

# Isolation and Characterization of Circulating Type 1 Vaccine-Derived Poliovirus from Sewage and Stream Waters in Hispaniola

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Twenty-one cases of acute flaccid paralysis (AFP) were reported on the island of Hispaniola in 2000. Laboratory analysis confirmed the presence of circulating vaccine-derived poliovirus (cVDPV) type 1 in stool samples obtained from patients. As a complement to the active search for cases of AFP, environmental sampling was conducted during November and December 2000, to test for cVDPV in sewage, streams, canals, and public latrines. Fifty-five environmental samples were obtained and analyzed for the presence of polioviruses by use of cell culture followed by neutralization and reverse-transcription polymerase chain reaction. Of the 23 positive samples, 10 tested positive for poliovirus type 1, 7 tested positive for poliovirus type 2, 5 tested positive for poliovirus type 3, and 1 tested positive for both poliovirus type 2 and type 3. By sequence analysis of the complete viral capsid gene 1 (VP1), a 2.1%–3.7% genetic sequence difference between 7 type 1 strains and Sabin type 1 vaccine strain was found. Phylogenetic analysis showed that these viruses are highly related to cVDPV isolated from clinical cases and form distinct subclusters related to geographic region. Our findings demonstrate a useful role for environmental surveillance of neurovirulent polioviruses in the overall polio eradication program.

In the summer of 2000, cases of acute flaccid paralysis (AFP) were reported in both the Dominican Republic and Haiti [1]. Stool samples obtained from the patients were positive for poliovirus type 1, and further characterization of the isolates by sequencing of the complete major capsid surface protein VP1 (viral capsid gene 1) indicated that they were circulating vaccine-derived polioviruses (cVDPV) and that they had a substantially lower degree of sequence similarity (97.4%–98.1%) than is normally observed (>99.5%) [2]. To

detect additional cases of poliovirus, surveillance for AFP was intensified, and, from July 2000 to July 2001, a total of 156 cases of AFP were reported; 21 of the cases were confirmed as polio cases, and most of them involved patients who were unvaccinated or incompletely vaccinated [2]. All isolates from the Hispaniola outbreak were recombinants, with noncapsid sequences obtained from other species C enteroviruses and from at least 4 different enteroviruses recombined with the type 1 cVDPV during its circulation in Hispaniola [2, 3]. Three additional outbreaks of cVDPV have been reported recently [4–6], stressing the importance of maintaining high levels of vaccine coverage. The World Health Organization (WHO) recommends oral poliovirus vaccine (OPV) for worldwide eradication of poliomyelitis, with the goal of vaccinating at least 90% of infants by age 1 year [7]. As long as vaccination is continued and vaccine coverage is high, it is expected that populations will be safe or at very low risk of

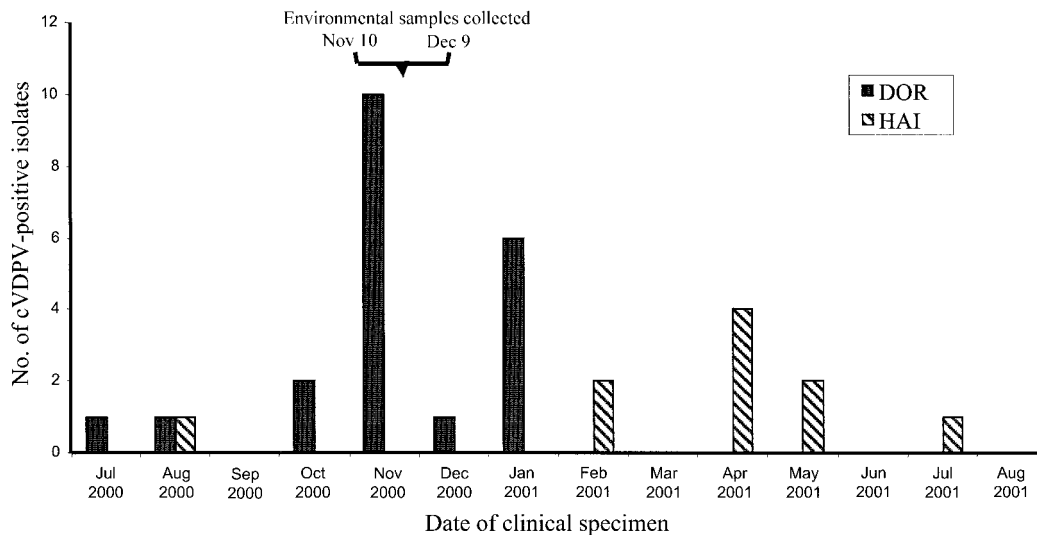
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**Figure 1.** Time (arrow) of environmental sample collection (Dominican Republic [DOR], 10 November–9 December 2000; Haiti [HAI], 6–9 December 2000) in relation to the peak of the outbreak in Hispaniola, on the basis of the no. of type 1 circulating vaccine-derived poliovirus isolates from case patients and their healthy contacts [2].

infection with neurovirulent polioviruses. However, until the termination of OPV immunization in the near future, cases and outbreaks caused by cVDPV must be considered, especially because the excreted viruses could change gradually during replication in the human gut, from attenuated to virulent [8, 9], ultimately acquiring the transmission and neurovirulence characteristics of wild-type (*wt*) polioviruses [2].

