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# Molecular Characterization of a Wild Poliovirus Type 3 Epidemic in The Netherlands (1992 and 1993)

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**An outbreak of poliomyelitis due to wild poliovirus type 3 (PV3) occurred in an unvaccinated community in The Netherlands between September 1992 and February 1993. The outbreak involved 71 patients. The aim of this study was to characterize the virus at the molecular level and to analyze the molecular evolution of the epidemic virus. Molecular analysis was carried out by sequencing the VP1/2A junction region (150 nucleotides) of 50 PV3 strains isolated in association with this outbreak and the entire VP1 gene of 14 strains. In addition, the sequence of the VP1/2A junction region of strains from geographical regions endemic for PV3 (Egypt, India, and Central Asia) was analyzed and compared with the nucleotide sequence of the epidemic strain from The Netherlands. The earliest isolate was obtained from river water sampled 3 weeks before diagnosis of the first poliomyelitis patient and was found by VP1/2A sequence analysis to be genetically identical to the strain isolated from the first patient. Sequence divergence among the strains from the epidemic in The Netherlands was less than 2%. The closest genetic similarity (97.3%) was found with an Indian isolate (New Delhi, December 1991), indicating the likely source of the virus. A more than 99% sequence similarity was found in the VP1/2A region. Finally, the sequence information was used to design primers for the specific and highly sensitive molecular detection of PV3 strains during the epidemic.**

Since 1988, the World Health Organization has been committed to the global eradication of poliomyelitis before the turn of this century (21). To realize this goal, two criteria have to be met: (i) no new cases of poliomyelitis due to wild poliovirus infection, and (ii) no more circulation of wild-type poliovirus in humans and in the environment. Improvement of vaccination coverage and surveillance of cases of acute flaccid paralysis, aided by virological laboratory analysis, are crucial elements of the eradication program. Since 1988, a substantial increase in vaccination coverage and a concomitant decrease in cases have been obtained (22). Nevertheless, epidemics continue to occur, even in countries with high vaccination coverage, such as The Netherlands. One of the reasons for this is the existence of communities with orthodox religious beliefs which refuse vaccination. As a result, members of these communities are at a continuous risk for the introduction of wild poliovirus. Epidemics in The Netherlands occurred throughout the 1960s; in 1971; in 1978, with 110 reported cases due to poliovirus type 1 (17); and again in the autumn of 1992, with 71 cases caused by wild poliovirus type 3 (14). None of the patients in these epidemics had been vaccinated.

Although conventional virological techniques are adequate to characterize a clinical isolate as vaccine derived or wild type, they are not suitable for establishing relationships among strains or to pinpoint the source of a virus. For these purposes, genomic analysis of a 150-nucleotide fragment encompassing the VP1/2A junction region has been used (15). Because poliovirus genomes have been reported to evolve rapidly upon passage through humans (1 to 2% nucleotide changes per year [13]), transmission pathways can be inferred from the patterns of nucleotide variation among strains. Genetic similarity of 98% or more is considered to imply a direct epidemiological link. Genotypes of poliovirus have empirically been defined as groups of strains that show at least 85% sequence similarity in the VP1/2A junction region (15). Molecular epidemiological findings are also used to study the progress of the eradication initiative and to identify reservoirs sustaining virus transmission (6).

In addition to sequence analysis of the VP1/2A junction region, analysis of larger regions of the genome, e.g., the entire 900-nucleotide major capsid protein gene VP1, may give additional information on the molecular evolution of the virus during an epidemic, e.g., clonal spread of the virus versus multiple lineages and antigenic drift (4), and on transmission mechanisms within an epidemic (6). Also, specific primers and probes for rapid molecular detection and characterization of the epidemic strain in clinical and environmental samples can be designed, which in turn provides an easy tool to monitor silent transmission of the epidemic virus.

