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Isolation of Sabin-Like Polioviruses from Wastewater in a Country Using Inactivated Polio Vaccine▽

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From 2001 to 2004, Switzerland switched from routine vaccination with oral polio vaccine (OPV) to inactivated polio vaccine (IPV), using both vaccines in the intervening period. Since IPV is less effective at inducing mucosal immunity than OPV, this change might allow imported poliovirus to circulate undetected more easily in an increasingly IPV-immunized population. Environmental monitoring is a recognized tool for identifying polioviruses in a community. To look for evidence of poliovirus circulation following cessation of OPV use, two sewage treatment plants located in the Zurich area were sampled from 2004 to 2006. Following virus isolation using either RD or L20B cells, enteroviruses and polioviruses were identified by reverse transcription-PCR. A total of 20 out of 174 wastewater samples were positive for 62 Sabin-like isolates. One isolate from each poliovirus-positive sample was analyzed in more detail. Sequencing the complete viral protein 1 (VP1) capsid coding region, as well as intratypic differentiation (ITD), identified 3 Sabin type 1, 13 Sabin type 2, and 4 Sabin type 3 strains. One serotype 1 strain showed a discordant result in the ITD. Three-quarters of the strains showed mutations within the 5′ untranslated region and VP1, known to be associated with reversion to virulence. Moreover, three strains showed heterotypic recombination (S2/S1 and S3/S2/S3). The low number of synonymous mutations and the partial temperature sensitivity are not consistent with extended circulation of these Sabin virus strains. Nevertheless, the continuous introduction of polioviruses into the community emphasizes the necessity for uninterrupted child vaccination to maintain high herd immunity.

The global polio eradication initiative was launched in 1988. Substantive progress has been made toward this goal, but further work is still required (11, 12, 21, 29). Successful eradication of the cause of an infectious disease, exemplified by that of *Variola virus*, has at least four fundamental requirements: (i) absence of an animal reservoir; (ii) effective vaccines and, in the case of live-attenuated viruses, genetically stable vaccines; (iii) absence of long-term virus carriers; and (iv) sensitive tools to detect the presence of the causal agent. In the case of poliovirus eradication, these requirements have not been completely met. The basic strategy of the polio eradication initiative involves achieving high levels of routine immunization, mass vaccination, supplementary mop-up immunization activity, and poliovirus surveillance based on virological investigation of acute flaccid paralysis (AFP) cases (51, 56). There are two effective vaccines: inactivated polio vaccine (IPV), originally developed by Jonas Salk and colleagues, and the live-attenuated oral polio vaccine (OPV) of Albert Sabin (39). Both vaccines provide effective protection from poliomyelitis. However, IPV induces less mucosal immunity in the gut than OPV, a prerequisite for reducing intestinal reinfection, virus shedding, and transmission to susceptible contacts (33, 37, 41, 44, 45). OPV, which better protects from infection, suffers from other inadequacies that complicate the ongoing initiative (11, 12, 21, 29). One inadequacy is the emergence of virulent vaccine-derived polioviruses (VDPVs) upon long-term replication in immunodeficient persons (iVDPV) or following sustained circulation in populations with immunity gaps (cVDPV). Ambiguous VDPVs, such as environmental isolates, represent a category of virulent polioviruses that cannot easily be assigned to iVDPV or cVDPV (29). Additional challenges for the initiative are poorly understood deficiencies in vaccine efficacy (24) and the low clinical attack rate following infection with virulent polioviruses (≤1:100 paralytic case/infections). This calls for specific and sensitive virus-tracking tools. AFP surveillance is presently the gold standard for meeting those requirements (56).

The three serotypes of poliovirus are members of the family *Picornaviridae* (20, 40, 55, 61). The viruses possess a single-stranded RNA genome of approximately 7,400 nucleotides (nt). Upon infection, the genomic RNA is translated as a polyprotein, which is then processed by proteases into functional proteins. The structural proteins are located within the N-terminal P1 region and the nonstructural proteins within the succeeding P2 and P3 regions of the polyprotein. Similar to some other RNA viruses, the virus-encoded RNA-dependent RNA polymerase is error prone and lacks proofreading activity. This results in a high mutation frequency for these viruses. The molecular evolution of polioviruses is characterized by selective propagation of virus variants that are generated through mutations and heterotypic or intragenus recombination (29). During wild-type (wt) poliovirus infection, a steady accumulation of mostly synonymous mutations is the major contribution to genetic evolution (23). In addition, an