Environmental poliovirus surveillance (EPS) is a powerful method for monitoring the circulation of polioviruses in large populations, and there are several examples of detection of circulating *wt* poliovirus in the absence of reported AFP [10, 11]. Moreover, EPS has been shown to be an extremely sensitive method, capable of detecting a strain of poliovirus if only 1 of 10,000 people is excreting the virus [12]. The properties of polioviruses isolated from the environment directly reflect the virus excreted from humans after OPV immunization or during an epidemic [13–16]. Therefore, detection of cVDPV in the environment, as a potential cause of poliomyelitis, should raise concerns, especially with regard to vaccine coverage and the number of susceptible individuals potentially at increased risk of infection.

During the recent poliomyelitis outbreak on the Caribbean island of Hispaniola, as a complement to surveillance of AFP, EPS was implemented between November and December 2000. In the present study, we report the results of this EPS study and describe the detection, characterization, and neurovirulence of cVDPV strains isolated from river water and sewage samples.

## MATERIALS AND METHODS

**Environmental water and waste samples.** Environmental samples from sewage, creeks, canals, and latrines were obtained in several geographically different areas of Haiti and the Dominican Republic, both near villages where either cases of AFP or laboratory-confirmed cases of polio had been reported and at sites where there was no evidence that cases of AFP had occurred in the nearby community. Fifty-five 1-L samples (12 from Haiti and 43 from the Dominican Republic) were obtained during November–December 2000, 4 months after the first case was reported in the Dominican Republic and during the peak of the outbreak (figure 1), and were stored at  $-20^{\circ}\text{C}$  for 3 months until shipment on dry ice to the University of North Carolina at Chapel Hill.

**Processing of samples.** Environmental water and sewage samples were processed as described elsewhere [17], with several modifications. In brief, the pH of the samples was adjusted to 7.2, and the samples were centrifuged at 5000 g for 30 min at  $4^{\circ}\text{C}$  to pellet the solids. The supernatant was retained, and the sediment was extracted twice with 5 parts (vol/vol) 3% beef extract and 3 parts (vol/vol) chloroform. After centrifugation at 5000 g for 30 min at  $4^{\circ}\text{C}$ , the aqueous supernatants from these extractions and initial supernatant were combined, and viruses were concentrated by use of polyethylene glycol (PEG) precipitation (8% [wt/vol] PEG 8000 and 0.3 mol/L NaCl) for 18 h at  $4^{\circ}\text{C}$ . After centrifugation at 6700 g for 30 min at  $4^{\circ}\text{C}$ , the PEG pellet was resuspended in a small volume of sterile PBS (5–20 mL) and was extracted with a 0.5 volume of chlo-

roform. After centrifugation at 5000 *g* for 30 min at 4°C, the chloroform phase was re-extracted with 1 part of 3% beef extract. After the aqueous supernatants were combined, 3% fetal calf serum (FCS), 30 µg/mL gentamycin, and 50 µg/mL kanamycin were added, and the concentrates were incubated for 30 min at room temperature and then were stored at -80°C.

**Virus isolation.** Environmental concentrates were inoculated onto confluent monolayers of Rhabdomyosarcoma (RD) cells and L20B cells (mouse fibroblast cells with the poliovirus receptor [PVR]) [18, 19] that were grown in Eagle minimal essential medium (MEM) containing 10% FCS. Two to five 25-cm<sup>2</sup> flasks of each cell line were inoculated with 1 mL/flask for each sample. After absorption for 60 min at 37°C, maintenance medium (MEM containing 2% FCS) was added, and flasks were incubated at 37°C and observed for cythopathic effect (CPE) for 7–10 days. All cultures were freeze-thawed 3 times, centrifuged at 2000 *g* for 10 min, filtered through 0.2 µm pore size filters (Gelman Scientific), and reinoculated onto 25-cm<sup>2</sup> flasks to confirm CPE positivity. Additional experiments included incubation at 40°C on L20B cells, to select for polioviruses that contain the reproductive capacity at supraoptimal temperature (RCT) marker [20].

**Characterization of poliovirus by use of microneutralization and intratypic differentiation (ITD) assay.** For the identification of poliovirus isolates, CPE-positive isolates from L20B and RD cells were tested by use of microneutralization using a pool of poliovirus antiserum (National Institute of Public Health and the Environment, The Netherlands), as described elsewhere [21]. All polioviruses were further characterized by previously validated ITD assays, which are used to investigate the origin (i.e., *wt* or vaccine-related) of poliovirus field isolates [22]. Neutralization with a mixture of Sabin-specific monoclonal antibodies (MAbs) of the 3 polio serotypes (MAbs 955, 1233, and 889 for PV1, PV2, and PV3, respectively) was performed in 96-well tissue-culture plates in MEM with 5% FCS, as described elsewhere [23], by use of a 1:100 dilution of the trivalent MAb ascites mixture. Final microscopic reading of the assay was done after 4 days. Each strain that was not neutralized by the MAb pool was considered to be non-Sabin.

**Diagnostic reverse-transcription polymerase chain reaction (RT-PCR) for poliovirus.** All CPE-positive samples were tested for poliovirus by use of a RT-PCR kit for the ITD of polioviruses (CDC) that included separate reactions with primers that were pan-enterovirus, pan-poliovirus [24], serotype specific [25], and specific for the Sabin type 1, 2, and 3 viruses [26]. Amplified products were loaded on premade 10% polyacrylamide gels (Bio-Rad) and were electrophoresed for 2 h at 100 V. The PCR products were visualized after staining for 5 min in 2 µg/mL ethidium bromide.

