similar to the pattern of the temperate countries. This clearly shows that even though climatic weather plays a role in the distribution pattern of NPEVs, movement of individuals from one country to the other and many other factors may also influence the NPEV patterns. Low period of transmission of NPEVs was observed between December to February with high periods within the rainy seasons. Similar findings have been reported in India [25] with low transmission in winter and high peaks from June to September. This condition is probably due to high degree of humidity with elevated temperature that facilitates NPEV transmission.

Table 1. Age and sex distribution of NPEVs isolated from AFP cases from 2010-2014 in Ghana

Year	2010	2011	2012	2013	2014	Total	p-value
AFP Cases	217	275	199	342	389	1422	
NPEV (%)	13.2	22.2	13.6	18.6	14.9	16.5	0.03
Sex							
M	14	16	17	11	9	67	0.9
F	11	10	9	6	7	43	
Age							
<2	13	11	9	6	5	44	0.975
≥2-<6	9	13	14	9	9	54	
≥6-≤14	3	2	3	2	2	12	

This study revealed a high serotypes diversity of EVs circulating in Ghana over the 5-year period

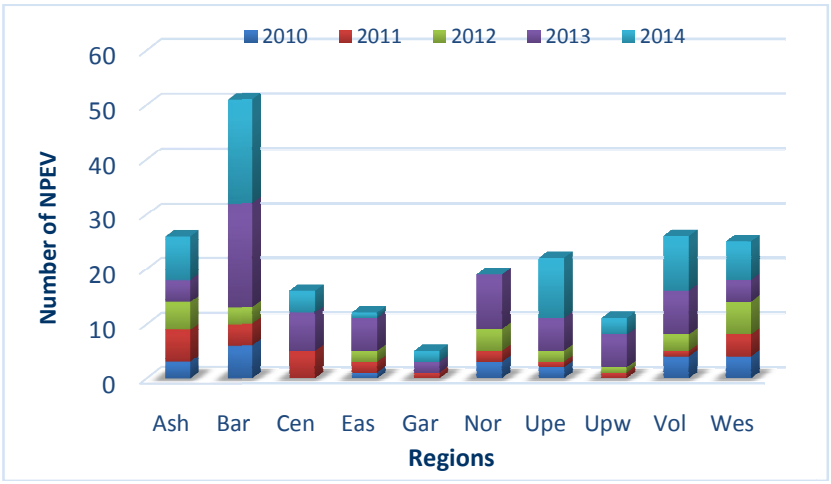

Fig. 1. Spectrum of NPEV serotypes from NP-AFP across Ghana, 2010-2014. Ash: Ashanti, Bar: Brong Ahafo, Cen: Central, Eas: Eastern, Gar: Greater Accra, Nor: Northern, Upe: Upper, Vol: Volta and Wes: Western