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early burst of nonsynonymous mutations and recombination is characteristic of the evolution of the Sabin strains in the guts of vaccinees and their susceptible contacts. This helps the virus to regain replicative fitness (64) and to revert to the virulent phenotype with an increased possibility of causing neurologic disease. There are a number of serotype-specific nucleotide positions associated with attenuation (29). Therefore, observation of the reversion of these attenuating mutations at these positions implies an increase in virulence. Nonetheless, other factors not solely associated with these single-nucleotide changes could also play a role in virulence (28). The number of synonymous mutations within the region coding for the capsid protein viral protein 1 (VP1) is used to estimate the "age" of poliovirus strains, i.e., the duration of in vivo replication in single or successive hosts (4, 14, 15, 23, 26). VDPVs are defined as viruses with a 1 to 15% nucleotide sequence difference within the VP1 region compared to the parental Sabin vaccine strain of the same type (11, 29). Viral isolates that display <1% nucleotide sequence difference from the parental vaccine strain are defined as vaccine- or Sabin-like (SL). In principal, both VDPVs and SL viruses can cause poliomyelitis, but at different rates on average. In settings with low vaccination coverage, however, the VDPVs are of growing concern in the efforts toward global polio eradication (12).

There are a few European countries that have relied exclusively and successfully upon IPV to control both poliomyelitis and the circulation of wt polioviruses. This success has been achieved by maintaining high vaccination coverage (17, 41). Similar to other countries, Switzerland before 2001 used mainly OPV. Between 2001 and 2004, Switzerland used a combination of IPV and OPV for safety reasons, i.e., to prevent cases of vaccine-associated paralytic poliomyelitis. In 2004, there was a switch to the IPV-only vaccination schedule. The reported vaccination coverage of children less than 2 years old with three doses of IPV is 94% (9). Following the switch from OPV to IPV, the Swiss population now consists of an older age group that was immunized with OPV and a growing younger age group that is immunized only with IPV. The national AFP surveillance system is important but not optimal, because a low number of stool samples are submitted for virological examination (59).

Since IPV provides only limited protection against intestinal infection, a possible risk is the undetected circulation of imported wt or vaccine poliovirus in persons with only IPV-induced immunity. Furthermore, vaccine viruses silently circulating among individuals lacking mucosal immunity could be a potential source for generation of cVDPV. Consistent with reports of the international spread of poliovirus, such strains could provoke a poliomyelitis outbreak in a setting with inadequate immunity. This prompted the initiation of a study to investigate the prevalence of poliovirus in municipal wastewater samples in Switzerland following the cessation of routine OPV use. In this study, we characterized poliovirus-positive sewage samples collected between January 2004 and October 2006 by employing a new protocol for virus concentration and either RD or L20B cells for primary virus isolation.

MATERIALS AND METHODS

Wastewater samples. A total of 174 wastewater samples were collected between January 2004 and October 2006 from two sewage treatment plants in Werdhoelzli (Wh) and Kloten-Opfikon (KO). The Wh plant serves Zurich, the largest city in Switzerland, with approximately 340,000 inhabitants. The KO plant serves a community of approximately 55,000 persons near the Zurich international airport. The grab samples for virus isolation were usually taken once a week, transported to the laboratory within 1 to 2 h, and processed immediately according to the WHO guidelines (57).

Sample preparation. The sample-processing protocol has been described in detail elsewhere (3, 34, 53). 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 (5), the samples were stirred for 30 min, followed by centrifugation at room temperature and 1,500 × *g* for 5 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 (B-4888; Sigma Chemie GmbH, Buchs, Switzerland). After centrifugation for 5 min at 4°C and 1,500 × *g*, the supernatants were adjusted to pH 7.5 and treated three times with 3 ml chloroform with rocking for 10 min each. After a final centrifugation, the concentrates were used to inoculate cell cultures.