**VP1 amplification and sequencing.** VP1 sequences were determined by use of PCR cycle sequencing, as described else-

where [27]. RT-PCR products were generated by use of primers Q8 and Y7 [11]. In brief, 1 µL of clarified L20B lysate was incubated for 5 min at 95°C, to release virion RNA. Poliovirus RNA was then amplified by use of a One-Step RT-PCR kit (Qiagen) and 5 U of RNase inhibitor (Promega), according to the manufacturers' protocol. For RT, the samples were incubated for 30 min at 50°C, and, after incubation for 15 min at 95°C. PCR consisted of 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. RT-PCR products were purified by use of a QIAquick PCR purification kit (Qiagen). The following PCR primers (and additional sequencing primers) were used for sequencing: VP1551R (5'-GCTGTTCCGTAGGTGTA-3') and VP1F449 (5'-CCTTAAATCAAGTGACCAA-3'). To minimize possible sequence ambiguities during RT-PCR, duplicate amplicons were independently amplified from RNA templates or clones.

**Sequence analysis of 5' noncoding region (NCR) and non-structural genomic region.** To determine specific mutations at genome positions 480 and 525, viral RNA from strains H9A, D21A, and D55A was amplified by use of RT-PCR using pan-enterovirus primers [28]—for 35 cycles of 1 min at 94°C, of 1 min at 55°C, and of 1 min at 72°C—and RT-PCR products of 196 bp were sequenced in both directions. To identify that the poliovirus strains isolated from the environmental samples were, like the clinical strains, recombinant viruses, we cloned and sequenced the region between VP1 and the 3' end of strain H9A. cDNA was prepared by use of SuperScript RT (Life Technologies), as described elsewhere [29]. Amplicons of 3.6 kb were generated by 2 rounds of PCR by use of the Expand template PCR kit (Roche), were cloned in the TA vector (Invitrogen), and were sequenced by use of primer walking.

**Phylogenetic analysis.** To determine the VP1 nucleotide sequence diversity of the environmental isolates from Hispaniola, we compared complete VP1 sequences (906 nt) from 10 sewage-derived Hispaniola strains, 29 strains isolated from either patients with clinical cases or their contacts [2], and the Sabin type 1 OPV reference strain. A multiple alignment was constructed by use of ClustalW (version 1.4; available at: <http://www.ebi.ac.uk/clustalw/>) and was imported into the Treecon software package [30]. To compile a phylogenetic tree, distances of 100 bootstrapped data sets were calculated by use of the Jukes and Cantor correction for evolutionary rate, and tree topology was inferred by use of neighbor-joining, with Sabin type 1 as an outgroup.

**Neurovirulence test.** The neurovirulence of 3 environmental isolates (H9A, D21, and D55) was compared with that of reference strains Sabin type 1, Mahoney *wt* poliovirus strain, and 2 clinical strains from the outbreak in Hispaniola, by use of transgenic (Tg) 21-Bx mice [31]. The poliovirus strains were purified by 3 rounds of limiting (end-point) dilution infectivity assay, followed by nucleotide-sequence analysis, to confirm

**Table 1. Laboratory data of circulating vaccine-derived poliovirus type 1 strains detected in environmental samples from Hispaniola.**

Isolate no.	Country	City; location	Collection date	Cell culture		RCT marker		VP1 sequence <sup>a</sup>	MAB NT <sup>b</sup>
				RD	L20B	37°C	40°C		
9	HAI	Port-au-Prince; Jobel creek	8 Dec 2000	CPE	CPE	CPE	CPE	Vaccine derived	No
13	HAI	Delmas; Orphelin canal	9 Dec 2000	CPE	CPE	CPE	No CPE	Sabin	Yes
21	DOR	Santiago; lift station	30 Nov 2000	CPE	CPE	CPE	CPE	Vaccine derived	No
22	DOR	Santiago; lift station	30 Nov 2000	CPE	CPE	CPE	CPE	Vaccine derived	No
51	DOR	Santiago; Arroyo Salado Gurabo stream	30 Nov 2000	CPE	CPE	CPE	CPE	Vaccine derived	No
55	DOR	Santo Domingo; waste-treatment plant	8 Dec 2000	CPE	CPE	CPE	CPE	Vaccine derived	No
56	DOR	Santo Domingo; La Cienaga waste-treatment plant	8 Dec 2000	CPE	CPE	CPE	No CPE	Sabin	Yes
59	DOR	Santiago; Navarrete channel	30 Nov 2000	CPE	Neg	CPE	CPE	Vaccine derived	No
62	DOR	Santo Domingo; Guagimia channel	8 Dec 2000	CPE	CPE	CPE	No CPE	Sabin	Yes
63	DOR	Santiago; La Mosca	10 Nov 2000	CPE	CPE	CPE	CPE	Vaccine derived	No

**NOTE.** All isolates were confirmed as poliovirus type 1 by use of reverse-transcription polymerase chain reaction, and all of them had the G→A substitution at nt 480. CPE, cytopathologic effect; DOR, Dominican Republic; HAI, Haiti; L20B, mouse fibroblast cell line with poliovirus receptor; Neg, negative; RCT, reproductive capacity at supraoptimal temperature [20]; RD, rhabdomyosarcoma cell line.

