Biosafety and Health 5 (2023) 174–180

Contents lists available at ScienceDirect

Biosafety and Health

journal homepage: www.elsevier.com/locate/bsheal

Original Research

A comparative study on environmental surveillance of enterovirus: Using a two-phase separation method and a filtration method with a mixed cellulose ester (MCE) membrane

Ling Fang [a,1], Meizhong Chen [a,1], Shuangli Zhu [b,1], Wei Zhang [a], Dongmei Yan [b], Xiaolei Li [b], Shufen Huang [a], Caixia Li [a], Xue Guo [a], Hanri Zeng [a], Bixia Ke [a], Hui Li [a], Wenbo Xu [b,2], Changwen Ke [a], Xiaoling Deng [a], Yong Zhang [b,2,*], Huanying Zheng [a,*]

[a] Guangdong Provincial Center for Disease Control and Prevention, Guangdong Workstation for Emerging Infectious Disease Control and Prevention, Guangzhou 511430, China
[b] WHO WPRO Regional Polio Reference Laboratory and Ministry of Health Key Laboratory for Medical Virology, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

ARTICLE INFO

Article history:
Received 20 December 2022
Revised 5 April 2023
Accepted 6 April 2023
Available online 11 April 2023

Keywords:
Mixed cellulose ester
Two-phase separation
Environmental surveillance
Poliovirus
Enterovirus

ABSTRACT

This study aimed to compare the sensitivity of two-phase separation and the filtration method using a mixed cellulose ester (MCE) membrane to detect enteroviruses in sewage samples. From December 2015 to July 2016, four domestic sewage samples (1 L/sample) were collected monthly from the Guangzhou Liede Sewage Treatment Plant, and each sewage sample was divided into two aliquots (500 mL). The sewage sample was concentrated using the two-phase separation and the filtration method using an MCE membrane, and the treated solutions were inoculated into cells for enterovirus isolation. Polymerase chain reaction amplification, *VP1* sequencing, and enterovirus molecular typing were performed on the positive isolates. The detection rates of poliovirus (PV) and non-polio enterovirus (NPEV) obtained using the filtration method using an MCE membrane were higher than those using the two-phase separation method. McNemar's test showed that the detection rates of PV, NPEV, type 1 Sabin-like (SL1), type 2 Sabin-like (SL2), and type 3 Sabin-like (SL3) strain were not statistically significant ($P > 0.05$). In Guangdong Province, China, the detection rates for PV and NPEV were 53.13% and 62.50% (20/32), respectively. Twenty-seven PVs were isolated, three highly variable strains of the type 1 vaccine, with seven nucleotide substitutions in the *VP1* region, compared with the type 1 Sabin strains. Eighty-seven strains of NPEV were isolated and nine serotypes were identified, among which coxsackievirus B3 (CVB3), echovirus 6(E6), and echovirus 11(E11) were the dominant strains. The filtration method using an MCE membrane is more sensitive than two-phase separation and can be used as a robust, sensitive, and cost-effective method to isolate enteroviruses from sewage.

1. Introduction

Human enteroviruses (EV) are members of the Picornaviridae. Enteroviruses have traditionally been divided into four categories, coxsackievirus A, coxsackievirus B (CVB), echovirus and poliovirus (PV). According to the molecular typing method, they are classified into four species, EV-A to EV-D. PV is the causative agent of poliomyelitis, and in addition to PV, other enteroviruses are collectively referred to as non-polio enterovirus (NPEV) [1].

With the implementation of the Global Polio Eradication Initiative (GPEI), the number of polio cases worldwide has decreased by 99% since 1988, and the global health burden of poliomyelitis has dramatically reduced. However, only Afghanistan and Pakistan have not blocked the indigenous transmission of wild poliovirus (WPV) [2]. The acute flaccid paralysis (AFP) case surveillance system is the gold standard for poliovirus surveillance [3]; however, the “silent” transmission of poliovirus in the population makes environmental surveillance a crucial auxiliary tool for poliovirus surveillance [4]. Environmental surveillance has at least two functions. First, direct detection of poliovirus in the environment is used to supplement

* Corresponding authors: WHO WPRO Regional Polio Reference Laboratory and Ministry of Health Key Laboratory for Medical Virology, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China (Y. Zhang); Guangdong Provincial Center for Disease Control and Prevention, Guangdong Workstation for Emerging Infectious Disease Control and Prevention, Guangzhou 511430, China (H. Zheng).

E-mail addresses: yongzhang75@sina.com (Y. Zhang), zhenghy.gdcdc@gmail.com (H. Zheng).

[1] These authors contributed equally to this study.

[2] Given their role as Editorial Board Members, Yong Zhang and Wenbo Xu had no involvement in the peer-review of this article and had no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Editor Zexin Tao.

https://doi.org/10.1016/j.bsheal.2023.04.001
2590-0536/

HIGHLIGHTS

Scientific question

Expanding the Global Polio Eradication Initiative (GPEI) and establishing environmental surveillance systems in countries at risk of poliovirus transmission warrants the development of additional virus enrichment methods with reduced costs and shorter turnaround times.