Virus isolation. RD and/or L20B cells (60) were used throughout the experiments. Both cell lines were cultivated in minimal essential medium supplemented with 7.5% fetal calf serum. Two different cell culture procedures were used during the course of the studies, differing in the cell lines initially inoculated and the nature of subsequent passages (Fig. 1). Environmental concentrates (3 ml) were adjusted to 19 ml with cell culture medium, and cell monolayers in two 96-well plates were inoculated with 0.1 ml per well of this suspension. On each plate, one well was mock infected as a negative control. The plates were incubated for 6 to 8 days at 36°C. Blind and second passages were typically performed in 24-well plates. With a focus on the biohazard and to avoid possible cross-contamination during laboratory work, a standard operating procedure was strictly followed throughout.

Determination of virus recovery efficiency. The in-house SiO_2 protocol was compared in parallel with the two-phase separation protocol (dextran 40 and polyethylene glycol 6000) recommended by WHO (57). A total of seven experiments with spiked wastewater were performed. One experiment was defined as spiking two 500-ml samples with 10,000 50% cell culture infective doses ($CCID_{50}$) of the Mahoney strain, and the virus recovery efficiency was determined based on the measured infectivity by titration on L20B cells using a series of $\log_5$ dilutions and six inoculated cultures for each viral dilution.

Molecular characterization of viruses. RNA was purified, using a Qiamp Viral RNA Mini Kit (Qiagen GmbH, Basel, Switzerland), from the supernatants of wells that showed cytopathic effect. Reverse transcription (RT) and PCR were done with a one-tube, two-step protocol (20 μl for RT and 50 μl for PCR; both from Promega AG, Dübendorf, Switzerland). RT was performed with specific antisense primers. All primers used (Table 1) were purchased from Microsynth GmbH, Balgach, Switzerland. Following agarose gel electrophoresis, the PCR products were purified (Qiaex II; Qiagen GmbH) and subjected to sequencing (Microsynth GmbH). Sequences were routinely obtained from both strands, and multiple-sequence alignments were carried out with the program CLUSTALW (http://www.ebi.ac.uk/clustalw).

Two generic primer pairs targeting the 5′ untranslated region (5′ UTR) (HuEV2.re and HuEV2.fw) (1) and the VP1 region (All-EV.re and All-EV.fw) (43) were used to detect enterovirus RNA. Three primer pairs were used as the basis for poliovirus characterization. The first pair (Y7 and Q8) amplified the entire VP1 coding region to allow complete sequencing of the region (54). The second pair (APAN and UG15) targeted the P3 coding region of the nonstructural proteins (6, 25). The third pair (HuEV2.re and UG52) allowed amplification and sequencing of the partial poliovirus 5′ UTR. Reactivity with Y7 and Q8 indicated the presence of a poliovirus RNA, and sequencing of the resulting amplicons identified both the serotype (1, 2, or 3) and some virulence attributes, namely, SL versus VDPV versus wt poliovirus. The primers used in this study are summarized in Table 1. VP1 sequences were compared with the respective Sabin prototype strains (GenBank accession no. AY082688.1, AY082679.1, and AY082683.1). The numbers of synonymous and nonsynonymous nucleotide substitutions were determined with the program DIVERGE (GCG package, version 10.3; Genetics Computer Group, Madison, WI). Based on this, the "ages" of the isolates were calculated (15, 64). Sequencing of the amplicons from the 5′ UTR gave additional information regarding reversion of attenuation. Within the 5′ UTR the most important determinant of attenuation for Sabin type 1 is the nucleotide "G" at position 480; for Sabin type 2, it is "A" at position 481, and for Sabin type 3, it is "U" at position 472. PCR amplicons of the P3 genomic region were subjected to restriction fragment length polymorphism analysis (SpeI) to screen for recombinants (6). Strains with altered SpeI patterns were sequenced and characterized based on multiple sequence alignment with published sequences (GenBank accession no. V01150, X00595, and X00925).

