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ORIGINAL PAPER

Isolation of Sabin-like Polioviruses from Sewage in Poland

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Abstract

As a complement to the active search for cases of acute flaccid paralysis, environmental sampling was conducted from January to December 2011, to test for any putative polio revertants and recombinants in sewage. A total of 165 environmental samples were obtained and analyzed for the presence of polioviruses by use of cell culture (L20B, RD and Caco-2) followed by neutralization and reverse-transcription polymerase chain reaction. Out of the 31 CPE positive samples, 26 contained one and 5 two different serotypes, yielding a total of 36 PVs. The microneutralization test revealed the presence of 7, 10 and 19 strains belonging to poliovirus serotype 1, 2 and 3, respectively. The genomic variability of 36 poliovirus strains was examined by the restriction fragment length polymorphism assay (RFLP). By combined analyses of two distant, polymorphic segments of the viral genome, one situated in the capsid protein VP1 coding region and the other in the 3D-polymerase coding region, we screened for the putative poliovirus revertants and recombinants. All detected PVs were classified as vaccine strains on the basis of RFLP-VP1 test. None of wild-type PVs or vaccine derived polioviruses were detected. RFLP assay also revealed the presence of 11 recombinants in 3D-polymerase coding region. Nine isolates appeared to be S3/S2, one S3/S1 and S1/S2 recombinant in analyzed $3D_{pol}$ region. This study revealed, through environmental monitoring, the introduction of SL PVs into the population associated with the routine use of OPV in Poland before the April 2016. Our findings demonstrate the usefulness of environmental surveillance in the overall polio eradication program.

Key words: environmental surveillance, oral polio vaccine (OPV), poliovirus recombinants and revertants in sewage

Introduction

The three serotypes of poliovirus (PV) are members of the family *Picornaviridae*. The viruses possess a single-stranded RNA genome of approximately 7,400 nucleotides (nt). Poliovirus is a causative agent of poliomyelitis, commonly known as polio (Landsteiner and Popper 1909). Polioviruses are transmitted by the fecal-oral route, they multiply in the gastrointestinal tract and are excreted in large numbers in the feces of infected persons, whether or not they are symptomatic. Virus infects sensitive cells of lymphoid tissue in the mouth, nose and throat. The incubation period lasts from 2 to 35 days. It leads to a transient viremia and the virus spreads to the reticuloendothelial system without causing clinical symptoms (Sabin, 1956). In very rare cases, 1–2% of infected individuals, the virus enters the central nervous system (CNS) and replicates in motor neurons within the spinal cord, brain stem, or motor cortex.

The Global Polio Eradication Initiative (GPEI) was launched in 1988. The basic strategy involves high levels of immunization, routine vaccination and poliovirus surveillance based on investigation of acute flaccid paralysis (AFP) cases. Although AFP surveillance is still the gold standard for GPEI, under certain circumstances supplementary information can be obtained by environmental poliovirus surveillance (ES). The rationale for ENV is based on the fact that infected individuals, whether they presenting the symptoms or not, excrete poliovirus in faeces up to several weeks. ES is used for monitoring of PV transmission in human populations by examining environmental samples contaminated by human faeces.

About 30 years ago, the effective control of poliomyelitis was achieved by the introduction of two polio vaccines: inactivated poliovirus vaccine (IPV) and the oral polio vaccine (OPV), both of which contain all three PV serotypes. The OPV consists of live attenuated Sabin strains obtained from wild PVs (Sabin and Boulger 1973). Since 2004, three doses of IPV and one dose of OPV have been used in Poland. According to WHO Global Action Plan III recommendations, Poland was obligated to switch to the IPV-only schedule before April 2016.