Evidence before this study

The acute flaccid paralysis (AFP) case surveillance system is the gold standard for poliovirus surveillance; however, the "silent" transmission of poliovirus in the population makes environmental surveillance a crucial auxiliary tool for poliovirus surveillance.

New findings

The filtration method using a mixed cellulose ester (MCE) membrane is more sensitive than two-phase separation and can be used as a robust, sensitive, and cost-effective method to isolate enteroviruses from sewage.

Significance of the study

The filtration method using a mixed cellulose ester (MCE) membrane allows sensitive monitoring of the dynamic changes of enteroviruses in the environment. Additionally, this method enables researchers to track the actual dynamics of enterovirus transmission in human communities and provide early warnings for diseases in the population.

disease-based surveillance, AFP case surveillance, identification of local WPV in endemic areas and imported WPV in non-endemic areas, and early detection of poliovirus transmission between infected individuals without paralysis symptoms [5]. Second, during the transition period from oral polio vaccine (OPV) to inactivated polio vaccine (IPV) use, it provides scientific data for the elimination of Sabin-related vaccine viruses from the environment [6].

The current technology/methodology for environmental surveillance of polio recommended by the World Health Organization (WHO) Polio Lab Net is based on grab sampling and two-phase separation [7]. However, many other methods are available for sewage concentration, among which the most successful is the filtration method using a mixed cellulose ester (MCE) membrane with a pore size of 0.45 μm [8]. This method has been successfully used for environmental surveillance of poliovirus in both Japan and China [8–12]. In Guangdong Province, China, we have adopted the filtration method using an MCE membrane for routine environmental surveillance of enteroviruses since 2008 and established a well-developed environmental surveillance system. With this in mind, we carried out a study to compare the sensitivity of the two-phase separation and the filtration method using an MCE membrane to detect enteroviruses in sewage. In addition, environmental samples usually contain a large amount of NPEV, so inoculating sewage sample concentrate to cells for viral isolation may mask the growth of PV on cells. Therefore, to consider the detection sensitivity of PV and NPEV together, we optimized the viral isolation method using the MCE membrane filtration method in routine environmental surveillance. We discussed the virus detection results to analyze whether our optimized method has a satisfactory overall performance of environmental surveillance.

2. Materials and methods

2.1. Sample source

2.1.1. Sample collection from wastewater treatment plant

From December 2015 to July 2016, four 1 L sewage samples were collected monthly from the water inlet of the Liede Wastewater Treatment Plant in Guangzhou, China, and then transported to the Polio Laboratory of Guangdong Center for Disease Control and Prevention (CDC) within two hours of collection. Each sewage sample was divided into two aliquots of 500 mL and concentrated using the two-phase separation and the filtration method using an MCE membrane.

2.1.2. Proficiency test samples provided by the World Health Organization

In March 2022, we participated in the 2021 proficiency test (PT) for the poliovirus environmental surveillance laboratory organized by the World Health Organization (WHO). The WHO uniformly prepared the PT samples; the sample number was ESPT-2021-1, including four samples, each containing a 10 mL suspension. After receiving the PT samples, the 10 mL suspension was added to a conical flask containing 490 mL distilled water and mixed well. The samples were further concentrated using the filtration method using an MCE membrane, as shown below.

2.2. Sample concentration treatment

2.2.1. Two-phase separation method

Centrifuge 500 mL of sewage sample at 1,940*g* at 4 ˚C for 30 min. Transfer the supernatant into a conical flask and store the dry pellets in a 4 ˚C refrigerator. Then, add 1 mol/L NaOH solution to adjust the pH of the supernatant to neutral (7.0–7.5). We added 39.5 mL of 22% dextran, 287 mL of 29% polyethylene glycol (PEG) 6000, and 35 mL of 5 mol/L NaCl solution into the conical flask and used a magnetic stirring rod to stir for 1 h to homogenize the solution continuously. Transfer the mixture to a 1L conical aseptic separating funnel and incubate it overnight at 4 ˚C. The next day, we opened the valve of the separating funnel and slowly dropped the entire lower layer and interphase into the sterile centrifuge tube (7–15 mL extraction volume per 0.5 L sample). Resuspend the pellet into the interphase. Add 20% volume of chloroform and sterile glass beads into the mixed concentrate and shake vigorously for 20 min with a conventional horizontal oscillator. Centrifuge the concentrated solution at 1,940*g* for 30 min. Collect the upper aqueous phase in a sterile tube and add antibiotics to the 100 mg/mL final concentration. Inoculate the final extracted concentrate (15–19 mL/per sample) into sensitive cells and store it in refrigeration at −40 ˚C.

