

Original Research Paper

Isolation and Public Health Significance of Non-Polio Enteroviruses in Healthy Nigerian Children

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Abstract: Non-Polio Enteroviruses (NPEVs) are in circulation all over the world. Some of these viruses have been associated with several chronic diseases such as cardiopathy, myositis, acute flaccid and spastic paralysis in children which mimic poliomyelitis. This study sought to ascertain whether Non-Polio Enteroviruses (NPEVs) are silently shed by apparently healthy school children in Bauchi state, Nigeria. This cross-sectional study involved 200 stool samples collected from 170 (85%) vaccinated and 30 (15%) unvaccinated apparently healthy school children from Bauchi, Katagum and Misau local government areas of Bauchi state, Nigeria. All samples were processed and inoculated onto Rhabdomyosarcoma (RD) and L20B cell-lines. Inoculated cell-lines were monitored for Cytopathic Effects (CPE) for 10 days with 1 subculture after first 5 days. None of the samples came down with CPE on L20B however, three (3) samples were positive for NPEVs on RD cell lines. One (1) coxsackie B virus was from a 7 years old male child, and 2 other untypeable isolated were from a male and a female child respectively. All the 3 (1.6%) positive samples were from children not immunized with Oral Polio Vaccine (OPV). The coxsackie B virus was identified by micro neutralization test using polyclonal sera as described by the World Health Organization. Findings from this study indicate the presence of relatively few NPEVs in the study participants. Since NPEVs can implicate persistent fecal-oral transmission and present with serious pathology, it should be considered as serious public health issue. This justifies the need for periodic surveillance of NPEVs in our communities in order to prevent unforeseen NPEVs-associated diseases and promote public health policies that will encourage environmental sanitation programs.

Keywords: Coxsackie B Virus, Microneutralization Test, NPEVs, Nigeria

Introduction

Among the enteroviruses, poliovirus was the most successful pathogen that caused man a lot of suffering. The persistent use of Oral Polio Vaccine (OPV) has resulted to remarkable global eradication of poliovirus except in three countries i.e., Nigeria, Pakistan and Afghanistan. However, Nigeria was recently certified polio-free by the World Health Organisation (WHO) because there was no any case of poliovirus strain reported in the country for over 12

calendar months (WHO, 2015). Considering the widespread use of Oral Polio Vaccines (OPV), more attention has been given to other enteroviruses that could cause aseptic meningitis, cardiopathy and poliomyelitis-like illnesses (Afifi *et al.*, 2009). Enteroviruses have been isolated from patients with variety of illnesses and occasionally from apparently normal people (Juliana *et al.*, 2014).

NPEVs are endemic worldwide and cause very common infections of childhood. Apparently healthy children are the major reservoir for enteroviruses

(Dhole *et al.*, 2009). Because of poor standard of hygiene, NPEVs infections are very common in developing countries (Rifqiyah *et al.*, 2009). They are transmitted through fecal-oral route and via respiratory droplets during acute infections. Enterovirus shedding in the faeces of persons continue for three weeks after being infected. Infections occur all year round with higher rates during rainy seasons (Juliana *et al.*, 2014). Although 70-80% infection are often asymptomatic, these viruses have also been associated with occasional outbreaks in which larger than usual number of patients develop clinical diseases with severe and fatal consequences (Juliana *et al.*, 2014). Baba *et al.* (2012) indicated that non-polio enteroviruses were in circulation in Borno and Adamawa states of Nigeria. In their study, twenty two (22) of the four hundred and nine samples collected from children aged 5-16 years were positive for non-polio enteroviruses.

To ensure that wild poliovirus transmission has been completely interrupted, surveillance for Non-Polio Enteroviruses (NPEVs) among apparently healthy children in the population to supplement Acute Flaccid Paralysis (AFP) surveillance programme is essential. This helps to identify gaps where poliovirus transmission could occur undetected and allow the timely detection of NPEVs in a previously polio-free area (WHO, 2013).

Since NPEVs have the propensity to induce chronic diseases, this study was instigated in order to identify apparently healthy school children who harbor and shed these viruses in the study area and to enlighten health policy makers on the need to implement, promote and sustain environmental sanitary programs in Bauchi state, Nigeria.

Materials and Methods

Study Area

This cross-sectional study aimed to identify children with faecal shedding of non-polio enteroviruses in three local government areas of Bauchi State. These local government areas include; Bauchi from the south, Katagum from the north and Misau from central zones. Bauchi state has been known and established to be one of the major states with highest polio incidence in Nigeria. Climatically, there is considerably varied weather condition across Nigeria, while it is humidly hot during the early part of the rainy season in the south, the hot and dry dusty weather predominate the northern part of Nigeria.