In this report, we describe the molecular characterization of poliovirus strains isolated during the poliovirus type 3 epidemic in The Netherlands from 1992 to 1993. The possible source of importation and the intraepidemic sequence variation within the VP1/2A junction region and for the entire VP1 gene are presented. Moreover, results with primers designed to

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TABLE 1. Primers used in this study

| Primer | Sequence[a] | Position[b] | Fragment (length in bp)[c] |
|---|---|---|---|
| M3s (B) | GTC AAT GAT CAC AA (C/T) CC[d] | 3206–3222 | a (290) |
| 2A | AAG AGG TCT CTA TTC CAC AT[e] | 3476–3495 | |
| M1 | tgt aaa acg acg gcc agt TTT GTG TCA GCG TGT AAT GA[f] | 2399–2418 | b (678) |
| M23 | cag gaa aca gct atg acc TGC CAI GTG TA (A/G) TC (A/G) TCC | 2980–2997 | |
| M9 | tgt aaa acg acg gcc agt TTC AC (C/A) TAI TCI AGN TTT GA | 2843–2862 | b′ (643) |
| M6 | cag gaa aca gct atg acc AAG AGG TCT CTA TTC CAC AT[e] | 3476–3495 | |
| NET1 | CGC CAA ACC ATC CTT GTA | 3232–3250 | c (115) |
| NET2 | TAC ATC AAA GGT GCG AAT C | 3329–3347 | |

[a] The M-13 sequence is given in lowercase.
[b] The numbering is according to reference 18.
[c] The fragments are as described in the legend to Fig. 1.
[d] As published in reference 10.
[e] Published previously without the M-13 tail (15).
[f] Published previously without the M-13 tail (1).

specifically detect and characterize the epidemic virus are shown.

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## MATERIALS AND METHODS

**Background on the epidemic.** After 14 years without endemic cases of poliomyelitis, an outbreak of wild poliovirus type 3 infections occurred in The Netherlands between September 1992, and February 1993 (14). A total of 71 cases of poliomyelitis were reported to the national public health authorities. As in previous outbreaks in 1971 and 1978, all cases, except one, occurred among members of a closely knit, orthodox religious denomination which rejects vaccination (14, 17). Likewise, the epidemic in 1992 and 1993 was confined to a distinct geographical area stretching from the southwest to the northeast of the country, where communities with a vaccination coverage rate well below 90% exist (14). Cases were virologically confirmed by wild-type poliovirus type 3 isolation from feces or throat swabs (90% of cases) and/or by detection of poliovirus type 3-specific immunoglobulin M antibodies by an enzyme immunoassay (91% of cases [12]).

**Viruses.** Fecal samples and throat swabs as well as concentrates of environmental specimens were examined by standard procedures for virus isolation, titration, and typing (14). Intratypic differentiation was carried out by an enzyme-linked immunosorbent assay that uses cross-absorbed rabbit antisera specific for either vaccine-derived or wild-type polioviruses to establish their wild-type character (20). A total of 50 poliovirus isolates were characterized in the VP1/2A junction region; 48 were from cases that were reported throughout the epidemic and 2 were from environmental samples of river water obtained 3 weeks prior to diagnosis of the index patient (EnvA/NET92) and from sewage (EnvB/NET92) obtained 4 days after diagnosis of the index patient (19). Fourteen isolates from the cases had their entire VP1 gene sequenced. For validation of the reverse transcriptase-PCR (RT-PCR), we used strains that had been sent to the National Institute of Public Health and the Environment between 1977 and 1994 for virological characterization and intratypic differentiation.

**Analysis of the VP1/2A junction region.** Analysis of the VP1/2A junction region was carried out as described previously (10). Alternatively, a slightly modified primer, M3sB (5′-GTC AAT GAT CAC AAT CC-3′), was used instead of M3s when amplification efficiency was found to be low. Sequence analysis across the recognition sequence of primer M3s in poliovirus type 3 strains from The Netherlands revealed a mismatch at the third position from the 3′ end of this primer.

**Analysis of the VP1-gene. (i) RNA isolation.** For cDNA synthesis, RNA was purified from 100 μl of cell culture supernatant as described previously (23).

**(ii) Primers.** For sequence analysis of the VP1 gene, the region was amplified in two fragments with sizes of 678 bp (M1 and M23) and 634 bp (M9 and M6), with a 143-bp overlap (Table 1 and Fig. 1; fragments b and b′, respectively). Primers M1 and M6 have been published previously without the M13 tails as UG1 (1) and 2A (15), and they were used as primers for the amplification of all poliovirus genomes. Inner primers M9 and M23 were selected from regions in the VP1 gene that are conserved in the prototype reference strains (Table 1 and Fig. 1). To overcome possible primer mismatches due to the expected high level of sequence variation within this gene, either deoxyinosine or multiple nucleotides were incorporated at certain positions (third base wobble).

