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Volume 2 • Issue 4 • 2018

Research Article

Clinical Biotechnology and Microbiology

ISSN: 2575-4750

Environmental Surveillance of Polioviruses: Five Years' Experience in Malaysia Urban Population Before and After Withdrawal of Oral Polio Vaccine

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Received: July 03, 2018; **Published:** July 12, 2018

Abstract

Introduction: After unsuccessful eradication of polio in 2000, the year 2018 was choosen as a new date for global eradication of poliomyelitis. The success of this programme relies on laboratory based surveillance of acute flaccid paralysis (AFP) cases to monitor the circulation of wild poliovirus in the population and the sufficient vaccination coverage in the community. However, as poliovirus can survive in the sewage environment, detection of poliovirus through environmental surveillance (ES) in the absence of clinical cases is very important to detect any silent transmission of polioviruses as well as circulating vaccine derived poliovirus (cVDPV). This study aimed to assess and determine polioviruses existence in sewage plants covering the urban population in Malaysia between 2012 to 2017.

Method: Sewage water was collected using the grab method once a month at identified locations. Samples were processed according to World Health Organization (WHO) standard protocol and viruses were concentrated by two-phase separation method using polyethylene glycol and dextran. Two cell lines, human rhabdomyosarcoma (RD) and mouse cell line expressing the gene for the human cellular receptor for poliovirus (L20B) were used for virus propagation and isolation. Polioviruses were identified by real time reverse transcriptase polymerase chain reaction (rRT-PCR) for intratypic differentiation (ITD) and vaccine derived poliovirus (VDPV) whereas non-polio enteroviruses (NPEVs) were identified by PCR and sequencing.

Results: A total of 13 polioviruses and 103 NPEVs were isolated throughout the study. All polioviruses were detected before the cessation of oral polio vaccine (OPV). No poliovirus was detected after the introduction of injectable polio vaccine (IPV) implemented in immunization programme. Real time RT-PCR revealed that all belonged to Sabin-Like virus and no wild poliovirus (WPV) or circulating vaccine derived poliovirus (cVDPV) was detected. Analysis of the VP4 gene of NPEV showed all were grouped in human enterovirus B (HEV-B).

Discussion/Conclusion: This is the first ES study conducted in Malaysia. It indicates the usefulness of ES for indirect monitoring and detecting the introduction and silent circulation of WPV and cVDPV before it reached the community.

Key Words: *Environmental surveillance; Poliovirus; sewage; Wild poliovirus; Circulating vaccine derived poliovirus; Polio eradication programme*

Volume 2 Issue 4 July 2018

Introduction

Poliomyelitis is a crippling and highly infectious disease cause by the poliovirus (http://www.who.int/mediacentre/factsheets). The disease mainly affects children under the age of 5 years old and is caused by one of the three serotypes of polioviruses (PV): PV1, PV2 and PV3 [1]. Now only WPV1 and WPV3 still circulating whereas the WPV2 has been eradicated globally in September 2015 with the last case was detected in India in 1999 (http://www.who.int/mediacentre/factsheets/fs114/en/). Till now, only WPV1 has been reported circulating in Afghanistan, whereas WPV3 has not been reported in the three endemic countries since 2017 (http://polioeradication.org/polio-today/polio-now/this-week/).

After global polio eradication initiative was launched in 1988 [2-4], the incidence of WPVs transmission has declined up to 99% [5]. Today, wild poliovirus (WPV) still remain endemic in two regions; South East Asia Region (Afghanistan, Pakistan) and Africa Region (Nigeria). Until PVs transmission is completely interrupted in these countries, risk of PVs importation is high, especially in countries with weak public health programme, low immunization coverage and has travel or trade links to endemic countries.

The standard surveillance for polio eradication is through clinical detection of acute flaccid paralysis (AFP) cases in children followed by laboratory confirmation [6]. The sensitivity of AFP surveillance is limited because in a fully susceptible population only a small fraction of infections leads to AFP and in immunised communities, ratio infection to paralysis is even higher [7]. Therefore detection of poliovirus circulation by environmental surveillance (ES) in the absence of clinical cases is very important to detect the silent transmission of PV [7,8]. Confirmation that poliovirus circulation has stopped must be based on detection of PV itself rather than its clinical manifestations as PV could be consistently isolated from the environment in regions with no recorded cases of flaccid paralytic [9,10] .