Replication capacity at supraoptimal temperature. In contrast to wt poliovirus, SL viruses do not grow well at the elevated temperature of 40°C. The

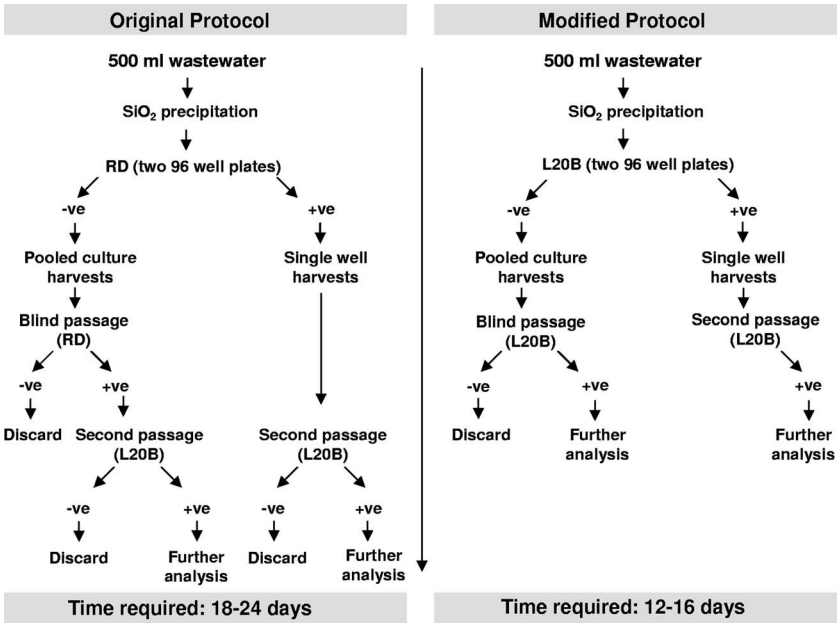

FIG. 1. Flowchart of poliovirus isolation protocols. For primary virus isolation, either RD or L20B cells were used. The second passage was on L20B cells. +ve, cytopathic effect within 6 to 8 days; −ve, no cytopathic effect within 6 to 8 days.

infectivities of isolates were measured in parallel by titration at 36°C and 40°C on L20B cells (7, 42). Following inoculation, the 96-well plates were sealed in plastic bags and submerged in a water bath at the specified temperature. The results were recorded 3 days postinoculation. A titer reduction of 2 $\log_{10}$ units at elevated temperature was observed with the reference strain Mahoney of poliovirus type 1. Therefore, a difference of >2 $\log_{10}$ units was considered to reflect temperature sensitivity.

Intratypic differentiation (ITD). The strains were characterized by an antigen-based enzyme-linked immunosorbent assay (ELISA) using type-specific cross-absorbed anti-poliovirus sera and by a molecular test, using Sabin-specific primers, according to WHO guidelines (60).

Nucleotide sequence accession numbers. The VP1 sequences determined during this study have been deposited in GenBank (accession no. DQ841124 to DQ841140 and EF490669 to EF490681).

RESULTS

Efficiency of virus recovery. The in-house SiO_2 protocol and the two-phase separation protocol were compared in parallel using spiked sewage samples. Virus recovery using our SiO_2 protocol (60 ± 30%) was significantly higher ($P < 0.05$; U test) than with the two-phase separation protocol (30 ± 25%) recommended by WHO. The observed variability of both protocols between samples was likely due to varied properties of the sewage samples and was not investigated further. All further studies were based on the in-house SiO_2 protocol.

Environmental poliovirus isolations. A total of 20 out of 174 (11.5%) wastewater samples were positive for any poliovirus isolation and resulted in recovery of 62 poliovirus strains, namely, 46 from 2004 to 2005 and 16 in 2006 (Table 2). The former 46 strains were recovered upon primary isolation on RD cells from 13 out of 88 samples. The latter 16 strains were found in 7 out of 86 samples by primary isolation on L20B cells. Sequencing of the VP1 region invariably identified these strains as SL (see below). During 2004 and 2005, there were 410 and 1,250 virus isolations, respectively, indicated by growth in a well on RD cell plates. Of these, 10 and 39 could be further propagated on L20B cells (Fig. 1 and Table 2). Two of the three nonpolioviruses from 2005 that grew on L20B cells were identified as human enteroviruses based on amplification with the enterovirus generic primers HuEV2.re and HuEV2.fw, leaving one strain with undetermined identity.