**(iii) RT-PCR.** Reverse transcription was carried out for the VP1/2A junction region as described previously (10), except that the $MgCl_2$ concentration was lowered to 1.5 mM. The PCR mixture was subjected to 40 cycles of denaturation at 94°C for 45 s, annealing at 42°C for 60 s, and extension at 60°C for 90 s. Analysis and purification of PCR products were carried out as described previously (10).

**(iv) Sequence analysis of PCR products.** PCR products were sequenced by dideoxy chain termination cycle sequencing with fluorescein-labeled M13 primers, and the sequencing was carried out according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, Calif.). Sequences of the VP1/2A junction region and the entire VP1 gene were edited and aligned with SEQED (version 1.0.3; Applied Biosystems Inc.) and converted to the GENEWORKS format (Intelligenetics, Mountain View, Calif.). Genetic relationships were estimated on the basis of the 150-nucleotide VP1/2A junction region with phylogenetic trees generated by the UPGMA (unweighted pair group with arithmetic mean) method (11).

**RT-PCR for the detection of poliovirus type 3 from The Netherlands.** (NET-PCR). Primers for the PCR were selected from regions at the 3′ end of the VP1 gene (Fig. 1 [fragment c] and Table 1) that were sufficiently different from those of representative strains of the different genotypes. Forward primer NET1 is located within the immunodominant site 3a (9). Reverse primer NET2 is located at the most outer 3′ end of VP1. A single-tube RT-PCR was started by adding 1 μl of freeze-thawed, clarified virus supernatant to a premixture containing all the necessary reagents except the enzymes and incubating the mixture for 30 min at 65°C and for 5 min at 95°C in order to disrupt the virus particles and release the RNA. After the mixture was chilled on ice, the enzymes were added and reverse transcription directly followed by the PCR was carried out in 20 μl with the following constituents: 0.5 μM (each) primers NET1 and NET2, 1.0 mM (each) deoxynucleoside triphosphates, 50 mM Tris HCl (pH 8.8), 50 mM NaCl, 6 mM $MgCl_2$, 0.1 μg of bovine serum albumin (Boehringer Mannheim Biochemicals) per μl, 2.0 mM dithiothreitol (Merck, Darmstadt, Germany), 50 mU of avian myeloblastosis virus RT per μl, 64 mU of RNase inhibitor per μl, and 28 mU of *AmpliTaq* DNA polymerase per μl. The cycling program was 1 cycle at 42°C for 30 min and 1 cycle at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. The 115-bp PCR product was detected on an ethidium bromide-stained, 12% polyacrylamide gel (16). In order to evaluate the specificity and sensitivity of the PCR, we used a well-characterized panel containing 30 recent isolates

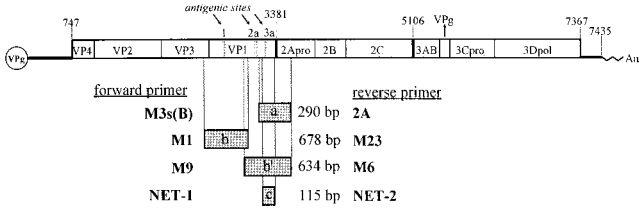


FIG. 1. Locations of the regions in the poliovirus genome that were amplified as described in Materials and Methods. Fragment a is a 290-bp fragment encompassing the 150-nucleotide VP1/2A junction region, fragments b and b′ are overlapping PCR products spanning the entire 900-nucleotide VP1 gene, and fragment c is a 115-bp region that is located at the 3′ end of VP1 and that was amplified by the PCR specific for the virus involved in the epidemic in The Netherlands.