Studies have demonstrated the usefulness of ES to determine the presence of PVs circulating in the communities [11,12]. These had been proven in many countries such as Nigeria where WPV1 was isolated from multiple sewage samples. In Egypt, WPV1 was isolated from two samples collected in Cairo in 2012 [13] although WPV has not been detected in persons with AFP since 2004 [14]. Israel has detected 67 WPV1 from sewage samples [15,16], without single isolation of WPV in AFP patients.

The World Health Organization (WHO) has included ES in the new Strategic Plan of the Global Polio Eradication Initiative, as a supplement to AFP surveillance. Combination of these surveillances could be more sensitive to detect low circulation of WPV and circulating vaccine derived poliovirus (cVDPV), in order to sustain poliovirus eradication.

In a polio free country like Malaysia, ES is very valuable to determine whether the virus is still circulating in a community that appears healthy. Malaysia has been free of indigenous and imported WPV circulation since 1984 and 1992 respectively and attained polio free status together with other countries in the Western Pacific Region in 2000. Nevertheless the risk of importation of WPV from endemic countries such as Pakistan, Afghanistan and Nigeria is a real threat to Malaysia as it has migrants and visitors from those countries.

So, in this paper, we discussed about ES in Malaysia, where not only to detect WPV but also cVDPV which can cause paralysis and trigger the AFP outbreaks. This virus could survive in sewage treatment plants and in the environment for several months [7] and this will give us indicators for successful of polio eradication programme.

Materials and Methods

Sampling sites

In Malaysia, although AFP surveillance and immunization coverage were good, but there were certain states/areas with poor achievement for the AFP surveillance indicator which was 1 per 100,000 children under 15 years old. Certain areas in the state of Selangor, Kuala Lumpur, Negri Sembilan and Sabah were identified for ES based on the poor achievement in AFP surveillance indicator and have a risk of WPV importation as these areas also had high mobility of foreign workers and migrants.

Sampling technique and schedule

The grab method protocol introduced by WHO was used for sampling purposes [17]. Briefly, 1 liter of flowing sewage water was collected at peak morning flow (6-10 am) once a month at three (3) selected plants. These plants had high population coverage including the residents of migrants and foreigners. Samples were maintained at 4°C or reverse cold chain until reaching IMR laboratory.

Processing sample and isolation of poliovirus

Processing samples and laboratory testing were done following [17] protocols. It was done separately from the work space for AFP cases to avoid any cross contamination. Briefly, about half (500 ml) of the collected raw sewage specimens were aliquoted 50ml each into 5 centrifuged tubes and centrifuged for 10 minutes at 1000g. The other half was kept at 4°C as a backup. Supernatants were pooled in a 1 liter Erlenmyer flask and the pellet was kept at 4°C.

The pH of supernatants were adjusted to 7.0-7.5. Later 39.5 ml of 22% dextran, 287 ml of 29% PEG6000, and 35 ml 5N NaCl were added, mixed thoroughly and kept in constant agitation for 1 hour at 4°C using a horizontal shaker or magnetic stirrer. The mixtures were poured into 1 liter separation funnel attached to the stand and left overnight at 4°C. The entire lower layer and the interphase were collected slowly into a sterile tubes, then mixed with the pellet that already harvested previously. Extraction with chloroform was done following the WHO protocol for extraction of stool samples [18]. Finally, 500ul of extracted samples were inoculated into 3 flasks (25 cm^2 flask) of each L20B and RD-A cells.

Poliovirus Intraypic differentiation (ITD)

All L20B positive cultures following new algorithms of PVs isolation were characterized using a RT-PCR kit [19]. This kit was run on Real-time ITD Assay using 5 set of primers: Quadriplex (Pan EV, Sabin 1, Sabin 2, Sabin 3), Pan Polio (PV 1, 2 and 3), Duplex WPV1 (AFR WPV1, SOAS WPV1), AFR WPV3 and SOAS WPV3. Isolates confirmed as Sabin-like viruses were run on Real-time VDPV Screening Assay to determine whether it is a real Sabin-Like or discordant Sabin-Like. Any Sabin-like discordants were further characterized by sequencing of the full VP1 gene. Identification of non-polio enterovirus (NPEV) isolates were done by amplifying and sequencing the VP4 gene, a short DNA fragment for serotyping entroviruses [20]. Sequence data were then analysed to determine the serotypes by using BLAST-search in GenBank.

