TABLE 6 Components of DTaP and Tdap vaccines

Vaccine	Manufacturer	PT (μg)	FHA (μg)	Pertactin (μg)	Fimbriae (μg)	D (Lf)	T (Lf)
DTaP, 0.5 ml	Kaketsuken	8	32			≤16.7	≤6.7
DTaP, 0.2 ml	Kaketsuken	3.2	12.8			≤6.7	≤2.7
Tdap, 0.5 ml (Boostrix)	GlaxoSmithKline Biological	8	8	2.5		2.5	5
Tdap, 0.5 ml (Adacel)	Sanofi Pasteur	2.5	5	3	5	2	5

the Adacel Tdap (12), which is used abroad (14) (Table 6). However, immunogenicities against PT in this study were sufficient despite 0.2 ml of DTaP, results which are very similar to those of previous reports (12–14, 21).

The most common local reactions reported in our study were erythema, swelling, and pain. The frequencies of these reactions were similar to those reported in Japanese adolescents aged 11 to 12 years (14) who had received 0.2 or 0.5 ml of DTaP. Compared to randomized clinical trials for Tdap, the frequency of injection site pain was similar, whereas there were more cases of erythema and swelling in our study. The frequencies of pain, swelling, and erythema in American adults who were vaccinated with the Adacel Tdap (12) were 65.7%, 21%, and 24.7%, respectively, and in those who had the Boostrix Tdap (13) were 61.0%, 21.1%, and 17.6%, respectively. These discrepancies in the frequencies of erythema and swelling between Japan and the United States may be due to the mode of injection. In the United States, Tdap is injected intramuscularly, while DTaP vaccine was administered subcutaneously in this study. The frequencies of other adverse reactions in this study did not differ from those in the clinical trials of Tdap (12, 13).

Several studies have examined the effects of vaccine antigen contents on immunogenicity and reactogenicity, and although immunogenicities differed between the studies, all demonstrated that local reactions can be reduced by decreasing the amount of antigen (14, 18, 22, 23). Knuf et al. assessed DTP vaccines with reduced amounts of antigens in the fourth dose in the second year in Germany and reported that the immunogenicity was adequate, whereas reduced amounts of antigen induced lower antibody concentrations (22). Hendrikx et al. examined IgG responses in children following revaccination at age 4 and found that a booster vaccine with higher pertussis antigen levels induced higher antibody levels than a vaccine containing low antigen levels (23). Okada et al. compared 0.2 and 0.5 ml of DTaP in children aged 11 to 12 years in Japan and observed that GMC and seropositivity rates were similar between the groups (14). Blatter et al. reported that GMCs for anti-PT and anti-FHA were approximately 2-fold higher in the Boostrix group than in the Adacel group 1 month after vaccination, and these differences remained apparent even 1 year after vaccination, although the magnitudes of difference decreased (18). It is difficult to know which dose is appropriate for booster vaccinations. On one hand, a lower dose might be suited to booster vaccinations, as it induces seropositivity levels of antigens with a lower rate of local reactions. On the other hand, a higher dose might be better if it induces not only higher immunogenicity but also longer persistence of this immunogenicity. Studies have demonstrated that antibody concentrations 1 month after vaccination strongly predicted antibody persistence (24) and hence, a higher dose vaccine may contribute to longer persistence of the antibody. However, to our knowledge, there is no study that has compared the long-term persistence of antibodies induced by various doses of antigens.

In the United States, the Advisory Committee on Immunization Practices (ACIP) recommends a Tdap vaccine booster dose for all adolescents aged 11 to 18 years, ideally at 11 to 12 years, and for adults aged 19 to 64 years who have not received a dose since 2005 (25, 26). In 2010, ACIP further recommended a booster dose of Tdap for unvaccinated adults aged 65 years and older who are in close contact with an infant aged <12 months (27). In 2012, ACIP approved the use of Tdap for all adults aged 65 years and older (28). In comparison, no additional DTaP vaccine after a single booster dose in childhood is recommended in Japan. We suspected that the lack of immunization with DTaP during adolescence at 11 to 12 years of age may be the leading cause of the recent resurgence of pertussis among young adults in Japan. To reduce the incidence of infant death as a result of severe pertussis, vaccination among pregnant women may have a greater impact than vaccination among adolescents; however, alteration of vaccination schedules is very difficult. Therefore, adolescents in Japan are currently expected to receive a low dose of DTaP instead of DT (14). However, this may increase the susceptibility to pertussis among adults, including pregnant women, because the immunity induced by DTaP decreases with time. In addition to vaccination in adolescents, repeat booster vaccinations of DTaP may be required to substantially reduce or eliminate the incidence of pertussis. With regard to longer persistence of immunity, a higher dose of booster vaccination may be suitable. Furthermore, the development of a more immunogenic and efficacious pertussis vaccine that requires considerably fewer doses and induces long-term durable protective immunity is required (29).

The present study had several limitations. First, although our relatively small sample size was sufficient to evaluate immunogenicity, it was less than ideal for the detection of adverse events. However, much larger clinical trials investigating Tdap have also not reported serious adverse events (12, 13). Second, study subjects were restricted to young adults only. In the present study, we enrolled students who were exposed to a pertussis outbreak in the previous year and did not exclude individuals who had been exposed to pertussis or had contact with pertussis patients. This may have influenced the vaccination response, although we excluded subjects who developed pertussis in the previous year, after which no outbreak was noted. A healthy group of individuals who were not previously exposed to pertussis would be a better cohort to assess the actual vaccination response in a population experiencing waning immunity from the last vaccination in infancy. However, several outbreaks of pertussis have been reported at college and university campuses in the current decade, and therefore, we consider individuals in this age group as one of the target populations for control of pertussis in Japan. In addition, booster immunization is considered essential at any age in those who have not received it previously. Further studies in other age groups and specific risk groups, such as pregnant women, their newborns, and health care workers, are needed.

In conclusion, 0.2 and 0.5 ml of the DTaP vaccine can induce

antibodies in young adults without severe adverse reactions that affect their daily ordinary activities; thus, both doses can be used for booster immunizations to control pertussis in Japan.

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Development of real-time PCR to detect oral vaccine-like poliovirus and its application to environmental surveillance

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ABSTRACT

In order to perform environmental surveillance to track oral poliovirus vaccine-like poliovirus sensitively and conveniently, real-time PCR was developed and applied to a raw sewage concentrate. The real-time PCR method detected 0.01–0.1 $TCID_{50}$ of 3 serotypes of Sabin strain specifically. The method also detected the corresponding serotypes of oral poliovirus vaccine-like poliovirus specifically, but detected neither wild poliovirus, except Mahoney for type 1 and Saukett for type 3, nor other enteric viruses, as far as examined.

When real-time PCR was applied to environmental surveillance, the overall agreement rates between real-time PCR and the cell culture were 83.3% for all serotypes. Since real-time PCR has the advantages of rapid detection of viruses and minimum requirement of sampling volume as compared with ordinary cell culture, it is suitable to monitor oral poliovirus vaccine-like poliovirus in the environment, especially in areas where an oral vaccine is being replaced by an inactivated vaccine.

1. Introduction

Poliovirus, a member of the genus *Enterovirus* of the *Picornaviridae*, is a human pathogen causing poliomyelitis with irreversible paralysis. As no specific therapy is available, prevention by vaccination is the basic strategy against this disease. The World Health Organization (WHO) initiated a global polio eradication program in 1988, and the number of polio cases and endemic areas have been decreasing gradually (WHO, 2011); however, wild poliovirus is still circulating in some polio endemic countries, and its exportation from endemic areas has been a great concern regarding the complete eradication of polio, since microbes can cross borders easily by global transportation. The transmission of highly divergent neurovirulent vaccine-derived poliovirus (VDPV) is also another concern generated from prolonged replication of oral poliovirus vaccine in immunodeficient individuals (DeVries et al., 2011), and communities with low vaccination coverage allow its circulation in an area (Alexander et al., 2009). To prevent transmission of poliovirus in the population, it is therefore important to maintain high herd immunity against poliovirus by vaccination (see review: Kew et al., 2005).

Two effective vaccines, live-attenuated oral and inactivated poliovirus vaccines, are employed throughout the world (Ghendon and Robertson, 1994). While both vaccines protect humans effectively from poliomyelitis upon infection, the inactivated vaccine is reported to induce less mucosal immunity, which may allow asymptomatic infection and virus shedding of long duration (Ghendon and Robertson, 1994; Onorato et al., 1991). Although oral poliovirus vaccine is highly effective, vaccine-associated paralysis or VDPV arising occasionally in a recipient or person exposed to the virus is an inevitable concern; therefore, oral poliovirus vaccine has been replaced by an inactivated vaccine (Alexander et al., 2004).

Poliovirus often infects humans asymptomatically and the virus shed in feces may persist in the environment. Sabin-like poliovirus strains or VDPVs have been found in sewage water even in the areas where inactivated poliovirus vaccine has been used (Zurbriggen et al., 2008; Roivainen et al., 2010); therefore, environmental surveillance is necessary even after the oral live vaccine has been replaced by an inactivated vaccine.

In Japan, acute poliomyelitis has been declining since the last outbreak in 1960 due to the introduction of an oral poliovirus vaccine for children in 1961 (Takatsu et al., 1973; Shimojo, 1984). Wild poliovirus strains have not been isolated since they were detected in a patient with poliomyelitis in 1980 and in two patients without AFP in 1984 and 1993 (Infectious Agents Surveillance

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Center in Japan, 1997; Shimizu, 2009). Oral poliovirus vaccine was replaced by an inactivated vaccine in Japan in September 2012 due to vaccine-related paralysis; however, oral poliovirus vaccine-like poliovirus possibly remaining in an area or being transmitted from outside should be monitored even after the transition has been completed, as described above.

Oral poliovirus vaccine-like poliovirus has been isolated from raw sewage and river water in spring and fall when the live vaccine is administered routinely to babies in Toyama, Japan, indicating that environmental surveillance detected circulating poliovirus in the community efficiently (Matsuura et al., 2000); however, a more rapid and sensitive method is desired since the ordinary cell culture method takes at least 2 weeks to identify poliovirus. In the present study, real-time PCR was developed to detect oral poliovirus vaccine-like poliovirus specifically with high sensitivity, and was verified by applying to environmental surveillance.