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Both vaccines (OPV and IPV) provide protection from poliomyelitis. However, OPV strains actively multiply in the gut and induce local secretory IgA response that is not seen with IPV. A major problem with the OPV is that it is genetically unstable. Sabin strains are subject to genetic variation during their replication in human intestine. Mutation in VP1 region encoding capsid protein may result in recovery of the capacity for higher neurovirulence of OPV strains. The neurovirulent revertants – vaccine derived polioviruses (VDPVs) may cause paralysis in under-immunized population and develop sustained circulation. VDPVs display 1–15% nucleotide difference within VP1 from parental vaccine strain (CDC 2007; Kew *et al.* 2005). Such changes were observed also by alterations of indirect markers such as thermosensitivity (ts-phenotype). Higher capacity to replication at supraoptimal temperature (40°C) of VDPVs and wild type (WT) polioviruses has been attributed to the loss of the ts-phenotype (Zurbriggen *et al.* 2008). Genetic recombination is another mechanism frequently observed in Sabin strains. The administration of trivalent OPV provides optimal conditions for recombination between heterotypic viral genomes. Thus, poliovirus strains are subject to genetic variation during their multiplication in humans (Kew and Nottay 1984). This prompted the initiation of the a study to investigate the prevalence of poliovirus in sewage samples collected in Poland. The study led us to create a comprehensive monitoring system for the environmental surveillance of PVs. Our results provide valuable information about the prevalence of PVs in sewage samples collected from 14 locations in Poland during 12-month period. Our study highlights the importance of environmental surveillance for detection of PVs during the OPV-to-IPV transition period.

Experimental

Materials and Methods

Sewage samples. A total of 165 raw sewage samples were collected between January and December 2011, one sample per month, from 14 sewage disposal systems in Poland. The sample-processing protocol has been described earlier (Zurbriggen *et al.*, 2008). To describe the process briefly, $AlCl_3$ (final concentration, 0.5 mM) was added to 500 ml of sewage sample, and the pH was adjusted to 3.5. Following the addition of 250 µl of an SiO_2 slurry, the samples were stirred for 30 min followed by centrifugation at room temperature and 1500 × g for 10 min to pellet the SiO_2. The virus was recovered by rocking the pellet for 20 min with 3 ml of 50 mM glycine (pH 9.5) containing 3% (wt/vol) beef extract. After centrifugation for 5 min at 4°C and 1500 × g, the supernatants were treated with 3 ml chloroform with rocking for 20 min. After a final centrifugation, the concentrates were used to inoculate cell cultures.

Isolation and identification of viruses. RD, L20B and Caco-2 cells were used throughout the experiment. RD and L20B cells were cultivated in minimal essential medium supplemented adequately with 3.5 and 10% fetal bovine serum (FBS). Caco-2 cells were cultivated in EMEM supplemented with 10% FBS. A volume of 200 µl sewage concentrate was inoculated into tubes with RD and L20B cells according to WHO procedure (WHO 2004) and additionally into tubes with Caco-2 cells. The tubes were incubated for 7 days at 36°C and examined daily for the appearance of cytopathic effect (CPE). After 7 days, the tubes were frozen at –20°C, thawed and 200 µl volumes passaged in cultures of the same cell type. Each specimen underwent three passages in RD, L20B and Caco-2 cells. Isolates obtained from specimens that are negative in L20B cells but positive in RD or Caco-2 cell line were repassaged in L20B to include the possibility of poliovirus. Samples demonstrating characteristic viral CPE were identified by neutralization assay using a selected set of polyclonal antisera against poliovirus type 1, 2 and 3 developed by National Institute of Public Health and the Environment, the Netherlands. The typing of polioviruses was performed according to WHO polio laboratory manual (WHO 2004). Briefly, using the micro-neutralization (microtitre plate) technique, the serum/isolate mixtures are incubated for one hour at 36°C to allow the antibodies to bind to the virus. Subsequently, suspensions of L20B cells are added to the microtitre plates which are examined daily for the presence of CPE. The antiserum that prevents the development of CPE indicates the identity of the virus.