Results

The ES was started in 2012 with 3 sampling sites in the Federal Territory of Kuala Lumpur. All sites had population more than 300,000 with foreigners and migrants from endemic countries. Thirteen PVs (0PV1, 6PV2, 7PV3) were isolated together with NPEV. All PVs (PV2 and PV3) were confirmed as Sabin-Like. NPEVs were isolated at every plant through out the year and comprised of ECHO viruses (E5, E6, E7, E30) and Coxsackie viruses (CB4 and CB5) (Table 1).

Location	Jan	Feb	Mac	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bandar Tun Razak	E7	P3	P3	P3	P2	CB4	CB5	CB4	CB4	CB4	CB4	E7
Taman Mawar, Puchong	E7	E7	E30	CB4	P2	P2	E11	P2	P2	E7	P2	CB5
Pantai Dalam	E6	P3	P3	E6	E6	E5	P3	P3	CB5	CB5	CB4	E7

Table 1: *Types Of Poliovirus And Npev Isolated In Kuala Lumpur Sewage Plants In 2012.*

In 2013, due to logistic problems, the ES was only commenced in February with 3 new plants in the state of Selangor. PVs were detected in 2 plants, Bandar Sunway (PV3) and Taman Sri Muda (PV1 and PV2) while another plant which is in Subang Damai had only NPEV isolated (E6, E11, CB2, CB3, CB4) (Table 2). PV1 and PV2 isolated from Taman Sri Muda in June and September respectively were Sabin-Like discordant by rRT-PCR. These samples were sent to Victoria Infectious Disease Reference Laboratory (VIDRL) in Melbourne Australia to sequence the complete VP1 gene. There were nucleotides changes but the changes were not significant to conclude as Non-Sabin-like viruses (NSL). Changes of ≥ 10 nucleotides for Sabin 1 and ≥ 6 nucleotides for Sabin2 compared to the prototype strains considered as NSL virus. Others PVs isolated were also confirmed as Sabin-Like.

Location	Jan	Feb	Mac	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bandar Sunway	Ns	E33	CB1	P3	NS	E6	E11	E6	E7	E11	E6	E6
Taman Sri Muda	Ns	CB4	CB4	CB2	CB2	P1*	E11	P2	P2*	CA9	E6	CB5
Subang Damai	Ns	E11	Ns	CB2	CB4	CB4	CB3	CB4	E11	Neg	E6	CB4

Table 2: *Types of poliovirus and NPEV isolated in Selangor sewage plants in 2013.*

Notes:
NS = No sample taken
Neg = No virus isolated
* = Sabin-Like discordant

The ES continued in 2014 with the 2 plants in Selangor with poliovirus isolated in the previous year. A new plant in Mantin Negeri Sembilan was included in the survey. PVs were isolated at each plant together with NPEVs (Table 3) and All PVs were confirmed Sabin-Like.

Location	Jan	Feb	Mac	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bandar Sunway	Ns	Ns	E11	P1	P2	E3	E3	Neg	E3	E3	CB4	E6
Taman Sri Muda	Ns	Ns	E3	P3	P2	P2	P1 + P2	Neg	P3	E6	E6	CB5
Mantin NS	Ns	Ns	CB3	P3	E6	CB5	Neg	Neg	Neg	P1	E13	E7

Table 3: *Types of poliovirus and NPEV isolated in Selangor and Negeri Sembilan sewage plants in 2014.*

Notes:
NS = No sample taken
Neg = No virus isolated

The ES was stopped for a while in 2015 and resumed in mid-2016 with Likas in Sabah was choosen as a new location. From June to December 2016, only NPEVs were detected without any poliovirus (Table 4) and similar finding was observed in 2017, where no PVs were detected except NPEVs (Table 5).