2.2.2. The filtration method using a MCE membrane

The 500 mL sewage samples were centrifuged at 4 ˚C, 1,940g for 30 min, and the supernatant was placed in a conical flask. We added 10 mL 2.5 mol/L MgCl2 solution for a final Mg^{2+} concentration of 0.05 mol/L. Moreover, a 0.5 mol/L HCl solution was slowly added to adjust the pH to 3.5–4.0, and the supernatant was thoroughly mixed. Sewage samples were filtered using a device containing a 0.45 μm nitrocellulose membrane. After filtration, the nitrocellulose membrane was cut into pieces and added to a centrifuge tube containing 10 mL of 3% beef extract at pH 9.6. Ultrasonic oscillation elution was performed twice to obtain the first and second eluate. Next, we added 0.5 mol/L HCl to adjust the pH to 7.0, and the eluent was centrifuged at 4 ˚C, 1,940g for 30 min. The supernatant was filtered and sterilized using a 0.22 μm sterile filter membrane. After treatment, the concentrated sewage sample was inoculated into sensitive cells and refrigerated at −40 ˚C.

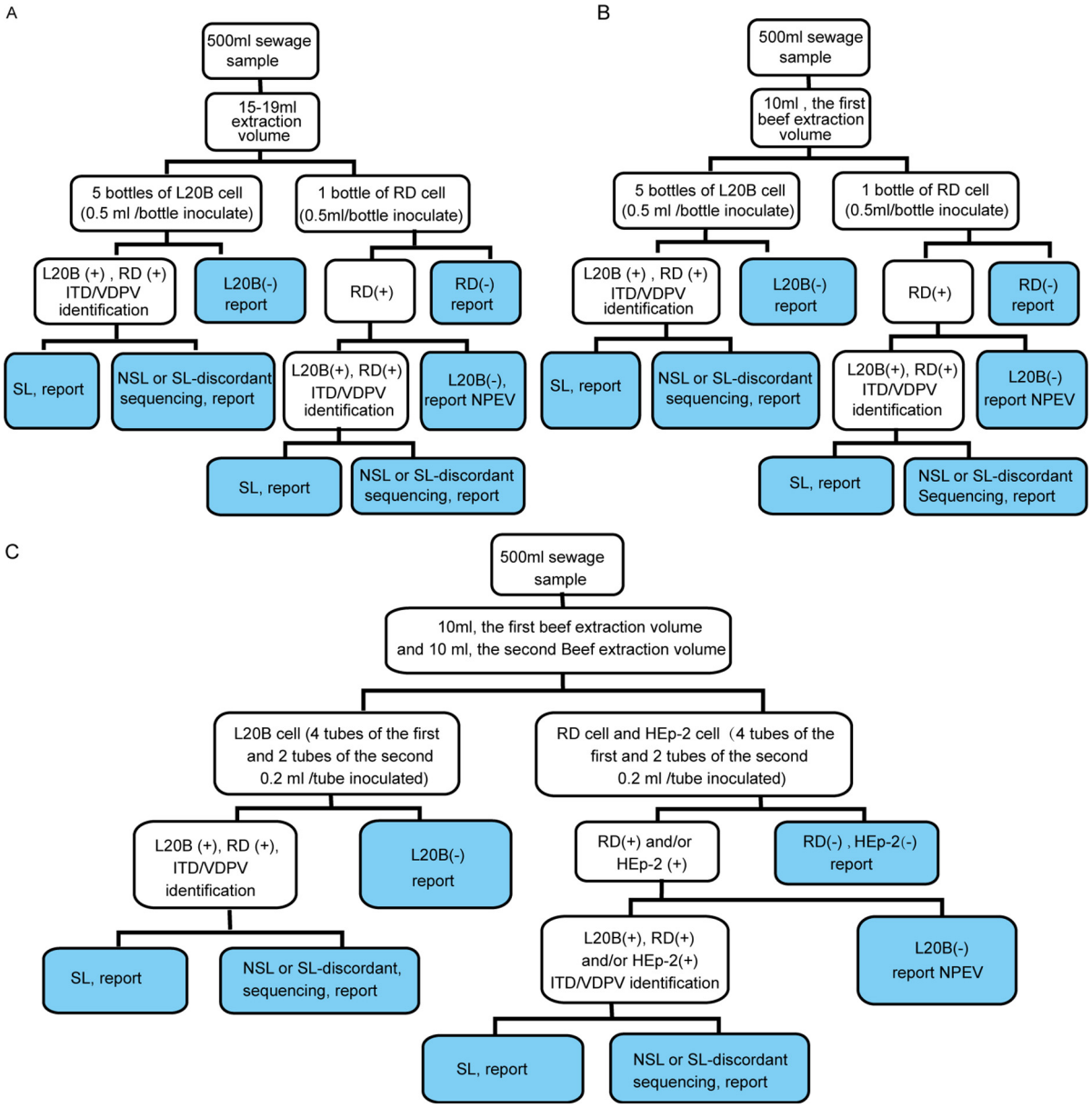

Fig. 1. Comparative flowchart of two-phase separation and acid treatment-filtration membrane method. A) Two-Phase Separation Method; B) acid treatment-filtration membrane method; C) optimized viral isolation using the filtration method using a mixed cellulose ester (MCE) membrane for routine environmental surveillance. Abbreviations: L20B, mouse cell line with poliovirus receptor CD155; RD, human rhabdomyosarcoma cell; HEp-2, human epidermoid carcinoma cell; ITD, intra-typic differentiation; VDPV, vaccine-derived poliovirus; SL, Sabin-like; NSL, non-Sabin-like; NPEV, non-polio enterovirus.

2.3. Viral isolation

2.3.1. Viral isolation using the two-phase separation method

The interphase treated by the two-phase separation method was inoculated into human rhabdomyosarcoma (RD) and mouse cell line expressing the gene for the human cellular receptor for poliovirus (L20B) cells lines to isolate the virus (Fig. 1A). The concentrates were inoculated into five L20B-T_{25} flasks and one RD-T_{25} flask (10 mL volume /flask) by using the algorithm recommended by WHO PolioLabNet. The cultures were incubated in a CO_2 incubator (5% CO_2 concentration) to observe the cytopathogenic effects (CPE) daily for one week. The L20B cultures with CPE are enriched in RD-T_{25} flasks, while RD cultures with CPE are first inoculated into L20B-T_{25} cell flasks before enrichment in RD-T_{25} cell flasks. All inoculations are carried out by adding 0.5 mL of the concentrates/flask. The CPE-positive isolates were stored in a −20 °C refrigerator.

2.3.2. Viral isolation using the filtration method using a MCE membrane

The first eluent treated by the filtration method using an MCE membrane was inoculated into RD and L20B cells lines to isolate the virus (Fig. 1B), and the concentrates were inoculated into five L20B-T_{25} cell flasks and one RD-T_{25} cell flask (10 mL volume /flask) by using the algorithm recommended by WHO PolioLabNet. The cultures were incubated in a CO_2 incubator (5% CO_2 concentration) to observe the CPE daily for one week. The L20B cultures with CPE are enriched in RD-T_{25} cell flasks, while RD cultures with CPE are first inoculated into L20B-T_{25} cell flasks before enrichment in RD-T_{25} cell flasks. All inocu-