Molecular characterization of viruses. Viral RNA was purified from supernatants of tubes that showed cytophatic effect, using Qiamp Viral RNA Mini Kit following the manufacturer's instructions. RT-PCR for intratypic differentiation (ITD) was carried out with Sabin-type specific primers according to WHO manual (WHO 2004). Three primer pairs (Sabin1.re/Sabin1.fw, Sabin2.re/Sabin2.fw, Sabin3.re/Sabin3.fw) targeting the sequence of VP1 region characteristic for each Sabin strain. These primers allow the amplification of genomic fragments of 97 bp, 71 bp, 53 bp from PV Sabin 1, Sabin 2 and Sabin 3 strains, respectively. RT-PCR was performed: 1 cycle of reverse transcription at 45°C for 20 min; 1 cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 70°C for 30 s followed by 1 cycle of elongation at 70°C for 7 min. Amplification products were analyzed in 2% agarose gels, stained with GelRed and visualized under UV light.

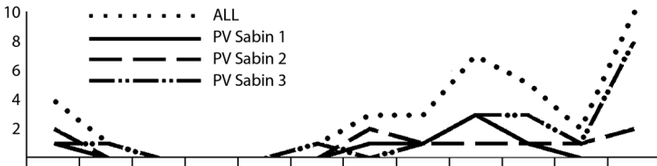
Restriction Fragments Length Polymorphism (RFLP) assays. Recombinant genomes were detected with a double RFLP assay that was performed as described elsewhere (Balanant *et al.* 1991, Furione *et al.* 1993) with slight modifications. Briefly, two distant regions of the viral genome were subjected to RT-PCR amplification followed by restriction enzyme digestion. The first pair of primers UC1 and UG1 was used for amplification of a 480 bp segment in VP1 capsid-encoding region in the RFLP-VP1 test. Another set of primers UC8 and UG7, was used to amplify a 291 bp segment in the 3D polymerase-coding region (RFLP-3D assay). The one step RT-PCR amplification was performed: 1 cycle of reverse transcription at 42°C for 30 min, 1 cycle of denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 10s, annealing at 45°C for 60 s, elongation at 70°C for 60s followed by 1 cycle of elongation at 70°C for 5 min. Amplification products of 490 bp and 291 bp were analyzed in 2% agarose gels, stained with GelRed and visualized under UV light.

Restriction enzymes were selected to differentiate between the genomes of the recombinants and the parental Sabin strains. RFLP-VP1 patterns of poliovirus strains were obtained by digesting a fraction of 480 bp amplified fragment with *Hae*III (10U), *Dde*I (5U) and *Hpa*II (14U). The 291 bp amplicon was digested by HaeIII (10U), DdeI (10U) and RsaI (10U) to obtain the RFLP-3D profiles. Reaction mixtures were incubated overnight at 37°C and digestion products were analyzed by electrophoresis in 3% agarose gel.

Determination of temperature sensitive (ts) phenotype. Tubes with confluent monolayer of L20B cells were inoculated with 200 µl of undiluted virus stocks: poliovirus isolates (no 1–36) and Sabin 1, 2 and 3 strains. After adsorption for 1 h at 36°C or 40°C, the unabsorbed virus inoculum was removed. The maintenance medium was added and the tubes were incubated in parallel at 36°C or 40°C. After 24 h, the tubes were frozen and thawed three times. The infectivity of isolates and Sabin strains was measured by titration on L20B cells according to WHO procedure (WHO 2004). In a previous study Zubriggen *et al.* (2008) showed a titer reduction of 2 $\log_{10}$ units with reference wild type PV1/Mahoney strain. Therefore, the titer reduction of >2 $\log_{10}$ units was considered to reflect temperature sensitivity.