TABLE 1. Primers used in this study for amplification and sequencing

Primer	Sequence (5′–3′)	Polarity	Position[a]	Product size (kb)	Target sequence[b]	Reference
HuEV2.re	ACCGGATGGCCAATCCAA	Antisense	638–621			
HuEV2.fw	CCTCCGGCCCCTGAATG	Sense	444–460	0.2	EV/5′ UTR	1
UG52	CAAGCACTTCTGTTTCCCCGG	Sense	162–182	0.48	PV/5′ UTR	25
Q8	AAGAGGTCTCTATTCCACAT	Antisense	3504–3485			
Y7	GGITTTGTGTCAGCITGCAATGA	Sense	2399–2421	1.11	PV/VP1	54
ALL-EV.re	CICCIGGIGGIAYRWACAT	Antisense	2969–2951			
ALL-EV.fw	MIGCIGYIGARACNGG	Sense	2613–2628	0.36	EV/VP1	43
APAN	TTTTTTTTTTTTTTTTTTCTCCG	Antisense	7436>			6
UG15	CTGTCACCAACCAGCAAACTT	Sense	4936–4956	2.53	PV/P3 region	25

[a] Positions are relative to the genome of Sabin 1 (GenBank accession no. V01150).
[b] EV, enterovirus; PV, poliovirus; P3, viral nonstructural protein region.

TABLE 2. Isolation of (polio)viruses from two sewage treatment plants[a]

Yr	No. of samples with poliovirus/no. of samples tested (%)	No. of cytopathic strains in P_1 (RD)	No. of poliovirus strains/total no. of strains (%) in:	
			P_2 (L20B)	$P_{1,2}$ (L20B)
2004	3/30 (10)	410	10/10 (100)	ND
2005	10/58 (17.2)	1,250	36/39 (92.3)	ND
2006	7/86 (8.1)	ND[b]	ND	16/58 (27.6)
Total	20/174 (11.5)	1,660	46/49 (93.9)	16/58 (27.6)

[a] Using either RD or L20B cells for primary isolation (P_1) and L20B cells for a second passage (P_2). Sewage sample concentrates were used to inoculate cells grown in two microtitration plates.
[b] ND, not determined.

The modified protocol implemented during 2006 using L20B cells for primary isolation (Fig. 1) resulted in 58 cytopathic-effect-positive cultures (Table 2). Of these, 16 (27.6%) were subsequently identified as polioviruses. Surprisingly, none of the remaining 42 strains reacted with the enterovirus generic primers HuEV2.re and HuEV2.fw. A second primer pair with panenterovirus specificity (All-EV.re and All-EV.fw), which targets the VP1 genomic region (43), was then used successfully to amplify the RNAs of 10 of these 42 nonpolioviruses, leaving 32 strains with undetermined identities. Amplicons from 6 of the 10 nonpoliovirus enteroviruses were sequenced, and a BLAST search showed that the most closely related sequences were from bovine enteroviruses (data not shown). In conclusion, the modified protocol detected, in addition to polioviruses, both presumptive bovine enteroviruses and a substantial number of viruses with undetermined identities. This finding with L20B cells thus contrasted with the results that were obtained when RD cells were used for primary isolation.

Two of the 20 poliovirus isolations (Wh/77P and Wh/4P) could be achieved in cell culture only after one blind passage (samples 2 and 13, in RD and L20B, respectively). In the remaining samples, the numbers of strains isolated during the first passage ranged from 1 (in 10 samples) to 16 in sample no. 6 (Table 3). Only four of the poliovirus-positive samples had more than two strains isolated. Considering the efficiency of virus recovery with the SiO_2 protocol (60%), the original titer was estimated to be $\leq$30 $CCID_{50}$/500 ml. Based on the nucleotide sequence of the VP1 region, the strains recovered from a given sample were basically identical ($\pm$1-nt difference), i.e., there was no evidence of heterotypic virus isolations.

Molecular characterization of isolated polioviruses. A total of 20 strains, 1 strain from each poliovirus-positive sample, were characterized in more detail (Tables 3 and 4). The numbers of synonymous mutations within the VP1 region were $\leq$1, 3, and 5 in 17, 2, and 1 strains, respectively. This indicated that the strains were either "newborn" (no synonymous substitution) or "young" (1 to 10 synonymous substitutions), i.e., the ages of these strains were invariably less than 1 year (64). In particular, the calculated ages of these strains were $\leq$6 weeks for 17 strains and 3.7 $\pm$ 2.2 months (Wh/596), 4.1 $\pm$ 2.2 months (KO/42.3), and 6.5 $\pm$ 3.0 months (Wh/4P) for the remaining strains. Most strains had reversed the attenuating mutations (29) in both the VP1 region and the 5′ UTR. Three strains