lations are carried out by adding 0.5 mL of the first eluent/flask. The CPE-positive isolates were stored in a −20 ℃ refrigerator.

2.3.3. Optimized viral isolation using the filtration method using a MCE membrane

According to the "Polio Laboratory Manual" (WHO 2004 4th edition) [13], the first and the second eluent were inoculated into RD, human laryngeal epidermoid carcinoma cell (HEp-2), and L20B cells lines to isolate the virus, which was termed optimized viral isolation process based on the filtration method using an MCE membrane (Fig. 1C). The first eluent was inoculated into four RD-tubes, four HEp-2 tubes, and four L20B tubes (1 mL volume /tube). The second eluent was inoculated into two RD tubes, HEp-2 tubes, and two L20B tubes (1 mL volume /tube). All inoculations are carried out by adding 0.2 mL of the eluent/flask. The cultures were incubated in a CO_2 incubator (5% CO_2 concentration), and the CPE was observed daily for one week. If no CPE was present, two consecutive blind passages were carried out for all three cell lines. When CPE was detected, RD or HEp-2 cells were additionally passaged on L20B cells for poliovirus detection. The CPE-positive isolates were stored at −20 ℃.

2.4. Extraction of viral RNA and identification of PVs and NPEVs

RNA of the positive isolates was extracted using the QIAamp Viral RNA Mini Kit. Next, the enterovirus was screened and identified using the universal nucleic acid detection kit for enterovirus [real-time polymerase chain reaction (PCR) method] from Shenzhen Aodong. After being identified as enterovirus, the serotype of PV was further determined using the nucleic acid detection kit for poliovirus type 1, type 2, and type 3 (Bioperfectus Technologies, Taizhou, China) (real-time PCR method). Additionally, intra-typic differentiation (ITD) was performed using quantitative real-time PCR (qRT-PCR) according to the WHO Polio Lab Net rRT-PCR protocols [14]. The National Polio Laboratory provided cells and reagents for ITD of poliovirus were provided by WHO.

2.5. Nucleotide sequencing of the VP1 coding region of poliovirus and other enteroviruses

Nucleic acid samples identified as poliovirus were amplified using a one-step RT-PCR kit (Qiagen). Nucleic acid samples identified as enteroviruses were amplified by semi-nested polymerase chain reaction (Sn-PCR) established by Nix et al. in 2006. RNA reverse transcription was performed using the QuantiTect Reverse Transcription Kit and semi-nested PCR using the Invitrogen Platinum Green Hot Start PCR Master Mix (2X) kit. The amplified product was examined using 1.5% agarose gel electrophoresis, and Guangzhou Haotian Gene Technology Co., Ltd sequenced the excellent products.