<sup>a</sup> Vaccine-derived >1% nucleotide sequence difference, compared with Sabin type 1 poliovirus.

<sup>b</sup> MAB NT, monoclonal antibody neutralization of CPE by a pool of poliovirus serotype-specific antisera.

their genotype. The mice were inoculated intramuscularly (left hind limb) with 50  $\mu$ L of 10-fold viral dilutions, and daily clinical score was monitored for 14 days [31]. Eight mice (4 male and 4 female) were used for each dilution. The 50% paralytic dose (PD<sub>50</sub>) of each strain was determined by use of the probit method and was compared with that of Sabin type 1, Mahoney *wt* poliovirus strain, and 2 strains isolated from clinical cases during the outbreak in Hispaniola.

## RESULTS

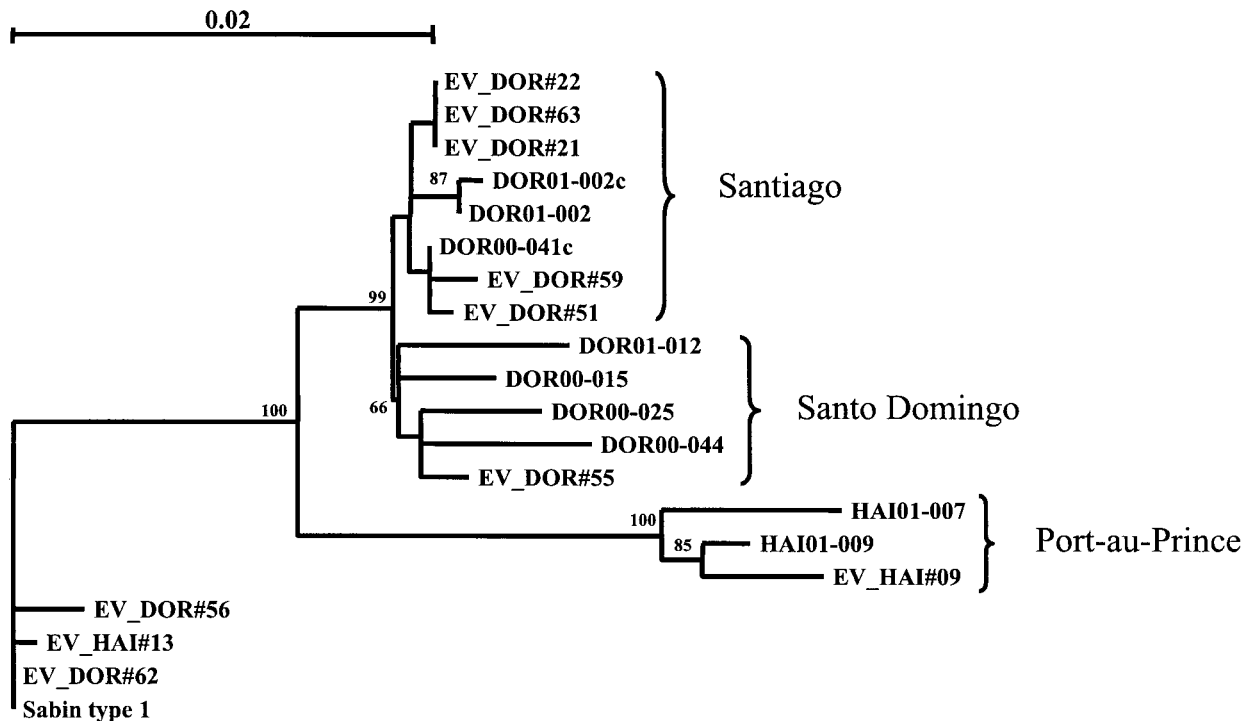
**Prevalence and properties of poliovirus isolated from environmental samples in Hispaniola.** In total, 55 environmental samples obtained during November and December 2000 were concentrated by use of PEG precipitation and were analyzed by use of cell culture on RD and L20B cells. Of these, 47 (85%) were CPE positive on RD cells, and 20 (36%) were CPE positive on L20B cells. One (8%) of the 12 samples obtained in Haiti was CPE positive on L20B cells, whereas 19 (44%) of the 43 samples obtained in the Dominican Republic were positive on L20B cells. Of all environmental samples, 12 (48%) of the 25 sewage samples, 1 (6%) of the 18 canal samples, 2 (33%) of the 6 stream-water samples, and 0 of the 6 latrine samples were positive on L20B cells. All 20 L20B-positive samples tested positive for enteroviruses by use of pan-specific primers. The presence of polioviruses could be confirmed in 19 (95%) of the 20 L20B-positive cell culture isolates by use of RT-PCR using pan-poliovirus-specific primers. In addition, 4 samples that were RD positive but L20B negative showed CPE after passaging on L20B cells and tested positive for poliovirus. Environmental samples obtained in the Dominican

Republic had a higher nonpolio enterovirus (NPEV) detection rate (91%) than did those obtained in Haiti (58%).

Of the 23 enterovirus isolates that were further tested by use of RT-PCR using pan-poliovirus-, serotype-, and Sabin-specific primers, 10 tested positive for poliovirus type 1, 7 tested positive for poliovirus type 2, 5 tested positive for poliovirus type 3, and 1 tested positive for both poliovirus type 2 and type 3. In addition, when the 10 poliovirus type 1 isolates were further screened by use of cell culture infectivity assay on L20B cells at 40°C, 7 (70%) were able to grow. The same 7 isolates were also able to grow in the presence of a mixture of Sabin-specific MABs (table 1).

