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Isolation of epidemic poliovirus from sewage during the 1992–3 type 3 outbreak in the Netherlands

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SUMMARY

To examine the extent of wild poliovirus circulation during the 1992–3 epidemic in the Netherlands caused by poliovirus type 3, 269 samples from sewage pipelines at 120 locations were examined for the presence of poliovirus. The epidemic virus strain was found in 23 samples, all from locations inside the risk area which contained communities that refuse vaccination for religious reasons. By sewage investigation, the wildtype virus was shown to be present in the early phase of the epidemic at two locations, one week before patients were reported from that area. The wild type 3 poliovirus was also detected retrospectively in a river water sample collected for other reasons three weeks before notification of the first poliomyelitis case, at a site a few kilometres upstream the home village of this patient. Oral poliovirus vaccine (OPV) virus was found at 28 locations inside or at the border of the risk area. Trivalent OPV was offered to unvaccinated or incompletely-vaccinated persons living in this region as part of the measures to control the epidemic.

INTRODUCTION

The method of choice for the virological confirmation of poliovirus infection is isolation and characterization of the virus from faecal specimens of a suspected case of poliomyelitis [1]. Faecal excretion of poliovirus by persons infected with wildtype poliovirus may last for up to 4 months [2] and stool samples may contain more than 10^6 viral particles/g of faeces [3]. Poliovirus can survive in the environment for several months [4], especially under cold or ambient temperatures in a moist environment, or when absorbed to solid material [5, 6]. More than 95% of the infections in non-vaccinated persons do not result in severe clinical symptoms. Consequently monitoring of the environment by examination of sewage for the presence of wildtype poliovirus provides an elegant way to document the extent of a poliomyelitis outbreak [7, 8] and may even demonstrate presence of virus in the community before cases of poliomyelitis are notified [9, 10].

Virological examination of sewage has also been used to document the effect of vaccination campaigns [11], because persons who are vaccinated with the live, attenuated oral poliovirus vaccine (OPV) also shed faecal virus into the environment for a considerable time [2]. Environmental surveillance will also be

Fig. 1. Map of the Netherlands, indicating the region with communities at risk, which refuse vaccination for religious reasons, and that was affected by poliomyelitis epidemics (shaded) and local outbreaks during 1961–71 after the start of the Dutch Poliomyelitis Prevention Programme (○).

essential to document the absence of wild poliovirus circulation for a prolonged period, as required in the certification phase of the WHO campaign for the global eradication of poliomyelitis and its cause, the wild poliovirus, by the year 2000 [12].

Inactivated poliovirus vaccine (IPV) has been used in the Dutch poliomyelitis prevention programme since the early 1960s [13–15]. The number of cases of poliomyelitis dropped dramatically almost immediately after the implementation of the programme. A vaccination coverage of more than 97% has been reached [15]. However, several municipalities, all situated in a narrow belt that stretches over the country from the southwest to the northeast (Fig. 1), have a vaccination coverage of $< 90\%$ because of the presence there of communities which refuse vaccination for religious reasons. These communities of unvaccinated persons were affected by local poliomyelitis outbreaks confined to single villages from 1961–71 (Fig. 1) and by a large epidemic in 1978 caused by poliovirus type 1 with 110 cases throughout the whole risk area [15].

An outbreak of 71 cases caused by poliovirus type 3 occurred between

September 1992 and February 1993. As in previous outbreaks, no patients were vaccinated, all but one for religious reasons [16–18]. The present investigation was designed to determine the extent of wild poliovirus circulation amongst the unvaccinated communities in the early phase of the 1992–3 epidemic, by means of virological analysis of sewage samples. In the later phase of the epidemic, a similar strategy was used to determine whether the epidemic virus had also spread amongst the IPV-vaccinated population. If this was the case, there would be a risk for unvaccinated persons living throughout the country outside the risk area, who were presumed to be protected by herd immunity.

