Non-polio enteroviruses among healthy children aged zero to five (0-5) years old in the Philippines

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Abstract

Background One of the indicators of a properly functioning Acute Flaccid Paralysis (AFP) surveillance is the proportion of stool samples in which non-polio enteroviruses (NPEV) are detected. Typically, this proportion is expected to be above 10% in tropical countries, however, the Philippines only averages 6.5% NPEV isolation rate. With continued threat of possible importation of wild poliovirus, the current NPEV rate prompted concerns about the quality of the AFP surveillance, in particular, stool sample management and transportation being performed in the field. To know whether the low NPEV rates gathered from AFP cases is a reflection of the true prevalence in the Philippines, a stool survey of healthy children 0-5 years old from three urban communities was conducted in 2015.

Methods Two stool samples were collected from three hundred sixty healthy children. Virus isolation and Polymerase Chain Reaction was performed to identify enteroviruses in the samples. The results were compared to the prevalence and isolation rate among AFP cases the same year.

Results Results show a prevalence of 24.7% which is higher than 12.4% prevalence rate among AFP cases the similar year. However, analysis of AFP cases between 0-5 years old showed prevalence of 20.8% (p-value 0.33) which is not significantly different when compared to the study.

Conclusion This study supports the idea that the observed low NPEV rate in the country may be due to low number of NPEV prevalence. However, further investigations on all possible data sources for NPEV such as among hand, foot, and mouth disease (HFMD), and aseptic meningitis cases, may be necessary to fully understand the occurrence of NPEVs in the country.

Introduction
In 1988, the World Health Organization (WHO) initiated its goal of eradicating poliovirus globally [1, 2]. This campaign is considered the largest public health initiative and after more than 30 years, from 125 countries endemic of poliovirus, it has gone down to just three countries, in Afghanistan and Pakistan with a decrease of 99% of cases since the start of the initiative – from over 350,000 cases to just 22 cases recorded in 2017 [3]. The Western Pacific Region, the Philippines included, obtained its certification as a polio-free region in October 2000 [4]. Despite this achievement, countries are still vulnerable to poliovirus importation due to the continued circulation of the wild poliovirus in three remaining endemic countries: Nigeria, Afghanistan, and Pakistan [3]. The emergence of vaccine-derived poliovirus (VDPV) also serves as a threat to polio eradication efforts worldwide [5].

In the Philippines, AFP surveillance was established in 1991 under the National Epidemic Sentinel Surveillance System (NESSS) to detect any poliovirus importation and circulation. In 2008, this surveillance program was integrated into the Philippine Integrated Disease Surveillance and Response (PIDSR) system [6]. This surveillance, together with routine oral polio vaccination of children under the Expanded Program on Immunization (EPI), has been successful in keeping the Philippines free from wild poliovirus since the isolation of its last case in 1993.

Through this surveillance, all AFP cases detected by disease reporting units throughout the country are screened for the presence of poliovirus or other enteroviruses in their stool samples. Virus isolation is the recommended testing by WHO to all national reference laboratories for poliovirus detection. However, one of the limitations of this method is its sensitivity as this can be affected by improper sample management, particularly, sample exposure to heat [7, 8]. One of the parameters to gauge this method’s sensitivity is the determination of the NPEV isolation rate among AFP cases.
NPEVs are incidental isolation of enteroviruses other than poliovirus in stool samples among AFP cases, thus, its isolation may be an indicative of proper sample handling and transportation [7]. Enteroviruses of the Picornaviridae family are clustered into species EV-A to EV-L including Rhinovirus A, B and C, of which, four are isolated exclusively in humans (EV-A to EV-D) [9, 10]. Human enteroviruses cause common infections among children, from asymptomatic to even fatal diseases [11, 12]. They are spread mainly through fecal-oral route with highest risk among children due to poor hygiene and low immunity levels. In areas where silent circulation is suspected, asymptomatic children are considered as the main reservoir of enteroviruses [13].