TABLE 3. Origins and overview of genotypic and phenotypic characteristics of environmentally derived polioviruses

Sample no.	Yr/wk	No. of strains isolated[a]	Origin/strain studied	Genotype[b]	No. of synonymous/all mutations in VP1[c]	Nucleotide at position[d]	Recombinant[e]	RCT[f]	ITD[g]	
									ELISA	PCR
1	2004/19	2	KO/50	Sabin 1	1/1	480**G**		6.0	SL	SL
2	2005/25	1	Wh/77P	Sabin 1	1/6	480A		4.0	NSL	SL
3	2006/14	1	Wh/14.13	Sabin 1	0/4	480A		5.5	SL	SL
4	2004/16	6	Wh/167	Sabin 2	1/2	481G		4.0	SL	SL
5	2005/26	1	Wh/80	Sabin 2	0/0	481**A**		4.75	SL	SL
6	2005/36	16	Wh/596	Sabin 2	3/4	481G		4.5	SL	SL
7	2005/39	2	Wh/679	Sabin 2	0/2	481G		4.5	SL	SL
8	2005/40	1	Wh/777	Sabin 2	0/1	481G		5.5	SL	SL
9	2005/41	11	Wh/786	Sabin 2	0/1	481G		4.5	SL	SL
10	2005/43	1	Wh/870	Sabin 2	0/2	481G		4.75	SL	SL
11	2005/47	1	Wh/939	Sabin 2	1/4	481**A**		5.75	SL	SL
12	2005/50	1	Wh/998	Sabin 2	0/1	481G	S2/S1	5.0	SL	SL
13	2006/04	1	Wh/4P	Sabin 2	5/9	481G	SpeI$^-$	4.0	SL	SL
14	2006/30	1	KO/30.1	Sabin 2	0/0	481**A**		5.5	SL	SL
15	2006/31	9	Wh/31.32	Sabin 2	0/2	481G		4.25	SL	SL
16	2006/42	1	KO/42.3	Sabin 2	3/5	481G		5.0	SL	SL
17	2004/09	2	Wh/70	Sabin 3	0/0	472C		3.5	SL	SL
18	2005/36	1	KO/565	Sabin 3	0/0	472**U**		5.25	SL	SL
19	2006/02	2	Wh/2.1	Sabin 3	0/1	472C	S3/S2/S3	3.75	SL	SL
20	2006/03	1	Wh/3.5	Sabin 3	0/1	472C	S3/S2/S3	4.25	SL	SL

[a] Number of cultures (out of 190) with poliovirus growth (see Materials and Methods). Multiple strains recovered from a given sample were essentially identical as deduced from the nucleotide sequence of the VP1 capsid region (data not shown).
[b] According to the nucleotide sequence of the VP1 gene.
[c] Compared with the respective Sabin sequence.
[d] Letters in boldface indicate Sabin-type nucleotides.
[e] Sabin-type sequences determined following restriction enzyme (SpeI) fingerprinting. SpeI$^-$, mutational loss of cleavage site.
[f] RCT, reproductive capacity at supraoptimal temperature: $\log_{10}$ ($CCID_{50}$ at 36°C/$CCID_{50}$ at 40°C). In this assay, PV1 (Mahoney) showed a 2.0 $\log_{10}$ reduction in titer.
[g] ITD according to WHO guidelines (60). NSL, non-SL.

TABLE 4. Nucleotide and amino acid changes observed within the VP1 gene of environmentally derived polioviruses