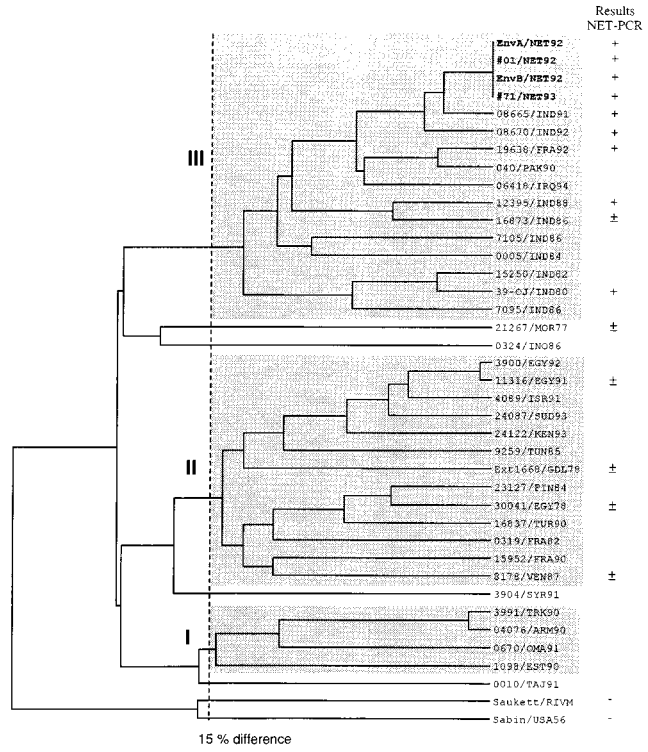


FIG. 2. Dendrogram based on the 150-nucleotide sequence of the VP1/2A junction region in the poliovirus type 3 genome (positions 3289 to 3438 [18]). Included are 4 strains isolated during the epidemic in The Netherlands during 1992 and 1993 (bold), 33 wild poliovirus type 3 strains isolated between 1977 and 1993 in different parts of the world, and the prototype strains used in the oral and inactivated poliovirus vaccines (Sabin/USA56 and Saukett/RIVM). The dotted line indicates 15% nucleotide variation and signifies a genotype (15), with a genotype being defined as a cluster of sequences of different strains with a sequence diversity of less than 15%. The three genotypes (I, II, and III) are shaded. The reactivity of the strains tested by the PCR (NET-PCR) are also given (+, positive; ±, weak positivity; −, negative). (Country abbreviations: ARM = Armenia, EGY = Egypt, EST = Estonia, FRA = France, FIN = Finland, GDL = Guadeloupe, IND = India, INO = Indonesia, IRQ = Iraq, ISR = Israel, KEN = Kenya, MOR = Morocco, NET = Netherlands, OMA = Oman, PAK = Pakistan, SUD = Sudan, SYR = Syria, TAJ = Tadjikistan, TRK = Turkmenistan, TUN = Tunisia, TUR = Turkey, USA = United States of America, VEN = Venezuela.)

from each poliovirus serotype from all over the world and the prototype strains used in vaccine production. In addition, we used 21 other enterovirus isolates.

## RESULTS

**Reproducibility of PCR sequence analysis.** The reproducibility of the cycle-sequencing method was tested by comparing repeated sequencings of the VP1/2A junction region of independently obtained RT-PCR products at one virus passage level (five sequences) and at different passage levels (three sequences). The index case isolate was sequenced eight times at three different passages and at three different time points over a period of 18 months by two different individuals. All sequences were identical. Fourteen other strains isolated during the epidemic were sequenced twice from independently obtained amplicons. Only one strain showed a 1-nucleotide difference in the entire 253-bp interval between primers M3s(B) and 2A. The overlap between the VP1/2A junction region and the VP1 gene was also used to compare sequences obtained by different methods (dye-terminator and dye-primer protocols). A comparison of the sequences of the 154-bp overlapping region located at the 3′ end of VP1 for both fragments

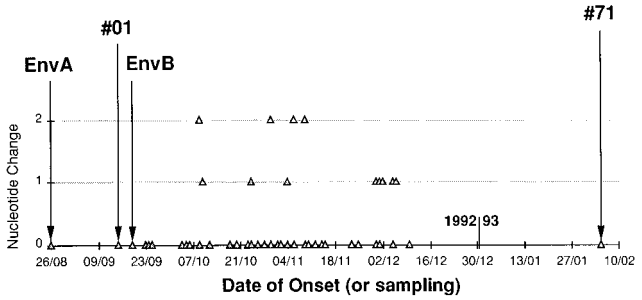


FIG. 3. Nucleotide sequence variation in the VP1/2A junction region for a total of 50 strains, as judged on the basis of the sequence of the first case isolate (#01). The number of nucleotide changes is shown; the horizontal axis shows the dates of the onset of illness for the case isolates and the dates of isolation for the other strains. Two environmental isolates (EnvA and EnvB [19]) have been included.

revealed some discrepancies, with an average difference of 1.76 nucleotides (1.1%; range, 0 to 4; standard deviation = 1.48; $n$ = 18).