Because the poliomyelitis outbreak in Hispaniola was associated with a derivative of the type 1 OPV strain [2], the genetic variability of the 10 type 1 isolates was further investigated by determining the nucleotide sequence of the complete VP1 capsid gene (906 nt). In total, 7 isolates were relatively closely related (96.3%–97.9% sequence identity) to the Sabin type 1 OPV strain and to each other (97%–100% sequence identity), but their sequence relatedness is substantially lower than that normally observed (>99.5%) in isolates from cases of AFP or vaccine-associated paralytic poliomyelitis. The VP1 sequences of the remaining 3 strains had 99.6%–100% sequence identity to Sabin type 1 OPV (figure 2). Phylogenetic analysis showed that polioviruses isolated from clinical cases and environmental samples formed distinct subclusters related to geographic origin (Santiago, Santo Domingo, and Port-au-Prince; figure 2).

**Neutralizing antigenicity of cVDPV.** By microneutralization using a pool of Sabin-specific MABs, 7 of 10 poliovirus type 1 isolates could not be neutralized (table 1). The complete VP1 nucleotide sequence of the 3 type 1 strains that were neu-



**Figure 2.** Nucleotide sequence relatedness (VP1 gene; 906 nt) among circulating vaccine-derived poliovirus (cVDPV) type 1 strains isolated from clinical samples (DOR01-002, DOR01-002c, DOR01-012, DOR00-015, DOR00-025, DOR00-041c, DOR00-044, HAI01-007, and HAI01-009 [2]) and environmental samples (EV\_DOR#21, EV-DOR#63, EV-DOR#22, EV-DOR#51, EV-DOR#59, EV\_DOR#55, EV-HAI09, EV\_DOR#56, EV\_HAI#13, and EV\_HAI#62) obtained in Hispaniola in 2000 and 2001. Sabin type 1 virus was used as an outgroup. Genetic clusters of strains that were isolated from 3 distinct geographic regions (Santiago, Dominican Republic [DOR]; Santo Domingo, DOR; and Port-au-Prince, Haiti [HAI]) are indicated.

tralized was identical to that of Sabin type 1 OPV. In total, 3 of the initial RD-positive and L20B-negative isolates were neutralized by a pool of polio-specific antisera. VP1 sequence analysis revealed that 1 isolate was a Sabin type 2 poliovirus and that 2 isolates were Sabin type 3 polioviruses.

**Sequencing of 5' NCR and noncapsid region.** Nucleotide-sequence analysis of a region of the 5' NCR revealed that all 7 type 1 cVDPV isolates could not be neutralized by Sabin-specific MAbs (table 1). The cVDPV isolates had the G→A substitution at nt 480 and the U→C substitution at position 525, which is known to be associated with increased neurovirulence [32]. To analyze whether the isolates were, like the viruses isolated during the outbreak in Hispaniola, recombinant polioviruses, a 3.6-kb nucleotide sequence corresponding to the 3' region of the genome coding for the noncapsid sequences of isolate EV\_HAI09 was determined. The region included the 2A, 2B, 2C, 3A, 3B, 3C, and 3D nonstructural genes and the 3' NCR. The genetic relationship of the noncapsid sequences was compared with those of clinical Hispaniola isolates and showed highest sequence similarity (93%) with strain HAI01-007, which was isolated from a patient who died in April 2001 in Port-au-Prince [2].

**Neurovirulence assay in PVR-Tg21 mice.** To measure the

neurovirulence of the cVDPV isolates from environmental samples, 3 isolates (H9A, D21A, and D55A; figure 2) were inoculated into Tg21-Bx transgenic mice [31], and their PD<sub>50</sub> values were compared with those of the Mahoney strain, 2 strains obtained from clinical cases of the outbreak of poliomyelitis in Hispaniola (DOR00-015 and DOR00-016 [2] and Sabin type 1; table 2). All 3 Hispaniola strains had PD<sub>50</sub> values that were either similar to or lower (more potent) than the neurovirulent Mahoney reference strain, and they were clearly much more neurovirulent than the Sabin type 1 virus strain.

## DISCUSSION

Surveillance of AFP has been a key factor in the successful efforts to achieve eradication of poliomyelitis. The outbreak in Hispaniola demonstrated that low levels of vaccine coverage, in combination with poor surveillance of AFP, could result in undetected and prolonged circulation of cVDPV in a community for up to 2 years before cases of poliomyelitis emerge [2]. In the present study, sampling of surface water or sewage in selected regions of Hispaniola where clinical cases had been reported demonstrated the supplemental value of EPS as a complement for surveillance of AFP in monitoring the presence

**Table 2. Results of a neurovirulence assay, performed in poliovirus receptor–transgenic 21 mice, of poliovirus strains obtained from environmental and clinical samples collected during an outbreak in Hispaniola.**

Strain	PD <sub>50</sub> (95% CI)
Sabin 1 <sup>a</sup>	>8.0 <sup>b</sup>
Mahoney <sup>c</sup>	5.9 (5.4–6.6)
DOR00-015 <sup>d</sup>	6.5 (6.3–6.7)
DOR00-016 <sup>d</sup>	6.4 (5.9–6.8)
H9A <sup>e</sup>	4.5 (3.5–5.5)
D55A <sup>e</sup>	5.5 (5.0–6.0)
D21A <sup>e</sup>	6.1 (5.6–6.6)

**NOTE.** Estimates of the 50% paralytic dose (PD<sub>50</sub>) and associated 95% confidence intervals (CIs) were calculated by use of the probit method [31].