As a tropical developing country, it is expected that the Philippines’ AFP surveillance should have an NPEV isolation rate greater than or equal to 10% [14, 15]. This value was used as one of the indicators of a properly functioning AFP surveillance, particularly of proper stool management and transportation [14]. However, the average NPEV isolation rate among AFP cases in the Philippines from 2000 to 2015 is only 6.5%, as reported by the National Reference Laboratory for Poliovirus and other Enteroviruses of the Research Institute for Tropical Medicine (NRL Polio and other Enteroviruses-RITM). This consistently low NPEV isolation rate raises curiosity on the actual prevalence of NPEV in the country. Several studies in other countries, particularly in China, Ghana, and Egypt, have been conducted to establish enteroviral prevalence on healthy children and the results among these countries suggest 10 to 24% NPEV detection rates [16-19].

To address this gap in knowledge, this study aimed to establish a baseline prevalence of NPEV among healthy children aged zero to five (0-5) years old in three selected sites in the Philippines in 2015.

Methodology

Study design
A descriptive, cross-sectional study was designed to determine the prevalence of NPEV among healthy children aged zero to five (0-5) years old in three major urban cities in the Philippines (Figure 1). Specifically, the study was conducted in urban areas in three different regions: (1) National Capital Region (NCR) in Barangay Addition Hills, Mandaluyong, Metro Manila; (2) Region VII in Barangay Carreta, Cebu City; and (3) Region XI in Barangay Buhangin, Davao City, Davao Philippines.

These sites were purposively selected as they were near the Department of Health-Regional Offices and had a barangay health center with sufficient cold storage equipment to store the collected stool samples. The minimum sample size computed for this study was 292. This was based on a 10.6% NPEV prevalence from a similar study conducted in China [18], 95% level of confidence, 5% precision estimate, design effect of 2. Additional 68 children were added to compensate for possible non-response, thus, a total of 360 healthy children (120 per site) aged zero to five years old were randomly selected from a sampling frame gathered through a survey of the health workers in their assignment areas. Only one child per household was permitted to join the study and the current health status of the participants was established to allow only healthy children to participate.

“Healthy child” was defined as a child who, by clinical history and physical examination, did not present with symptoms that may be associated with enteroviral infections such as acute flaccid paralysis, diarrhea, fever, cough, colds, conjunctivitis, and hand, foot and mouth disease.

The study protocol was submitted to and approved by the RITM- Institutional Review Board and was conducted in compliance with the principles of the Declaration of Helsinki. Since the study participants are children aged zero to five years old, written informed consent was sought from the children’s parent or legal guardian. After the completion of viral testing, parents and guardians were informed of their ward’s results and those found to be
positive for enteroviruses were advised to visit their health center physician for proper clinical management.

**Study Procedures**

**Stool survey**

Standard physical examination and history taking were performed by the study physician to assess the child’s health status. Signs and symptoms that may indicate current enteroviral infection, such as acute flaccid paralysis, gastroenteritis, influenza-like illness, encephalitis, myocarditis, hand, foot, and mouth disease, and conjunctivitis were evaluated. If the child was found healthy, the parents or guardians were instructed to collect two stool samples from their child, at least 24 hours apart. Two stool samples were required to compensate for the intermittent shedding of the virus [8, 20]. To ensure that cold chain is maintained, parents and guardians were provided appropriate sample containers, ice pack, and a detailed instruction on proper sample collection. After collection, they were advised to forward samples to the barangay health center. From there, the samples were shipped by the Regional Epidemiology and Surveillance Unit staff to the NRL Polio and other Enteroviruses-RITM for testing.

**Virus isolation**

Stool samples were labeled with the study identification numbers prior to sample processing and testing. Virus isolation was performed at the NRL Polio and other Enteroviruses-RITM, following the WHO standard procedures for poliovirus isolation. Briefly, all stool extracts were inoculated in RD-A and L20B cell lines and observed for a total of 10-14 days. The infected tissue culture fluids (ITCF) showing the characteristic cytopathic effect (CPE) of enterovirus (rounding necrosis) were harvested and stored for subsequent testing [7].

**Sequencing**
EV positive ITCFs were selected and sent to the National Institute for Health- Korea Center for Disease Control and Prevention (NIH-KCDC) for partial VP1 gene sequencing according to the institute’s protocol. In case both stool samples of the child yielded NPEVs, only the first stool isolate was included for sequencing.