MATERIALS AND METHODS

Collection and treatment of sewage samples

Samples of sewage water (0·5 l) were taken at various locations, from residential quarters in cities and villages. The sampling sites were chosen so that the risk of industrial pollution toxic for cell culture was as small as possible. Samples were taken on rainfree days to prevent a dilution effect. Samples were collected in sterile dark flasks, stored at 4 °C and transported to the laboratory within 24 h. Upon arrival at the laboratory, samples were treated immediately by thorough shaking for 30 min with chloroform (10% final concentration). This denatures lipid-containing biological material, removes some of the chemical substances that are toxic for cell cultures, and increases the number of infective poliovirus particles detectable by dissociating clumps of viral particles [19]. After centrifugation for 30 min at 1500 g, the clarified water phase was collected and stored at 4 °C. Clarified sewage samples were concentrated 200–300 fold by filtration through Diaflo® PM 10 ultrafiltration membranes (10000 MW cut off) in an ultrafiltration device (Amicon Division, Beverly, MA).

Isolation and characterization of viruses

Concentrated sewage samples were inoculated onto confluent monolayers of Hep-2 Cincinnati cells which were observed for the appearance of cytopathological effect (CPE) for up to 10 days. To exclude cytotoxic effects a second passage was made from cultures with rapidly-developing CPE. Viruses from cultures showing CPE were typed with poliovirus- and enterovirus-specific antisera. Polioviruses were characterized as wild or vaccine-derived viruses by an ELISA using type-specific cross-absorbed antisera [20–22]. In addition, virus-positive samples were tested with PCR-based assays, using primer sets specific for enteroviruses [23] or for Sabin viruses [24]. A primer pair (NET-1: 5′-TACATCAAAGGTGCGAATTC-3′ and NET-2: 5′-CGCCAAACCATCCTTGTA-3′) was developed, which reacted specifically with the epidemic type 3 strain in a PCR test format similar to that used for the Sabin virus PCR test [24]. The sequence of the 115 base pair-long amplificate is co-linear with nucleotides 3232–3346 of the poliovirus Sabin 3 strain Leon [25]. All enteroviruses that could not be typed in neutralization assays were analysed with this PCR assay to confirm the absence of the epidemic virus. The presence of adenoviruses in some of the virus-positive cultures was confirmed by an ELISA that used a set of monoclonal antibodies specific either for adenoviruses or for the adenovirus types 40 or 41 [26].

Table 1. *Virus isolation from sewage water during 1992–3 poliomyelitis epidemic in the Netherlands*

	Week number	Week after start of epidemic	Number of specimens	Polioviruses*				Other viruses†									
				PV1 SL	PV2 SL	PV3 SL	PV3 NSL	Enteroviruses E6	E7	E11	E30	CB1	CB4	CB5	NT	Adeno-virus	Nega-tive
Before	32	−5	1	—	—	—	—	—	—	—	—	—	—	—	1	—	—
epidemic	34	−3	1	—	—	—	1	—	—	—	—	—	1	1	1		
Early	38	1	3	—	—	—	2	—	—	—	—	—	1	—	—	—	1
phase	39	2	64	1	4	—	4	1	—	12	2	—	1	1	2	—	36
epidemic	41	4	23	—	6	1	8	—	—	1	1	—	—	—	—	—	8
Late	50	13	16	—	6	1	1	—	—	1	—	—	—	—	3	—	7
phase	51	14	14	—	3	—	2	1	1	2	—	—	1	—	—	—	6
epidemic	52	15	16	3	1	—	1	—	—	—	—	—	—	—	1	—	11
	1	17	14	—	1	—	1	—	—	5	—	—	—	—	1	—	7
	2	18	16	—	—	—	—	—	—	2	—	—	—	—	—	—	14
	3	19	13	—	1	—	—	—	—	—	1	—	—	—	—	1	10
	4	20	16	—	1	—	—	—	—	1	—	—	—	—	—	—	14
	5	21	14	—	—	—	—	—	—	2	1	—	—	—	—	1	10
	6	22	16	—		—	—	—	—	1	—	—	—	—	2	2	11
	7	23	14	—	1	—	2	—	—	2	—	—	—	—	—	2	8
	8	24	16		—	—	1	—	—	2	—	1	—	—	—	—	12
	9	25	14	—	—	—	—	—	—	2	—	—	—	—	—	1	11
	Total		271	4	24	2	23	2	1	33	5	1	4	2	11	7	166

* PV, poliovirus; SL, Sabin like; NSL, non-Sabin like.
† E, ECHO; CB, Coxsackie B; NT, not typable.