Strain (Sabin type)	Nucleotide substitution at position[a]	Change to amino acid[a]	Contained in antigenic region[b]
KO/50 (S1)	G3313A		
Wh/77P (S1)	G2741A	A088T	+
	A2749G	I090M	+
	A2775C	K099T	+
	A2795G	T106A	+
	G3058A		
	C3171U	A231V[c]	+
Wh/14.13 (S1)	G2502A	S008N[c]	−
	G2776U	K099N	+
	A2795U	T106S	+
	A3059U	I194F	−
Wh/167 (S2)	A2908G	I143V	−
	A3342G		
Wh/80 (S2)			
Wh/596 (S2)	G2715A		
	U2909C	I143T	−
	G3258A		
	C3318U		
Wh/679 (S2)	U2909C	I143T	−
	C2962U	P161S[c,d]	−
Wh/777 (S2)	U2909C	I143T	−
Wh/786 (S2)	U2909C	I143T	−
Wh/870 (S2)	A2527G	N016D[c]	−
	U2909C	I143T	−
Wh/939 (S2)	A2785G	S102G[c,d]	+
	U2909A	I143N	−
	A2986G	K169E[c]	−
	A3297G		
Wh/998 (S2)	U2909C	I143T	−
Wh/4P (S2)	A2513U		
	C2568U	E011V[c,d]	−
	A2610G		
	G2742A		
	U2909C	I143T	−
	A2950U	I157L[c,d]	−
	C3146U	S222L	+
	A3255G		
	G3267A		
KO/30.1 (S2)			
Wh/31.32 (S2)	A2527G	N016D[c]	−
	U2909C	I143T	−
KO/42.3 (S2)	U2565C		
	G2631A		
	G2772A		
	U2909C	I143T	−
	A2986G	K169E[c]	−
Wh/70 (S3)[e]			
KO/565 (S3)[e]			
Wh/2.1 (S3)[e]	G2636A	A054T	−
Wh/3.5 (S3)[e]	G2636A	A054T	−

[a] Left-hand letter, Sabin original nucleotide/amino acid; right-hand letter, nucleotide/amino acid recorded with the isolated strain; number, nucleotide position and amino acid residue within VP1.

[b] Based on Yakovenko et al. (64). +, yes; −, no.

[c] Amino acid substitution not described by Yakovenko et al. (64).

[d] Amino acid substitution not located in the NCBI data bank.

[e] With Wh/70 and KO/565, the nucleotide at position 2493 is C; with Wh/2.1 and Wh/3.5, it is U. This is explained by vaccine seeds that contain a mixture of both strains (47).

were heterotypic recombinants within the nonstructural coding region. Strain Wh/998 (Sabin 2; zero synonymous changes in VP1) recombined between nt 6248 and nt 6282, revealing an S2/S1 genotype. The same site of recombination was previously observed in an immunocompromised poliomyelitis patient (15). Strains Wh/2.1 and Wh/3.5 (Sabin 3; both zero synonymous changes in VP1) recombined between nt 6988 and nt 7001 from S2 to S3. Since both strains are S3 in the VP1 region, as revealed by sequence determination, these two strains have an S3/S2/S3 genotype. The S3/S2/S3 genotype has also previously been observed in stool samples from healthy vaccinated children (19). An additional strain (Wh/4P) had lost one of the two type 2-specific SpeI cleavage sites within the same region. All 20 strains remained, at least partially, temperature sensitive (Table 3). Upon ITD, a single strain (Wh/77P) was recorded with a discordant result by ELISA, indicating some antigenic change and the need for further characterization. This antigenic result was consistent with the finding of five nonsynonymous substitutions within the VP1 region.

The 20 SL strains had accumulated zero to nine substitutions within the VP1 region, giving rise to a total of 46 mutations (Table 4). Fifteen of these were synonymous, and 31 were nonsynonymous. In contrast to the findings of Yakovenko et al. (64), only a minority of the observed amino acid changes (9/31) were located within the antigenic region of VP1. These changes in antigenic-site amino acids were also seen in only four positive samples and five of nine observed from a single isolate (Wh/77P). In addition, there was an indication of a discordant nucleotide consensus at position 2493 (boldface) among the type 3 SL viruses, namely, "A**C**U" in Wh/70 and KO/565 and "A**U**U" in Wh/2.1 and Wh/3.5. Consequently, the deduced amino acids were either threonine or isoleucine, respectively. This discordance may reflect the presence of corresponding type 3 viral genotypes in diverse vaccine seeds (47). This study revealed a number of amino acid changes that were not previously recorded in derivates of OPV (Table 4). The possible significance of these changes cannot be judged due to limitations in the number of observations in this study. This may be addressed in future work.