In brief, viral ribonucleic acid (RNA) was extracted using Tecan Freedom Evo™ (Tecan Group Ltd, Männedorf, Switzerland). Enterovirus gene was amplified through polymerase chain reaction (PCR) using iNtRON iNNOPLEX™ Enterovirus VP1 detection kit (Bulldog Bio, Inc., Portsmouth, New Hampshire). Amplified products were then sent to Macrogen Korea and to Cosmogenetech Korea for Sanger sequencing. Sequencing results were cleaned and aligned using DNA Star™ software (DNASTAR, Inc., Madison, Wisconsin) and MEGA software v.7 A BLAST search was then conducted to identify the enterovirus serotype [21]. Sequence generated from the study were submitted to GenBank with accession numbers: MK959771 to MK959836 and MK977636 to MK977640

**Statistical Analysis**

All questionnaires and signed informed consent forms were checked in the field for completeness. Data entry was performed using Epi Info v. 3.5.3 (Centers for Disease Control and Prevention, Atlanta, Georgia). Comparison of sex, age group and location with the NPEV prevalence as well as comparison in between groups was analyzed by using Chi-square test, and p-values <0.05 were considered statistically significant. The data gathered from the study were compared to AFP surveillance cases among 0-5 years old and to the AFP surveillance results 2015.

**Results**

**Prevalence of NPEV**

A total of 360 healthy children from three major urban sites (120 per site) in the Philippines were enrolled in the study in order to determine baseline NPEV prevalence in
their area. The mean age of the participants is 2.4 and an almost equal proportion of males and females, 51% and 49%, respectively.

Of the 720 total collected stool samples from 360 children, NPEVs were isolated in 129 (17.9 %) samples from 89 cases. Of these 129 samples, 126 were single isolates of enteroviruses while three were mixed with a poliovirus type 3, Sabin-like strain. NPEVs were isolated in 89 children (89/360) or 24.7%. Among these positive cases, 50.6% (45/89) are females and 60.7% (54/89) belong to the 1 to 3 years old age group. Among the three study sites, Region VII had the highest detected case prevalence with 51 out of the 120 study participants (42.5%) yielded at least 1 NPEV from their stool samples.

NPEV case prevalence was different among the study sites ($X^2=32.81$, p-value=$<0.001$, df=2). Likewise, the NPEV case prevalence in Region VII was highest followed by NCR and Region XI, respectively. Analysis showed also that there was no significant difference in NPEV case prevalence among males and females and also between age groups in the study [Table 1].

Table I. Demographic information of participants, 2015

| Characteristics          | NPEV Positive No. | NPEV Negative No. | X^2 test |
|--------------------------|-------------------|-------------------|----------|
|                          | %                 | %                 |          |
| **Sex**                  |                   |                   |          |
| Males                    | 44                | 141               | 0.09     |
| Females                  | 45                | 130               |          |
| **Age Group**            |                   |                   |          |
| <1 year                  | 13                | 47                | 3.71     |
| 1-3 years                | 54                | 133               |          |
| 4-5 years                | 22                | 91                |          |
| **Geographic Location**  |                   |                   |          |
| NCR                      | 24                | 96                | 32.81    |
| Region VII               | 51                | 69                |          |
| Region XI                | 14                | 106               |          |

Comparative analysis on the NPEV case prevalence [Table 2] and NPEV isolation rate [Table 3] reported by the study with those reported by the AFP surveillance in 2015 was also done. The NPEV case prevalence as well as the NPEV isolation rate for all study sites was higher than those reported from the overall AFP surveillance prevalence among cases
and isolates in the country (p-value <0.001; \( \alpha = 0.05 \)). NPEV case prevalence (p-value 0.010, \( \alpha = 0.05 \)) and NPEV isolation rate (p-value <0.001, \( \alpha = 0.05 \)) among healthy children from Region VII were higher than those reported from the AFP surveillance of the same region. However, there is no difference between the case prevalence of NPEV and NPEV isolation rate from the study and AFP surveillance from NCR and Region XI as their p-values are greater than 0.05 [Table 2 and 3].

Comparing the NPEV rates from the study and AFP surveillance of similar age, there was no significant difference in both NPEV case prevalence and NPEV isolation rate except for Region XI where there was a difference in terms of NPEV isolation rate but among cases (p-value 0.046) [Table 2 and 3].