Treatment and analysis of river water samples

River water samples of (180 l) were processed by filtration and organic flocculation to a volume of 3–5 ml as described previously [27]. The concentrates were inoculated onto BGM-cells, which were then overlaid with agar. Plaques with enterovirus-characteristic morphology were selected. Plaque-purified viruses were typed as described above.

RESULTS

Investigations in the vicinity of the first case

The first poliomyelitis patient in the 1992–3 epidemic was a boy, age 14, from the village of Streefkerk (Fig. 1), which is situated on the River Lek, and has a large community that refuses vaccination for religious reasons. Immediately after virological confirmation of the clinical diagnosis of poliomyelitis, the extent of virus circulation in the area was determined by examination of sewage samples that were collected in Streefkerk and also in Rotterdam, close to the secondary school that was attended by the patient. The two sewage samples taken in Streefkerk contained the epidemic virus. One of them also contained Coxsackie B4 virus. No virus was isolated from the sample collected in Rotterdam.

Two river water samples (each 180 l), collected as part of a pilot study on the microbiological control of recreational surface water were also available. The samples had been taken 5 and 3 weeks before notification of the first patient, at a site a few kilometres upstream from his home village. The oldest sample contained a non-polio enterovirus, as determined by neutralization assays and PCR tests. The sample collected 3 weeks before the polio outbreak started, contained the epidemic wildtype polio 3 virus, as well as Coxsackie B4 virus, Coxsackie B5 virus and a non-typable enterovirus.

Investigations at the start of the epidemic

In the second week of the epidemic (8–9 days after notification of the first polio patient) sewage water samples were taken at 58 sites in the south-western part of the area with communities at risk. Two samples from locations close to Streefkerk were found to be positive for wild poliovirus type 3, while five samples collected in the extreme west of the sampling area, contained one of the vaccine viruses which reflected the start of OPV vaccination a few days earlier. In 19 samples a variety of non-polio enteroviruses was detected, 12 of which contained ECHO 11 viruses (Table 1, Fig. 2).

On the same days, six sewage samples were collected from the north-eastern part of the risk area, in cities and villages that had polio cases during earlier outbreaks in the 1960s and 1970s. At the time of collection of the samples no cases of poliomyelitis had been reported yet from this area. Two of these six samples contained the epidemic poliovirus type 3 (Fig. 2), indicating that virus circulation at that time was not confined to the region around the first case and that cases of poliomyelitis could be expected from the whole risk area. Cases of poliomyelitis were notified from this area 1 week later (week 3). Further samples were collected from locations in this north-eastern part of the risk area during the following week (Fig. 3). Seven out of 22 samples contained the wild type 3 poliovirus, as did a

Fig. 2. Regions in which sewage samples were collected during week 2 of the 1992–3 poliomyelitis epidemic (shaded). Locations where wild type 3 poliovirus (●), or Sabin vaccine virus (○) was found.

sample taken from the region in the vicinity of the first case (Table 1). Seven samples contained Sabin vaccine virus, mainly type 2.

Investigations of sewage samples outside the risk area

Almost all sewage samples analysed in the first month of the epidemic were collected at locations from within the area with communities at risk. To determine whether or not the circulation of the epidemic virus was restricted to this region, sewage samples were taken in the second half of the epidemic every 2 weeks for 3 months (i.e. weeks 13–25) at 30 locations from all over the Netherlands. Ten sampling sites were chosen outside the risk area, ten at its border and ten inside the risk area (Fig. 4). No wildtype or vaccine poliovirus could be detected in any of the samples from outside the risk area. In 8 samples from 4 different locations inside the risk area, epidemic wildtype 3 poliovirus was found, while 18 samples from 12 locations contained one of the Sabin vaccine viruses, predominantly type 2 (Table 1). Six of these samples were collected at the border of the risk area. Non-polio enteroviruses and adenoviruses were found in 33 and 7 samples, respectively, with no preference for any of the three groups of locations. The relatively high

Fig. 3. Regions in which sewage samples were collected during week 4 of the 1992/3 poliomyelitis epidemic (shaded). Locations where wild type 3 poliovirus (●), or Sabin vaccine virus (○) was found.

number of wildtype poliovirus-positive samples at several locations coincides with the number of polio patients from these regions at the time the samples were taken. The last sample, that still contained the wildtype polio 3 virus was collected in the last week (week 24) of the epidemic.