2. Materials and methods

2.1. Viruses

Mahoney, Sabin 1 of poliovirus type 1, Sabin 2, Lansing of poliovirus type 2, Sabin 3, Saukett of poliovirus type 3, Hill of echovirus type 9 (E9), Gregory of E11, Del Carmen of E13, Metcalf of E18, Kowalik of coxsackievirus (Cox) A10, Ohio-1 of CoxB2, BrCr of enterovirus 71 (EV71), Lang of reovirus type 1 (Reo1), and Amy of Reo2, were obtained from the National Institute of Infectious Diseases (NIID), (Tokyo, Japan), as were cDNAs of wild poliovirus type 1 strains of NV86-92, M128-82, M174-82, Mb25-82, Lao11-96, HEN041-83, NV134-93, NV102-92, NV106-92, NV99-92 (Yoshida et al., 1997). Strains of G4-12/Toyama/1993, Fu-May-7/Toyama/2008, Fu-Dec-24/Toyama/2008 of poliovirus type 1, G18-2/Toyama/1994, Fu-May-13/Toyama/2009, Fu-Jun-13/Toyama/2009 of poliovirus type 2, G5-1/Toyama/1993, Fu-Jun-9/Toyama/2008, Fu-Jul-5/Toyama/2008, and Fu-Oct-12/Toyama/2009 of poliovirus type 3, were isolated from raw sewage in Toyama Prefecture, Japan. Strains of 309-FC/Toyama/2004 of poliovirus type 2, Hu/409/Toyama/2006 of norovirus GII.4, and Hu/Ni22/Toyama/2009 of sapovirus GI.1 were detected in healthy children in Toyama Prefecture (Matsuura et al., 2000; Iwai et al., 2009a, 2009b).

2.2. Raw sewage

Raw sewage was collected monthly from January 2008 to December 2009 at the threshold point of a waste tank in a sewage disposal plant located in Toyama Prefecture (Iwai et al., 2009a).

2.3. Concentration of viruses in raw sewage

More than 1 L of raw sewage was centrifuged at 3000 rpm for 30 min (4 °C), and 1 L of the supernatants was used for subsequent concentration of viruses using the filter adsorption and elution method as described previously (Matsuura et al., 1984). Briefly, $MgCl_2$ (final 0.05 M) was added to 1 L of the supernatant of the sewage sample, and the pH was adjusted to 3.5 with HCl. The supernatant was filtered through a mixed cellulose ester-type membrane (ADVANTEC^R A0456A142C, Toyo Roshi Ltd., Tokyo, Japan) to adsorb viruses. Then, the membranes were soaked in 10 mL of 3% beef extract solution and the viruses were eluted. The eluted solution was used for isolation of viruses and extraction of viral RNA.

2.4. Isolation and identification of viruses in raw sewage

Two hundred microliters per well of raw sewage concentrates were inoculated into Vero, MA104, RD-18S, and HEp-2 cell lines using 24-well plates. The cell cultures were then incubated at 34 °C in 5% CO_2 air and observed for 14 days to monitor the cytopathic effect (CPE). If CPE was not observed by the end of the observation period, a blind passage was performed once. Isolates were identified by a neutralization test with type-specific rabbit antisera against poliovirus and enterovirus (25 U; Denka-Seiken, Tokyo, Japan). The isolates were used for further genetic analyses.

2.5. Virus titration

The titers of poliovirus and enterovirus in the concentrated sewage were determined by the 50% tissue culture infectious dose ($TCID_{50}$/25 μL) using Vero, MA104, RD-18S, or HEp-2 cell lines in 96-well cell culture plates. The plates were incubated at 37 °C for 7 days in a 5% CO_2 air and were then observed for CPE.

2.6. RNA extraction, RT-PCR, and nucleotide sequencing

Viral RNA was eluted in a total volume of 60 μL from 140 μL raw sewage concentrates or poliovirus isolates using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Twelve microliters of extracted RNA were treated with 5 U of DNase I (TaKaRa, Otsu, Japan), and cDNA was synthesized by SuperScript III Reverse Transcriptase in a reaction volume of 30 μL (Invitrogen, Carlsbad, CA, USA) with a random hexamer, according to the manufacturer's instructions. The cDNA was used for polymerase chain reaction (PCR) and real-time PCR. The PCR was carried out using primers as described previously (Rico-Hesse et al., 1987; Balanant et al., 1991), which amplify 1126 bp corresponding to nucleotides (nt) from 2402 to 3527 of poliovirus type 1 (Sabin 1; GenBank ID: V01149). The PCR products were used directly for nucleotide sequence analysis using a Big Dye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences in the complete VP1 region of the isolates were compared with those of oral poliovirus vaccine strains.

2.7. Real-time PCR

The primers and probes for oral poliovirus vaccine-like poliovirus were designed as shown in Table 1. Real-time PCR was carried out in a reaction mixture of 25 μL containing 2.5 μL cDNA, 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), a 400 nM concentration of each primer, and 200 nM of each probe. PCR amplification was performed with an ABI Prism 7500 under the following conditions: incubation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, and then 45 cycles of amplification with denaturation at 95 °C for 15 s, and annealing and extension at 56 °C for 1 min. For enterovirus, the primers of pan-entero+ (sense; 5′-CCT CCG GCC CCT GAA TG-3′) and pan-entero- (antisense; 5′-ACC GGA TGG CCA ATC CAA-3′), which amplify 195 bp corresponding to nucleotides (nt) 444–638 of poliovirus type 1 (GenBank ID, AY184219) and the probe, (FAM)-CCG ACT ACT TTG GGT GTC CGT GTT TC-(TAMRA), were used as previously described (Katayama et al., 2002).

To prepare control plasmids of poliovirus, the capsid regions of poliovirus strains of Sabin 1, 2, and 3 were amplified by PCR using the newly designed primers (Table 1). The amplicons were verified by sequencing and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid DNAs were purified with a QIA prep Spin Miniprep kit (QIAGEN), according to the manufacturer's instructions, and their concentrations were determined by measuring the A_{260}. Standard curves were generated by 10-fold serial dilution (10^6 to 10^1 copies) of the plasmids. The number of genomic copies in the sample was estimated by comparing its Ct value with those on the standard curve. Molecular cloning was

Table 1
Alignment of nucleotide sequences of the primers and probes for real-time PCR to detect three serotypes of oral poliovirus vaccine-like poliovirus.

Real-time PCR for poliovirus type 1

	Strain	Accession No.	Primer (PolioS1F) 3187 3213 CTTCGGTATTTTGGCTGTTAGAGTAGT	Probe (PolioS1_Probe) 3219 3244 FAM-ATCACAACCCGACCAAGGTCACCTCC-BHQ	Primer (PolioS1R) 5'-GACGCGGGCACCAGACT-3' 3280 3296 AGTCTGGTGCCCGCGTC
Polio 1	Sabin1	AY184219	.....	.....	.....
	Mahoney	V01149	.....	.....	.....
	NV86-92	AB053958	T.....C..CC....C..G.....G..	.C.....T..A..T.TA.....T	...G.....A..C.
	G4-12/Toyama/1993	AB061286	.....	.....	.....
	Fu-May-7/Toyama/2008	AB629948	.....	.....	.....
	Fu-Dec-24/Toyama/2008	AB629949	.....	.....	.....
Polio 2	Sabin2	AY184220	T..T..ATCAC.....C.C..G..	.....C..GCG.C.....	.....A..A.
	G18-2/Toyama/1994	AB061293	T..T..ATCAC.....C.C..G..	.....C..GCG.C.....	.....A..A.
	Lansing	M12197	...T..ATCAC.....C....G..	.C.....T..G..AC....T..A	...G.....T.....A.
	309-FC/Toyama/2004	AB629950	T..T..ATCAC.....C.C..G..	.....C..GCG.C.....	.....T..A..A.
	Fu-May-13/Toyama/2009	AB629951	T..T..ATCAC.....C.C..G..	.....C..GCG.C.....	.....A..A.
	Fu-Jun-13/Toyama/2009	AB629952	T..T..ATCAC.....C.C..G..	.....C..GCG.C.....	.....A..A.
Polio 3	Sabin3	X00596	...T...G.A.....A...C.T..T..	.....C..T..A..A.....	T.....TA.A.
	G5-1/Toyama/1993	AB061352	...T...G.A.....AA..C.T..T..	.....C.....A..A.....	T.....TA.A.
	Saukett	AY082685	...T...G.A.....AA..C....T..	.....C..T..A..A.....	T.....TA.A.
	Fu-Jun-9/Toyama/2008	AB629953	...T...G.A.....A...C.T..T..	.....C..T..A..A.....	T.....TA.A.
	Fu-Jul-5/Toyama/2008	AB629954	...T...G.A.....A...C.T..T..	.....C..T..A..A.....	T.....TA.A.
	Fu-Oct-12/Toyama/2009	AB629955	...T...G.A.....A...C.T..T..	.....C..T..A..A.....	T.....TA.A.

Real-time PCR for poliovirus type 2

	Strain	Primer (PolioS2F) 1499 1519 GTGGAGTGCTGGTAGGGAATG	Probe (PolioS2_Probe) 1544 1573 FAM-TAAACCTGCGCACTAACAACTGTGCTACGC-BHQ	Primer (PolioS2R) 5'- TTTGTCATGCTATCTATTGAGAGTGAGTT -3' 1591 1619 AACTCACTCTCAATAGATAGCATGACAAA
Polio 2	Sabin2	.....	.....	.....
	G18-2/Toyama/1994	.....	.....	.....
	Lansing	.C.....A....CG..A....	.T..T.....C.....C...T	..T.....T..C.....C.....
	309-FC/Toyama/2004	.....	.....	.....
	Fu-May-13/Toyama/2009	.....	.....	.....
	Fu-Jun-13/Toyama/2009	.....	.....	.....
Polio 1	Sabin1	A...CAC.T.AT.G.....	.....A..G..C.....A.	.....C.....G.....T...GT...
	Mahoney	A...CAC.T.AT.G.....	.....A..G..C.....A.	.....C.....G.....T...GT...
	NV86-92	.....C.G..A....	.T...T..A.A.....T..TA....C..T.	..TG.T.....T..C...TCA...GTC..
	G4-12/Toyama/1993	A...CAC.T.AT.G.....	.....A..G..C.....A.	.....T.....G.....T...GT...
	Fu-May-7/Toyama/2008	A...CAC.T.AT.G.....	.....A..G..C.....A.	.....C.....G.....T...GT...
	Fu-Dec-24/Toyama/2008	A...CAC.T.AT.G.....	.....A..G..C.....A.	.....C.....G.....T...GT...
Polio 3	Sabin3	....G...T.AC.G..A....	.T...T...A.G..C.....A.C..A..TA	..TG.TT.GG.C..T...TCA...GTT..
	G5-1/Toyama/1993	....G...T.AC.G..A....	.T...T...A.G..C.....A.C..A..TA	..TG.TT.GG.C..T...TCA...GTT..
	Saukett	.C..G...T..C.G..A....	.T...T...A.G..C..T...A.C..A..CA	..TG.TT.AG.T..T..CTCA...GTT..
	Fu-Jun-9/Toyama/2008	....G...T.AC.G..A....	.T...T...A.G..C.....A.C..A..TA	..TG.TA.GG.C..T...TCA...GTT..
	Fu-Jul-5/Toyama/2008	....G...T.AC.G..A....	.T...T...A.G..C.....A.C..A..TA	..TG.TT.GG.C..T...TCA...GTT..
	Fu-Oct-12/Toyama/2009	....G...T.AC.G..A....	.T...T...A.G..C.....A.C..A..TA	..TG.TT.GG.C..T...TCA...GTT..

