The progress of the Global Polio Eradication Initiative is monitored by acute flaccid paralysis (AFP) surveillance supplemented with environmental surveillance in selected areas. To assess the sensitivity of environmental surveillance, stools from (re)vaccinated elderly persons with a low seroprevalence and from wastewater were concurrently collected and analyzed in the Netherlands over a prolonged period of time. A total number of 228 healthy individuals with different levels of immunity were challenged with monovalent oral polio vaccine serotype 1 or 3. Poliovirus concentrations were determined by the titration of fecal suspensions on poliovirus-sensitive L20B cells and of sewage concentrates by L20B monolayer plaque assay. Almost half of the individuals (45%) shed poliovirus on day 3 after challenge, which peaked (57%) on day 8 with an average poliovirus excretion of 1.3 × 10^5 TCID_{50} per g of feces and gradually decreased to less than 5% on day 42. The virus concentrations in sewage peaked on days 6 to 8 at approximately 100 PFU per liter, remained high until day 14, and subsequently decreased to less than 10 PFU per liter on day 29. The estimated poliovirus concentration in sewage approximated the measured initial virus excretion in feces, within 1 log_{10} variation, resulting in a sensitivity of detection of 100 infected but mostly asymptomatic individuals in tens of thousands of individuals. An additional second peak observed in sewage may indicate secondary transmission missed by enterovirus or AFP surveillance in patients. This enables the detection of circulating poliovirus by environmental surveillance, supporting its feasibility as an early warning system.

The worldwide eradication of poliomyelitis as resolved by the World Health Assembly in 1988 (41) is well in progress, although the final stage has proven to be challenging. In little more than 2 decades after the global eradication goal was set, the number of countries in which poliovirus is endemic has decreased from more than 125 to 4 (Afghanistan, India, Nigeria, and Pakistan), and the estimated number of polio cases has decreased from 350,000 to fewer than 1,351 in 2010 (9, 10, 22). Despite many efforts to eradicate (wild) poliovirus, it is still circulating in regions where many people are not vaccinated and live close together in poor hygiene and sanitation conditions (especially in regions hit by war or disaster), and it is causing occasional outbreaks. Especially in countries with large numbers of nonimmune people, or in developed countries that have elderly with waning immunity (11), these circulating polioviruses can represent a high risk. The Global Polio Eradication Initiative (GPEI) strategic plan specifically names environmental surveillance as a means to identify reservoir areas (10). In areas in which polio is endemic, this type of surveillance provides important additional surveillance data, whereas in polio-free regions it provides insights into the international spread of poliovirus.

Vaccination with either inactivated polio vaccine (IPV) or with oral attenuated live polio vaccine (OPV) will protect people against poliomyelitis. The use of these vaccines has eliminated poliomyelitis from most countries in the world. However, after vaccination, the vaccine viruses will still circulate in the population and in the environment. In rare cases, the attenuated polioviruses can regain their virulence after genetic changes by replication in the gut of the vaccinated individual, resulting in paralytic disease (vaccine-associated paralytic poliomyelitis). Therefore, vaccination programs with OPV can result in the circulation of vaccine-derived poliovirus (VDPV) strains in the environment; if these strains regain their neurovirulence, they might cause poliomyelitis cases. Several outbreaks of poliomyelitis caused by VDPVs have occurred when the conditions for the spread of VDPVs were favorable, including sufficiently susceptible individuals and unsanitary living conditions (8, 14, 19, 23, 32, 40). In unvaccinated populations, infection by wild-type poliovirus may be symptomatic in only 0.1 to 1% of the infected individuals or even less in partially immune groups (11, 16, 36). However, infected individuals, whether they are with or without symptoms, will shed high levels of poliovirus in their feces for several weeks, and these viruses will subsequently end up in the environment (3). In fact, in immune-deficient patients a prolonged poliovirus excretion is frequently seen, which can continue for years (20). The consequence is that these poliovirus strains can cause frequent new infections when susceptible individuals come in contact with these strains.

Vaccine strains, VDVPs, and even wild-type poliovirus strains may remain infectious for as long as 2 months in sewage depending upon inactivation by sunlight, high temperature, and other environmental factors (6, 31). High concentrations of circulating polioviruses in the environment may facilitate a more sensitive detection method via environmental surveillance than virus and/or disease surveillance in the human population. Using simulation models, it was shown that a small poliovirus outbreak may go unnoticed by AFP surveillance in a vaccinated population (33).
In contrast, these models hypothesize that environmental surveillance even at low sampling frequencies can detect 1 infected excreting individual in a population of 10,000. Indeed, the detection of wild-type poliovirus in the environment has been described in the absence of AFP cases (16, 27), but the sensitivity and specificity of environmental surveillance are difficult to assess in wild-type poliovirus outbreaks, since the onset of the epidemic and the number of asymptomatic infections are not known.