2.6. Statistical analysis

Excel 2019 was used for data collation, and SPSS 22.0 was used for statistical analysis. The counting data were expressed as rate or composition ratio, and the difference in rate was compared using McNemar's test. The test level was $\alpha = 0.05$, and when $P < 0.05$, the difference was considered statistically significant.

3. Results

3.1. Viral isolation using the two-phase separation and the filtration method using a MCE membrane

From December 2015 to July 2016, 32 sewage samples were collected by the Guangdong Polio Laboratory, and each sewage sample was divided into two aliquots, which were treated by the two-phase separation and the filtration method using an MCE membrane (Fig. 1A and B).

For the concentrate obtained using the two-phase separation method, the positive rates of NPEV and PV in sewage were 43.75% (14/32) and 21.88% (7/32), respectively. Additionally, 21 EV strains were isolated, including 14 NPEV and 7 PV strains. PVs were all Sabin-like (SL) vaccine strains, including two Sabin-like type 1 (SL 1), two type 2 Sabin-like, and three type 3 Sabin-like (SL3) strains. For the concentrate obtained using the filtration method using an MCE membrane, the positive rates of NPEV and PV in the sewage samples were 53.13% (17/32) and 31.32% (10/32), respectively. Additionally, 28 EV strains were isolated, including 17 NPEV strains and 11 PV strains. The PVs were all Sabin-like vaccine strains, including 2 SL1, two SL2, 6 SL3, and 1 SL1 + SL2 mixed strain (Fig. 2). WPV1, WPV3, and vaccine-derived poliovirus (VDPV) were not isolated using either method, as shown in Fig. 2.

The two-phase separation method was used to concentrate 500 mL sewage samples to 15–19 mL, whereas the filtration method using an MCE membrane yielded 10 mL samples. The concentration ratios of the two methods were 33.33–26.32 and 50.00, respectively. McNemar's test showed no significant difference in the positive rates of NPEV and PV between the two methods ($P = 0.55$ and $P = 0.61$, respectively). The detection rates of different PV serotypes in two sewage concentration methods were compared. The positive rates of SL1, SL2, and SL3 were not statistically significant ($P = 0.99$, $P = 0.99$, and $P = 0.45$, respectively), as shown in Table 1.

3.2. Routine environmental surveillance between December 2015 and July 2016

During routine environmental surveillance (Fig. 1C) between December 2015 and July 2016, the positive rates of PV and NPEV were 53.13% (17/32) and 62.50% (20/32), respectively. Furthermore, 118 viral strains were isolated, including 27 PV strains, 87 NPEV strains, and 4 Non-Type (NT) strains. PV1, PV2, and PV3 accounted for 14.81% (4/27), 14.81% (4/27), and 70.38% (19/27) of the PV isolates, respectively. In February 2016, three highly variant strains of the type I vaccine were isolated from sewage. Compared to the type I Sabin strain's reference strain, there were seven base mutations; the remainder were Sabin-like vaccine strains. Among the NPEV isolates, nine serotypes were identified, among which coxsackievirus B3 (CVB3), echovirus 6 (E6), and echovirus 11 (E11) were the dominant strains, accounting for 28.74% (25/87), 22.99% (20/87), and 18.39% (16/87), respectively. Echovirus 12 (E12), echovirus 3 (E3), CVB2, coxsackievirus B2 (CVB2), coxsackievirus B4 (CVB4), and coxsackievirus B5 (CVB5) accounted for 11.49% (10/87), 9.20% (8/87), 3.44% (3/87), 2.30% (2/87), 2.30% (2/87) and 1.15% (1/87) of the NPEV isolates, respectively, as shown in Fig. 3.

3.3. PT for environmental surveillance in Guangdong Province, China in 2021

In February 2021, Guangdong Polio Laboratory participated in the PT of environmental surveillance, and the WHO uniformly prepared environmental surveillance PT samples (ESPT). Four 1 L sewage samples were received. After acid treatment and membrane filtration, the eluent was inoculated into six tubes containing RD and six tubes containing L20B cells for viral isolation and culture. In sample one, six SL1 strains and three NPEV strains were identified. In sample two, three NPEV strains were identified. In sample three, seven SL1 and one SL1 and SL3 hybrid strains were identified. Finally, one SL3 strain and two NPEV strains were identified in sample four. The WHO noted that although NPEV was not added to the water samples, the substrate contained low levels of NPEV (<50 per sample), as shown in Table 2.