<sup>a</sup> Type 1 oral poliovirus vaccine strain.

<sup>b</sup> Virus titers are expressed as log<sub>10</sub> of CCID<sub>50</sub>.

<sup>c</sup> Wild-type virus parent of Sabin 1.

<sup>d</sup> Virus isolate from a clinical case from the outbreak in Hispaniola.

<sup>e</sup> Virus isolate from environmental sample obtained in Hispaniola.

of polioviruses in fecally contaminated surface waters and community fecal waste and, thus, in the population. Type 1 cVDPV was detected in environmental samples from Haiti and the Dominican Republic and was antigenically and genetically similar to the viruses isolated from cases of AFP during the outbreak of poliomyelitis in Hispaniola [2]. These viruses were readily detectable in environmental samples from regions where vaccine coverage is low. The results of the present study demonstrate that environmental assessment for polioviruses in geographic areas at increased risk for cVDPV circulation may be an important tool for the worldwide poliomyelitis eradication program in its final stages.

A high rate of detection of NPEV in environmental samples is considered to be a reliable indicator that the environmental samples were obtained, processed, and analyzed appropriately and that the cold chain used during specimen transportation was effective in preserving virus infectivity. Environmental samples obtained in the Dominican Republic had a higher rate of detection for NPEV isolates (91%) than did those obtained in Haiti (58%). Reported sampling and transport problems for some of the sampling sites (latrines) in the northern part of Haiti could explain the relatively low rate of detection for NPEV. Overall, among the sites of sample collection, the rate of detection for poliovirus was highest in sewage and streams. However, in regions in the world where community sewage systems do not exist, EPS as it is currently conducted is not always feasible. Nonetheless, methods for the detection of polioviruses

in solid fecal waste have been developed [15, 33, 34]. Furthermore, nonliquid waste, such as night soil, is obtained and composed for treatment on a community basis in some parts of the world, and even latrine wastes can be sampled in a systematic way to obtain composite samples representative of communities and neighborhoods. Therefore, systematic and effective approaches for sampling of solid waste should be further developed if we choose to implement EPS in regions where no sewage systems are present.

Various methods have been described for the concentration of poliovirus from environmental samples, including ultrafiltration, 2-phase separation, and PEG precipitation [13, 16, 35, 36]. The method we used for concentrating viruses from sewage and water samples in the present study included PEG precipitation, followed by chloroform extraction, and was based on many different protocols that have been developed in our laboratory over the years [17, 33, 37–39]. Enterovirus recovery rates average 21%–88%, depending on the type of sample (e.g., water and municipal sewage, airplane holding tank sewage, and latrine waste), and recently have been shown to be highly sensitive in detecting *wt* polioviruses in a high background of OPV [36]. The technical skill, effort, time, and cost needed to perform the methods for poliovirus isolation from environmental samples are not likely to be appreciably greater than those used to detect polioviruses in clinical samples. Except for the initial steps of sample centrifugation and PEG precipitation, both of which are simple methods requiring modest skills and little actual sample processing time by a laboratory technician, the steps in poliovirus isolation and characterization are similar to those now used for clinical specimens of feces. The only factor responsible for additional cost is the possible need for a large-capacity high-speed centrifuge and rotor for those laboratories currently lacking such equipment.

At present, environmental sampling is not recommended for surveillance of poliovirus in countries where poliovirus is endemic. The major reason has been that the method involved (i.e., concentration) is not easily implemented in a clinical virologic laboratory and that poliovirus can be rapidly inactivated at ambient temperature in tropical areas [40]. However, our EPS study and a recent study in India [36] demonstrate that poliovirus can be detected easily when samples are stored frozen or analyzed rapidly (<24 h) after they are obtained.

Cell culture on L20B cells at elevated temperature (39.5°C–40°C) has been used successfully for the selection of nonvaccine poliovirus strains against a high background of Sabin viruses [11, 14]. This RCT assay [20] is based on the relatively small difference (<2 log<sub>10</sub>) in virus titer for *wt* polioviruses grown at normal temperature (36°C), compared with those grown at elevated temperature (40°C), and the large (>4 log<sub>10</sub>) titer differences for Sabin viruses grown under the same conditions. Hence, growth at elevated temperature can be used to select

for *wt* polioviruses against a high background of Sabin viruses, which is particularly useful when screening environmental samples [11]. However, this RCT marker is not definitive for neurovirulent polioviruses, and, when used as a selector, it will allow growth of 1% of vaccine (Sabin) type 1 and 2 isolates and 30% of type 3 isolates, while selecting against 30%–35% of the type 1, 2, and 3 isolates [20]. For this reason, it is probably best used as a supplemental and secondary selection and characterization marker and not for primary isolation of viruses in cell cultures.

Another useful tool to select for non-Sabin polioviruses in a background of vaccine polioviruses is the use of Sabin-specific MABs, which have been shown to effectively neutralize Sabin viruses in ITD assays [22, 31]. Among the cVDPV strains detected in the present study, there was complete concordance between the presence of the RCT marker and the lack of neutralization by Sabin-specific MABs.