Real-time PCR for poliovirus type 3

	Strain	Primer (PolioS3F) 2636 2654 TGGCACCATCCGACACAG	Probe (PolioS3_Probe) 2657 2680 FAM-AAACGCGCCACGTAGTCCAACGAC-BHQ	Primer (PolioS3R) 5'- CCGCGTGCGAAGAATGAT -3' 2706 2723 ATCATTCTTCGCACGCGG
Polio 3	Sabin3	.....	.....	.....
	G5-1/Toyama/1993	..A.....	.....	.....T.....
	Saukett	...T.....	.....T...A.....G.	.....T.....
	Fu-Jun-9/Toyama/2008	...T.....	.....	.....
	Fu-Jul-5/Toyama/2008	..A.....	.....	.....
	Fu-Oct-12/Toyama/2009	..A.....	.....	.....
Polio 1	Sabin1	.A.TC..T..T..T....	....CA.A..T..T..A....ATA	G..T.....G..G..
	Mahoney	.A.TC..T..T..T....	....CA.A..T..T..A....ATA	G..T.....G..G..
	NV86-92	.A.TT..C..A.....	.G..TA.A..T..CA.T....ATA	G..C.....T..
	G4-12/Toyama/1993	.A.TC..T..T..T....	....CA.A..T..T..A....ATA	G..T.....G..G..
	Fu-May-7/Toyama/2008	.A.TC..T..T..T....	....CA.A..T..T..A....ATA	G..T.....G..G..
	Fu-Dec-24/Toyama/2008	.AATC..T..T..T....	....CA.A..T..T..A....ATA	G..T.....G..G..
Polio 2	Sabin2	...TG..T..G.....C.	.....T..CA....GA...	G.....T...A.A..
	G18-2/Toyama/1994	...TG..T..G.....C.	.....T..CA....GA...	G.....T...A.A..
	Lansing	...TG..T..A.....C.	....T..T.....CA.....AAG.	G..T.....A.A..
	309-FC/Toyama/2004	...TG..T..G.....C.	.....T..CA....GA...	GG.....T...A.A..
	Fu-May-13/Toyama/2009	...TG..T..G.....C.	.....T..CA....GA...	G.....T...A.A..
	Fu-Jun-13/Toyama/2009	...TG..T..G.....C.	.....T..CA....GA...	G.....T...A.A..

[a] FAM: 6-carboxyfluorescein reporter dye; BHQ: Black Hole Quencher.
[b] The positions are numbered according to the nucleotide positions of Sabin 1, 2, and 3 (GenBank accession number: AY184219, AY184220, and X00596, respectively). Nucleotides in the target sequence different from those in the primer and probe are shown.

approved by the Committee for Genetic Modification Safety of Toyama Institute of Health.

2.8. Seeding experiment

Known amounts of poliovirus Sabin strains of 1, 2, and 3 (1.0×10^5, 1.0×10^4, and 1.0×10^5 $TCID_{50}$, respectively) were seeded in control sewage concentrates in which poliovirus was verified to be free. RNA extraction, RT-PCR, and real-time PCR were performed as described in Materials and methods. Reaction mixtures of real-time PCR were calculated to contain 1.7×10^3, 1.7×10^2, and 1.7×10^3 $TCID_{50}$ of poliovirus Sabin strains of 1, 2, and 3, respectively.

2.9. Nucleotide sequence accession numbers

Nucleotide sequences of poliovirus genomes determined in this study were deposited in GenBank under ID: AB629948 to AB629955.

3. Results

3.1. Development of real-time PCR for detection of oral poliovirus vaccine-like poliovirus

Serotype-specific primer sets and fluorescent probes were designed based on the nucleotide sequences in the capsid region of Sabin strain without degeneracy to maximize specificity (Table 1). The positions of each primer and probe are close to the neutralization antigenic sites (N-Ags) II and IIIa of poliovirus type 1, N-Ag IIb of poliovirus type 2, and N-Ag I of poliovirus type 3 (Minor et al., 1986, 1991; Patel et al., 1993; Jorba et al., 2008). Nucleotide sequences of each primer and probe differed by less than 3 bases from those of the corresponding serotypes of oral poliovirus vaccine-like poliovirus, VDPV isolated in Toyama, and wild poliovirus strains of Mahoney and Saukett, while 3–7 bases of those of wild poliovirus strains of NV86-92 and Lansing (Table 1).

Standard curve of poliovirus type 1, 2, or 3 was generated with 10-fold serial dilutions of plasmid DNA carrying each cDNA to evaluate the dynamic range of real-time PCR (Fig. 1). This method is able to detect each poliovirus cDNA ranging from 10^1 to 10^6 copies with good linearity. The correlation coefficients (R^2) between the amount of plasmid DNA and the threshold cycle (Ct) value were 0.995, 0.996, and 0.991 for poliovirus types 1, 2, and 3, respectively. Ten copies of plasmid DNAs of poliovirus types 1, 2, and 3 could be detected repeatedly and reliably. Values tended to deviate from standard curve below 10 copies.

The serotype specificity of real-time PCR was examined using RNAs purified from Sabin strains with known concentrations (Table 2). Real-time PCR detected poliovirus types 1, 2, and 3 of Sabin strain in serotype-specific manner. The detection limits of the PCR were 0.1 $TCID_{50}$ of poliovirus type 1 and 0.01 $TCID_{50}$ of types 2 and 3. One $TCID_{50}$ of poliovirus types 1, 2, and 3 were estimated to contain 0.60×10^2, 3.39×10^2, and 3.02×10^2 of genomic copy numbers according to the standard curve, respectively. Known amounts of poliovirus Sabin strains of 1, 2, and 3 (1.0×10^5, 1.0×10^4, and 1.0×10^5 $TCID_{50}$, respectively) were also seeded in control sewage concentrates in which poliovirus was verified to be free, and viral RNAs were extracted as described in Materials and methods. Reaction mixtures of real-time PCR were calculated to contain 1.7×10^3, 1.7×10^2, and 1.7×10^3 $TCID_{50}$ of poliovirus Sabin strains of 1, 2, and 3, respectively, if sample loss during the process was disregarded. Real-time PCR showed the genomic copy numbers of 6.02×10^4, 1.02×10^4, and 2.51×10^4 of each strain according to the standard curve, respectively. The percent recoveries of each strain were calculated as 58.8, 17.7 and 4.9% when genomic copy numbers measured were compared with those estimated from $TCID_{50}$ in the seeds, respectively. Samples may be lost during the extraction of viral RNA.

The reactivity of real-time PCR was verified further using 3 strains of oral poliovirus vaccine, 10 strains of oral vaccine-like poliovirus, 1 VDPV strain, and 4 strains of wild poliovirus with known concentrations (Table 3). Real-time PCR detected specifically the corresponding serotypes of oral vaccine-like poliovirus. It detected neither wild poliovirus strains, except Mahoney for type 1 and Saukett for type 3, nor echovirus 9, 11, 13, and 18, coxsackievirus A10 and B2, enterovirus 71, reovirus 1 and 2, norovirus genogroup II genotype 4 (GII.4), and sapovirus GI.1. Cross reactivity to Mahoney and Saukett was anticipated by the close sequence similarity of the probes and primers to the targets (Table 1). There were significant differences between genomic copy numbers measured by real-time PCR and the virus titers measured by cell culture among strains. Although the values of Sabin strains were equivalent to those in Table 2, those of oral poliovirus vaccine-like strains and wild strains showed much higher genomic copy numbers than the $TCID_{50}$ values. This phenomenon might be because oral poliovirus vaccine-like strains and wild strains generate a greater amount of replication incompetent viruses than Sabin strains.

Nine additional isolates of wild poliovirus type 1 (M128-82, M174-82, Mb25-82, Lao11-96, HEN041-83, NV134-93, NV102-92, NV106-92, NV99-92) were not detected by real-time PCR, and their nucleotide sequences in the VP1 region differ 20.4–22.0% from those of the vaccine strain (Yoshida et al., 1997) (data not shown).

3.2. Application of real-time PCR to environmental surveillance

To assess the feasibility of real-time PCR for environmental surveillance, it was applied to raw sewage collected in 2008–2009, and detection profiles of poliovirus were compared with those by cell culture. In addition, enterovirus was assessed by real-time PCR and cell culture. Poliovirus types 1, 2, and 3 were detected by real-time PCR in May to July, and October to December when oral poliovirus vaccine was administered in Toyama Prefecture (Fig. 2A). The genomic copy numbers of the poliovirus ranged from 0 to 4.43 (Log_{10}) copies/L raw sewage, as estimated by the standard curves. Poliovirus was isolated simultaneously by cell culture. Numbers of the isolates are indicated in Fig. 2B. All these isolates were defined as oral poliovirus vaccine-like poliovirus since the nucleotide sequences of the VP1 region (31 isolates) differed 0–0.56% from those of oral poliovirus vaccine strains (Sabin). Enterovirus was detected simultaneously from these raw sewage samples by the real-time PCR with increased genomic copy number in June to December and decreased in January to April (Fig. 2A). The fluctuation of genomic copy number (0–5.83 (Log 10) copies/L) seems to reflect prevalence of enterovirus in Japan (NIID, 2009). Consistently, various serotypes of enterovirus were isolated from May to December by cell culture (Fig. 2B).

The agreement rate, defined as the rate of the sum of the numbers of months when poliovirus was either detected or undetected from raw sewage by both real-time PCR and cell culture to the total number of months in 2008 and 2009, was 83.3% (20/24) in all serotypes (Table 4). Poliovirus types 1, 2, and 3 were detected in 2, 8, and 9 months during the two years by real-time PCR, while they were detected in 4, 4, and 7 months by cell culture, respectively; thus, poliovirus types 2 and 3 were more frequently detected by real-time PCR than cell culture, whereas poliovirus type 1 was the converse. This seems to be consistent with the difference in the sensitivity of real-time PCR among the 3 types, as shown in Table 2; however, since it is important to confirm not only the existence but also the inexistence of the viruses, high overall agreement between the two methods supports the feasibility of real-time PCR for environmental surveillance.

Table 2
Genomic copy numbers in the known amount of virus solutions of Sabin strains measured by real-time PCR.