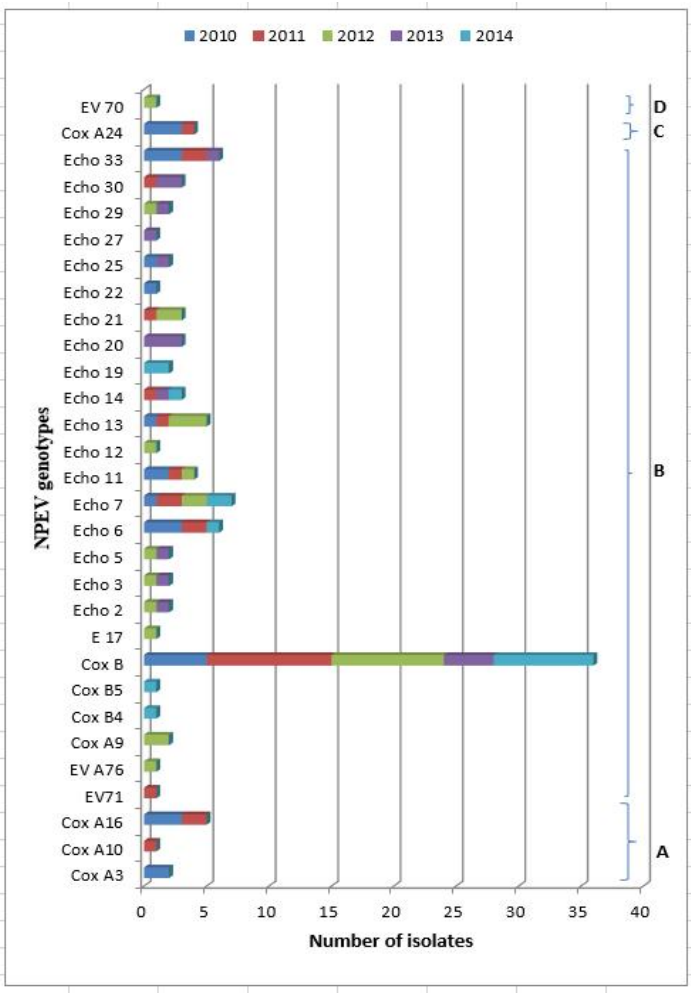

Fig. 2. Different serotypes of non- polio enterovirus isolated in Ghana 2010-14

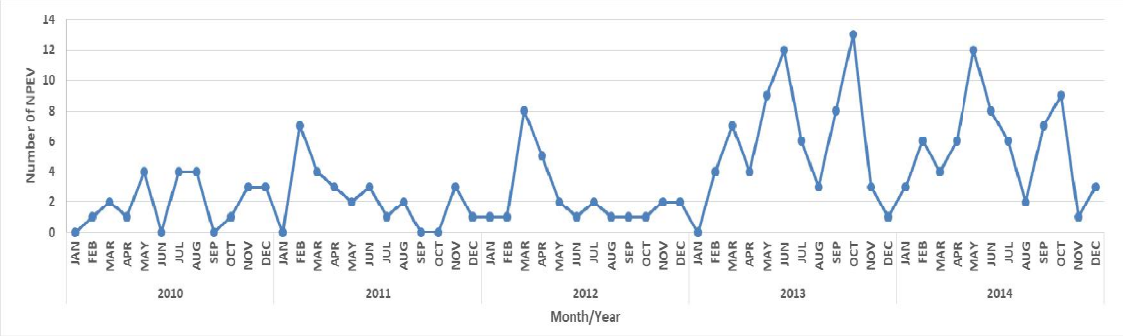

Fig. 3. Seasonal distribution of NPEVs in AFP patients over a five-year period, 2010-2014 in Ghana

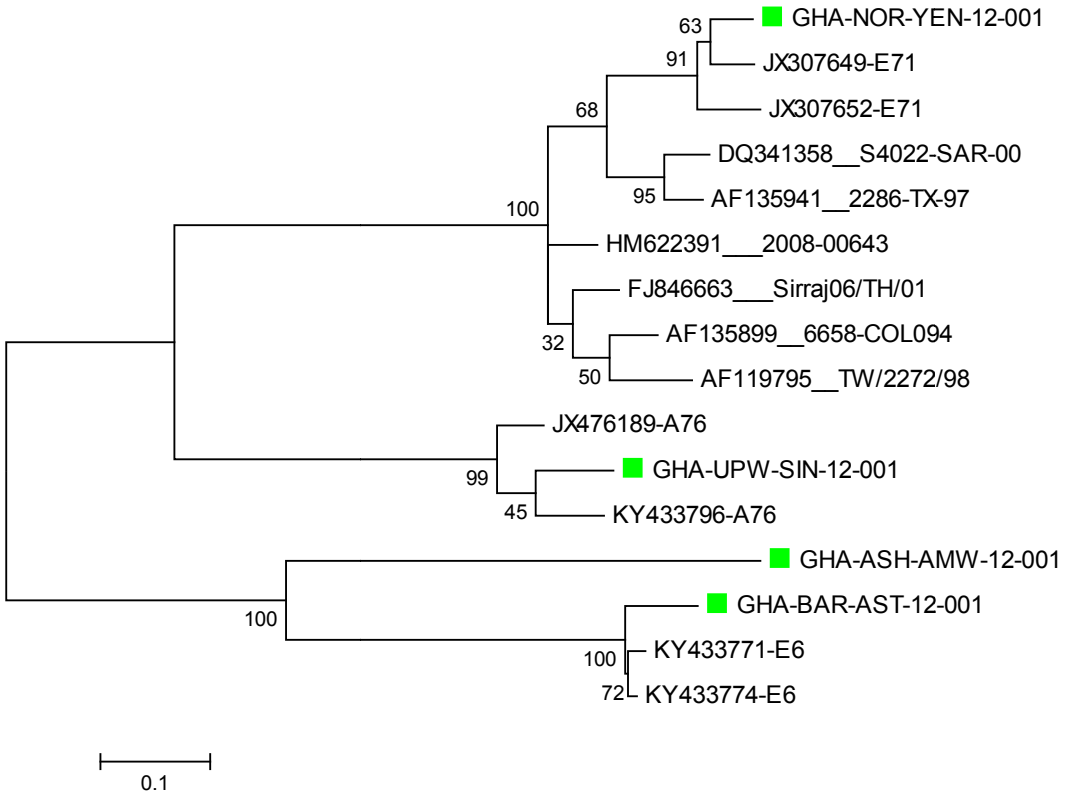

Fig. 4. Neighbor-joining tree representing phylogenetic analyses of NPEV serotypes isolated in Ghana between 2010 and 2014. Relationship between partial VP1 coding region of the NPEVs from Ghana and reference strains. Numbers at nodes indicate the percentage of 1000 bootstrap pseudoreplicates supporting the cluster

5. CONCLUSION

The study provided definitive evidence on the circulation of different patterns of NPEVs serotypes in a polio-free country and the importation of new serotypes implicated in AFP. It also indicated the importance of monitoring NPEVs that mimic polio as we approach polio-free world and continuous vaccination for interruption of transmission.

ETHICAL APPROVAL

This study was conducted on specimens collected upon conclusion of routine examinations and stored as anonymous. Patient

identifiers including personal information (name, address) and hospitalization number were removed from these samples to protect patient confidentiality and neither did they appear in any part of document in this study. The research protocol was approved by the Institutional Review Board (IRB reference number DF. 22), Noguchi Memorial Institute for Medical Research. IRB waived the need for consent because the samples were de-identified.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26729