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Original Article

Poliovirus Detection and Genetic Characteristic from Sewage in Heilongjiang Province from 2013 to 2016

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SUMMARY: By monitoring the sewage system in Heilongjiang province from 2013 to 2016, this study aimed to analyze the epidemiological tendency and genetic mutation of poliovirus (PV) found in the environment in order to setup a warning system for vaccine-derived poliovirus (VDPV) and the spread of wild poliovirus. In this study, we collected 139 sewage samples from 8 regions in Heilongjiang province. Poliovirus was identified from 72 samples, and the positivity rate was 51%. A total of 263 PV strains were isolated, including 22 strains of type 1 PV, 104 strains of type 2 PV, and 137 strains of type 3 PV. As a result of intratypic differentiation, using real-time PCR and nucleotide sequencing, 3 type 1 pre-VDPV, one type 2 VDPV, and 2 type 3 pre-VDPV strains were isolated. Interestingly, one type 1 strain with 5 nucleotide deletions and one type 3 recombinant on VP1 were isolated. By continuously monitoring the poliovirus in the environment, we aimed to recognize the VDPV or wild poliovirus with high neurovirulence from large-scale circulation and set up a warning system to avoid morbidity and virus transmission.

INTRODUCTION

Polioviruses (PVs) mainly spread by fecal-oral transmission, and PVs usually cause inapparent infection after infecting a human host. However, in some cases, PV can cause numbness or even permanent disability. Thanks to the widespread use of oral poliomyelitis attenuated live vaccine (OPV), the global effort to eradicate poliovirus has made a significant progress. However, in some neighboring countries of China (such as in Pakistan and Afghanistan), there are still transmissions of wild poliovirus (WPV) which have not been blocked at all. Hence, China remains at risk of cross-border transmission of WPV (1,2). A poliovirus vaccine strain undergoes gene mutation and genetic recombination during the replication process in the human body. Revertant mutations occurring in some attenuated positions may increase neurovirulence, which are known to have caused several outbreaks of paralytic poliomyelitis in the different parts of the world (3,4). Therefore, the emergence of vaccine-derived poliovirus (VDPV) is gaining more attention in many countries. PV has a strong ability to survive in the environment, and a large number of viruses excreted into the environment will survive for a certain period and remain infectious (5). Continuous monitoring of the external environment is helpful to understand the distribution of poliovirus in the environment and for the early detection of WPV and circulating VDPV (cVDPV). In addition, it can provide a solid basis for prevention and control of poliovirus outbreak.

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MATERIALS AND METHODS

Monitoring area, sampling frequency, and sample quantity between 2013 and 2016: As the capital city of Heilongjiang Province, Harbin city has a vast floating population; hence, the status of PV found in the environment should be monitored. We set major urban area and Shuangcheng County as the 2 sampling sites. In the urban area of Harbin City, samples were collected once a month and a total of 48 samples were collected. In Shuangcheng County, 40 sewage samples were collected. Along the border area and the area that was affected by flood in 2013, 6 sampling sites, with higher risk of WPV spread, were chosen. The samples were collected in summer because the sewage water during this season was more suitable for poliovirus survival. In Fuyuan county of Jiamusi city, Tongjiang county of Jiamusi city, and Mudanjiang city, 9 samples were collected in each area. In Aihui District of Heihe city, 12 samples were collected. In Zhaoyuan county of Daqing city and Suifenhe county of Mudanjiang city, 6 samples were collected (Table 1). The distribution of sampling sites is illustrated in Fig. 1. One liter of sewage water was collected from the entry point of local sewage treatment plants at each sampling site in continuation with the environmental monitoring work.