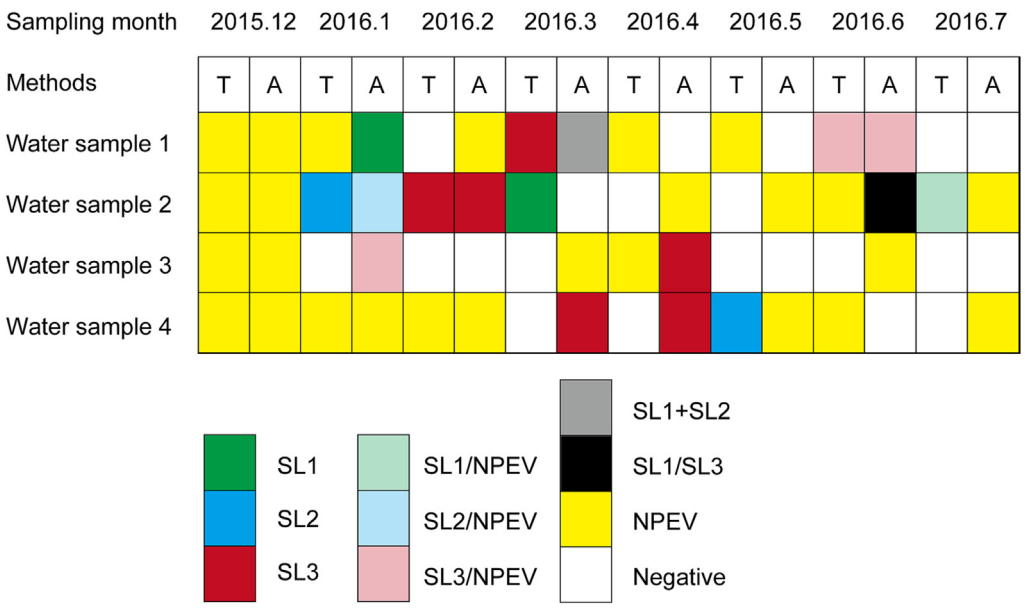

Fig. 2. Detection of poliovirus (PV) and non-polio enterovirus (NPEV) in water samples using the two-phase separation and the filtration method using a mixed cellulose ester (MCE) membrane. T = two-phase separation method; F = filtration method using a MCE membrane, SL1 = Sabin-like type 1, SL2 = Sabin-like type 2, SL3 = Sabin-like type 3.

Table 1
Comparison of NPEV and PV detection in matching two-phase separation and anionic membrane adsorption methods.

The concentration method using a MCE membrane		Two-phase separation method		OR (95%CI)	*P* value
		+	–		
PV	+	3	7	1.93 (2.27,12.84)	0.55
	–	4	18		
	total	7	25		
NPEV	+	8	9	1.33 (1.00,6.76)	0.61
	–	6	9		
	total	14	18		
SL1	+	0	3	1.07 (0.10,2.26)	0.99
	–	2	27		
	total	2	30		
SL2	+	1	2	6.75 (6.33,117.46)	0.99
	–	2	27		
	total	3	29		
SL3	+	1	5	2.40 (2.22,34.28)	0.45
	–	2	24		
	total	3	29		

Notes: +, positive detection; -, negative detection; OR, odds ratio; MCE, mixed cellulose ester; SL1, Sabin-like type1; SL2, Sabin-like type 2; SL3, Sabin-like type 3; PV, poliovirus; NPEV, non-polio enterovirus; *P* value calculated using the McNemar mid-p test.

4. Discussion

Poliovirus environmental surveillance is an essential tool to evaluate the progress of poliovirus eradication and obtain final certification. Many countries carry out environmental surveillance as a supplement to AFP surveillance [4,15–17]. For early detection of WPV importation and VDPV transmission, the two-phase separation method recommended by the WHO is an "aqueous two-phase system," developed by Norrby and Albertsson [18], which is used to concentrate poliovirus while maintaining the integrity and infectivity of the viral particles. In this method, polymers of PEG and dextran are distributed into two separate aqueous phases using the physical and chemical properties of reagents and/or solutes. Furthermore, hydrophobicity, surface charge, concentration, size, and biological affinity play a significant role in the distribution of viral particles [16]. The two-phase separation method is relatively simple to operate and does not require complicated equipment. However, this method is costly (50 USD/sample) and time-consuming, requiring two days to treat water samples. In addition, only two companies produce dextran worldwide, and the dextran and PEG are prepared to remain viable at 4 °C for up to two weeks [19]. The expansion of GPEI and the establishment of environmental surveillance systems in countries at risk of poliovirus transmission warrants the development of additional enrichment methods to reduce costs and shorten turnaround times. The filtration method using an MCE membrane with a 0.45 mm pore size nitrocellulose membrane was developed based on the existing filtration method. $MgCl_2$ was added to charge the virus positively, and the positively charged viral particles were adsorbed onto the nitrocellulose membrane by ion interaction and then eluted by adjusting the pH value. The cost of consumables was low (10–20 USD/sample), and it required one day to treat water samples. All reagents were easily obtained from global markets and suppliers and were easy to store. The 3% beef extract at pH 9.6 can