Serotype	Strain	$TCID_{50}$/reaction tube					
		1×10^3	1×10^2	1×10^1	1×10^0	1×10^{-1}	1×10^{-2}
Polio 1	Sabin 1	0.91×10^5[a]	0.98×10^4	0.81×10^3	0.60×10^2	0.36×10	–
	Sabin 2	–	–	–	–	–	–
	Sabin 3	–	–	–	–	–	–
Polio 2	Sabin 1	–	–	–	–	–	–
	Sabin 2	3.63×10^5	4.17×10^4	3.31×10^3	3.39×10^2	2.51×10	2.75×10^0
	Sabin 3	–	–	–	–	–	–
Polio 3	Sabin 1	–	–	–	–	–	–
	Sabin 2	–	–	–	–	–	–
	Sabin 3	2.19×10^5	3.02×10^4	3.02×10^3	3.02×10^2	2.04×10	4.37×10^0

–: undetected.
RNA was purified from Sabin strain of known concentration and cDNA was synthesized as described in Materials and methods. cDNA that corresponded to the 10^3 $TCID_{50}$/reaction tube was serially diluted 10-fold and applied to real-time PCR.
[a] Average number of genomic copies/reaction tube in the duplicated test was calculated according to the standard curve as shown in Fig. 1.

Table 3
Reactivity of real-time PCR to three serotypes of oral poliovirus vaccine-like poliovirus, vaccine-derived poliovirus, wild poliovirus and other enteric virus.

Virus[a]	Strain	Definition of poliovirus[b]	Virus titer	Genomic copy number/25 μL detected by real-time PCR[c]		
			($TCID_{50}$/25 μL)	Polio 1	Polio 2	Polio 3
Polio 1	Sabin 1	OPV	1.0×10^5	+[d] (9.3×10^6)	–	–
	G4-12/Toyama/1993	OPV-like	5.6×10^6	+(2.0×10^9)	–	–
	Fu-May-7/Toyama/2008	OPV-like	1.0×10^5	+(8.1×10^8)	–	–
	Fu-Dec-24/Toyama/2008	OPV-like	1.0×10^5	+(4.4×10^9)	–	–
	Mahoney	Wild	1.0×10^6	+(1.1×10^8)	–	–
	NV86-92	Wild	2.0×10^7	–	–	–
Polio 2	Sabin 2	OPV	1.0×10^5	–	+(2.8×10^7)	–
	G18-2/Toyama/1994	OPV-like	3.2×10^6	–	+(5.7×10^8)	–
	Fu-May-13/Toyama/2009	OPV-like	3.2×10^5	–	+(1.3×10^9)	–
	Fu-Jun-13/Toyama/2009	OPV-like	3.2×10^5	–	+(1.6×10^9)	–
	309-FC/Toyama/2004	VDPV	1.0×10^6	–	+(7.1×10^7)	–
	Lansing	Wild	1.8×10^6	–	–	–
Polio 3	Sabin 3	OPV	1.0×10^5	–	–	+(2.7×10^7)
	G5-1/Toyama/1993	OPV-like	5.6×10^6	–	–	+(3.4×10^8)
	Fu-Jun-9/Toyama/2008	OPV-like	3.2×10^5	–	–	+(2.9×10^9)
	Fu-Jul-5/Toyama/2008	OPV-like	1.0×10^6	–	–	+(2.4×10^9)
	Fu-Oct-12/Toyama/2009	OPV-like	3.2×10^5	–	–	+(3.4×10^8)
	Saukett	Wild	3.2×10^6	–	–	+(6.5×10^6)
Echo 9	Hill		3.2×10^6	–	–	–
Echo 11	Gregory		1.0×10^6	–	–	–
Echo 13	Del Carmen		5.6×10^5	–	–	–
Echo 18	Metcalf		3.2×10^4	–	–	–
Cox A10	Kowalik		5.6×10^4	–	–	–
Cox B2	Ohio-1		5.6×10^3	–	–	–
Entero 71	BrCr		1.8×10^4	–	–	–
Reo 1	Lang		3.2×10^1	–	–	–
Reo 2	Amy		5.6×10^1	–	–	–
Noro GII.4	Hu/409/Toyama/2006		NT[e]	–	–	–
Sapo GI.1	Hu/Ni22/Toyama/2009		NT	–	–	–

[a] Echovirus (Echo); coxsackievirus (Cox); enterovirus (Entero); reovirus (Reo); norovirus (Noro); sapovirus (Sapo).
[b] Poliovirus was defined by the difference in the nucleotide sequence of the whole VP1 region in each strain from that in the corresponding serotype of oral poliovirus vaccine (OPV). Less than 1%: oral poliovirus vaccine-like poliovirus (OPV-like); 1–15%: vaccine-derived poliovirus (VDPV); more than 15%: wild poliovirus (Wild).
[c] RNA was extracted from each virus solution with the indicated titer and its genomic copy number detected by real-time PCR was estimated by the standard curve, as shown in Fig. 1.
[d] +: detected; –: undetected.
[e] NT: not tested. Viral genome was confirmed by conventional PCR.

Table 4
Numbers of months when poliovirus was either detected or undetected from raw sewage by real-time PCR and/or cell culture in 2008 and 2009.

	Cell culture											
	Polio 1				Polio 2				Polio 3			
		+	–	Total		+	–	Total		+	–	Total
Real-time PCR	+[a]	1	1	2	+	4	4	8	+	6	3	9
	–	3	19	22	–	0	16	16	–	1	14	15
	Total	4	20	24	Total	4	20	24	Total	7	17	24
Agreement rate[b]		83.3% (20/24)				83.3% (20/24)				83.3% (20/24)		

[a] +: detected; –: undetected.
[b] Agreement rate was calculated as the ratio of the sum of the number of months when poliovirus was either detected or undetected from raw sewage by both real-time PCR and cell culture to the total number of months in 2008 and 2009.

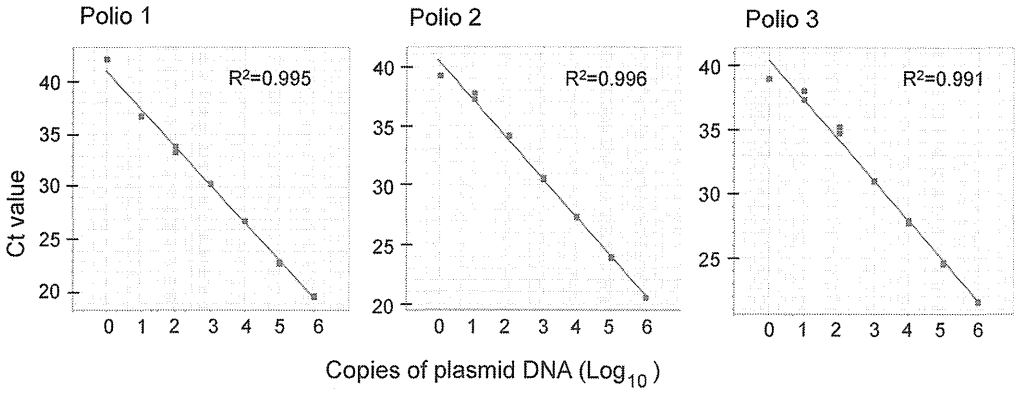
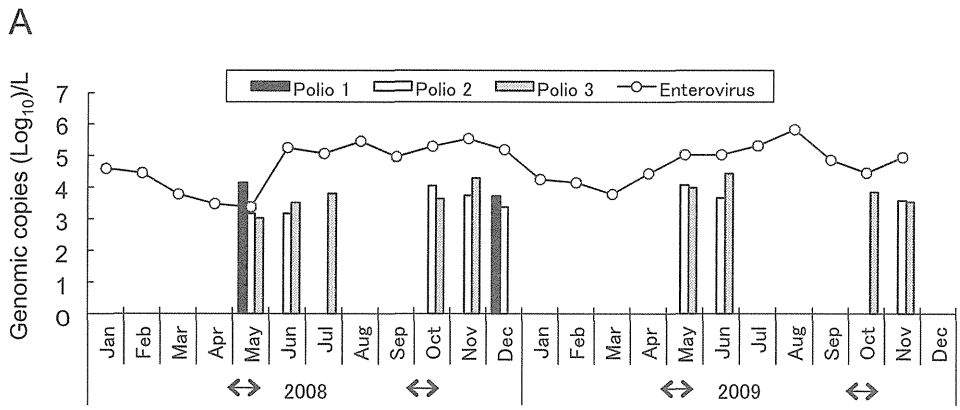


Fig. 1. Standard curves of poliovirus types 1, 2, and 3 by real-time PCR. Standard curves were generated by 10-fold serial dilution (10^6 to 10^1 copies) of control plasmids carrying cDNAs which encode partial capsid regions of Sabin 1, 2, and 3. Threshold cycles (Ct) are plotted against copy numbers of plasmid DNA. Reactions were tested in duplicate simultaneously and the test was conducted at least three times. Representative data were shown. The Ct value is the cycle number at which a positive amplification reaction is measured; the straight line is the regression line. R: correlation coefficient.

4. Discussion

The WHO has included environmental surveillance in the global polio eradication initiative strategic plan 2010–2012 to detect poliovirus strains, including those asymptomatically circulating in an area comprehensively (WHO, 2010). Environmental surveillance has also been employed to monitor the importation and transmission of poliovirus among inhabitants in European countries where inactivated poliovirus vaccine has been introduced (Zurbriggen et al., 2008; Roivainen et al., 2010). Even though VDPV has been



B

Virus	2008 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	2009 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Polio1					2					3	2											2		
Polio2					1						1						3						1	
Polio3					1	2	1				4					3		4				1		
CoxB3										4	2	5	2	2	1		2		1	1				
CoxB4									2			5											1	
CoxB5	1					1																		
Echo3										1											1			
Echo5									1															
Echo6																			1	4				
Echo9							1																	
Echo11								5							1					1	3	2	5	1
Echo18							1																	
Echo25												3		1										
Echo30	1	2			2	4				1	4	1										3		4

Fig. 2. Detection profiles of poliovirus and enterovirus from raw sewage in 2008–2009. (A) Genomic copy number of poliovirus or enterovirus of raw sewage was quantified by real-time PCR as described in Materials and methods. Black, white, and gray bars indicate the genomic copy numbers/L of poliovirus types 1, 2, and 3, respectively. White circles with line indicate those of enterovirus. (B) Poliovirus and enterovirus strains were isolated from raw sewage by cell culture. Raw sewage concentrates were inoculated into Vero, MA104, RD-18S, and HEp-2 cells as described in Materials and methods. Isolates were identified by a neutralization test with type-specific rabbit antisera against poliovirus or enterovirus. Numbers of isolates are shown. Arrows indicate the periods of administration of oral polio vaccine to babies in the drainage area in Toyama, Japan (21 April to 19 May and 16 September to 31 October 2008, and 20 April to 18 May and 16 September to 13 October 2009).

isolated occasionally (Roivainen et al., 2010), most of the isolates have been Sabin-like strains (Zurbriggen et al., 2008). Therefore, sensitive surveillance to detect oral polio vaccine-like virus will be helpful in monitoring and warning of the importation and transmission of poliovirus among inhabitants. For this purpose, a real-time PCR method was developed in this study.