Sample transportation and treatment: Within 48 h, all the sewage samples were placed in refrigerated transportation and sent to the polio laboratory of Heilongjiang Provincial Center for Disease Control and Prevention (Heilongjiang CDC), and the sewage samples were concentrated and enriched using the filter adsorption and elution method. Approximately 500 mL of each sample was centrifuged at 3,000 rpm for 30 min at a temperature

Table 1. Environment surveillance situation in Heilongjiang province between 2013 and 2016

Sampling sites	Sampling period	Sample no.	Sample no. of PV+	PV+ rate%	Type 1 strains	Type 2 strains	Type 3 strains	Total strains	Sample no. of NPEV+	No. of NPEV isolate
Haerbin	Jan. 2013–Dec. 2016	48	32	66.7	18	53	58	129	38	135
Shuangcheng	Sep. 2013–Dec. 2016	40	15	37.5	0	22	13	35	13	45
Heihe	Jul., Aug., Sep. of 2013–2016	12	7	58.3	1	6	13	20	8	15
Fuyuan	Jul., Aug., Sep. of 2013, 2014 and 2016	9	6	66.7	0	17	31	48	2	13
Tongjiang	Jul., Aug., Sep. of 2013, 2014 and 2016	9	4	44.4	1	0	8	9	6	35
Mudanjiang	Apr., May, Jun. of 2013; Jul., Aug., Sep. of 2013–2014	9	3	33.3	0	5	6	11	5	19
Suifenhe	Jul., Aug., Sep. of 2013 and 2016	6	3	50.0	0	0	5	5	6	42
Zhaoyuan	Jul., Aug., Sep. of 2014 and 2016	6	2	33.3	2	1	3	6	4	8
Total	—	139	72	51.8	22	104	137	263	82	312

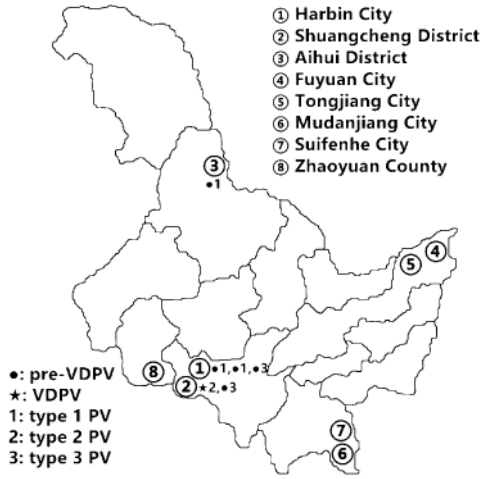

Fig. 1. Distribution of sampling sites and poliovirus strains isolated in Heilongjiang Province. Each dot indicated one VDPV or pre-VDPV strain.

of 4°C. About 2.5 M of $MgCl_2$ solution was added into the supernatant to make a final concentration of 0.05 M, and 0.5 N HCl was used to adjust the total pH value to 3.5. A cellulose nitrate membrane filter (0.45-μm pore size; model no. 31023150; Toyo Roshi, Tokyo, Japan) was used as a virus adsorbent utilizing a multi-position filtration system (BioVac321B; Chemvak, Germany). The membranes were cut into pieces after collection. The viruses adsorbed on the membrane fragments were eluted into 10 mL of 3% beef extract solution with a pH value of 9 using a vortex mixer. After the collection of eluents, another 10 mL of 3% beef extract was added into the rest of the membrane fragments for the second elution process. The first eluent and the second eluent were centrifuged at 3,000 rpm for 30 min. The supernatant was collected after filtration using a 0.45-μm filter for virus isolation experiments (6).

Isolation and identification of the poliovirus: The sewage concentrates were inoculated in human rhabdomyosarcoma (RD) cells, mouse cell lines expressing the human cellular receptor gene for poliovirus (L20B), and human laryngeal carcinoma (HEp-2) filled with monolayer cells to detect and isolate the poliovirus. The first eluent was inoculated in 5 tubes of each cell line, and the second eluent were inoculated in 3 tubes. For each tube, 200 μL of eluent was inoculated. Cultures with enterovirus cytopathic effects in RD or HEp-2 cells were re-inoculated onto L20B cells. Positive L20B isolates were collected, and the poliovirus type-specific polyclonal antiserum (produced by RVIM, provided by the WHO and China CDC polio laboratory) was used for identification (7). All PV isolates were confirmed by intratypic differentiation (ITD). PV isolates collected before December 2014 were shipped to China CDC for ITD confirmation process and after January 2015, ITD for PV isolates was conducted in Heilongjiang CDC.