Results

Isolation and identification of polioviruses. The wastewater samples were collected monthly from 14 sampling sites in Poland between January and December, 2011. In total, 31 out of 165 (18.8%) sewage samples were positive for poliovirus isolation in L20B, RD, Caco-2 cells and resulted in recovery of 36 PV strains. Polioviruses were isolated from samples collected in all months except April, May and June (Fig. 1). The highest monthly percentage of positive samples was observed in December (57.1%) and September (42.9%). The percentage of samples positive in cell culture



	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEPT	OCT	NOV	DEC	Total PVs/ sampling site	Poliovirus positive samples %
Koszalin									▼			▼	2	16.7
Bielsko-Biała							■						1	8.3
Pilchowice	■	▼					■		●●	▼▼		■	8	50.0
Opole	●								●	●■		▼	5	33.3
Warka										▼			1	8.3
Radom									▼		▼	▼	3	25.0
Piotrków Tybunalski						▼			▼				2	16.7
Mińsk Mazowiecki	■								■				2	16.7
Białystok								■					1	8.3
Jelenia Góra							●						1	8.3
Warszawa Południe				✖			✖	▼		✖		▼	2	16.7
Warszawa Czajka	▼											▼	2	16.7
Gdynia								●			■	▼▼	4	25.0
Chełm												■▼	2	8.3
Total PVs month	4	1	0	0	0	1	3	3	7	5	2	10	36	
Poliovirus positive samples %	28.6	7.1	0.0	0.0	0.0	7.1	23.1	21.4	42.9	23.1	14.3	57.1		

● PV Sabin 1 ■ PV Sabin 3 ▼ PV Sabin 3 ✖ lack of sample

Fig. 1. Seasonal distribution of polioviruses isolated in cell culture from sewage samples in Poland.

isolation at the different sampling sites ranged between 8.3% and 50.0% (Fig. 1).

Out of the 31 CPE positive samples, 26 contained one and 5 two different serotypes, yielding a total of 36 PVs. All poliovirus strains were isolated in three cell lines: L20B (17 strains), RD (2 strains) and Caco-2 (17 strains). The identification of poliovirus isolates, was performed using a selected set of polyclonal antisera against poliovirus types 1, 2 and 3. The microneutralization test revealed the presence of 7, 10 and 19 strains belonging to poliovirus serotype 1, 2 and 3, respectively.

Intratypic differentiation of polioviruses. Further characterization of isolates was carried out by intratypic differentiation using Sabin type-specific primers. The results obtained by RT-PCR assay for ITD confirmed the serotypes identified in microneutralization test and showed that all isolates are poliovirus Sabin-like (SL) strains (Table I).

Most of identified PVs come from samples collected during the June-December period (Fig. 1). The most frequently identified PV type 3 was detected in the following months: January, February, June, August (7.1%), September (21.4%), October (23.1%) and November (7.1%) with a peak on December (57.1%). Detection of PVs type 2 was mainly observed from July to December. PVs type 1 were isolated almost on the same level as PVs type 2, without a clear peak. All samples collected in March, April and May were negative for polioviruses.

The geographical distribution of polioviruses identified in sewage samples is shown in Figure 2. The number of detected PVs differed from 8 to 1, depending on the sampling site.

Determination of ts phenotype. In contrast to WT polioviruses, SL strains display ts phenotype and do not grow well at the elevated temperature of 40°C. For this reason, all isolates were tested for the replication capacity in supraoptimal temperature. Almost all poliovirus strains were temperature sensitive as expected, with titer reduction of ~2 $\log_{10}$ in 36°C/40°C. Only two strains showed < 2 $\log_{10}$ reduction in titer.

Identification of vaccine (Sabin) origin by RFLP-VP1. The vaccine origin of isolates was determined first by ITD assay. To confirm the vaccine origin of the strains, viral genomes were analyzed by RFLP-VP1 test. A 480-nucleotide fragment in the VP1 capsid protein-coding region was amplified by PCR from the viral genome. RFLP patterns specific for the isolates were generated by digestion of the PCR product with *Hae*III, *Dde*I, and *Hpa*II endonucleases and compared with the patterns obtained with the reference strains: Sabin type 1, 2, 3 (Table I). The RFLP profiles of all iso-