Surveillance programs will be important tools in evaluating the progress of poliovirus eradication and ultimately certification. Besides the WHO golden standard of acute flaccid paralysis (AFP) surveillance, environmental surveillance can contribute to monitoring the effectiveness of the polio vaccination strategies and to the detection of the remaining level of circulation of vaccine poliovirus strains and of VDPVs (4, 29, 34, 37, 44, 45). It can also help monitor the reemergence of wild-type polioviruses (12, 13, 15, 17, 26). The early detection of poliovirus circulation is critical for an effective response to outbreaks and to prevent the further spread of the virus and therefore possibly prevent new poliomyelitis cases.

In the Netherlands, a study was undertaken to determine the level of protection against poliovirus in the elderly population (1). A selection of seronegative elderly persons was challenged with monovalent OPV (either type 1 or 3) in a generally IPV-vaccinated population, which enabled us to perform a quantitative assessment of the sensitivity of environmental poliovirus surveillance based on the concentrations of polioviruses in stools and in sewage. In this study, we longitudinally determined the virus titer in the feces of the vaccinated individuals and the concentration of infectious poliovirus in the nearby sewerage. A quantitative model was fitted to the derived data to estimate the sensitivity of environmental poliovirus surveillance.

**Materials and Methods**

**Vaccine.** The monovalent vaccines Oral-Virelon T1 type 1 (OPV1) and type 3 (OPV3; Chiron Behring, Marburg, Germany) contained $5.8 \times 10^5$ and $1.0 \times 10^5$ 50% tissue culture infective doses (TCID$_{50}$), respectively. The vaccines were stored at 4°C until oral administration in 1.0-ml volumes.

**Study groups.** In Table 1, a summary of the study groups is shown as described extensively elsewhere (1, 5). Prechallenge serum-neutralizing antibody levels for poliovirus types 1, 2, and 3 and exclusion criteria were used to select elderly, healthy individuals (177 individuals born between 1925 and 1945, before the start of the IPV vaccination in the Netherlands [in 1957]) with different levels of immunity, as well as seronegative, naturally immune, and IPV-vaccinated persons (51 individuals born between 1946 and 1950) (1). Seronegative persons were challenged with a serotype of the polio vaccine, and seropositive persons were randomly assigned to a challenge with OPV1 or OPV3. A total of 228 participants were challenged orally with either monovalent OPV1 or OPV3, and they remained resident in the area during the study period. Participants signed an informed consent, and those with major medical problems and/or with a prior OPV vaccination history were not eligible for the study. The study proposal was approved by the Medical Ethical Committee of the Netherlands: Organization for Applied Scientific Research (TNO), Leiden, the Netherlands.

**Sampling and analysis of feces.** Stool samples were collected at 3, 7, 14, 21, 28, 35, 42, 49, and 56 days after challenge. Fecal suspensions were made according to the WHO protocol (41). A recombinant murine cell line permanently expressing the human polio virus receptor was used for virus detection in feces, and the presence/absence of cytopathogenic effects were read out (41). The L20B cells are susceptible to poliovirus but are nonpermissive to most other human enteric viruses (30).

**Sampling, concentration, and analysis of water.** Grab 10-liter samples of the sewage, taken primarily in the morning, were collected at a pumping engine station (population size, ~37,000). One 10-liter sewage sample was collected 7 days before the start of the vaccination to determine the baseline poliovirus circulation in the sewage. In the first 9 days, 10-liter sewage samples were collected daily, followed by sampling on days 11, 14, 16, 21, 28, 35, and 62.

Viruses in the water were concentrated by the use of a modified conventional filter adsorption-elution method (25) followed by ultrafiltration. Briefly, magnesium chloride was added to 10 liters of water to a final concentration of 0.05 M to enable the formation of a virus-magnesium complex. By reducing the pH to 3.8 ($\pm 0.2$) and adding hydrochloric acid to a final concentration of 0.5 M, these complexes adsorb to a negatively charged cartridge filter (1.2 µm nominal; Millipore, Etten-Leur, the Netherlands). Viruses were eluted from the filter with a 3% beef extract (Difco Laboratories, Detroit, MI) solution, pH 9.0, and the precipitate was dissolved and neutralized with a concentrated acetic acid buffer (pH 5.0) to a final pH of 7.2 ($\pm 0.2$). The resulting eluate, with a volume of approximately 650 ml, was further concentrated using an ultrafiltration method. This final concentrate, with an average volume of 20 ml, was stored at −70°C until the final concentrate was inoculated onto L20B cells.