Sequencing of VP1 region: The primer set of UG1/UC11 was used for amplification of VP1 region with the sequence of UG1:5′-TTTGTGTCAGCGTGTAATGA-3′, and UC11:5′-AAGAGGTCTCTATTCCACAT-3′. The sequencing work was conducted by Sangon Biotech (Shanghai) Co., Ltd. Three levels of the national criteria for originated strain of Sabin vaccine were used to assess the risk for polio. Three levels of diagnosis for poliomyelitis in China are Sabin like (SL), pre-VDPV, and VDPV. The sequencing results of Sabin strains were compared as follows: in the VP1 coding region, the nucleotide sequence variations of VDPV types 1 and 3 were ≥ 10 and < 135 (mutation rate: > 1% and < 15%), respectively, while those of VDPV type 2 were ≥ 6 and < 135 (mutation rate: > 0.6% and < 15%). In the VP1 coding region, if the nucleotide sequence variations of types 1 and 3 were between 6 and 9, they were judged as high-mutant strains (pre-VDPV) (8). PV strains whose mutation standards did not qualify for VDPV and pre-VDPV were classified as Sabin like (SL).

Sequence analysis: The Sequencher 5.2.4 software was used to analyze the sequencing results. In the comparative analysis, Sabin 1, 2, and 3 strains had completed sequences on Genbank respectively: AY184219.1, AY184220.1 and AY184221. The VP1 sequence codes of Sabin 1, 2, and 3 strains on GenBank were AY082688.1, AY082679.1, and AY082683.1, respectively.

RESULTS

Isolation of PVs from sewage samples collected from the external environment: Between 2013 and 2016, a total of 139 sewage samples were collected from monitoring sites around Heilongjiang province, and the presence of PV was detected in 72 samples, with a positivity rate of 51.8%. Among all samples, 82 contained

non-polio enterovirus (NPEV), with a positivity rate of 59.0%. Among the sewage samples, 263 strains of PV and 312 isolates of NPEV were isolated, including 22 strains of type 1 PV (PV1), 104 strains of type 2 PV (PV2), and 137 strains of type 3 PV (PV3). The monitoring sites are shown in Table 1.

Mutations in PV detected from sewage samples: According to the updated definitions of VDPV and pre-VDPV (8), in 2013–2016, one strain of type 2 VDPV, 3 strains of type 1 pre-VDPV, 2 strains of type 3 pre-VDPV, one strain of PV1 with gene deletion, and 1 strain of type 3 + 2 with gene recombination were identified. Other strains of PV were identified to be SL. Sampling sites, in which the VDPV and pre-VDPV were isolated

Table 2. Differences of nucleotide sequences between the genomes of Sabin strains and isolates strains

Serotype	Isolated strain and mutation type	No. of mutations in VP1 Region-	Mutation position	Change of nucleotide	Change of amino acid
Type 1	2013003L1-3	5, deletion	nt2698	A → U	Ser
	Gene deletion		nt2749	A → G	Ile → Met
			nt2783–nt2788	GAUAAG → /	Asp, Lys → /
			nt2790	U → C	Leu → Pro
			nt2860	U → C	Tyr
			nt3229	G → A	Pro
	2013007R1-3	7	nt2645	G → A	Val → Ile
	pre-VDPV		nt2747	A → U	Ile → Leu
			nt2775	A → U	Lys → Met
			nt2901	A → G	Asn → Ser
			nt2914	U → C	Thr
			nt3151	A → G	Leu
			nt3358	C → U	Pro
	2013015L1-1	6	nt2747	A → U	Ile → Leu
	pre-VDPV		nt2755	G → A	Val
			nt2775	A → U	Lys → Met
			nt2854	C → U	Phe
			nt2975	G → A	Val → Met
			nt3079	U → C	Gly
	2014010H1-5	9	nt2614	C → U	Thr
	pre-VDPV		nt2638	U → C	Asn
			nt2645	G → A	Val → Ile
			nt2747	A → U	Ile → Leu
			nt2774	A → G	Lys → Glu
			nt2901	A → G	Asn → Ser
			nt3064	G → A	Ser
			nt3085	G → A	Ser
			nt3358	C → U	Pro
Type 2	2013033R1-5	7	nt2548	U → C	Ser → Pro
	VDPV		nt2712	U → C	Val
			nt2909	U → C	Ile → Thr
			nt2932	U → C	Leu
			nt2958	U → C	Tyr
			nt2986	A → G	Lys → Glu
			nt3108	U → C	Tyr
Type 3	2014009R1-3	6	nt2493	C → U	Thr → Ile
	pre-VDPV		nt2637	C → U	Ala → Val
			nt2790	U → C	Met → Thr
			nt3217	C → U	His
			nt3253	C → U	Tyr
			nt3346	C → A	Asp → Glu
	2014012L1-2	1, recombination	nt2493	C → U	Thr → Ile
	Gene recombination		nt3353–nt3376[1)]	[2)]	/
	2014032R1-2	6	nt2493	C → U	Thr → Ile
	pre-VDPV		nt2637	C → U	Ala → Val
			nt2790	U → C	Met → Thr
			nt2977	A → G	Ser
			nt3292	G → A	Pro
			nt3328	C → U	Asp