Phylogenetic analysis of VP1 sequences from cVDPV strains isolated from environmental samples and from cases of AFP in Hispaniola shows that they are highly genetically related in this region of the poliovirus genome. In addition, VP1 sequences from strains isolated from clinical cases and environmental samples obtained in different geographic areas (Port-au-Prince in Haiti and Santo Domingo and Santiago in the Dominican Republic) form distinct subclusters. Further sequence analysis of the nonstructural genes of 1 environmental strain from Haiti confirmed that this strain, like the clinical strains, consisted of a recombinant genome with noncapsid sequences obtained from other species C enteroviruses [2] and had the highest sequence similarity with a clinical strain from that region.

The environmental cVDPV isolates were as neurovirulent as poliovirus strains isolated from cases of AFP from the outbreak in Hispaniola when tested in PVR-Bx transgenic mice expressing the human receptor for poliovirus [31]. Although cVDPV isolated from environmental samples have been shown previously to be neurovirulent in a mouse model [32], our finding confirms an association with paralytic cases in humans. One of the type 1 cVDPV strains was isolated from a stream-water sample from Haiti that was obtained 4 months before genetically similar viruses were found in cases of AFP.

In summary, the present study has documented the presence of cVDPV with >1% sequence difference from Sabin vaccine strain polioviruses in environmental samples obtained from regions with reported AFP or paralytic cases caused by similar, if not identical, cVDPV strains. These findings demonstrate that EPS is a sensitive method for detecting cVDPV in a background of OPV. The finding that cVDPV can, under suitable conditions, acquire neurovirulence and transmission characteristics similar to those of *wt* polioviruses has important implications for current and future strategies of the WHO initiative to eradicate polio worldwide. Because of concerns that

inadequate vaccine coverage and the potential circulation of poliovirus may occur in other locations worldwide, greater efforts are being made to use EPS for active surveillance of polioviruses in high-risk settings. Recently, EPS was applied to the isolation and identification of polioviruses in high-risk areas in Ecuador (J.V., unpublished data).

EPS has the potential to detect polioviruses in the environment, even before paralytic cases occur, which may lead to decisions to increase surveillance of AFP, to look for gaps in immunity, to identify risk groups, and, ultimately, to immunize children, thereby possibly preventing cases and outbreaks that would otherwise be inevitable. Although EPS never can replace surveillance of AFP, now that the endgame of polio eradication has begun, EPS might become a critical component when the exact conditions of Hispaniola are repeated in many countries that are not likely to switch to inactivated polio vaccine. In this scenario, it is likely that some children will be excreting OPV into an environment where there are suddenly many children who are not immunized. Therefore, environmental surveillance may, as a complement to surveillance of AFP, become an important tool for the early detection of both *wt* poliovirus and cVDPV in the final stages of the poliovirus eradication program.

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## References

- Centers for Disease Control and Prevention. Outbreak of poliomyelitis—Dominican Republic and Haiti, 2000. *MMWR Morb Mortal Wkly Rep* **2000**; *49*:1094, 1103.
- Kew O, Morris-Glasgow V, Landaverde M, et al. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. *Science* **2002**; *296*:356–9.
- Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol* **1999**; *37*:1288–93.
- Centers for Disease Control and Prevention. Acute flaccid paralysis associated with circulating vaccine-derived poliovirus—Philippines. *MMWR Morb Mortal Wkly Rep* **2001**; *50*:874.
- Centers for Disease Control and Prevention. Circulation of a type 2 vaccine-derived poliovirus—Egypt, 1982–1993. *MMWR Morb Mortal Wkly Rep* **2001**; *50*:41–2.
- Rousset D, Rakoto-Andrianarivelo M, Razafindratsimandresy R, et al. Recombinant vaccine-derived poliovirus in Madagascar. *Emerg Infect Dis* **2003**; *9*:885–7.
- Hull BP, Dowdle WR. Poliovirus surveillance: building the global Polio Laboratory Network. *J Infect Dis* **1997**; *175*(Suppl 1):S113–6.
- Abraham R, Minor P, Dunn G, Modlin JF, Ogra PL. Shedding of virulent poliovirus revertants during immunization with oral poliovirus vaccine after prior immunization with inactivated polio vaccine. *J Infect Dis* **1993**; *168*:1105–9.