Location	Jan	Feb	Mac	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bandar Tun Razak	Ns	Ns	Ns	Ns	Ns	E6	E7	E6	E6	E6	E6	E7
Cyberjaya	Ns	Ns	Ns	Ns	Ns	E6	E6	Neg	E6	E19	E19	Neg
Pusat Pembentongan Likas	Ns	Ns	Ns	Ns	Ns	Neg	Neg	Neg	Neg	Neg	CB1	E6

Table 4: *Types of poliovirus and NPEV isolated in Selangor and Sabah sewage plants in 2016.*

Location	Jan	Feb	Mac	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bandar Tun Razak	E6	E7	E7	E7	E7	E7	E7	CB5	E7	E7	E3	E3
Cyberjaya	E7	E6	CB1	E7	CB5	E6	E7	CB5	E7	E7	E3	E3
Pusat Pembentongan Likas	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	E6	Neg	Neg	Neg

Table 5: *Types of poliovirus and NPEV isolated in Selangor and Sabah sewage plants in 2017.*

Notes:
NS = No sample taken
Neg = No virus isolated

Discussion

Malaysia together with other countries in the Western Pacific Regions (WPR) had been certified polio free on 29th October 2000 (http://www.wpro.who.int/topics/poliomyelitis/en/). The last indigenous WPV case was reported in 1984 but in 1992, Malaysia detected imported cases of WPV. Sequencing data showed that the strain was similar to WPV from India. Today, with three countries, Afghanistan, Pakistan and Nigeria still endemic for polio (http://www.wpro.who.int/topics/poliomyelitis/en/), therefore all countries in the WPR have risk of importation of WPV as reported in Singapore in 2006 where the virus was proven to be from Nigeria. Australia and China in 2007 and 2011 respectively also had reported cases of WPV from Pakistan (http://www.wpro.who.int/mediacentre/releases/2010/).

Even though Malaysia has been declared as WPV free country in 2000 and so far no report of cVDPV till now, but surveillance of AFP in clinical cases is on-going. AFP is required to be notified administratively. Case definition used is any child under 15 years olds presented with AFP cases for whatever causes except injuries must be fully investigated with 2 stool specimens collected at least 24-48 hours apart but within 14 days of onset of paralysis. This surveillance programme is vital to exclude WPV or cVDPV circulating in Malaysia via importation from endemic countries.

Together with clinical cases surveillance, ES was introduced in 2012 in order to expand the polio surveillance system. It could be considered as a powerful tool to detect circulating WPV and cVDPV before the virus reach community and probably invoke the outbreaks. It also provides an early detection of viruses before emerging as clinical cases as conducted in Nigeria where ES findings were used as a guide in public health response to polioviruses [21] .

More importantly it could verify the existence of WPV in the absence of clinical AFP cases as shown in studies in India [22] and Nigeria in 2011-2012 [23]. Currently, ES has become an important strategy in the hunt for the final reservoir for poliovirus [7, 24].

In the 5 years study of ES in Malaysia before cessation of oral polio virus vaccine (OPV) and after introduction of injectable polio virus vaccine (IPV), results showed different isolation of PVs and NPEVs. A total of 9 sewage disposal plants located in urban highly

populated areas in Kuala Lumpur (3), Selangor (4), Sabah (1) and Negeri Sembilan (1) were investigated. A total of 27 Sabin-like PVs were isolated consisting of 4 PV1, 12 PV2 and 11 PV3. All these were detected in 2012, 2013 and 2014, when trivalent oral polio vaccine (tOPV) was still used in the National Immunization Program (NIP) for 7-year old school children. Inactivated polio vaccine (IPV) was first introduced in 2008, covering only 8 states and 3 Federal Territories. It was given as prime doses at the age of 2, 3 and 4-5 months of life. The policy changed in 2010, where 4 doses of IPV (at 2,3,4-5 and 18 months) was introduced nationwide and tOPV were given as booster at the age of 7 years old. In mid 2015, tOPV was withdrawn from NIP as well as private and universities health facalities. Only IPV was used for any age throughout the vaccination programme.

The ES detected PVs for three consecutive years from 2012 to 2014 probably due to the usage of tOPV in NIP. OPV contains a live attenuated polio virus which is excreted through the faeces to the sewage systems. After the transition of tOPV to IPV, PV isolation rate was reduced significantly because there was no more live poliovirus excreted to the sewage systems. This was shown in the study as no PVs were isolated in 2016 and 2017. This finding were similar to what were reported in Mexico and Indonesia [25,26] during the OPV to IPV transition period and within 2 to 3 months after the cessation of OPV administration.