[1)]: The recombined gene sequence of type2 PV is nt3361–nt3384.
[2)]: The recombined RNA sequence is CCAGAAAAGGGAUUAACGACUUAU.

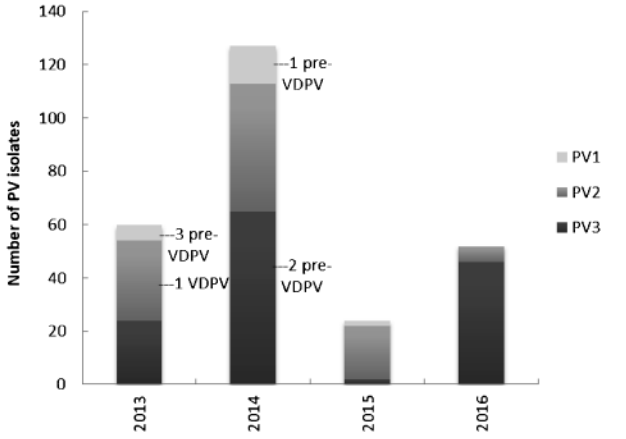

Fig. 2. The time distribution of PVs, pre-VDPV and VDPV.

from, are shown in Fig. 1, and mutation details are shown in Table 2. VDPV and pre-VDPV were mostly isolated from the samples collected from the urban area in Harbin city and Shuangcheng District. In addition, gene deletion strains and gene recombination strains were isolated from the samples collected from Harbin city. The time distribution of the 3 types of PV, pre-VDPV, and VDPV are shown in Fig. 2.

Gene mutation positions: In Table 2, 4 strains of PV type 1 had non-synonymous mutations, with amino acid substitution at position 90 (nt2747 and nt2749) of the VP1 region. Among the abovementioned strains, 3 had mutations from Ile to Ler. One strain changed from Ile to Met, which led the amino acid at this point to reverse to Mahoney (the wild strain). The amino acid at this position was changed to Lys from Thr in the process of attenuation from Mahoney strain to Sabin strain. Previous researches proved that the mutation at this point had no obvious change in neurovirulence (9). Mutations at other positions led to the substitution of amino acid, but they are all different from the positions of attenuated from wild virus strain to Sabin strain. Type 1 pre-VDPV, 2013007R1-3 strain, and 2014010H1-5 strain had 7 and 9 nucleotide variations, while 4 points had the same nucleotide variations. Compared with all PV1 detected in the sewage samples, the 4 point mutations were not considered as mutation hotspots (data were not shown). 2013007R1-3 was isolated from the sewage samples collected in Harbin on May 28, 2013, while 2014010H1-5 came from the samples obtained from the same location on May 26, 2014.

There was only one strain of type 2 VDPV isolated from the environment within the surveillance period. This strain had 7 nucleotide mutations on VP1 region, and 3 of them led to the change in amino acid: from Ser to Pro of nt2548 mutation, from Ile to Thr of nt2909 mutation, and from Lys to Glu of nt2986 mutation. Meanwhile, nt2909 mutation occurred at the amino acid position 143, which relates to the decrease of neurovirulence of type 2 wild virus strain.