Real-time PCR has an advantage over cell culture as the former can detect poliovirus within a day, while the latter takes about 2–4 weeks. Real-time PCR requires less raw sewage concentrate (140 μL) than cell culture (approximately 4 mL), which makes the transportation of samples much easier. A real-time PCR has been developed previously to discriminate between vaccine-related and wild poliovirus isolates (Kilpatrick et al., 2009), using highly degenerate primers that correspond to wide varieties of genomic sequences of wild poliovirus. The PCR primers had been designed based on the VP1 nucleotide sequences of wild poliovirus strains (at least 20 independent poliovirus isolates) (Kilpatrick et al., 1998), and their specificities were confirmed against large collection of wild poliovirus isolates of serotypes 1 and 3, and VDPVs of three serotypes (Kilpatrick et al., 2009). The sensitivity of the real-time RT-PCR assay was reported to be equivalent to that of their conventional RT-PCR assay that detected at least 100 fg of RNA of Sabin type 1 (equivalent to 2.5×10^4 genomic copies) (Kilpatrick et al., 1996). Another method has been developed to distinguish the revertant of oral poliovirus vaccine-like poliovirus from the nonrevertant using characteristic point mutations in the internal ribosome entry site of the 5′ untranslated region as a target (Troy et al., 2011). The separate primer and probe sets were generated for the three serotypes and their revertant forms according to the principles of the mismatch amplification assay (Li et al., 2004). The method is reported to be able to detect 37 $CCID_{50}$/100 μL or less poliovirus (Troy et al., 2011).

As the primers and probes in this study match to the nucleotide sequences of Sabin strains completely, the real-time PCR method detected at least 10 genomic copies of cDNA and 0.01 to 0.1 $TCID_{50}$ of 3 types of Sabin strains specifically. However, the method cross-reacted with wild poliovirus of Mahoney for type 1 and Saukett for type 3 due to the same or similar nucleotide sequences positioned in the primers and probe, as described; therefore, the poliovirus detected by real-time PCR should be confirmed by the nucleotide sequence. In contrast, as real-time PCR does not cross-react with wild poliovirus of other strains and VDPVs due to their highly divergent sequences, cell culture will be required in parallel to detect these viruses.

The genomic copy number detected by the real-time PCR seems to be higher than the viral titer measured by cell culture. Similar results were observed previously in other RNA viruses, such as rhinovirus (*Picornaviridae*) and respiratory syncytial virus (*Orthomyxoviridae*) (Perkins et al., 2005; Sachs et al., 2011). This phenomenon might be because the specimens contain virus particles unable to replicate and/or inhibitors of viral growth in cell culture. Alternatively, using plasmids as a standard may overestimate the copy number of the RNA genome.

When known amounts of Sabin strains were seeded in control sewage concentrates and viral RNAs were extracted, the percent recoveries of the strains of 1, 2, and 3 measured by the real time PCR method were calculated as 58.8, 17.7 and 4.9%, respectively. When known amounts of coxsackievirus B1, poliovirus type 1, echovirus 6, and Coxsackievirus A9 were seeded in non-concentrated sewage and viruses were recovered from concentrates, the rates of recovery were estimated to 89 to 100, 100, 79.4, and 31.5%, respectively by $TCID_{50}$ titration (Matsuura et al., 1984). Therefore, virus seemed to be lost mainly after the process of RNA extraction rather than concentration process.

As oral poliovirus vaccine was replaced by an inactivated vaccine as a routine immunization in Japan in September 2012, many children have not received poliovirus vaccine during the transition; therefore, the risk of the circulation of wild poliovirus, VDPV or progeny of the live vaccine strains may increase due to the gap in polio immunity. Oral vaccine-like poliovirus should be monitored for as long as it remains in the area. The real-time PCR method in this study will be used not only to detect but also to confirm the eradication of oral poliovirus vaccine-like poliovirus. Further studies will be required to validate the real-time RT-PCR method according to the guidelines (Armbruster and Pry, 2008; Burd, 2010).

In conclusion, real-time PCR was developed to detect oral poliovirus vaccine-like poliovirus and was applied to environmental surveillance. The sensitivity and specificity of real-time PCR was equivalent to cell culture. Since real-time PCR has the advantages of rapid detection and minimum requirement of sampling volume, it will be suitable to monitor the transmission of oral poliovirus vaccine-like poliovirus in areas where the oral vaccine is being replaced by an inactivated vaccine.

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Prevalence of Nonpolio Enteroviruses in the Sewage of Guangzhou City, China, from 2009 to 2012

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Prevalence of Nonpolio Enteroviruses in the Sewage of Guangzhou City, China, from 2009 to 2012

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The human-pathogenic viruses in urban sewage have been extensively monitored to obtain information on circulating viruses in human communities. Enteroviruses (EVs) excreted by patients who present with diverse clinical syndromes can remain infectious in the environment for several weeks, and limited data on circulating environmental EVs are available. A 4-year (2009 to 2012) surveillance study was conducted to detect nonpolio enteroviruses (NPEVs) in the urban sewage of Guangzhou city, China. After the viruses in the sewage samples were concentrated and isolated, molecular identification was used to detect and type the NPEVs. During the 4-year study, 17 different NPEV serotypes were identified in the sewage of Guangzhou city. The most common serotypes were echovirus 11 (ECHO11), ECHO6, ECHO7, and ECHO12 and coxsackie group B viruses 5 (CVB5) and CVB3. The predominant serotypes were influenced by spatial and temporal factors and differed each year. CVB5 was commonly detected in 2009 and 2010 but was rarely isolated in 2011 and 2012. In contrast, CVB3 was not observed in 2009 and 2010 but was increasingly detected in 2011 and 2012. Our study provides an overview of the serotype distribution and circulation patterns of NPEVs in the sewage of Guangzhou, China. In the absence of a systematic EV disease surveillance system, the detection and characterization of sewage-borne NPEVs will help us better understand the changes in EV disease trends and the epidemic background of circulating EVs, which could help interpret the EV trends and warn of future outbreaks in this area.

Human enteroviruses (EVs) are members of the genus *Enterovirus* within the order *Picornavirales*, family *Picornaviridae*, and consist of 4 species: *EV-A*, *EV-B*, *EV-C*, and *EV-D* (1). Based on their pathogenicity in humans, EVs were initially classified into 4 subgroups: polioviruses, type 1 (PV1) to PV3; coxsackie group A viruses, type 1 (CVA1) to CVA22 and CVA24; coxsackie group B viruses, type 1 (CVB1) to CVB6; and echoviruses, type 1 (ECHO1) to ECHO7, ECHO9, ECHO11 to ECHO27, and ECHO29 to ECHO34 (2). EVs are associated with diverse clinical syndromes, ranging from mild fever; headache; herpangina; and hand, foot, and mouth disease (HFMD) to severe and potentially fatal illnesses, such as aseptic meningitis, encephalitis, myocarditis, and acute flaccid paralysis (3–6). In recent years, outbreaks of different EV infections and related diseases have been frequently reported in China (7–10); however, only limited data are available on the circulation patterns of EVs in the environment (11, 12).

Although different serotypes of EVs can cocirculate, the predominant serotype is determined by spatial and temporal factors. For instance, in Beijing, China, CVA21 and EV-D68 were the predominant serotypes in patients with acute EV respiratory infections from 2006 to 2010 (13). However, in France and Spain, ECHO11 and ECHO6 are the most frequently detected agents in human-EV-positive adults with acute respiratory tract infections (14, 15). The high incidence of aseptic meningitis that occurred in Alberta, Canada, in 2010 was caused by CVA9, and an aseptic meningitis outbreak in Korea in 2008 was mainly caused by ECHO6 and ECHO30 infections (16, 17). For this reason, determining the temporal and geographic patterns of EV circulation, especially the dynamics of EV serotype shifts, is critical.

The presence of human-pathogenic viruses in urban sewage has served as an indicator of their existence in a given population (18–21). In urban populations with no or questionable surveillance, monitoring the viruses in circulating sewage can provide valuable supplementary information, especially when persistent virus circulation or frequent reintroduction is suspected. In this study, we provide an overview of the nonpolio enteroviruses (NPEVs) circulating in urban sewage of Guangzhou, China, from 2009 to 2012. During this 4-year period, 17 cocirculating NPEVs were isolated from sewage samples, and the circulation patterns of the predominant NPEVs (ECHO6, ECHO7, ECHO11, ECHO12, and CVB5) were described. To our knowledge, this is the longest, most systematic study of EV prevalence in sewage in China, and these results will help us to better understand the changes in enteric disease trends and the potential risk of an enteric disease epidemic.

MATERIALS AND METHODS

Sewage sample collection. Raw sewage samples were collected monthly from January 2009 to December 2012 from the primary sedimentation

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tanks at the Liede wastewater treatment plant (WWTP) in Guangzhou City, China. This WWTP is located in the northern part of Guangzhou city and serves a population of about 2,150,000. Four samples (1 liter each) were obtained from the inlets of the primary sedimentation tanks on a routine basis each month. The samples were immediately transported to the laboratory, and sample treatment was started within 2 h after the samples arrived at the laboratory.

Virus concentration. Viruses in the sewage samples were concentrated using a previously described improved negative-charge filter membrane absorption and sonication method (22). Briefly, the collected sewage samples were centrifuged at 3,000 rpm for 30 min. Then, $MgCl_2$ was added to the supernatant at a final concentration of 0.05 M, and the pH was adjusted to 3.5 to 4.0 with HCl. The samples were then slowly passed through a negatively charged membrane filter (mixed cellulose ester membrane filter; Advantec Co. Ltd., Tokyo, Japan) under gentle positive pressure. To elute the viruses, the filter with adsorbed viruses was cut into pieces and sonicated for 1 min in 10 ml of a 3% beef extract solution (pH 9.6), followed by centrifugation at 1,940 × *g* for 30 min twice to yield 2 eluents (first and second eluents). Finally, the eluents were passed through a 0.22-μm syringe filter to remove bacteria and fungi.

Virus isolation. Human rhabdomyosarcoma (RD) and human laryngeal epidermoid carcinoma (HEp-2) cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and were used for virus isolation. For each cell line and concentrated eluent, six parallel cell tubes were set up, with four for the first eluents and two for the second eluents. A total of 200 μl of each concentrated eluent was used for inoculating the standard monolayer of cells. Each tube was examined microscopically for the appearance of cytopathic effect (CPE) daily for up to 7 days. The tube in which cells developed CPE was recorded, the culture supernatant was collected separately as a positive isolate, and molecular typing was performed independently.

RNA extraction and molecular typing. To type the NPEV isolates, nucleic acid was extracted from the collected cultures with a QIAamp Viral RNA minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Viral RNA was eluted by adding 60 μl of sterile nuclease-free water and stored at −80°C until use.