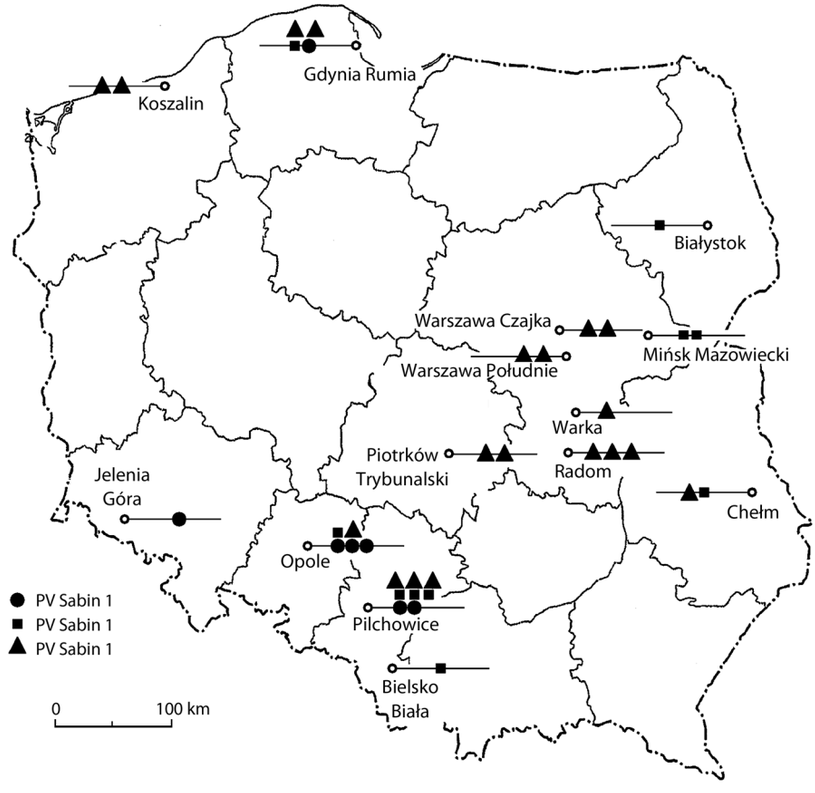


Fig. 2. Geographical distribution of polioviruses identified in sewage samples in Poland.

Table I
Polioviruses identified in sewage samples in Poland.