Infectious viruses were detected by use of a monolayer plaque assay (25). L20B cells were grown to a confluent monolayer in 75-cm$^2$ plastic flasks. Before inoculation, culture medium was removed and the concentrated sample was added to the flasks. After a 2-h incubation period at room temperature to allow virus adsorption to the cells, the cells were overlaid with Medium 199 with Earl’s salts (Life Technologies, Breda, the Netherlands) with 10% fetal bovine serum (Life Technologies), 0.9% Bacto agar (Difco, Amsterdam, the Netherlands), 0.2% bicarbonate, 100 IU penicillin, and 100 µg/ml streptomycin (Life Technologies). After 9 days of incubation at 37°C in an inverted position, the cells were stained with 0.03% neutral red (Sigma-Aldrich, Zwijndrecht, the Netherlands) in

<table>
<thead>
<tr>
<th>Group</th>
<th>n$^\text{a}$</th>
<th>Immune status</th>
<th>mOPV$^b$ received</th>
<th>Avg days of poliovirus excretion</th>
<th>Individuals excreting poliovirus (%)</th>
<th>Avg poliovirus concn in positive feces samples$^c$ (95% interval)</th>
<th>Total poliovirus excretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>Seronegative</td>
<td>1</td>
<td>13.3</td>
<td>96.7</td>
<td>$2.3 \times 10^4$ (2.0 \times 10^4 to 1.9 \times 10^6)</td>
<td>29.7</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>IPV vaccinated</td>
<td>1</td>
<td>1.3</td>
<td>14.3</td>
<td>$9.5 \times 10^4$ (4.7 \times 10^4 to 2.8 \times 10^5)</td>
<td>0.0080</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>Naturally immune</td>
<td>1</td>
<td>2.4</td>
<td>26.1</td>
<td>$2.2 \times 10^4$ (1.6 \times 10^4 to 9.7 \times 10^5)</td>
<td>0.038</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>Seronegative</td>
<td>3</td>
<td>21.8</td>
<td>85.1</td>
<td>$2.8 \times 10^4$ (2.4 \times 10^4 to 2.1 \times 10^5)</td>
<td>67.4</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>IPV vaccinated</td>
<td>3</td>
<td>5.7</td>
<td>31.0</td>
<td>$1.3 \times 10^4$ (1.1 \times 10^4 to 8.7 \times 10^4)</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>Naturally immune</td>
<td>3</td>
<td>5.0</td>
<td>33.3</td>
<td>$5.3 \times 10^4$ (4.6 \times 10^3 to 4.4 \times 10^4)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

$^a$ Number of participants.

$^b$ mOPV, monovalent oral polio vaccine.

$^c$ Virus concentration per gram feces.
RESULTS

Table 1 summarizes the average poliovirus concentration in positive feces, the percentage of polioviruses excreted in the feces, and the average days of excretion of the challenged individuals in the different groups. Almost half of the individuals (45%) shed poliovirus on day 3 after challenge, which peaked with shedding by 57% of the individuals on day 7 with an average poliovirus excretion of $1.3 \times 10^9$ TCID$_{50}$ per g of feces, gradually decreasing to less than 5% at day 42. The data are shown linearly in Fig. 1 and 2, since these represent the mean virus counts.

In sewage sampled at the pumping engine station, no poliovirus was detected prior to challenge. The virus concentrations peaked on days 6 to 8 at approximately 100 PFU per liter of sewage, remained high until day 14, and subsequently decreased to less than 10 PFU/liter at day 29 (data not shown).

The total numbers of OPV shed by the vaccinated population were estimated assuming that individuals produce 100 to 500 g feces per day. The linear graphs in Fig. 1 and 2, representing the mean virus counts, appear very similar, although an additional peak can be distinguished in sewage at day 22 (and possibly at day 36) that is not apparent in feces.

Vaccination with OPV1 and OPV3 was carried out in 61 and 67 PV-seronegative individuals, respectively. PV1 shedding appeared to precede the shedding of PV3 in feces. OPV1 and OPV3 counts in feces peaked on day 8 (Fig. 1 and 2) with mean concentrations of $1.3 \times 10^9$ (5.9 $\times 10^8$ to $2.6 \times 10^9$) and $2.6 \times 10^9$ (1.2 $\times 10^9$ to $4.6 \times 10^9$) TCID$_{50}$ in feces, respectively. In sewage, the estimated mean OPV counts were also highest on day 8, at $1.2 \times 10^9$ (1.0 $\times 10^9$ to $1.4 \times 10^9$) PFU OPV1 and $1.4 \times 10^9$ (1.2 $\times 10^9$ to $1.7 \times 10^9$) PFU OPV3. The primary, secondary, and possibly tertiary peaks can be even more clearly observed after typing the polioviruses in sewage on days 8, 22, and 36.