**Source of the epidemic virus.** We compared the sequence of the 150-nucleotide VP1/2A junction region in the genome of the index case isolate as well as those of 3 other strains related to the outbreak (the last case isolate, #71/NET93, and 2 environmental isolates, EnvA and EnvB [19]) with those of 33 wild poliovirus type 3 isolates and the 2 prototype vaccine strains (Sabin/USA56 and Saukett/RIVM). In the dendrogram, three distinct branches in addition to that of the vaccine strains can be seen (Fig. 2). Each branch represents one genotype (a more than 15% sequence difference [15]). The first main genotype (genotype I) is represented by four strains that were isolated in the Central Asian republics. Genotype II, represented by 13 isolates, is found mainly in the Mediterranean region and also includes the poliovirus type 3 strain from the Finland 1984 epidemic as well as 2 strains isolated in the Americas (8178/VEN87 and Ext1668/GDL78). The third main genotype (III) is represented by 18 strains and contains 6 isolates from the outbreak in The Netherlands. The closest similarity (146 of 150 nucleotides identical, 97.3%) to the VP1/2A sequence of the index case (#01/NET92) was found with a strain isolated in India in November 1991 (08665/IND91). Seven older strains from India (39-OJ/IND80, 15250/IND82, 0005/IND84, 16837/IND86, 7095/IND86, 7105/IND86, and 12395/IND88) also cluster in this genotype, illustrating that this genotype had been circulating in India in an endemic fashion at least since 1980. Another strain belonging to this genotype was isolated in France in 1992 (19638/FRA92). The VP1/2A nucleotide sequences of three other isolates taken during the epidemic in The Netherlands and shown in this dendrogram were identical to that of the index case isolate.

**Intraepidemic sequence variation within the VP1/2A junction.** To determine the molecular evolution of the RNA genome during the outbreak, the VP1/2A sequences of 48 strains from patients and 2 environmental isolates were compared with the sequence of the index case (Fig. 3). The maximum sequence variation was 2 nucleotides (<1.5%). Thirty-five strains isolated during all stages of the epidemic were identical to the index strain; nine strains had one nucleotide substitution in the VP1/2A junction and four strains had two nucleotide changes. Of the 17 nucleotide substitutions, 2 were transversions and 15 were transitions; 9 substitutions resulted in synonymous codon changes and 7 resulted in a different amino acid codon (three alanine-to-valine changes at position 275 in the VP1 gene, two serine-to-proline changes at position 293 in

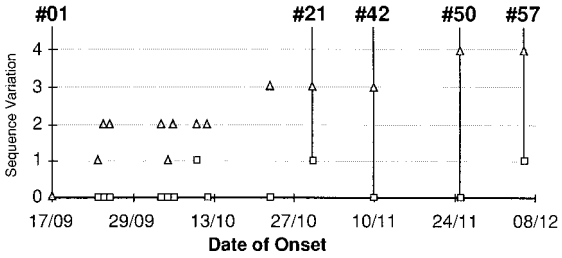


FIG. 4. Nucleotide (△) and amino acid (□) sequence variation for the entire 900 nucleotides of the VP1 gene of 13 strains isolated from patients (#21, #42, #50, and #57 as well as nine others) at different time points during the epidemic, as judged on the basis of the sequence of the first case isolate (#01). The vertical axis gives the amount of variation in nucleotides or amino acids; the horizontal axis gives the date of the onset of disease.

the VP1 gene, one tyrosine-to-phenylalanine change at position 10 in the 2A gene, and one threonine-to-alanine change at position 11 in the 2A gene). None of the amino acid codon changes were located in the antigenic site 3a (9).