Isolate no./Month	Cell line	City	ITD	RFLP assay					
				RFLP-VP1			RFLP-3D		
				HaeIII	DdeI	HpaII	HaeIII	DdeI	RsaI
1/VIII	CaCo-2	Gdynia	S1	S1	S1	S1	S1	S1	S1
2/VII	L20B	Jelenia Góra	S1	S1	S1	S1	S1	S1	S2
3/I	L20B	Opole	S1	S1	S1	S1	S1	S1	S1
4/IX	RD	Opole	S1	S1	S1	S1	S1	S1	S1
5/X	L20B	Opole	S1	S1	S1	S1	S1	S1	S1
6/IX	CaCo-2	Pilchowice	S1	S1	S1	S1	S1	S1	S1
7/IX	L20B	Pilchowice	S1	S1	S1	S1	S1	S1	S1
Control strain: PV Sabin 1			S1	S1	S1	S1	S1	S1	S1
8/VIII	CaCo-2	Białystok	S2	S2	S2	S2	S2	S2	S2
9/VII	RD	Bielsko-Biała	S2	S2	S2	S2	S2	S2	S2
10/XII	CaCo-2	Chełm	S2	S2	S2	S2	S2	S2	S2
11/XI	CaCo-2	Gdynia	S2	S2	S2	S2	S2	S2	S2
12/I	L20B	Mińsk Maz.	S2	S2	S2	S2	S2	S2	S2
13/IX	L20B	Mińsk Maz.	S2	S2	S2	S2	S2	S2	S2
14/X	CaCo-2	Opole	S2	S2	S2	S2	S2	S2	S2
15/I	L20B	Pilchowice	S2	S2	S2	S2	S2	S2	S2
16/VII	CaCo-2	Pilchowice	S2	S2	S2	S2	S2	S2	S2
17/XII	CaCo-2	Pilchowice	S2	S2	S2	S2	S2	S2	S2
Control strain: PV Sabin 2			S2	S2	S2	S2	S2	S2	S2
18/XII	CaCo-2	Chełm	S3	S3	S3	S3	S3	S3	S3
19/XII	CaCo-2	Gdynia	S3	S3	S3	S3	S2	S2	S2
20/XII	L20B	Gdynia	S3	S3	S3	S3	S2	S2	S2
21/IX	L20B	Koszalin	S3	S3	S3	S3	S3	S3	S3
22/XII	L20B	Koszalin	S3	S3	S3	S3	S2	S2	S2
23/XII	CaCo-2	Opole	S3	S3	S3	S3	S3	S3	S3
24/II	CaCo-2	Pilchowice	S3	S3	S3	S3	S2	S2	S2
25/X	CaCo-2	Pilchowice	S3	S3	S3	S3	S2	S2	S2
26/X	L20B	Pilchowice	S3	S3	S3	S3	S3	S3	S3
27/VI	L20B	Piotrków Tryb.	S3	S3	S3	S3	S3	S3	S3
28/IX	L20B	Piotrków Tryb.	S3	S3	S3	S3	S1	S1	S1
29/IX	L20B	Radom	S3	S3	S3	S3	S3	S3	S3
30/XII	L20B	Radom	S3	S3	S3	S3	S3	S3	S3
31/XI	CaCo-2	Radom	S3	S3	S3	S3	S3	S3	S3
32/X	CaCo-2	Warka	S3	S3	S3	S3	S2	S2	S2
33/I	L20B	W-wa Czajka	S3	S3	S3	S3	S2	S2	S2
34/XII	L20B	W-wa Czajka	S3	S3	S3	S3	S2	S2	S2
35/VIII	CaCo-2	W-wa Płd.	S3	S3	S3	S3	S3	S3	S3
36/XII	CaCo-2	W-wa Płd.	S3	S3	S3	S3	S2	S2	S2
Control strain: PV Sabin 3			S3	S3	S3	S3	S3	S3	S3

S1, S2, S3: Poliovirus Sabin type 1, 2 and 3, respectively.

lates, after cleavage with 3 endonucleases were identical to those of the Sabin type 1, 2 and 3 reference strains. However, one exception was recorded. The restriction profiles after cleavage with *Dde*I and *Hpa*II of the strain 25 were similar to those obtained with Sabin 3. Cleavage with *Hae*III yielded one additional DNA fragment (< 300 bp), different from those obtained with Sabin 3. This RFLP pattern may suggest genetic variation and introduction additional restriction site recognized by endonuclease *Hae*III.

Detection of recombinant strains by RFLP-3D. We screened for recombinant strains by RFLP-3D assay, which analyzes the 5' part of the 3D polymerase coding region. RFLP-3D profiles were generated by digestion of the 291 bp PCR product with *Hae*III, *Dde*I, and *Rsa*I endonucleases and compared with the patterns obtained with the reference strains: Sabin type 1, 2, 3 (Table I). Among the 7 strains belonging to PV1, 6 had RFLP-3D profiles similar to those obtained with the Sabin 1. One strain (2) had restriction profile similar to those of the Sabin 2 with one of the three endonucleases used (RsaI), which strongly suggested the it relatedness to the Sabin 2. None of the serotype 2 strains was found to have a recombinant genome. Out of the 19 Sabin 3-derived strains, only 9 had restriction profiles identical to those obtained with Sabin 3. Nine strains (19, 20, 22, 24, 25, 32, 33, 34, 36) had RFLP-3D profiles indistinguishable from those obtained with Sabin 2, suggesting that their genomes consisted of segments derived from both Sabin 3 and Sabin2. One strain (28) had restriction profile obtained after digestion with three endonucleases, identical to those of the Sabin 1, suggesting close relatedness.