Reverse transcription (RT)-PCR was performed according to the method developed by Nix et al. (23). The primer sequences used in this study are listed in Table S1 in the supplemental material. Briefly, cDNA was synthesized by using a Qiagen OneStep RT-PCR Kit (final volume, 20 μl) with 1 μM each cDNA primer (primers AN32, AN33, AN34, and AN35). Following incubation at 37°C for 60 min, 2.5 μl cDNA was then used in the first PCR (PCR1; final volume, 25 μl) with a *Taq* PCR Master Mix Kit (Qiagen) with 0.5 μM (each) primers 224 and 222, targeting a highly conserved motif in the VP3 and VP1 regions, respectively. After 40 cycles of amplification (95°C for 30 s, 42°C for 30 s, and 72°C for 45 s), 2.5 μl PCR1 products was used as a template in the second-round PCR with 0.5 μM (each) primers AN88 and AN89, targeting a partial VP1 region. After 40 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 45 s), the PCR products were analyzed on 1.2% agarose gels, and the positive products (~350 to 400 nucleotides [nt]) were purified using a QIAquick PCR purification kit (Qiagen) and sent for sequencing using primer AN88 or AN89. The sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) server at the National Center for Biotechnology Information (NCBI), and the serotype of each isolate was determined according to a previously described molecular typing method (24). In general, a pending EV was classified as the same serotype as the prototype strain if it had >75% nucleotide identity and >85% amino acid sequence identity in the VP1 coding region; the pending EVs were classified into different serotypes if they had <70% nucleotide identity and <85% amino acid sequence identity.

RESULTS

In total, 947 positive isolates were collected, comprising 916 NPEVs and 31 nontypeable viruses. Seventeen NPEV serotypes were identified based on the molecular typing of a 340-bp fragment sequence in the *VP1* region, and the number of EV serotypes ranged from 10 to 16 during the period from 2009 to 2012. The distribution of serotypes identified in EVs is presented in Table 1. Overall, the 6 most commonly identified EVs were ECHO11 (26.4% of all isolates), ECHO6 (24.1%), ECHO7 (13.4%), ECHO12 (13.3%), CVB5 (9.5%), and CVB3 (3.1%).

TABLE 1 NPEV serotypes detected in the sewage of Guangzhou City, China, each year from 2009 to 2012

Serotype[a]	No. detected				
	2009	2010	2011	2012	Total (%)
ECHO1	0	1	0	0	1 (0.1)
ECHO3	7	5	5	6	23 (2.5)
ECHO6	38	101	42	40	221 (24.1)
ECHO7	40	28	22	33	123 (13.4)
ECHO11	59	71	59	53	242 (26.4)
ECHO12	20	21	46	35	122 (13.3)
ECHO13	4	5	2	5	16 (1.7)
ECHO19	6	1	1	4	12 (1.3)
ECHO20	0	1	1	2	4 (0.4)
ECHO24	5	1	4	4	14 (1.5)
ECHO29	0	1	0	1	2 (0.2)
ECHO30	0	1	1	2	4 (0.4)
CVB1	0	1	9	0	10 (1.1)
CVB2	1	1	1	1	4 (0.4)
CVB3	0	1	6	21	28 (3.1)
CVB4	0	0	0	3	3 (0.3)
CVB5	34	43	2	8	87 (9.5)
Total	214	283	201	218	916

[a] Most common serotypes: 2009, ECHO11 (27.6%), ECHO7 (18.4%), and ECHO6 (17.5%); 2010, ECHO6 (34.8%), ECHO11 (24.5%), and CVB5 (14.8%); 2011, ECHO11 (29.4%), ECHO12 (22.9%), and ECHO6 (20.9%); 2012, ECHO11 (22.7%), ECHO6 (17.1%), and ECHO12 (15.0%).

To investigate the circulating NPEVs according to season, the number of NPEV isolates detected each month was determined. As shown in Fig. 1, from 2009 to 2012, the number of NPEVs typically increased from February on and generally peaked in July, except in 2010, when a large number (35 isolates) of NPEVs were isolated in June and only a few (9 isolates) were detected in July. However, some variations in the seasonal prevalence of NPEVs were observed. In 2009, there was another peak in April, and 36 (16.7%) NPEVs were isolated. In 2010 and 2011, numerous NPEVs were isolated in October (32 isolates) and November (26 isolates), but a similar pattern was not observed. Interestingly, our results differ from those of other studies (25, 26), in which the number of NPEVs in Guangzhou sewage samples did not remain high during the summer and fall and a sharp decrease in the number of NPEVs was observed in June, July, and August of 2009, 2010, and 2011, respectively. The circulation pattern of each NPEV serotype also differed. As shown in Fig. 2, a large number of ECHO6 and ECHO12 viruses were detected in June, whereas most ECHO7 and ECHO11 viruses were isolated around October.

The distribution patterns of NPEV serotypes also varied over the years, even though the total numbers of NPEVs isolated from sewage each year were similar. Among these NPEV serotypes, 10 were detected every year, and another 2 serotypes (ECHO1 and CVB4) were detected in only a single year. The predominant serotypes also differed each year. As shown in Table 1 and Fig. 2, ECHO6 and ECHO11 were the most prevalent serotypes during

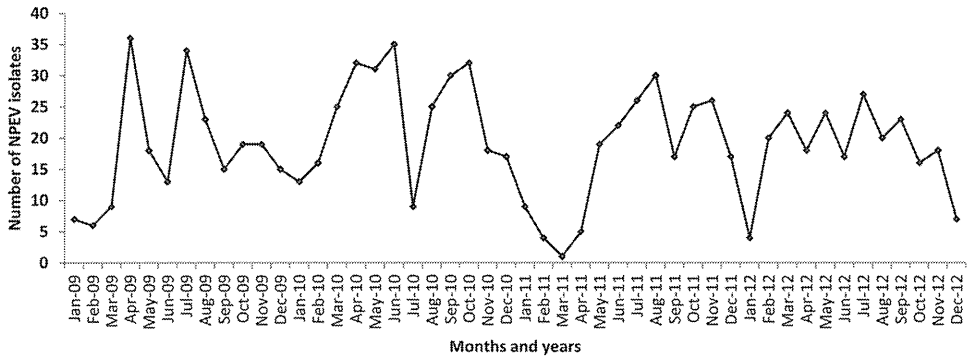

FIG 1 Number of NPEV isolates detected each month by virus isolation from 2009 to 2012.

the study period, whereas ECHO7 and CVB5 were prevalent only in 2009 and 2010, respectively. ECHO12 was increasingly detected starting in 2011 and became the most common serotype in 2011 and 2012 (Fig. 2d). While CVB5 was frequently isolated in 2009 and 2010 (15.7% and 14.8% of total isolates, respectively), it was rarely detected in 2011 and 2012 (Fig. 2e). Although only a single CVB3 isolate was detected in 2009 and 2010, an increasing number were detected in the subsequent years, and 21 isolates (10% of total isolates in 2012) were identified in 2012 (Fig. 2f).

In this study, different cell lines also exhibited different sensitivities to NPEVs. Our results indicated that RD cells were more sensitive to echoviruses, with 83.1% of echoviruses isolated from RD cells while only 16.9% were isolated from HEp-2 cells. Conversely, HEp-2 cells seemed to be better for CVB detection, since 59.8% of CVBs were isolated from HEp-2 cells, whereas 40.2% were isolated from RD cells.

DISCUSSION

Circulation of EVs in sewage is a proven indicator of their presence in a given community. Therefore, sewage surveillance is regarded as a complementary approach to determine the prevalence and duration of epidemic EVs in a human population (11, 22, 26, 27). For instance, in a serotype-based surveillance study performed by Sedmak et al., clinical isolates were compared with sewage isolates in Wisconsin from August 1994 to December 2002 (26). The study showed that the most commonly detected EV serotypes in sewage were similar to the most commonly detected EV serotypes in clinical samples. Also, the annual peaks in sewage EV titers were accompanied by peaks in clinical cases, which occurred in late summer or early fall. Moreover, the high sequence similarity between EVs from sewage and clinical samples provides substantial evidence at the molecular level. A study conducted by Iwai et al. from 2002 to 2003 demonstrated that the nucleotide sequences of ECHO13 isolated from sewage water were closely related to those isolated from patients with aseptic meningitis in Toyama Prefecture, Japan (22).

In this study, we reported an overview of NPEV prevalence in the sewage of Guangzhou city, the capital city of Guangdong Province, China. During the 4-year surveillance study, 10 common circulating NPEV serotypes were identified in Guangzhou, which were (in descending order) ECHO11, ECHO6, ECHO7, ECHO12, CVB5, CVB3, ECHO3, ECHO13, ECHO24, and ECHO19. In contrast, ECHO1, ECHO20, ECHO29, ECHO30, CVB1, CVB2, and CVB4 were only occasionally detected in sewage samples.

Currently, no surveillance system is set up to monitor NPEV clinical infection in China. Because most people infected with enterovirus do not show clinical symptoms, or show only mild symptoms, clinical data on NPEV infection are very limited and mainly from testing of patients with meningitis or meningoencephalitis and HFMD. ECHO6, a predominant NPEV in the sewage of Guangzhou, was also isolated in other regions of China, including Yunnan, Shandong, and Henan Provinces (11, 12, 28, 29). Correspondingly, outbreaks of ECHO6-associated aseptic meningitis and HFMD were reported in Anhui and Shandong Provinces in 2005 and 2011 (7, 30). CVB5, which was commonly detected in 2009 and 2010 in the sewage of Guangzhou, was reported to be the etiologic agent for an aseptic meningitis outbreak in Shandong Province, China, in 2009 (31). More direct evidence was provided by ECHO30. In this surveillance, ECHO30 was not detected until 2010. Although the number of positive isolates was low, the continuous identification of ECHO30 from 2010 to 2012 suggested its circulation in the environment in Guangzhou. Consistently, an outbreak of aseptic meningitis occurred in Luoding city, which adjoins Guangzhou city, in 2012. Our most recent study revealed that the newly emerged ECHO30 was the most commonly isolated EV serotype in cerebrospinal fluid samples from the patients (32, 33). These cooccurrences of NEPV in sewage and clinical samples prove the value of environmental surveillance for enteroviruses.

The results of this study also suggest that the circulation patterns of individual EVs change, along with temporal and spatial factors. Due to the different features of each EV, the seasonal pattern of the EVs differs over time, and the circulation patterns for the different serotypes might vary. In Guangzhou city, ECHO11 and ECHO6 were the most common serotypes in sewage during our 4-year surveillance study. Meanwhile, the months during which the largest numbers of ECHO6 and ECHO11 viruses were detected differed. Comparing our surveillance data with those from similar reports, we found that the NPEVs detected in this study were also identified in sewage surveillance studies from other areas, such as Shandong Province, China; Iran; France; and the United States, whereas the predominant NPEV serotypes in these different geographical regions varied (11, 25, 26, 34). These findings demonstrate the value of environmental surveillance of EVs in this area.