Two strains of type 3 pre-VDPV were isolated from the sewage samples from the environment. Sequencing detection showed that the RNAs of all type 3 PVs (including SL strain; not shown in Table 2) at nt2493 were all U, different from Sabin strain, which was C at this position. Hence, the amino acid was decoded as follows: Thr for Sabin strain and Ile for isolated strains. In addition, the 2 isolated type 3 pre-VDPV virus strains had the same mutation at nt2637 and nt2790.

Gene deletion and gene recombination: One strain of PV1 with 6 nucleotide gene deletions and one strain of type 3 + 2 with gene combination were detected. In the 2013003L1-3 virus strain, gene deletion occurred at nt2783–nt2788 and led to the missing of 2 amino acid residues, while in the Sabin strain, Asp was at position 102 and Lys was at position 103. In the VP1 region of 2014012L1-2 virus strain, the major sequence belonged to Sabin 3 strain. However, from nt3353, it changed to type 2 Sabin strain until the end of the VP1 region, with a total length of 24 nucleotides, and their sequence was exactly the same as that of Sabin 2 strain at the same position. No more sequencing detection test was performed for the following region.

DISCUSSION

In Heilongjiang province, a total of 139 sewage samples were collected from the external environment in 2013–2016. From these sewage samples, 22 strains of type 1, 104 strains of type 2, and 137 strains of type 3 were isolated. In other areas in China, similar situations were observed, that is, the number of types 2 and 3 PV isolates found in the environment were higher than that of type 1 (10,11). The excretion periods of types 2 and 3 strains were considered to be longer than that of type 1 (12). The time distribution of pre-VDPV and VDPV was concentrated in 2013–2014. However, the number of sewage samples obtained in 2013–2014 were higher than the number of sewage samples obtained in 2015–2016. Hence, we could not draw the conclusion that the number of VDPVs decreased every year (shown in Fig. 2). The Heilongjiang province implemented the large-scale OPV campaign and supplemental OPV campaign in 2014, in which trivalent attenuated live vaccine was used. Those were the possible reasons for the higher detected number of PVs in 2014.

Of all PV strains, one strain of type 2 VDPV, 5 strains of pre-VDPV (3 of type 1 and 2 of type 3), one gene deletion strain of type 1, and one gene recombination strain of type 3 + 2 were identified. As shown in Fig. 1, VDPV and pre-VDPV were detected in the relatively concentrated places. In addition to the fact that more samples were collected in Harbin city, the higher population density of Harbin city, as the capital city, may be the reason for the higher detection rate of PV gene mutations. Therefore, Heilongjiang province was at higher risk of PV gene mutations. According to the surveillance data of acute flaccid paralysis (AFP) from 2013 to 2016, no VDPV or pre-VDPV was detected in the human stool samples.

PV1 attenuated live vaccine (Sabin strain) is known to have acquired 57 nucleotide mutations and 21 amino acid substitutions in the process of attenuation from the neurovirulent parental Mahoney strain (13). Furthermore, nt mutations that were known to cause the attenuation occurred at nt935 (VP4), nt2438 (VP3), nt2795, and nt2879 (VP1) (13). In addition, the reverse mutation of nt2795, which is associated with a vaccine-associated paralytic poliomyelitis (VAPP) case in China (14), increased the neurovirulence of Sabin strains. In the

present study, no PV1 strain contained the abovementioned mutations. Among the 3 pre-VDPV strains, mutations of nt2774 and nt2775 correspond to amino acid substitution at position 99, and the mutation of nt2975 corresponds to amino acid substitution at position 166. The amino acids at positions 93–104 of VP1 of the Sabin strain were known to constitute a neutralization epitope, and previous researches showed that amino acids at positions 165–172 of VP1 might be a specific neutralizing site (15,16). The isolated time gap for 2013007R1-3 and 2014010H1-5 strains was one year, and the nt sequencing proved that these 2 strains have quite a few different mutations, suggesting that pre-VDPV-1 may have been circulating in the population for a while. However due to a potential weak neurovirulence and herd immunity in Heilongjiang province, no AFP patients correlated with these strains.