Discussion

Environmental surveillance has been used successfully in monitoring poliovirus circulation and assessing the extent or duration of epidemic poliovirus circulation in specific populations. In several countries, wild polioviruses and VPDVs have been detected in the sewage, sometimes in the absence of reported AFP cases. The list of countries routinely employing environmental surveillance includes Czech Republic, Egypt, Estonia, Finland, India, Israel, Japan, Latvia, The Netherlands, New Zealand, Pakistan, Russia, Slovakia and Switzerland. In Finland and Israel it is considered to be the main approach of PV surveillance (Hovi *et al.*, 2010). Polioviruses are frequently isolated from sewage samples in countries using OPV in routine immunization schedule. In Finland, where IPV is used exclusively for polio immunization, Sabin strains are isolated from environmental samples annually. This phenomenon involves traveling tourists from neighbouring Estonia and Russia, where OPV has been used recently. Moreover, importations of wild polioviruses was documented by ENV in the Israel and Gaza district in the absence of AFP cases (Manor *et al.*, 1999; Manor *et al.*, 2007). As a complement to the active search for cases of AFP, ENV has a role in the new WHO strategy (The Global Polio Eradication Strategic Plan, 2010–2012) for intensified efforts to complete poliomyelitis eradication.

After eradication of WPV transmission, the risk of re-emerge of polio remains is high (especially for under-immunized populations) as long OPV continues to be used routinely. Attenuated strains of the Sabin OPV tend to vary during their natural multiplication in the human gut and in rare cases cause vaccine-associated paralytic poliomyelitis (VAPP). Thus oral polio vaccine provides a potential source of circulating neurovirulent VDPV strains. Environmental contamination with feces and increasing use of wastewater in agriculture are likely risk factor for transmission of PVs (Dowdle *et al.*, 2006). Highly divergent and neurovirulent VDPVs have been isolated from sewage samples in several countries: Greece, Finland, Slovakia, Switzerland (Dedepsidis *et al.*, 2007; Roivainen *et al.*, 2010; Cernáková *et al.*, 2005). ENV can be important tool for monitoring the emergence of VDPVs, re-emergence of WT PVs or disappearance of Sabin strains after OPV cessation.

The present study used SiO_2-based protocol for concentration of sewage samples, followed by isolation of viruses in cell lines: L20B, RD and Caco-2. Compared to other methods of concentration, this protocol is simple, fast and easy to implement in environmental microbiology, especially for large volumes of the analyzed samples. Moreover, Zurbriggen *et al.* demonstrated a higher recovery rate (60%) in silicon dioxide assay than the two-phase separation protocol (30%) recommended by WHO (WHO 2003).

Culture-based systems for virus isolation have been the "gold standard" in clinical virology for decades. Although this technique is often slow and requires considerable technical expertise, WHO recommends L20B and RD cell lines for isolation of polioviruses (WHO 2004). In this study, Caco-2 cell line was used to obtain the maximum sensitivity for isolation of poliovirus from the sewage samples. Our earlier studies and other authors showed that Caco-2 cell line support growth of astroviruses, adenoviruses, rotaviruses and enteroviruses including poliovirus (Hamza *et al.*, 2011; Pinto *et al.*, 1995; Wieczorek *et al.*, 2013). In total, we isolated 36 poliovirus strains out of 165 (isolation rate 18.78%) sewage samples in L20B, RD, Caco-2 cells. Interestingly, 47% of poliovirus strains were isolated in Caco-2 cell line. The use of Caco-2 in the present study significantly increased the poliovirus detection.

The seasonal distribution of polioviruses is well documented, its presence being common in the late summer and fall. In our study the distribution of PVs followed the seasonal pattern with a clear peak in September and December. Polioviruses were isolated from samples collected in all sampling sites during 12-month period except April, May and June. In fact, scheduled OPV immunizations determine the frequency of Sabin-like PVs isolation. Other studies have reported a rapid decline in PV isolation around OPV-to-IPV transition period, with a dissappearance of PV vaccine strains from wastewater within 2 to 3 months after the cessa-

tion of OPV administration (Mueller *et al.*, 2009; Wahajuhono *et al.*, 2014).