In the present study, the detection results using 2 cell lines with different sensitivities to the different EV serotypes are more convincing (26). Therefore, it is reasonable to assume that our sewage

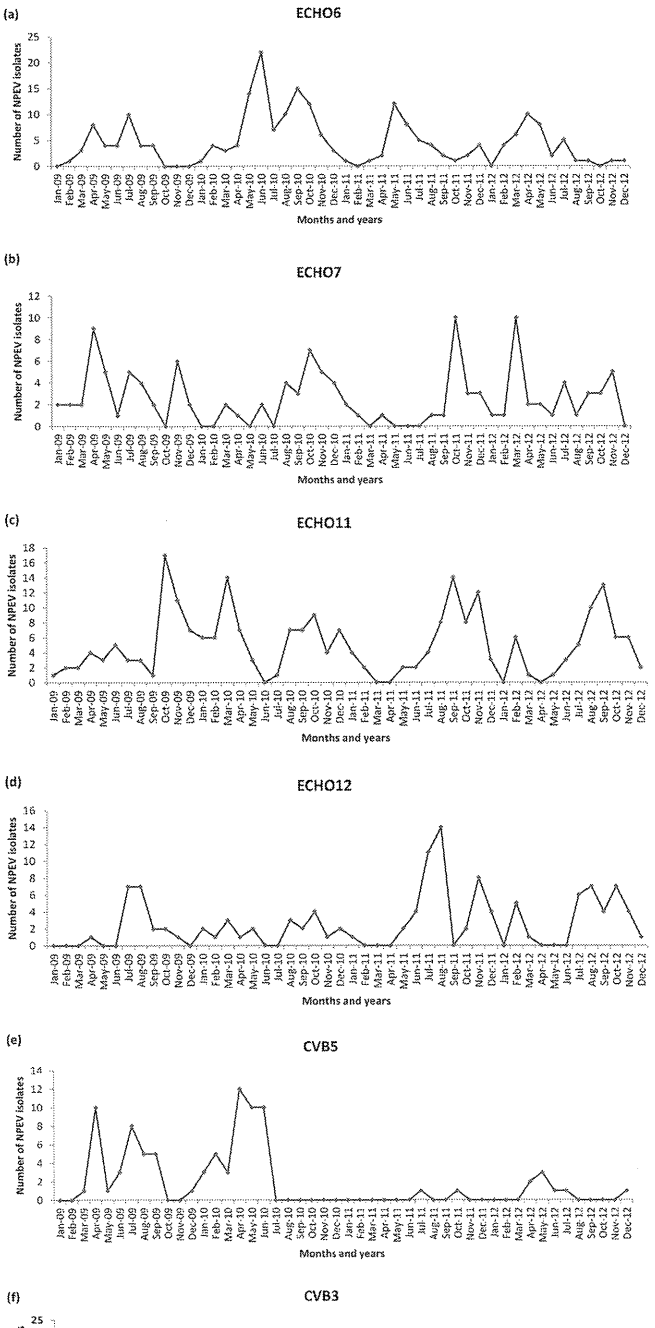

FIG 2 Seasonal patterns of 6 predominant circulating NPEVs in Guangzhou from 2009 to 2012.

testing is a reflection of local EV activity. However, it should also be noted that some EVs, especially EV-As, such as EV-A71, might have been missed in our study, because their growth rates in cells are lower than those of EV-Bs. Molecular typing of EVs is better for identifying strains that might have been classified as "untypeable" by the conventional neutralization method. Moreover, the sequences obtained allowed us to further analyze the evolution of the circulating NPEVs through phylogenetic assays.

In the absence of a systematic EV disease surveillance system in China, our study on the prevalence of sewage-borne NPEVs has at least two advantages. First, our study provides relevant area-specific epidemiological data on potential waterborne pathogenic viruses that will help public health practitioners determine the long-term circulation patterns of individual EVs and health-based targets (i.e., water or food quality targets for pathogens). Second, since NPEVs are occasionally related to serious diseases, such as myocarditis and aseptic meningitis, our description of the predominant NPEVs in sewage provides an epidemic background of the circulating EVs that can be used to interpret the trends in EV prevalence and to provide warning of possible enteroviral disease outbreaks.

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We declare that we have no competing interests.

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Isolation and Characterization of a Type 2 Vaccine-Derived Poliovirus from Environmental Surveillance in China, 2012

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Abstract

Environmental surveillance of poliovirus on sewage has been conducted in Shandong Province, China since 2008. A type 2 vaccine-derived poliovirus (VDPV) with 7 mutations in VP1 coding region was isolated from the sewage collected in the city of Jinan in December 2012. The complete genome sequencing analysis of this isolate revealed 25 nucleotide substitutions, 7 of which resulted in amino acid alteration. No evidence of recombination with other poliovirus serotypes was observed. The virus did not lose temperature sensitive phenotype at 40°C. An estimation based on the evolution rate of the P1 coding region suggested that evolution time of this strain might be 160–176 days. VP1 sequence analysis revealed that this VDPV strain is of no close relationship with other local type 2 polioviruses (n = 66) from sewage collected between May 2012 and June 2013, suggesting the lack of its circulation in the local population. The person who excreted the virus was not known and no closely related virus was isolated in local population via acute flaccid paralysis surveillance. By far this is the first report of VDPV isolated from sewage in China, and these results underscore the value of environmental surveillance in the polio surveillance system even in countries with high rates of OPV coverage.

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Introduction

Polioviruses (PVs) have three serotypes and belong to the *Enterovirus* genus, *Picornaviridae* family. Their infection is known to be associated with acute paralytic poliomyelitis. The global incidence of poliomyelitis has dropped by more than 99 per cent since the Global Polio Eradication Initiative (GPEI) was launched in 1988 [1]. No case due to type 2 wild poliovirus (WPV) has been identified since 1999 and the remaining two serotypes are limited to just a small number of endemic regions [2,3].

However, the GPEI has faced obstacles to the desired target. One is the re-emergence of WPV in previously polio-free countries, such as the WPV1 importation to mainland China in 2011. Another is the emergence of vaccine-derived polioviruses (VDPVs) (>1% divergent [PV1 and PV3] or >0.6% divergent [PV2]). Since the live, attenuated oral poliovirus vaccine (OPV) is a main tool used in polio eradication efforts, rare paralytic poliomyelitis cases caused by VDPVs can occur when neurovirulence reverses as the result of mutations. VDPVs have the potential for sustained circulation in areas with low OPV coverage and many outbreaks of circulating VDPVs (cVDPVs) were reported worldwide in recent years [4,5]. In Mainland China, VDPVs have also been identified through the acute flaccid paralysis (AFP) surveillance system [6]. One of the interesting observations from the recent outbreaks is that most of the cVDPVs reported in these outbreaks is Sabin2 associated. Most of the type2 VDPVs isolated from the field contain the G481A and U2909C mutation encoding a Ile to Thr substitution in VP1. These two mutations appear to be responsible for the neurovirulence reversion of the Sabin2 VDPVs [7].

The World Health Organization (WHO) strategy for monitoring the wild type and mutated vaccine poliovirus is to identify virus isolates from AFP cases and their contacts. Environmental surveillance serves as a supplementary method to monitor the PV transmission in human populations by examining sewage specimens which have been potentially contaminated by human

feces [8]. Surveillance for enteroviruses in sewage samples has been conducted in our laboratory since 2008. For the first time, a type 2 VDPV strain E12–221 was isolated from the sewage collected in December, 2012. In this report, we describe the genomic characterization, temperature sensitivity phenotype and phylogenetic analysis of this virus, and discuss the significance of environmental surveillance in GPEI.

Materials and Methods

Ethics Statement

The ethical approval was given by Ethics Review Committee of the Shandong Center for Disease Control and Prevention, and the study was conducted in compliance with the principles of the Declaration of Helsinki. Written informed consents for the use of their clinical samples were obtained from the parents or legal guardians of the patients. The permission for each sampling location was issued by Shandong Provincial Environmental Protection Department.

Shandong Province and sampling sites

Shandong is a coastal province located in the eastern part of China with an area of 156,700 km^2 and a population of 95.79 million (2010 census data). Jinan is the capital city of Shandong Province. Its metropolitan area and population is 296 km^2 and 2.6 million, respectively. Linyi is a city with frequently documented large outbreaks of enteroviral diseases in recent years [9–11]. Its metropolitan area and population is 178 km^2 and 1.9 million, respectively. The inlets to the sewage treatment plants of the two cities, namely, Jinan Everbright Water and Linyi Shouchuang Water, were chosen as the sampling sites.

Sampling and concentration

Collected monthly in Jinan and semimonthly in Linyi, the samples were from the inlet collector canal by grab sampling method between 2–3 pm. Approximately 1 liter of sewage sample was collected from flowing sewage by a stainless plastic bucket and maintained at about 4°C during sample transport, storage (<24 h), and processing.

Sewage samples were concentrated by membrane absorption/elution method as described previously [12,13]. Briefly, the sewage samples were centrifuged at 3000×*g* for 30 min at 4°C. 2.5 M $MgCl_2$ was added to the supernatant to a final concentration of 0.05 M. The pH value was adjusted to 3.5 by 0.5 M hydrochloric acid. Then the solution was filtered through a 0.45-μm-pore-size, mixed cellulose ester membrane filter (Advantec, Tokyo, Japan) by positive pressure pump. Absorbents on the filter were then eluted with 10 ml 3% beef extract solution followed by ultrasonication for 5 minutes, and the solution was centrifuged at 4000×*g* for 30 min. Subsequently the supernatant was filtered through a 0.22-μm-pore-size filter and was ready for cell inoculation.

Virus isolation and serotyping

L20B, RD and HEp-2 cell lines were used for virus isolation. All these cell lines were gifts from the WHO Global Specialized Laboratory in USA and were all originally purchased from the American Type Culture Collection (ATCC). A total of 200 μl of concentrated solution was added to each of the cell culture tube (18 tubes of each cell line for one sewage sample). Subsequently, the tubes were kept in 36°C incubator for 7 days and were examined daily. After 7 d, the tubes were frozen and thawed and re-inoculated into L20B, RD and HEp-2 cell lines. If a complete cytopathic effect (CPE) was observed in RD or HEp-2 cell lines, the cells in the tube were frozen and thawed and inoculated into L20B cells for isolation of PV.

According to standard protocols recommended by the WHO [14], PV serotyping was carried out via micro-neutralization assays in 96-well tissue culture plates using polyclonal antisera against PV types 1, 2 and 3 (National Institute for Public Health and the Environment, RIVM, the Netherlands). The antisera-virus mixtures were incubated for 1 h at 36°C. Subsequently, suspensions of cells were added to the plate which was subsequently examined daily for the presence of CPE. The antiserum that prevented the development of CPE indicated the identity of the virus.