Study Population

Two hundred (200) fresh stool samples were collected from apparently healthy primary school children in selected schools from Bauchi, Katagum and

Misau local government areas of Bauchi state. Forty-six samples were collected from Katagum, fifty-four from Misau and one hundred from Bauchi. The samples were transported in cold boxes (maintained at 4°C) to the World Health Organization national polio reference laboratory, University of Maiduguri Teaching Hospital (UMTH) for virological analysis.

Sample Size Calculation

The sample size was determined using data from a prevalence study conducted in Nigeria with a prevalence of 5.38%, as demonstrated by (Baba *et al.*, 2012). Therefore, the minimum sample size for a sample proportion with 5% margin of error and 95% confidence level was 80. However, in order to maximum the credibility of the results, we made use of 200 participants.

Sample Collection and Storage

Pea-size stool samples were collected from primary school children aged 3-12 years. It was placed in clean screw-capped universal container. It was labeled with the name of the pupil using a water resistant pen. The specimen was placed in a cold box below 8°C between frozen ice packs. The name, age and gender of the pupils were recorded in an exercise book. The samples were transported to WHO national polio reference laboratory, UMTH, Nigeria. Only apparently healthy male and female children aged 3-12 years were randomly recruited for the study because this age range represents individuals who are at high risk of contracting polio. However, crippled children were not selected for the study. Children below the age of 3 years or above 12 years were excluded and samples other than stool were not required for the study because of fecal-oral mode of polio transmission. Two children from Katagum, five from Misau and nine from Bauchi were excluded from the study because of their ages.

Passaging Cell Culture

The RD and L20B cells were examined for quality (i.e., an entire monolayer of healthy cells) and absence of contamination by microscopy. The growth medium was decanted from the cell culture flask and the confluent cell monolayer washed twice with Ca²⁺ and Mg²⁺ free Phosphate Buffered Saline (PBS). About 0.25% of trypsin solution was added into the PBS monolayer and dispersed evenly. The flask was placed in 36°C incubator until the cells detached from the surface. Completion was checked by examining under inverted microscope. The cells were re-suspended in growth medium to halt the action of trypsin (WHO, 2004). The suspension was gently aspirated 7 times through a fine pasture pipette to break up cell clumps. The cells were diluted with growth medium to desired concentration

based on pre-determined split ratio of 1:3. Fresh flasks were seeded, capped tightly and Placed in a 36°C incubator. The flasks were changed to maintenance medium when the monolayer was nearly confluent after two days. Flasks were subcultured after every 7 days at a split ratio (WHO, 2004).

Stool Extraction

Centrifuge tubes were labeled with stool sample numbers. About 4.5 mL PBS, 1 g of glass beads and 0.5 mL chloroform were added into each tube to kill/remove bacteria/fungi and to dissociate all forms of virus aggregates. About 2 g of each fecal sample was transferred into the labeled centrifuge tube. The tubes were tightly closed and vortexed for few seconds, the mixture was shaken for 30 min on a mechanical shaker and later spun at 1500 g for 30 min in a cold centrifuge. The supernatant was then transferred to cryovials and stored at -20°C.

Inoculation and Isolation

Inoculation of samples and isolation of virus on cell lines were conducted according to procedures contained in the World Health Organization polio laboratory manual (WHO/IVB/04.10).

Identification of Virus Growth in Cell Culture

According to the WHO laboratory manual for polio and NPEVs isolation, Cytopathic Effects (CPE) on L20B cell line characterized by rounding off of cells from their monolayer is typical for polio viruses. Samples are considered negative if there no CPE on L20B cell line for 10 days with 1 subculture after first 5 days. Samples with CPE on Rhabdomyosarcoma (RD) cells were presumptive for NPEVs. Thereafter, samples containing the unknown virus were identified by microneutralization using polyclonal sera containing specific antibodies to 21 of 68 known enteroviruses.

Microneutralization Test

All isolates which show CPE on RD cell lines were selected for microneutralization identification using standard typing polyclonal sera kit prepared by the National Institute of Public Health and Environment (RIVM), Netherlands. The identification was made by analyzing the pattern of inhibition of CPE by the antiserum pools. These procedures were carried out in BSCL-2 (WHO, 2004).

Informed Consent

The purpose of this work was explained to parents of the children before they voluntarily consented to allow their children to participate in the research. The consent forms were appropriated filled by children's class

teachers after which each parent/guardian signed their corresponding forms on behalf of their children. All data were analyzed confidentially throughout the study.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by ethical and human research committee of Bauchi state ministry of health before embarking on the research. The approval number was MOH/GEN/S/1409/I.