To be able to determine the value of environmental surveillance as a useful tool in global poliovirus eradication, the virus counts in sewage should correspond to the level of virus-infected individuals in the population. The excretion of OPV in feces generally was found to be quite high in individuals after challenge. By far, the highest virus counts were detected in fecal suspensions of seronegative individuals (97%) (Table 1). In the first week after challenge, the highest virus counts were shed by individuals in all groups, declining only approximately 10-fold on the individual level in the subsequent weeks.

DISCUSSION

Environmental surveillance may contribute to monitoring the remaining level of circulation of vaccine poliovirus strains, VDPVs, and wild-type polioviruses in the world. Despite high immunization coverage and good-quality supplementary immunization activities, for poliomyelitis cases, environmental surveillance can aid AFP or other types of surveillance (7, 11, 12). Overall immuniza-
tion rates may conceal unvaccinated individuals, thereby sustaining the circulation of polioviruses in a region for several years (7), as is the case in the Netherlands due to religious beliefs (in the so-called Bible Belt) (11). This region remains an area of attention with respect to the large susceptible population and therefore the higher chance of individuals contracting poliomyelitis, and they also are more likely to spread polioviruses. The vaccination coverage rate in these areas varies from less than 25% up to 85% and higher than the national vaccination coverage of at least 95% in the Netherlands. Vulnerable groups within a population such as vaccinated-but-immunodeficient individuals may contribute to poliovirus circulation because of prolonged shedding (20, 21, 28). Silent transmission may be missed by types of surveillance other than environmental surveillance, such as AFP or enterovirus diagnostics. Recombination with a strain different from those used in the vaccines may lead to poliomyelitis cases after a prolonged poliovirus-free status (7, 16). In rare cases, the circulation of vaccine-derived polioviruses results in strains with reverted neurovirulence and antigenicity, which may be picked up in sewage samples before the first case of poliomyelitis occurs (12, 18, 43, 45). When estimating the extent of VDPV outbreaks, the numbers of infected individuals appears to highly underestimate the spread of these viruses based on the number of reported clinical cases. The highly multidisciplinary modeling approaches described by Wringe et al. can provide significant aid in designing surveillance strategies to prevent or respond to future VDPV outbreaks (42). Undervaccinated populations are at continued risk for poliovirus spread, because they become susceptible to infection by VDPVs (2) and also wild poliovirus.

The detection and investigation of at least one case of non-polio AFP per 100,000 children aged less than 15 years has proven to be the key indicator of whether a surveillance system would be able to detect poliomyelitis (41). Our study showed that environmental PV surveillance is a very sensitive tool. In the case of high vaccination coverage of 95% with 1% naturally immune and 4% seronegative individuals, assuming that 100 persons are infected with an average shedding of 200 g feces per day, our data indicate that with a population size of 37,000 and an average sewage volume per day of 2.18E7 liters, approximately 25 polioviruses could be detected in 1 liter of sewage. Due to the ability to detect these numbers of polioviruses in sewage, this scenario would lead to an informative environmental poliovirus surveillance system. In a second plausible scenario (in a Bible Belt community) with a vaccination coverage of 60%, 8% naturallyimmune individuals, and 32% seronegative individuals, when 100 individuals are infected, approximately 750 polioviruses could be detected in 1 liter of sewage. These scenarios show that even at a very high vaccination coverage, environmental poliovirus surveillance is able to detect poliovirus-infected individuals, either symptomatic or asymptomatic, in a population of several tens of thousands of uninfected individuals if sampling is done regularly in strategic locations. Theoretically, based on our data, it would even be possible to detect polioviruses shed by, for instance, one individual, if he or she were seronegative. However, these scenarios were based on the data derived from this specific smaller-sized study population. In the case of a larger study population and/or a wider study area, the sensitivity of environmental surveillance may drop below the detection limit due to excessive dilution. When a high dilution effect is suspected, a possible solution is to sample multiple sites in the sewerage to monitor smaller population sizes per sample but with coverage of the whole targeted population. Critical factors in environmental surveillance include, for instance, sample location, sample frequency, and sample volume. Furthermore, results (especially negative results) should be considered carefully in light of these factors, since these results only indicate the presence or absence of polioviruses at a given moment in time. As described by Ranta et al. (33), simulation models may aid in the optimization of the performance of the environmental poliovirus surveillance.