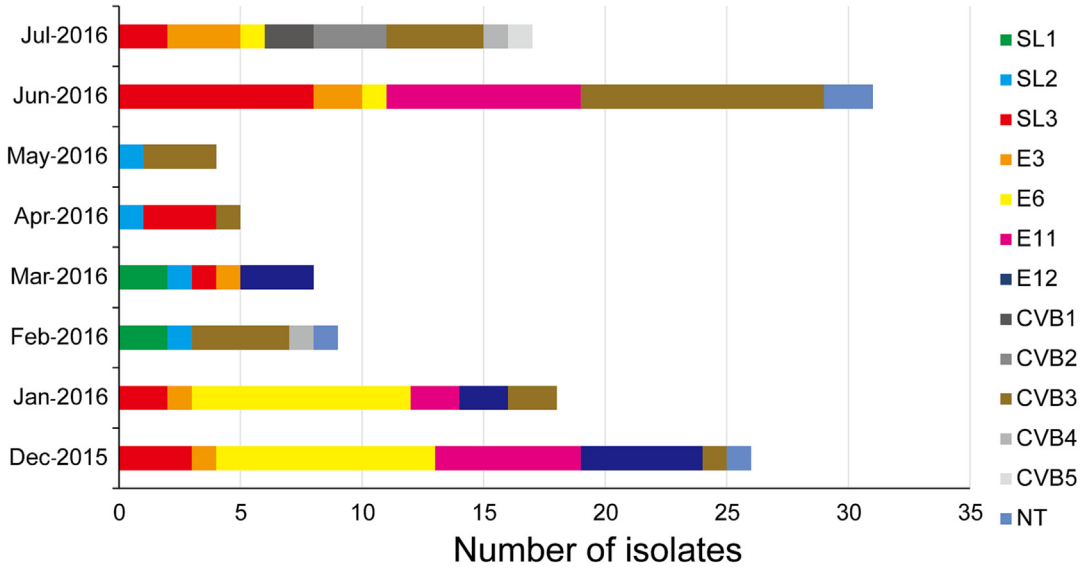

Fig. 3. The number of solates of poliovirus (PV) and non-polio enterovirus (NPEV) in routine environmental surveillance between December 2015 and July 2016. Abbreviations: SL, Sabin-like; E, echovirus; CVB, coxsackievirus B; NT, non-tested.

Table 2
Guangdong Province, China Poliovirus Laboratory report of WHO Global Polio Laboratory Network Proficiency Testing (PT) program.

Samples	Content ($CCID_{50}$/sample)	Expected VI result	Expected ITD result(s)	Result VI	Result(s) ITD
Sample 1	10^3 SL1 + 10^4 E33	L20B +	SL1	L20B +	NPEV + SL1
Sample 2	5 × 10^2 E33	NPEV	///	NPEV	///
Sample 3	10^3 SL1 + 10^3 SL3	L20B +	SL1 + SL3	L20B +	SL1 + SL3
Sample 4	3 × 10^2 SL3	L20B +	SL3	L20B +	NPEV + SL3

Abbreviations: +, positive detection; -, negative detection; VI, viral isolation; ITD, intra-typic differentiation; SL1, Sabin-like type1; SL2, Sabin-like type 2; SL3, Sabin-like type 3; PV, poliovirus; NPEV, non-polio enterovirus; E33, echovirus 33; L20B, mouse cell line with poliovirus receptor CD155.

be stored for three months at 4 °C, and $MgCl_2$ and HCl can be sealed at room temperature for long-term storage.

Furthermore, this method has been successfully employed for environmental surveillance in Japan and China. Since 2008, polio labs in Guangdong Province [9], Shandong Province [20], and other provinces have successively carried out environmental surveillance work. To further validate the filtration membrane method, the Guangdong Polio Laboratory conducted a study comparing the two-phase separation and the filtration method using an MCE membrane over eight months starting in December 2015.

In this study, we used different sample concentration treatment methods and the same virus isolation methods to isolate enteroviruses in sewages. We found that the positive viral detection rate in the filtration method using an MCE membrane was higher than that of the two-phase separation method, which may be because the concentration efficiency of the filtration method using an MCE membrane was slightly higher than that of the two-phase separation method. According to McNemar's test, the differences in the positive rates of PV, NPEV, SL1, SL2, and SL3 between the two methods were not statistically significant. In 2003, the WHO issued "Guidelines for Environmental Surveillance" [7], which suggested that the positive rate of NPEV in sewage can reflect the sensitivity of poliovirus detection and environmental surveillance sites. Moreover, an NPEV-positive rate in concentrated sewage samples >30% indicates that the environmental surveillance level in this area is high. The positive rates of the two-phase separation and the filtration method using an MCE membrane were 43.74% and 53.13%, respectively, which highlighted the increased sensitivity of the filtration method using an MCE membrane compared to that of the two-phase separation method. We suggest that the filtration method using an MCE membrane could be widely used as a robust, sensitive, and cost-effective method for enterovirus isolation from sewage.