All Sabin-Like PVs were subjected to rRT-VDPV PCR [19] to determine whether these isolates were true Sabin-Like viruses or have been mutated to form VDPV. A changes of ≥ 10 nucleotides for both PV1 and PV3, and ≥ 6 nucleotides for PV2 in the VP1 gene was considered as VDPV [27]. Two of ES isolates identified in 2013 were classified as Sabin-Like discordant P1 and P2 as rRT-PCR for ITD showed as Sabin-Like viruses but the rRT-PCR for VDPV showed as non Sabin-Like viruses. These two isolates were from Taman Sri Muda sewage plant isolated in June and August 2013 respectively. The results of full VP1 sequence from VIDRL, Melbourne Australia revealed that they were totally Sabin-Like viruses. What is more important from these data, it indicated that in Malaysia even though PVs were existed in environment, but all of them were Sabin-Like viruses and no evidence of imported WPV or VDPV in the sampling sites over the course of this study.

After the cessation of tOPV and full introduction of IPV, it is almost impossible for children to get vaccine associated paralytic polio (VAPP) or VDPV as there is no source of live PV. However, we are still facing risk of importation of PVs when visitors from OPV-using come to Malaysia. This must be closely monitored with a high quality surveillance system, because PVs can be silently transmitted and its viral genome can mutate during replication in the human gut, partly because of insufficient mucosal immunity when using IPV [8, 28]. Under these circumstances, environmental surveillance of PV plays a key role not only in monitoring the importation of WPV from areas where the disease is endemic but also in preparation against emerging VDPVs before the global cessation of OPV at the final stage of polio eradication.

Beside the PVs, a wide range of enteroviruses were also isolated in the study. NPEV isolation rate was 82.7%. Sewage plants in Peninsular Malaysia had higher NPEV isolation rate compared to a plant in Sabah. In Sabah the low isolation rate (15.8%) was probably due to the technical incapability as ES just started in the middle of 2016 and also less number of plants involved in ES. Technical staff have been trained to improve laboratory capability in managing ES samples and virus isolation.

NPEV isolation rate was quite consistent compared to the study in Mongolia [29] which was around 80%. A total of 103 NPEV were isolated and all of them were HEV-B (Coxsackie A9, Coxsackie B1-B6, E3, E5, E6, E7, E11, E13, E19, E30, E33). The finding was quite different with a study conducted in Singapore to detect prevalence of NPEV. The study found that 76.5% was of HEV-C (coxsackieviruses A-1, A-11, A-17, A22 and A-24), followed by HEV-A species 64.7% (EV71 and 89, coxsackievirus A2, A5 and A16). HEV-B species represented 41.2% whereas HEV-D species was not detected [30]. Study of ES of polio and NPEV in urban sewage in Dakar, Senegal found that Coxsackieviruses represented the majority (75% of serotyped viruses) with coxsackie A-7 and coxsackie A-11 being the more represented [31]. Some of these NPEVs are associated with diseases in human such as EV68 is related to severe respiratory diseases [32], EV 70 and Cox-A24 are the major etiological agents involved in acute hemorrhagic conjunctivitis (AHC) outbreaks worldwide [33,34].

ECHO-19 found recently associated to acute flaccid paralysis and fulminant hepatitis whereas CV-A3, CV-A4, CV-A11, CV-A13 and CV-A17 are associated with herpangina and Hand Foot and Mouth Disease [35].

The main target of ES is to look for PVs either WPVs and VDPVs, but the isolation of wide range of NPEVs may give some indication on the cleanliness of our environmental in related to public health concern. Some of them are of medical importance and can cause our-breaks as these viruses are transmitted through faecal-oral . This information can be used to strengthened the early warning system in prevention of faecal-oral transmitted diseases, and to highlight the importance of personal hygiene and environmental cleanliness. In some countries, the ES findings has triggered the speed of IPV introduction in the community as reported by [36] that the epidemiological and virological surveillance of the environment, even in polio-free countries, enables early detection of any reemergence of WPV or cVDPV strains, with different implications for public health measures. The best example was in Finland during the 1984–1985 outbreak of polio, WPV3 was revealed by ES in several provinces without the occurrence of paralytic cases [14].

In conclusion, ES is very important and crucial in detecting the introduction and silent circulation of WPV and cVDPV before the virus reaches the community.

Acknowledgement

The authors would like to thank the Director General of Health Malaysia and the Director of the Institute for Medical Research for permission to publish this paper. This project was funded by Operational Budget of Virology unit, IMR and a special grant from Centre of Disease Control, Ministry of Health, Malaysia.

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