We observed one virus peak in feces and two (possibly three) peaks in sewage for poliovirus type 1, 3, or both. The secondary peak in sewage on day 22 may indicate secondary transmission in the exposed population, although rules of hygiene were given to prevent the household spread of OPV. This again shows the usefulness of environmental surveillance in addition to other types of poliovirus surveillance.

In our study, it was estimated that the numbers of polioviruses in sewage approximated the measured initial virus excretion in feces within 1 log10 variation, taking into account the number of inhabitants who discharge on the sewerage and the average water flow at the sewage pumping engine station. One uncertainty concerned the daily feces production, and although this needs further attention, it was found that the estimated total numbers of virus particles excreted was insensitive to this variable, meaning that variation in the amount of feces produced by an individual per day (factor of 5 between 100 and 500 g) does not have such a great effect on the total numbers of virus compared to, for instance, the number of viruses produced per gram of feces (up to a 4 log10 difference). The issue of whether poliovirus levels indicate the level of poliovirus circulation in the population is multifactorial. At which phase of infection did an individual’s poliovirus enter the sewage? What was the specific immune status of the individual? What was the general immune status of the exposed individual? It is difficult to evaluate the sensitivity of environmental surveillance in the case of wild-type poliovirus outbreaks.

In the environmental surveillance study of Roivainen et al. (34), it was shown that VDPV strains which regained their neurovirulence are repeatedly detected in Finnish sewage. Nevertheless, no cases of suspected poliomyelitis have occurred in Finland since 1985. These VDPVs are probably shed for a long period of time by one or more chronically infected individuals in the population, and when the susceptible individuals are exposed to these poliovirus strains, transmission may increase. Nevertheless, because of the high rate of asymptomatic infections for poliovirus, the authors of that study, as well as those of the current study, emphasize the importance of maintaining high vaccination coverage, and we also highlight the importance of environmental surveillance. Environmental surveillance also can retrospectively identify the circulating polioviruses in the community. Although often no clinical cases are seen in areas where these altered viruses are seen (4, 16, 27, 44, 45), a high level of awareness should be retained, because when susceptible individuals are exposed to these viruses, secondary transmission could take place and subsequently infection could occur.

Savolainen-Kopra and Blomqvist (35) review the different studies in which it is shown that, among other things, recombination and the accumulation of point mutations are a recurrent phenomenon in the poliovirus life cycle. One of the many conclusions is that recombination, and especially the accumulation of point mutations, is a key factor in gaining increased fitness for attenuated poliovirus strains. Although the circulation of wild-
type polioviruses seems to have been enormously reduced, the circulation of the many different VDPVs highlights the emergence of a possible threat to global polio eradication, causing the occurrence of poliomyelitis cases in long-term disease-free states.

Vaccination with IPV or OPV protects people against poliomyelitis but not against poliovirus infection or shedding. The screening of waste waters has been recognized as a useful tool for monitoring the circulation of wild-type poliovirus in populations (12, 13, 15–17, 26, 27). We have established that in our elderly study population, the variation in the number of virus particles shed by the individual was highly dependent on the presence or absence of a memory response (1). Both the levels of virus shed and the number of individuals shedding was highest in the seronegative group. Therefore, the number of poliovirus-infected individuals may be estimated from the virus concentration in the sewage by the extrapolation of the vaccination coverage and the mean levels of virus shed by naïve and immune individuals. To this end, models need to be developed which take both the vaccination status and the age of the individuals into consideration.

Besides assisting in global polio eradication, environmental surveillance may also aid in the detection of the circulation of other emerging viruses, such as severe acute respiratory syndrome surveillance may also aid in the detection of the circulation of negative group. Therefore, the number of poliovirus-infected individuals and the number of individuals shedding was highest in the seronegative group. Therefore, the number of poliovirus-infected individuals may be estimated from the virus concentration in the sewage by the extrapolation of the vaccination coverage and the mean levels of virus shed by naïve and immune individuals. To this end, models need to be developed which take both the vaccination status and the age of the individuals into consideration.

Besides assisting in global polio eradication, environmental surveillance may also aid in the detection of the circulation of other emerging viruses, such as severe acute respiratory syndrome (SARS) coronaviruses. Half of the first cases of this serious emerging infectious disease excreted virus in their feces (24), pointing to the usefulness of sewage monitoring not only for enteric but also primarily respiratory pathogens. Our estimation of the sensitivity of environmental surveillance for poliovirus may give indications for the circulation of other human-pathogenic viruses. Policy decisions may be assessed through environmental surveillance for their efficacy in preventing the spread of pathogenic microorganisms and therefore diseases, especially for those serious diseases for which no preventive or curative measures can be taken.

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