**Molecular evolution in the VP1 gene.** The complete VP1 gene of 14 strains was sequenced and compared with that of the index case strain in order to further analyze the intraepidemic genomic variation; the strains had been isolated throughout the course of the epidemic (Fig. 4). A maximum sequence diversity of 4 nucleotides was found (<0.4%), resulting in a maximum amino acid sequence difference of 1 codon, located outside the antigenic sites.

**NET-PCR.** All strains isolated during the outbreak and tested by this assay gave the expected 115-bp product. Serotype 1 and 2 strains were all negative by this PCR assay, as were 21 nonpoliovirus enteroviruses (echovirus 24 gave a distinct but smaller fragment). Of the 33 type 3 panel strains, 8 were positive, although 3 strains (30041/78EGY, 11316/EGY91, and Ext1668/GDL78) were amplified only very poorly (data not shown). Sequence analysis of these eight strains revealed a high degree of similarity between the primer recognition sequences of these strains and that of the strain from the outbreak in The Netherlands (Fig. 5). In addition, three other strains not included in the panel (08665/IND91, 08570/IND92, and 19638/FRA92), were also positive by this assay. Six of the total 11 NET-PCR-positive poliovirus type 3 strains cluster in the same genotype (III); 5 of these strains were isolated between 1980 and 1992 in India (08665/IND91, 08670/IND92, 12395/IND88, 16873/IND86, and 39OJ/IND80) and 1 was isolated in France in the same year that the outbreak started (19638/FRA92). Four strains belong to genotype II (Fig. 5): 11316/EGY91, 8178/VEN87, 30041/EGY78, and Ext1668/GDL78. The remaining PCR-positive strain, strain 21267/MOR77, does not cluster in any of the genotypes.

The sensitivity of the NET-PCR for strains isolated during the epidemic in The Netherlands was compared with that for unrelated PCR-positive strains. Virus titers were determined for all strains, and serial 10-fold dilutions were tested by the PCR assay. The sensitivity of this test was defined as the amount of input virus that gave a visible PCR product on an ethidium bromide-stained agarose gel. Epidemic strain 16260/01/NET92 could be detected to a dilution of 10 50% tissue culture infective doses per RT-PCR reaction. The sensitivity of the PCR for the detection of strains 16873/IND86, 11316/EGY91, 8178/VEN87, 30041/EGY78, and 21267/MOR77 with two mismatches close to the 3′ end of primer NET1 (Fig. 5) was at least 1,000-fold less (data not shown).

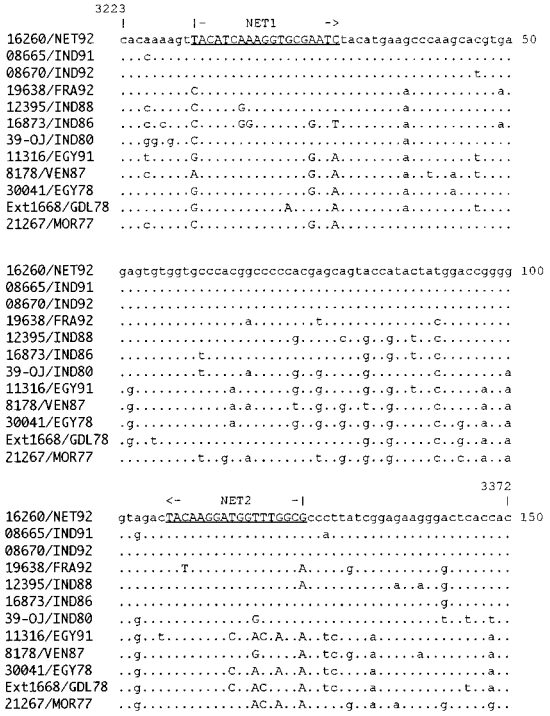


FIG. 5. Alignment of a 150-nucleotide region at the 3′ end of VP1 between positions 3223 and 3372 (18) and encompassing the recognition sequences (underlined, with the carets indicating direction) of the primers (NET1 and NET2) used in the NET-PCR with strains 08655/IND91, 08670/IND92, 19638/FRA92, 12395/IND88, 16873/IND86, 39-OJ/IND80, 11316/EGY91, 8178/VEN87, 30041/EGY78, Ext1668/GDL78, and 21267/MOR77. The reference sequence (top) is from the poliovirus type 3 isolated from the index case in the epidemic in The Netherlands (16260/01/NET92); the other sequences are from nonepidemic strains that gave a product by the NET-PCR.