Table II. Comparison of NPEV case prevalence among healthy children, AFP cases, and AFP cases 0-5 years old, 2015
| Case | NPEV Positive | NPEV Negative | \( \chi^2 \) test | p-value | Case | NPEV Positive | NPEV Negative | \( \chi^2 \) test | p-value |
|------|---------------|---------------|----------------|---------|------|---------------|---------------|----------------|---------|
| No.  | %             | No.           | %              |         | No.  | %             | No.           | %              |         |
| NCR  |               |               |                |         |      |               |               |                |         |
| HC   | 14            | 11.7          | 81             | 0.15    | HC   | 14            | 11.7          | 25             | *0.40    |
| AFP  | 9             | 7.0           | 3.3            |         |      | 7             | 2.6           |                |         |
| Region VII | | | | | | | | | |
| HC   | 51            | 42.2          | 69             | 6.7     | HC   | 51            | 42.2          | 69             | *0.38    |
| AFP  | 4             | 15.4          | 57             |         |      | 5             | 15.4          |                |         |
| Region XI | | | | | | | | | |
| HC   | 24            | 20.0          | 96             | 0.04    | HC   | 24            | 20.0          |                | *0.20    |
| AFP  | 8             | 31.6          | 81             |         |      | 8             | 31.6          |                |         |
| All sites/ Philippines | | | | | | | | | |
| HC   | 89            | 24.7          | 271            | 21.6    | HC   | 89            | 24.7          |                | 0.93     |
| AFP  | 60            | 7.1           | 424            |         |      | 60            | 7.1           |                | 0.334    |

*Fisher exact test p-value

Table III. Comparison of NPEV isolation rate among healthy children, AFP cases, and AFP cases 0-5 years old, 2015
| Isolate | NPEV Positive | NPEV Negative | $X^2$ test | p-value | Isolate | NPEV Positive | NPEV Negative | $X^2$ test | p-value |
|---------|---------------|---------------|------------|---------|---------|---------------|---------------|------------|---------|
|         | No. | %    | No. | %    |         | No. | %    | No. | %    |         |
| NCR     |     |      |     |      |         |     |      |     |      |         |
| HC AFP  | 20  | 8.3  | 220 | 91.7  | 0.01   | 0.931 | HC AFP 0-5 | 20  | 8.3  | 220  | 91.7  | *0.59  |
|         |     |      |     |      |         |     |      |     |      |         |
| Region VII |     |      |     |      |         |     |      |     |      |         |
| HC AFP  | 77  | 32.1 | 163 | 6.7   | 11.9   | <0.001| HC AFP 0-5 | 77  | 32.1 | 163  | 6.7   | *0.13  |
|         |     |      |     |      |         |     |      |     |      |         |
| Region XI |     |      |     |      |         |     |      |     |      |         |
| HC AFP  | 32  | 13.3 | 208 | 86.7  | 0.14   | 0.709 | HC AFP 0-5 | 32  | 13.3 | 208  | 86.7  | 0.046  |
|         |     |      |     |      |         |     |      |     |      |         |
| All sites/ Philippines |     |      |     |      |         |     |      |     |      |         |
| HC AFP  | 129 | 17.9 | 82.1 | 591  | 23.7   | <0.001| HC AFP 0-5 | 129 | 17.9 | 591  | 82.1  | 0.16   |

*Fisher exact test p-values

**Molecular Sequencing Result**

The study identified a total of 19 different enterovirus serotypes with majority belonging to species Enterovirus B (EV-B) with 11 different serotypes detected. The predominant circulation pattern of the EV-B species was seen in all sites – NCR at 57%, Region VII at 63% and Region XI at 34%. No subtype or serotype under EV-D was detected. Isolation from group A (16%) was higher than EV-C (9%). Coxsackievirus B1 is the most common as it comprised 29.9% of the NPEVs identified. In this study, due to the limitation of the method used, only 81% of the NPEVs isolated were characterized. The remaining 19% of NPEVs did not produce clean sequences and thus, were termed as untypable NPEVs (uEVs).