The serotypes of the isolates were determined by the microneutralization assays. The serotyping test revealed the presence of 7 and 10 strains belonging to poliovirus serotype 1, 2 respectively. The most frequently detected polioviruses were PV3 (19 strains) and these accounted 53% of total serotyped strains. Other authors also indicate that PV3 is frequently isolated from sewage samples (Sarijlou *et al.*, 2007; Yoshida *et al.*, 2000). In previous studies Stanway *et al.* (1984) showed that the difference between attenuated Sabin 3 and wild-type PV3 Leon strains is restricted to only 10 nucleotides and three amino acids. In addition, it has been shown that P1 domain of PV3 Sabin strain harbors only two nucleotide changes correlating with the apparent mitigation of neurovirulence. Therefore, most cases of VAPP result from vaccination with PV3 (Minor 2004).

Mutations known to be involved in restoring neurovirulence or eliminating the temperature-sensitive phenotype of vaccine derivatives are associated with capsid coding region of poliovirus genome (Macadam *et al.*, 1989; Westrop *et al.*, 1989; Ren *et al.*, 1991; Macadam *et al.*, 1993). The ts phenotype correlates with the attenuated Sabin strains. A titer reduction of $> 2 \log_{10}$ units at 40°C is considered to reflect temperature sensitivity (Zubriggen *et al.*, 2008). In the present study we showed a $\geq 2 \log_{10}$ reduction in titer for most of poliovirus isolates propagated at 40°C. All analyzed isolates grew poorly in supraoptimal temperature, what is characteristic for Sabin-like strains. Similar findings presented by other authors, also demonstrate that ts is an important viral phenotypic marker, as it may be involved in virulence of attenuated vaccine strains (Blomqvist *et al.*, 2003; Zurbriggen *et al.*, 2008). On the other hand, Bouchard *et al.* (1995) suggest that temperature sensitivity may not always be an acceptable method for evaluating the presence of attenuating mutations. Although intratypic differentiation revealed the presence of 36 SL PVs, two strains showed $< 2 \log_{10}$ reduction in titer. Our results also show that that determinants of attenuation and temperature sensitivity can be genetically separated.

Recombination is a very frequent event in poliovirus evolution (Agol 2006). Moreover, the trivalent OPV provides ideal conditions for intertypic recombination between the attenuated strains of the three PV serotypes. In order to understand the evolution of vaccine strains during their replication in humans, our study focused on the characterization of PV genomes by double RFLP assay and revealed the presence of 11 recombinants in 3D-polymerase coding region. Nine isolates appeared to be S3/S2, one S3/S1 and S1/S2 recombinant in analyzed $3D_{pol}$ region. The low degree of changes found in the genome of the 36 SL PVs may suggest that these strains have undergone only limited circulation in the population.

In summary, the isolation of 36 SL PVs demonstrated that environmental surveillance is an effective supplemental support to the AFP surveillance to verify the absence of wild or VDPV strains in the country. No WT and VDPV strains were isolated from the environmental samples examined during the study period. In Slovakia, high percentage of PV strains (SL and VDPV) were also observed in sewage water during 2001–2006. Thereafter, PV isolates were gradually replaced by non-polio enteroviruses (NPEVs). A transition was due to the change in the childhood vaccination programme in which OPV was substituted by IPV (Klement *et al.*, 2013). In contrast, other authors have showed the disappearance of SL PVs from the environment even before the OPV immunization has ceased (Nakamura *et al.*, 2015).

In conclusion, our study revealed, through environmental monitoring, the introduction of SL PVs into the population associated with the routine use of OPV in Poland before the April 2016. Detection of polioviruses in sewage samples of the local community reflects the presence of virus-shedding individuals. ENV should be continued in the critical period between interruption of WPV transmission and certification of polio eradication. Environmental surveillance should be also available in the post-eradication and OPV cessation periods. Even if the OPV-to-IPV transition is successful, the risk of PV infection for a susceptible population should be monitored with an appropriate surveillance system.

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