PV isolates from AFP surveillance

The specimens from AFP cases in Shandong Province were collected between 2012 and June 2013 and processed according to standard protocols recommended by the WHO [14].

VP1 sequencing and phylogenetic analysis

Total RNA was extracted from 140 μl of the infected cell culture using QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended procedure. RT-PCR was performed using Access RT-PCR System (Promega, USA). Primer pair UG1/UC11 [15] was used to amplify the entire VP1 coding region. PCR products were purified and sequenced directly with the BigDye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) by ABI 3130 genetic analyzer (Applied Biosystems). The PCR products were sequenced in both directions to avoid possible ambiguous nucleotides.

Nucleotide sequence alignments were carried out by BioEdit 7.0.5.3 software [16]. Phylogenetic trees were constructed by Mega 4.0 [17] using neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method [18]. A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0.

Full-length genome amplification

The complete genome of VDPV2 strain E12–221 was amplified by two long-distant PCR reactions using the TaqPlus Precision PCR system (Stratagene, USA). The primer pairs Y7/7500A and 0010S48/Q8 [19,20] were used to amplify a 5.28 kb and 3.57 kb fragment, respectively. The combined sequences of the two fragments yielded the entire genome sequence. Sequencing were performed with primers described previously [6].

Estimation of the date of the initial OPV administration

The time interval between the date of OPV administration and the date of sampling of the VDPV2 strain E12–221 was estimated via the calculation of Ks (synonymous substitutions per synonymous site) and Kt (all the substitutions per site) values in the P1 coding region. It is assumed that the evolution rates are 0.032 synonymous substitutions per synonymous site per year and 0.011 total substitutions per site per year [21].

Temperature sensitivity

Temperature sensitivity of strain E12–221 was tested on monolayered RD cells in 6-well plates as described before [22]. 200 μl of virus stocks was inoculated onto the cells. After absorption at 36 or 40°C for 1 h, the supernatant was removed, and 2.5 ml of maintenance medium was added and incubated at 36 or 40°C, separately. After 8, 24, and 48 h, the plates were harvested, and the $CCID_{50}$ were calculated in 96-well plates. More than 2 logarithms reduction of the titers at different

temperatures was considered to be temperature sensitive. In order to minimize experimental error, the assay was repeated three times.

Reported vaccination rate and AFP surveillance data

The vaccination rate of Jinan city in 2012 was obtained from the National Immunization Information Management System. The AFP surveillance data was obtained from the China Information System for Diseases Control and Prevention.

Rapid assessment of vaccination rate

Rapid assessment was conducted in the 10 counties of Jinan city from January 21 to 24, 2013. Two towns were randomly selected for each county, three villages were randomly selected for each town, and six children <4 years of age in each village were investigated for the OPV immunization history.

Nucleotide sequence accession numbers

The complete genome sequence of VDPV2 isolate E12–221 and VP1 sequences of other PV2 strains from sewage described in this study were deposited in the GenBank database under the accession numbers KF656732 and KF666568–KF666633, respectively.

Results

Analysis of the genome sequence

A virus strain named E12–221 was isolated from the sewage collected in Jinan in December 2012. Neutralization test showed that it was a type 2 poliovirus. Based on the full-length genome sequencing analysis, we found that, compared with Sabin 2 strain, E12–221 contains 25 nucleotide substitutions with 7 mutations resulting in amino acid alteration (Table 1). It should be noted that, in strain E12–221, the nucleotide A at position 481 in 5′ noncoding region has been mutated to G, which is a neurovirulent reversion mutation usually observed in the VDPV-associated paralytic poliomyelitis cases [7]. In the VP1 coding region of E12–221, there are 7 substitutions, 4 of which lead to amino acid change. However, the Ile at position of 143 in VP1, the other neurovirulent determinant of Sabin 2, didn't mutated. In addition, no recombination event was observed.

Table 1. Nucleotide and amino acid substitutions in the sewage isolate E12–221 in comparison to Sabin 2.

Region	Nucleotide			Amino acid		
	Position*	Sabin 2	E12–221	Position*	Sabin 2	E12–221
5′ NTR	398	U	C			
	481	**A**	**G**			
	735	C	U			
VP4	786	C	U			
VP2	960	A	G			
	993	A	G			
VP3	1905	G	A			
	2005	A	G	420	T	A
	2026	U	C			
	2256	A	G			
VP1	2548	U	A	601	S	T
	2557	A	G	604	S	G
	2595	C	U			
	2986	A	G	747	K	E
	3120	A	U			
	3145	U	C	800	S	P
	3339	U	C			
2C	4158	U	C			
	4188	C	U			
	5031	U	C			
3C	5578	G	A	1611	A	T
	5946	C	U			
3D	6209	A	G	1821	K	R
	6390	U	A			
	6765	C	U			

*Nucleotide and amino acid positions are numbered according to Sabin 2 (AY184220).
The nucleotide position described as being involved in Sabin 2 attenuation is in bold.
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Estimation of the evolution time of strain E12–221

The approximate evolution time of the VDPV2 strain after the initial vaccine dose was estimated by its difference from Sabin 2 strain in the P1/capsid coding region. The *Ks* and *Kt* values for P1 coding region was 1.4% and 0.53% respectively. Compared with the reported evolution rate of 3.2% (*Ks*) and 1.1% (*Kt*) in P1 region per year [21], we estimated the evolution time of the virus was 160 days (from the *Ks* estimate) or 176 days (from the *Kt* estimate). Assuming a one-week efflux of the virus in the sewage network, the administration time of the parental OPV might be June 2012. Nevertheless, it had to be admitted that the evolution time was just an estimate. The actual origin of the virus and its evolutionary experience in the sewage network might differ from the deduction.

Temperature sensitivity

The temperature sensitivity assay of VDPV2 strain E12–221 showed that there is a titer reduction of more than 2 logarithms at different temperatures (Table 2), suggesting that, similar to Sabin

Table 2. Temperature sensitivity of VDPV2 strain E12–221 and Sabin 2 strain.

Virus	Titers at 37°C			Titers at 40°C			Log titer reduction		
	8 h p.i.	24 h p.i.	48 h p.i.	8 h p.i	24 h p.i.	48 h p.i.	8 h p.i	24 h p.i.	48 h p.i.
Sabin 2	8.0	8.0	7.9	5.5	5.0	5.5	2.5	3.0	2.4
E12–221	7.9	8.5	8.0	5.9	5.6	5.0	2.0	2.9	3.0

The titers and its reduction at different temperatures are presented as logarithm 10.
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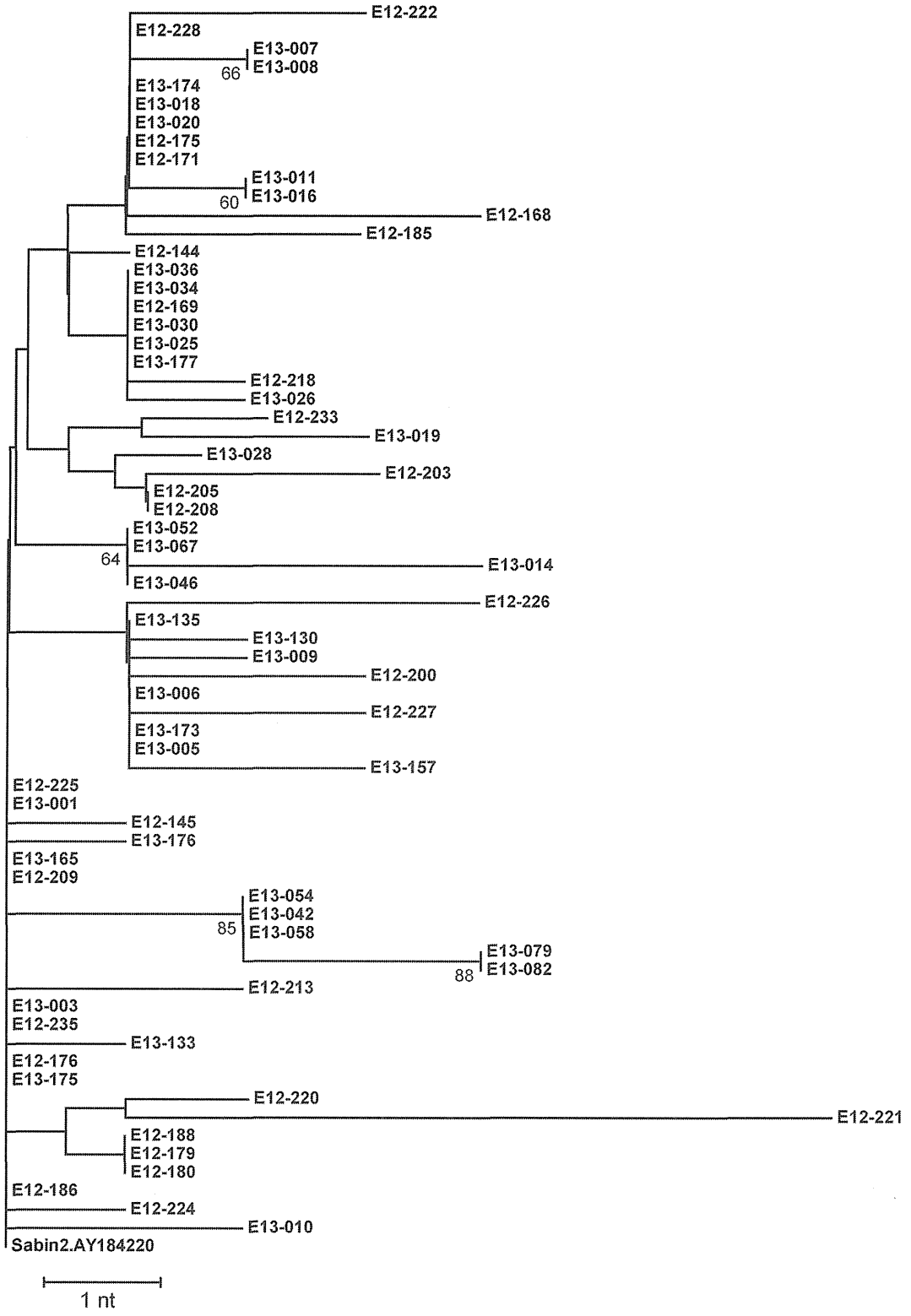

Figure 1. Phylogenetic tree based on VP1 sequences of all environmental polioviruses. The polioviruses in the tree were isolated from sewage in Shandong Province from May 2012 to June 2013. Strain E12–221 is a VDPV2 strain isolated in December 2012. The isolates are represented as E(SampleYear)-(serial no). The percentage number at the node corresponds to the resampling value, and the values lower than 60% are not shown.
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