Results

Virus isolation technique was used for all the treated and extracted stool samples. A total of 200 were collected from 170 (85%) vaccinated and 30 (15%) unvaccinated apparently healthy primary school children from randomly selected primary schools in Bauchi, Katagum and Misau local government areas of Bauchi state. Out of this number, 110 (55%) were males and 90 (45%) were Females. None of the samples was positive for poliovirus but three of the samples were found to be positive for non-polio enteroviruses. One (1) of the stool samples from a male child was identified as coxsackie B virus, one (1) from female and one (1) from another male had untypeable non-polio enteroviruses. All the 3 (1.6%) positive samples were from children not immunized with OPV. An 11 year old had 1 untypeable NPEV, a 7 year old had coxsackie B virus, while the other untypeable non-polio enterovirus was from a 5 year old child. All the 3 (1.5%) children with positive NPEVs were from Bauchi local government area of Bauchi State, Nigeria.

Discussion

The frequency of NPEVs isolated in this study was very low when compared with the 34 and 20% reported in India (Morens, 1978; Grassly *et al.*, 2010) and 5.38% previously reported in Nigeria (Baba *et al.*, 2012). The differences observed may be attributed to factors such as differences in the sample size, stage/degree of poliovirus endemicity in the study areas and years of studies. The implication of few NPEVs isolated this study indicates that wild polio viruses might be no more circulating in the study area. This is evident because there has been no case of polio from the time of this present study till date (WHO, 2015).

Virus isolation and neutralization assay which allow the identification of NPEVs serotypes are the World Health Organization standard protocol for enteroviruses investigation (WHO, 2004; Grassly *et al.*, 2010). This standard protocol was used in analyzing stool samples of apparently healthy school children from Bauchi state, Nigeria. NPEVs are common in children, however their

public health significance in Nigerian school children has not been well documented.

The coxsackie B virus isolated was from a male child. This conforms to a finding in India Rifqiyah *et al.* (2009) which shows that male children had higher propensity of being infected by enteroviruses than their female counterpart. This is also in conformity with a large body of data which reported that NPEVs infections and diseases occur more frequently in males than females (Laxmivandana *et al.*, 2013). They suggested that biological reasons such as longer duration of virus excretion and higher virus titer in stools were responsible for the higher infection rate among males. It has been found that age is one of the most important determinants of enteroviral infection outcomes, with different age groups having different susceptibilities to infection (Kuramitsu *et al.*, 2005). Due to the low frequency of NPEVs from this study, we cannot categorically establish gender predilection of NPEVs isolated from our subjects.

In this study, the coxsackie B virus isolated from a child of 7 years. This finding however contradicts the findings in India where the isolation rate was significantly higher in the age 0-2 years' group (Rifqiyah *et al.*, 2009). Other viruses including rheoviruses and adenoviruses could also not be ruled out especially in those classified as untypeable particles (Dhole *et al.*, 2009).

In consistence with our findings, studies involving serotyping of NPEVs isolated from stool specimens among healthy children in India and Egypt found Echovirus 6, 11, 9 and Coxsackie B virus to be most prevalent (Grist and Bell, 1984; Muir *et al.*, 1998). NPEVs differences observed in different geographical areas could be that within a given geographic locality, some serotypes may be endemic, with little or only gradual change in the range of serotypes present from year to year (Muir *et al.*, 1998).

Findings from the study of Juliana *et al.* (2014) showed that out of 273 stool samples processed, they detected 13 different serotypes of non-polio enteroviruses. Serotyping of the isolates yielded predominantly coxsackie B viruses followed by echoviruses 13 and 7. More than half of the isolates could not be typed by the antisera pools (untypeable). In our study, 2 isolates could not be identified by the antiserum pools provided by RIVM, Netherlands. Their failure to neutralize a given isolate could be due to the absence of corresponding antibodies in the antiserum pools (Kapoor *et al.*, 2001).

Coxsackie B virus is a naked virus that can be resistant to inactivation thus remains viable for long time. From our study, the child harbouring and shedding coxsackie B virus poses danger of person-to-person transmission particularly if personal hygiene, sanitary conditions of households and schools are poor. School children and parents need to be educated on hygienic measures such as

frequent hand washing before and after eating, more so indiscriminate defecation should be prohibited.

Conclusion

Findings from this study indicate the presence of few NPEVs in study participants. Since NPEVs can implicate persistent fecal-oral transmission and present with serious pathology, it should be considered as serious public health issue. This justifies the need for periodic surveillance of NPEVs in our communities in order to prevent unforeseen NPEVs-associated diseases and promote public health policies that will encourage environmental sanitation programs.

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Author's Contributions

Muhammad Sagir Shehu: Conceptualized the study design, conducted the laboratory analysis and participated in developing the initial draft manuscript.

Yunusa Thairu, Idris Abdullahi Nasir and Fatima Yahaya: Gave input in the study design, developed the initial draft manuscript, critically revised the manuscript before publication and during peer review processes. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there are no competing interests associated with this manuscript.

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