9. Wood DJ, Macadam AJ. Laboratory tests for live attenuated poliovirus vaccines. *Biologicals* **1997**; 25:3–15.
10. Hovi T, Cantell K, Huovilainen A, et al. Outbreak of paralytic poliomyelitis in Finland: widespread circulation of antigenically altered poliovirus type 3 in a vaccinated population. *Lancet* **1986**; 1:1427–32.
11. Manor Y, Handsher R, Halmut T, et al. Detection of poliovirus circulation by environmental surveillance in the absence of clinical cases in Israel and the Palestinian Authority. *J Clin Microbiol* **1999**; 37:1670–5.
12. Hovi T, Stenvik M, Partanen H, Kangas A. Poliovirus surveillance by examining sewage specimens: quantitative recovery of virus after introduction into sewerage at remote upstream location. *Epidemiol Infect* **2001**; 127:101–6.
13. Pöyry T, Stenvik M, Hovi T. Viruses in sewage waters during and after a poliomyelitis outbreak and subsequent nationwide oral poliovirus vaccination campaign in Finland. *Appl Environ Microbiol* **1988**; 54:371–4.
14. Shulman L, Manor Y, Handsher R, et al. Molecular and antigenic characterization of a highly evolved derivative of the type 2 oral poliovaccine strain isolated from sewage in Israel. *J Clin Microbiol* **2000**; 38:3729–34.
15. Tambini G, Andrus JK, Marques E, et al. Direct detection of wild poliovirus circulation by stool surveys of healthy children and analysis of community wastewater. *J Infect Dis* **1993**; 168:1510–4.
16. van der Avoort HG, Reimerink JH, Ras A, Mulders MN, van Loon AM. Isolation of epidemic poliovirus from sewage during the 1992–3 type 3 outbreak in The Netherlands. *Epidemiol Infect* **1995**; 114:481–91.
17. Shieh YS, Wait D, Tai L, Sobsey MD. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by the polymerase chain reaction. *J Virol Methods* **1995**; 54:51–66.
18. Mendelsohn CD, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequencing, and expression of a new member of the immunoglobulin superfamily. *Cell* **1989**; 56:855–65.
19. Pipkin PA, Wood DJ, Racaniello VR, Minor PD. Characterisation of L cells expressing the human poliovirus receptor for the specific detection of polioviruses in vitro. *J Virol Methods* **1993**; 41:333–40.
20. Nakano JH, Milford MH, Thieme ML, Nottay B. Parameters for differentiating vaccine derived and wild poliovirus strains. In: Melnick JL, ed. *Progress in medical virology*. New York: S. Karger, **1978**:178–206.
21. Kapsenberg JG. Picornaviridae: the enteroviruses (polioviruses, Coxsackieviruses, ECHOviruses). In: Barlows A, Hausler WJ, Lenette EH, eds. *Laboratory diagnosis of infectious diseases, principles and practise*. Vol. 2. New York: Springer Verlag, **1988**:692–722.
22. van der Avoort HGAM, Hull BP, Hovi T, et al. Comparative study to five methods for intratypic differentiation of polioviruses. *J Clin Microbiol* **1995**; 33:2562–6.
23. World Health Organization (WHO). *Manual for the virological investigation of poliomyelitis [WHO/EP/GEN/97.1]*. Geneva: WHO, **1997**.
24. Kilpatrick DR, Nottay B, Yang CF, et al. Group-specific identification of polioviruses by PCR using primers containing mixed-base or deoxyinosine residue at positions of codon degeneracy. *J Clin Microbiol* **1996**; 34:2990–6.
25. Kilpatrick D, Nottay B, Yang C, et al. Serotype-specific identification of polioviruses by PCR using primers at positions of codon degeneracy. *J Clin Microbiol* **1998**; 36:352–7.
26. Yang C, De L, Holloway B, Pallansch M, Kew O. Detection and identification of vaccine-related polioviruses by the polymerase chain reaction. *Virus Research* **1991**; 20:159–79.
27. Liu HM, Zheng DP, Zhang LB, Oberste MS, Pallansch MA, Kew O. Molecular evolution of a type 1 wild-vaccine poliovirus recombinant during widespread circulation in China. *J Virol* **2000**; 74:11153–6.
28. De Leon RD, Shieh Y-SC, Baric RS, Sobsey MD. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction. In: *Proceedings of the Water Quality Conference (San Diego)*. Vol. 18. Denver: American Water Works Association, **1990**:833–53.
29. Liu BL, Clarke IN, Caul EO, Lambden PR. Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. *Arch Virol* **1995**; 140:1345–56.
30. Van der Peer Y, De Wachter R. TREECON for windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **1994**; 10:569–70.
31. Martin J, Samoilovich E, Dunn G, et al. Isolation of an intertypic poliovirus capsid recombinant from a child with vaccine-associated paralytic poliomyelitis. *J Virol* **2002**; 76:10921–8.
32. Horie H, Yoshida H, Matsuura K, et al. Neurovirulence of type 1 polioviruses isolated from sewage in Japan. *Appl Environ Microbiol* **2002**; 68:138–42.
33. Shieh YS, Baric RS, Sobsey MD. Detection of low levels of enteric viruses in metropolitan and airplane sewage. *Appl Environ Microbiol* **1997**; 63:4401–7.
34. Francis T, Brown GC, Ainslie JD. Poliomyelitis in Hidalgo county, Texas, 1948: poliomyelitis and coxsackie viruses in privy specimens. *Am J Hyg* **1953**; 58:310–8.
35. Ramia S, Sattar SA. Second-step concentration of viruses in drinking and surface waters using polyethylene glycol hydroextraction. *Can J Microbiol* **1979**; 25:587–92.
36. Deshpande JM, Shetty SJ, Siddiqui ZA. Environmental surveillance system to track wild poliovirus transmission. *Appl Environ Microbiol* **2003**; 69:2919–27.
37. Schwab KJ, De Leon R, Sobsey MD. Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. *Appl Environ Microbiol* **1996**; 62:2086–94.
38. Tsai YL, Sobsey MD, Sangermano LR, Palmer CJ. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase–polymerase chain reaction. *Appl Environ Microbiol* **1993**; 59:3488–91.
39. Beller M, Ellis A, Lee SH, et al. Outbreak of viral gastroenteritis due to a contaminated well: international consequences. *JAMA* **1997**; 278:563–8.
40. Hurst CJ, Benton WH, McClellan KA. Thermal and water source effects upon the stability of enteroviruses in surface freshwaters. *Can J Microbiol* **1989**; 35:474–80.