DISCUSSION

Environmental poliovirus monitoring conducted over a 3-year period resulted in the detection of poliovirus in 20 samples from two locations in the Zurich area (Switzerland). All of the 20 SL strains showed partial temperature sensitivity, indicating some attenuated characteristics of the vaccine strains. This is exemplified by a recent report (10) showing that a type 2 SL isolate from an immunodeficient patient expressed temperature sensitivity. Four of the 20 SL strains showed no difference from the parental Sabin strains based on a comparison of mutational markers within the VP1 region and the 5′ UTR (Table 3). However, the remaining 16 strains displayed substitutions at attenuation sites within the 5′ UTR and the VP1 region, and three of these strains were also heterotypic recombinants (Table 3). Most of the 16 strains had accumulated additional nonsynonymous substitutions within VP1 (Table 4). Some of these mutations are known to contribute to the gain of viral fitness and to occur in vaccinees in the early phase of intestinal replication (13, 14, 23, 30, 31, 38, 64). It has been reported that OPV strains can accumulate mutations during in vitro propagation similar to those seen during in vivo replication (16, 48, 52). Even though the number of in vitro passages was restricted to two, we cannot rule out the possibility that

these mutations occurred during in vitro passage. However, sequencing of several first-passage amplicons (data not shown) confirmed our assumption that the observed changes reflected the original environmental strains or must have occurred from selection at the initial propagation step. Direct RT-PCR amplification from environmental samples without the passage of virus in cell culture would circumvent such uncertainty of the determined sequences (32). This possibility should be further investigated despite the low titer of virus in the sample inferred from this study.

Over the 3-year period of our study, neither wt polioviruses nor VDPVs were isolated from the two sewage treatment plants. Taking the wide international use of OPV and global human mobility into account, it may not be surprising to detect SL viruses in the sewage from IPV settings. However, the low number of positive findings (20/174 samples) may reflect procedural limitations rather than an estimate of true prevalence. It has been established that vaccinees typically shed OPV-like viruses no more than 2 months after vaccination (2, 32, 63). The majority (17/20) of the characterized SL viruses had ≤1 synonymous VP1 substitution (age, ≤6 weeks) and were therefore likely to have been shed by primary vaccinees. The remaining three strains had estimated ages of approximately 4 and 7 months. The prolonged time of replication or circulation of these strains could be explained by three scenarios. First, these strains represent multiple passages in the population. If this was the case, the observed strains originated from contacts of OPV vaccinees. These contacts could either be from abroad or from the Swiss setting. Second, on rare occasions, healthy vaccinees can shed virus for extended periods. This has been reported, notably, for up to 10 months (35, 50). Third, although it is highly unlikely, these viruses could be descendants of OPV strains previously used in Switzerland. However, if this were the case, a more distinct genetic drift would have been expected. In any case, it is possible that these three viruses represent limited circulation in the Zurich area for a few months.

The present study used an SiO_2-based protocol followed by culture on RD and/or L20B cells for the concentration and detection of poliovirus in sewage samples. This protocol demonstrated a higher recovery rate (60%) than the two-phase separation protocol (30%) that is recommended by WHO (57). The SiO_2 protocol is simple and fast, requires fewer chemicals, and is easy to implement in an environmental microbiology laboratory equipped with a centrifuge with a 500-ml sample capacity. To obtain maximum sensitivity for the isolation of poliovirus from the sewage samples, typically one cell passage on RD cells followed by one passage in L20B cells is used (27, 36, 46, 57, 58, 62). However, this protocol generates a substantial workload (Table 2) that could be considerably reduced by the use of L20B cells, which are selective for poliovirus propagation (62). These cells simplify isolation from clinical samples of human origin, whereas in environmental monitoring, additional viruses can be expected. Surprisingly, when employing only this protocol in the third year of our study, we detected numerous nonpoliovirus enteroviruses of presumptive bovine origin and additional viruses that could not be further classified.

In summary, the present study revealed through environmental monitoring continuous introduction of SL polioviruses into the community. However, there is little evidence that these viruses established long-term circulation in the community. The high hygienic standards possibly prevent the more efficient fecal-oral transmission and permit only the less efficient oral-oral transmission. This is in agreement with recent findings in the United States (22), as well as previous observations made in The Netherlands and in Sweden (8, 18, 49). The continuous detection of polioviruses in the wastewater of the local community reflects the regular presence of virus-shedding individuals who return or enter from an OPV setting. This, in turn, emphasizes the necessity of uninterrupted child vaccination in order to ensure sustained high herd immunity.

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