Isolation of non-polio viruses from sewage during the 1992–3 polio epidemic

Non-polio enteroviruses or adenoviruses were isolated from 66 of the 271 water samples collected during the epidemic (Table 1). Thirty-three of these isolates (50%) were typed as ECHO 11 virus. Eleven of the enteroviruses could not be typed with the set of antisera available in the laboratory. All these samples reacted positive in a enterovirus-specific polymerase chain reaction test [23], but negative in PCR tests using primer sets that are specific for the epidemic polio 3 virus or the three Sabin poliovirus types [24]. Seven samples collected at five different locations contained adenovirus. These viruses caused a rapidly growing adenovirus-typical CPE on HEp-2 cells and were confirmed as adenoviruses in an adenovirus-specific ELISA. The viruses did not react with adenovirus type 40- or 41-specific monoclonal antibodies in a similar ELISA [26].

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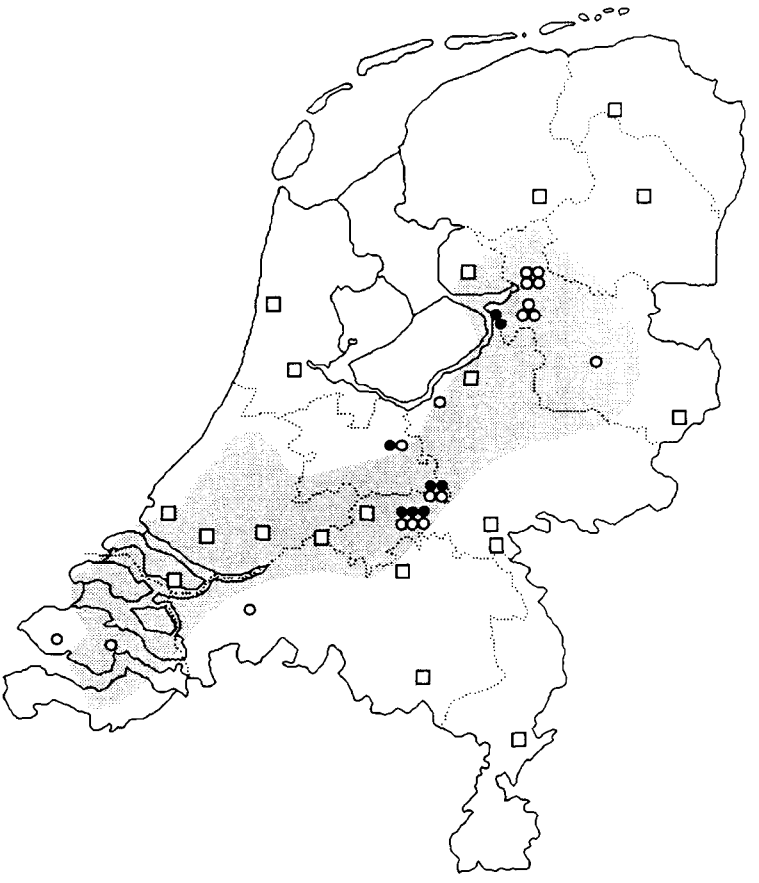

Fig. 4. The 30 locations where sewage samples were collected every 2 weeks during weeks 13–25 of the epidemic, showing locations where epidemic type 3 poliovirus was found (●), locations where one or more of the Sabin vaccine viruses was found (○), locations at which no poliovirus was found (□), and area at risk (shaded).

DISCUSSION

The present investigation describes the isolation of wild poliovirus type 3 in sewage samples collected during the 1992–3 poliomyelitis outbreak in the Netherlands. Information on the extent of the outbreak in its early phase, e.g. confinement to a single village as in the 1960s and early 1970s or extensive circulation as in 1978, would be of importance for measures to control the outbreak. Collection of a considerable number of samples of a manageable volume was initiated before the start of the OPV vaccination campaign to enable more rapid virological analysis. The presence of attenuated vaccine viruses in sewage samples would considerably complicate characterization of wild poliovirus from sewage samples. In particular, intratypic differentiation of polioviruses cannot be performed unequivocally on mixtures of poliovirus serotypes, or on mixtures of wild and vaccine strains from one serotype [21].