In this study, we optimized the process of viral isolation using the MCE membrane filtration method in routine environmental surveillance (Fig. 1C). According to the "Polio Laboratory Manual" (WHO 2004 4th edition), the combination of RD and L20B cells was selected to isolate the viruses, which provided great sensitivity and specificity for the detection of polioviruses. In the process of viral isolation flow recommended by the WHO, five L20B T25 cell flasks and one RD cell tube were set up for the inoculation concentrate on improving the isolation rate of PV. Different from WHO flows, Guangdong Provincial Polio Laboratory takes into account the detection sensitivity of both PV and NPEV in the environment, so we set up the same number of RD and L20B T_{25} cell flasks (four RD T_{25} cell flasks and four L20B T_{25} cell flasks for the inoculation of the first eluent, two RD T_{25} cell flasks and two L20B T_{25} cell flasks for the inoculation of the second eluent). In addition, we also added HEp-2 cells for viral isolation, which can improve the isolation rate of NPEVes, mainly when CVB is transmitted in the community. During the study period, the positive rates of PV and NPEV were 53.13% and 62.50%, respectively, which indicated that the conventional environmental surveillance level using the filtration method using an MCE membrane was sufficiently sensitive. Twenty-seven poliovirus strains were isolated, and the nucleotide sequence of the *VP1* coding region was determined. Among them, three strains were highly divergent from the type I vaccine, and there were seven base mutations. Eighty-seven NPEV strains were isolated, and nine serotypes were identified, all belonging to the EV-B group. Enteroviruses cause seasonal epidemics that usually peak in the summer and autumn. The number of viruses isolated in this study decreased significantly from February to March 2016, peaking in June

2016. Of note, ECHO30 caused an outbreak of aseptic meningitis in Luoding City, Guangdong Province, in 2012.

Furthermore, this virus shares high similarity (98.5% ~ 100%) in the nucleotide sequence with viral strains isolated from sewage in Guangzhou and Luoding City from 2010 to 2012 [21]. Furthermore, the nucleotide sequences of E11 domestic sewage isolates from Guangzhou in 2019 were highly consistent with those of E11 infections from Guangdong in 2019 [22], which shows that since 2008, routine environmental surveillance using the filtration method using an MCE membrane in Guangdong Province has allowed sensitive monitoring of the dynamic changes of enteroviruses in the environment. Additionally, this method has enabled researchers to track the natural dynamics of enterovirus transmission in human communities and provide early warnings for diseases in the population.

In order to evaluate the quality of poliovirus environmental surveillance laboratories in mainland of China, maintain and improve the sensitivity, timeliness, and accuracy in detecting WPV and VDPV in sewage samples, the National Polio Laboratory has organized 12 provincial polio laboratories to participate in the environmental surveillance PT of the WHO since 2018. The improved process aims to improve the detection sensitivity of NPEV without reducing the detection sensitivity of PV. The primary purpose of the WHO PT is to test the sensitivity of the environmental surveillance laboratory to detect PV, so in order to test the sensitivity of our improved process to detect PV, we participated in the WHO PT. In all provincial poliomyelitis laboratories participating in WHO PT, MCE membrane filtration was used to concentrate sewage samples, and PV and NPEV were identified. All laboratories passed the PT, indicating that the MCE membrane filtration method can meet the WHO requirements for the sensitivity of PV detection.

In conclusion, the filtration method using an MCE membrane is a sensitive, cost-effective, and simple method that can meet the needs of the expanding environmental surveillance systems in the final stage of polio eradication.

Acknowledgments

We thank the Liede Sewage Treatment Plant for supplying the sewage samples. This study was supported by the National Key Research and Development Program of China (Project No. 2021YFC2302003), and the Natural Science Foundation of Beijing (Project No. L192014).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Ling Fang: Writing – original draft. **Meizhong Chen:** Writing – original draft, Formal analysis. **Shuangli Zhu:** Writing – review & editing. **Wei Zhang:** Investigation. **Dongmei Yan:** Investigation. **Xiaolei Li:** Investigation. **Shufen Huang:** Investigation. **Caixia Li:** Investigation. **Xue Guo:** Investigation. **Hanri Zeng:** Investigation. **Bixia Ke:** Investigation. **Hui Li:** Supervision. **Wenbo Xu:** Supervision. **Changwen Ke:** Supervision. **Xiaoling Deng:** Supervision. **Yong Zhang:** Conceptualization, Writing – review & editing, Formal analysis, Project administration. **Huanying Zheng:** Conceptualization, Investigation, Resources, Project administration.

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