## DISCUSSION

In this report, we describe the molecular analysis of poliovirus type 3 isolates from the epidemic in The Netherlands in 1992 and 1993.

Sequence analysis of the VP1/2A junction region of the first poliovirus type 3 strain (16260/01/NET92) involved in the outbreak in The Netherlands revealed a similarity of 97.3% with that of a poliovirus type 3 strain isolated in Bombay, India, in November 1991 (08665/IND91), indicating that the epidemic virus was imported from this region. Since poliovirus is endemic to India, it is probable that genetically even more related strains were circulating but not sampled. High sequence diversity among poliovirus type 1 strains in such regions of endemicity as India and also Egypt, Pakistan, and the Caucasian republics underscores this possibility (5, 8, 10).

Within the epidemic, sequence variation among isolates was minimal: 48 strains isolated from patients during this epidemic, 2 environmental isolates taken either 3 weeks before or during the outbreak, and 2 isolates from contacts in southern Alberta, Canada (2), showed a sequence variation in the VP1/2A junction region of less than 1.5%. This indicates the clonal spread of one variant during the epidemic, which was further confirmed by sequence analysis of the entire VP1 region of 14 strains. As poliovirus had not been endemic to The Netherlands during the 14 years preceding the epidemic in 1992 and 1993, the spread of one imported poliovirus variant in a susceptible, nonvaccinated, and closely knit population is not surprising.

To determine whether the observed sequence variation was real or reflected misincorporation during amplification, several strains were analyzed more than once. By each method (VP1/2A sequencing and VP1 sequencing), sequences were found to be identical with only one exception, showing the reproducibility of the automated RT-PCR sequencing method for these regions. Surprisingly, however, when sequences generated by the two different methods (dye-terminator sequencing of the VP1/2A junction and dye-primer sequencing of the VP1 gene) were compared, the sequence variation in the overlapping 154-bp region was found to be significant. These findings show the need for cautious interpretation when sequences obtained by different methods are compared. It is unclear what actually caused this discrepancy. Previously, we showed that sequence analysis of the VP1/2A junction by automated cycle sequencing (both dye-primer and dye-terminator protocols) and conventional radioactive sequencing with avian myeloblastosis virus RT resulted in identical sequences (10).

The lack of sequence variation is somewhat surprising, since the poliovirus type 3 strain responsible for an outbreak in Finland during 1984 showed significant variation in and around the antigenic sites among isolates obtained from different patients (3, 4). This discrepancy may be explained by the differences between the populations in which these outbreaks occurred: in Finland, the outbreak occurred in a vaccinated community with insufficient immunity to the infecting strain, whereas the outbreak in The Netherlands involved people who had never been vaccinated and therefore had no preexisting immunity to the infecting strain. Therefore, the poliovirus type 3 strain causing the outbreak in Finland was subject to some degree of immune selection, whereas the strain in The Netherlands was not.

To develop a rapid and easy test to detect and characterize the strain responsible for the outbreak in The Netherlands, specific PCR primers were designed. Using these primers, we were able to amplify all epidemic strains (sensitivity, 100%). However, the specificity of the assay was lower for some genetically more distant strains. These strains were also amplified, though at a 1,000-fold lower level of efficiency. Subsequent sequence analysis of these strains revealed mismatches in the last four nucleotides at the 3′ end of primer NET1; the possibility of successful amplification despite such mismatches has been described previously (7). Despite these shortcomings, the NET-PCR was a helpful, reliable, easy, and fast tool to confirm classic serological and intratypic characterization (19) and to monitor silent transmission (2) during the epidemic.

Close virologic monitoring of endemic and epidemic strains is critical for the eradication program. Such molecular epidemiological methods as that described in this study can be used to establish routes of transmission and to find problem areas with multiple cocirculating virus lineages. These areas should be specifically targeted by the global poliovirus eradication campaign.

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