The choice of the methods used in the present investigation, i.e. ultrafiltration for concentration of samples and subsequent detection of polioviruses by cell

culture and neutralization and PCR assays, was influenced by the rapid and unexpected appearance of the epidemic after 14 years without cases of endemic poliomyelitis. We were able to detect wild poliovirus in relatively small volumes of sewage, although the method used was rather laborious: only 15–20 samples could be analysed in 1 week; the limiting step in the procedure being the concentration step, which took 12–72 h per sample. Application of PCR-based methods for the detection of polioviruses or other enteroviruses directly on concentrated samples was unsuccessful, most likely due to the presence of interfering substances.

Twenty-three samples were found to contain the epidemic type 3 virus, all from locations inside the risk area. In addition, 30 Sabin vaccine viruses were isolated. The majority (80%) of these were Sabin type 2 viruses, as might be expected from other studies [11, 27]. OPV polioviruses were isolated only from samples collected inside or at the border of the risk area. OPV virus was only administered to unvaccinated or not completely vaccinated persons living inside the risk area. Outside this region, IPV was given to unvaccinated individuals. The use of HEp-2 cells for the detection of polioviruses resulted in the isolation of non-polio enteroviruses of a limited number of serotypes. In future studies, use of the recently described and evaluated mouse L-cells expressing the poliovirus receptor will allow selective isolation of polioviruses from environmental samples [28, 29]. Three findings in the present investigation illustrate the potential of this type of work:

1. Wildtype polio 3 virus was found retrospectively a river water sample, collected 3 weeks before the first patient was notified, at a site a few kilometres upstream the village where the first patient lived. Hence, routine screening of sewage samples may herald outbreaks and thereby alert public health services to initiate preventive measures.

2. Wildtype polio 3 virus was detected prospectively in sewage samples, collected in the second week of the epidemic in villages, located 75–100 km from the village where the first patient lived, and where at that moment no patients had been found. This indicated the spread of the epidemic virus over the whole risk area. Thus, environmental sampling can be used to determine the extent of the virus circulation before occurrence of actual cases of poliomyelitis.

3. Wildtype polio 3 virus was not detected in any sample taken outside the risk area which contained people that refused vaccination for religious reasons. This was in agreement with other observations that also provided no evidence for circulation of the epidemic virus outside the region at risk. None of the virological laboratories in the Netherlands reported incidental isolation of the epidemic virus from any of 2775 faecal samples collected during the first 3 months of the epidemic from patients with clinical symptoms not characteristic for poliovirus infection and excluding patients belonging to the known risk groups. The only poliovirus reported in this survey was a vaccine poliovirus type 3 isolate from a stool specimen of a child recently adopted from Brazil [17]. Also, a population survey in which more than 3000 faecal samples were collected and analysed for the presence of the wild poliovirus, detected only eight positive samples, all from healthy children living inside the risk area, seven of whom had not been vaccinated for religious reasons [17].

The limited spread of the epidemic virus during the 1992–3 outbreak in the Netherlands contrasts with the data from poliovirus type 3 outbreaks in Finland [7] and Oman [30]. Although in both countries a high vaccination coverage was reached with IPV or OPV, respectively, widespread circulation of the epidemic virus among the whole population was observed. For the Finnish epidemic, it was suggested that this nation-wide spread was the result of a combination of low poliovirus type 3 protective antibody titres in the vaccinated population with the appearance of an antigenically aberrant virus [7]. In the Netherlands, the limited spread of the epidemic virus during the 1992–3 outbreak, confined to the regions with unvaccinated communities, supports the poliomyelitis prevention strategy, which uses only inactivated poliovirus vaccine (IPV) as part of the national vaccination programme. Apparently the routine vaccination programme, six doses of IPV before the age of 10, induces sufficient immunity in vaccinees to restrict widespread circulation of the wild poliovirus. The small percentage of unvaccinated individuals who live dispersed throughout the country is therefore adequately protected by herd immunity. As mandatory vaccination in the Netherlands is still not considered a viable option to protect the unvaccinated community against poliomyelitis, the only way to achieve this goal is global eradication of the causal agents, the wildtype polioviruses, as planned by the World Health Organization by the year 2000.

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