Through mouse experiments, Ren et al. demonstrated that the major attenuation position of wild type PV-2 for neurovirulence is amino acid position 143 in VP1 from Thr (Lansing strain) or Val to Ile (Sabin strain) (17). In this study, we found the reverse mutation (Ile to Thr) at nt2909 of the PV-2 VDPV isolate 2013033R1-5. This mutation occurred within the mutation hotspot of PV-2, as reported previously (11,14). Out of 10 PV-2 isolates in this study, 5 strains had mutation at nt2909.

With regard to the attenuation process, the major attenuated positions of type 3 WPV strains were nt472 (5′ noncoding region) and nt2034 (VP3). Nt472 was reported to be at the main attenuated position, which was responsible for 70%–80% attenuated effect (14). The Nt2493 nucleotide of wild strains was U. However, after attenuation, it mutated to C, with amino acid changing from Ile to Thr. Previous studies reported that the mutation of nt2493 might affect the level of neurotoxicity (3). Type 3 virus strains detected in present research were all expressed as U in this site, consistent with wild strains. The reason was that the PV3 strain used in OPV in China was not the same with Sabin strain. Two nucleotide variants existed (Beijing Biological Products Institute: nt2493 and nt2832; Chinese Academy of Medical Sciences Institute of Medical Biology: nt2493 and nt3353). The polio vaccine used in our province from 2012 to 2014 was produced by the Beijing Biological Products Institute. The poliovirus detected in the external environment was consistent with the Sabin strain at the nt3353 site. Due to the fact that the sequence of the polio vaccine produced by Beijing Biological Products Institute was not published, the vaccine sequence information in the study was derived from the third-party institutions in 2006. In the vaccine strains of the abovementioned 2 institutes, the nt2493 nucleotides of PV3 were all U (19). Both vaccines were qualified in the clinical trials, and their safety and reliability were confirmed in the long-term application, which could be concluded that the single reverse mutation of nt2493 site alone did not cause the increase of neurovirulence of PV3.

The nucleotides missing in the strain 2013003L1-3 with gene deletion led to deletion of amino acid at positions 102 and 103 in the VP1 region. Furthermore, due to the mutation of nt2790, the 104th amino acid transferred from Leu to Pro, which was located on the peptides composed by residues of the 93rd–104th amino acids. The location was the exact neutralizing epitope of type 1 PV. These mutations of amino acid residues may lead to antigenic drift or change (15). After isolation, the strains were used for serial passages and maintained the good viability in the laboratory, which proved that gene deletion at this position had no clear influence on the reproductive capacity of PV.

In PV strains, recombination among different serotypes always tended to occur between types 2 and 3 viruses in China, and it was demonstrated that virus strains with gene recombination could give increase virulence at diverse levels (20). At present, most of recombination positions of detected recombinant virus were located in the section of non-structural protein, especially at 2A to 2C and 3D areas (11). Changes in the polymerase genes promoted the occurrence of genetic recombination. There was no similar report for the recombination of PV2 and PV3 in the VP1 region.

During the response to the polio outbreak in 1992 in the Netherlands, Vanderavoort et al. found that the detection of the related PV strains in sewage samples was preceded by an index case. Therefore, the continuous monitoring of PV in the environment may help identify areas at risk for polio outbreak (21). In September 2013, after the detection of VDPV in sewage samples collected in Shuangcheng in Heilongjiang province, supplementary immunization with OPV was implemented in the local target crowd. More intense monitoring of the environment and AFP cases were implemented in Shuangcheng area. There were no more VDPVs detected and no AFP cases occurred due to cVDPV. Hence, early warning played an important role for the forecast and control of disease.

The Global Commission for the Certification of Polio Eradication certified the eradication of type 2 wild poliovirus in September 2015, and polio eradication went into the endgame. In May 2016, China and other countries implementing OPVs were called to simultaneously switch from using trivalent OPV (tOPV), containing live attenuated poliovirus types 1, 2, and 3, to bivalent OPV (bOPV), containing poliovirus types 1 and 3 (22). After this switch policy, surveillance of environment should be enhanced, especially for PV2. Before the switch, there were only 5 PV2 strains isolated in February 2016. After May, there was no PV2 strain isolated in the present study.

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Conflict of interest None to declare.

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