**Discussion**
The study aims to determine the baseline NPEV rate among healthy Filipino children aged five years old and below. In this study, the case prevalence is shown to be 24.7%, as NPEVs were isolated in the stool sample of 89 out of 360 enrolled participants. In contrast with the studies done in Indonesia and South-Western India [22, 23], the notion that males are more likely to contract EV infection was not established in this study. The study revealed that NPEV case prevalence among these age brackets (<1, 1-3 and 4-5) were almost equal indicating that the chances of NPEV infection among these age groups, from infancy to preschool age, is comparable. The NPEV isolation rate in this study is evidently higher than that of the average NPEV isolation rate of AFP surveillance in the Philippines which is at 6.5% from 2000-2015 or in similar year at 9.7% when the study was conducted. Taken in this context, this high NPEV isolation rate among healthy children could only mean that NPEVs are silently circulating in the community and may reflect that true NPEV infection may have gone unnoticed or underreported in the existing EV surveillance programs. With the disparity observed on NPEV isolation rate, there is a need for more exploratory surveys to identify risk factors such as environmental sanitation, population density in the area as well as immunity of the population. Likewise, from the surveillance side, inclusion of factors such as cold chain management and transportation time that might influence the disparity of NPEV isolation rates as seen in the two sets of population, AFP versus healthy children, must also be investigated.

Despite the result showing a higher case prevalence and isolation rate in the study compared to the AFP surveillance, this difference may be influenced by the site selection and proper management of cold chain from the point of collection to receipt of samples at the testing laboratory. The study was conducted on urban-poor communities, which increases the risk level of excreta related infections [15, 24] and this living condition is not reflective of the whole country unlike the AFP surveillance which is conducted
nationwide. Likewise, concerns on the condition of AFP samples received in the reference laboratory have been noted, such as unmaintained cold chain, that may explain the lower NPEV isolation rate in AFP surveillance as opposed to the study where the optimal cold temperature was sustained. Another factor which may clarify the lower NPEV rate in AFP is the time of sending of AFP samples from the date of collection, taking longer even up to 162 days according to surveillance data in 2015, which may have affected the viability of the enteroviruses present in the stool. Past hospital-based monitoring of AFP surveillance system revealed that stool specimens are kept at bedside for significant period of time as collection of specimens are relied to parent/caretaker of in-patients. These factors observed from AFP surveillance indicate poor reverse cold chain practices and low compliance to surveillance performance indicators. In this study, strict compliance to the maximum three days of transit time from collection to receipt in the reference laboratory was implemented.

A study conducted in Sweden from 2003 to 2007 has shown that the peak of EV isolation is among 18 month-old participants [25] and several other studies suggest that enteroviral infection is greater among younger children [16, 22, 26]. The fact that the study participants’ ages (0-5 years old) do not match those of the AFP surveillance cases (0-15 years old) might be a factor for the difference in NPEV case prevalence and isolation rate. To see if age played a role in the discrepancy in case prevalence, an analysis comparing the NPEV prevalence data from the study participants and from AFP surveillance among children aged 0-5 years old collected in 2015 was performed. There is no difference between the prevalence of NPEVs collected from AFP cases and healthy children with similar age bracket [Table 2]. This result, therefore, supports the findings of other studies that NPEV rates are indeed higher among children [16, 22, 26]. A study with similar demographic profiles including socio-economic conditions and age coverage of AFP cases
is encouraged to ascertain the true NPEV case prevalence and isolation rate in the Philippines.

Previous study on the characterization of enterovirus isolates from AFP cases in the Philippines showed that the circulating isolates are, in decreasing order, species B, followed by C then by A species [27]. This circulation pattern is also seen in other studies from Asian countries [28, 29]. This pattern was in contrast with the results of this study wherein a minor difference in the proportion was seen for EV-A and EV-C species. While all sites followed the isolation pattern of EV-B > EV-A > EV-C, there is a variation in the proportion of species detected per region especially in NCR where EV-A has almost the same as EV-B isolation. All of the detected EV serotypes among healthy children mirror the EV serotypes isolated from AFP cases and environmental samples in previous reports except for one isolate which is CV-A22. The detection of CV-A22 in the Philippines was first identified and documented in this study [27, 30].

Coxsackievirus A22 was first isolated from a healthy person from Chulman, Russia [31]. This type is rarely seen because of its difficulty to be isolated through tissue culture techniques [32]. In this study, the isolate came from a three years old child from Region XI.

Likewise, the isolation of CV-B1 in all sites is consistent with the previous study where CV-B was classified as one of the EV serotypes with endemic circulation in the Philippines [33]. More echoviruses were also detected than CV-A supporting other studies that indeed, the transmissibility of echoviruses is greater than CV-As [16, 34].

The study was able to detect EV-A71 and CV-A16 in healthy children and this finding is significant as these are usually associated with hand, foot and mouth disease [35-37]. This finding, however, is not exclusive to this study, as a similar result was seen in the survey of healthy children conducted in Shenzhen and Yunnan Province, China [18, 19]. These
enterovirus types were also detected in previous studies on NPEVs among AFP cases and environmental samples in the Philippines, which may be suggestive of indigenous circulation of these pathogens in the country [27, 30]. Further analysis indicated that the EV-A71 in this study belonged to genogroup C, specifically the C2 cluster which is genetically homologous to the EV-A71 C2 cluster reported among AFP cases [33]. While neighboring countries in the Asia Pacific region revealed high mortality rates caused by EV-A71 of the genogroup C like in China, [38] the Philippines has yet to report any fatal case. Data from a comprehensive and longitudinal hand, foot and mouth disease study on the overall EV-A71 epidemiology is crucial to conclude that C2 cluster of EV-A71 found in the Philippines is only causing mild disease or even asymptomatic infection.

The molecular method used in the study limits the detection of enteroviruses as uEVs accounts for 19% of the overall EV isolation. For a more comprehensive picture on diversity of EVs among healthy children, a more sensitive method that could identify all possible EV serotypes should be used for subsequent studies. Nonetheless, the method was able to detect newer enteroviruses like EV99.

Conclusion

The study was able to establish a baseline NPEV case prevalence of 24.7% among healthy children aged zero to five in three major urban sites in the Philippines and was able to determine EV-B as the most prevalent type. This NPEV case prevalence is higher compared to the NPEV prevalence among AFP cases in 2015 which is 12.4%. Though the NPEV prevalence in this study is higher, no significant difference was observed when compared with the same age group among AFP cases. The gap in the overall NPEV prevalence seen in healthy children versus in AFP, however, may be due to age difference as this study concentrated to the known affected age group of 0-5 while in AFP, it is diffused until 15 years of age. Another consideration may be due to the sites selected which were urban-
poor communities. Several other factors were also pointed out in AFP surveillance such as poor reverse cold chain practices and longer transit time from collection to receipt in the testing laboratory.

From this study, the high isolation of NPEV among healthy children signifies continuous fecal-oral transmission and given the multitude of diseases NPEV is causing, a routine surveillance targeting diseases or syndromes caused by EVs, such as HFMD, acute encephalitis and aseptic meningitis, is needed. Further investigation using a study population and site distribution similar to AFP cases is necessary in order to conclusively say that a rate of 10% NPEV rate can be consistently achieved. As this study was conducted in conformance to standards for specimen handling and transport which may have influenced the higher NPEV isolation rate compared with AFP, it is encouraged that AFP surveillance workforce in the field especially those directly involved in monitoring the flow of specimen – from collection to receipt in the laboratory – must firmly adhere to proper specimen management. This action entails training of field staff and all stakeholders from hospitals to the laboratory. Subsequently, it is recommended that a regular assessment on the compliance with good practices for handling and shipping specimens as regards to reverse cold chain be carried out by the Epidemiology Bureau-Department of Health (EB-DOH) as the oversight of the field-based implementation of AFP surveillance in the Philippines.

Declarations

**Ethics approval and consent to participate**

The study was approved by Research Institute for Tropical Medicine-Institutional Review Board on December 9, 2014 (RITM IRB 2013-038). A written informed consent in a language understood by the parents/guardians was given and explained prior to enrolling the participants.
Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are not publicly available due to data privacy protection of participants but are available from the corresponding author on reasonable request.

The partial VP1 sequences of the enterovirus were deposited in GenBank under accession numbers: MK959771 to MK959836 and MK977636 to MK977640

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

MDJ, AOT, MC, YJ, VR, MM, FN and CT formulated the goals and developed the framework of the research study. MDJ, AOT and LNGA coordinated and supervised study site activities. MDJ performed laboratory work and data cleaning and, together with LNGA, drafted the initial paper. All authors read and approved the final manuscript.

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Figures
Figure 1

NPEV isolates among healthy children, Philippines, 2015