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Molecular Detection of an Importation of Type 3 Wild Poliovirus into Canada from The Netherlands in 1993

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During the fall and winter of 1992-1993 an outbreak of wild poliovirus type 3-associated poliomyelitis involving 71 patients occurred in The Netherlands. Almost all of the individuals involved in the outbreak belonged to an orthodox religious denomination that prohibits vaccination. A surveillance was initiated to determine if there had been an importation of this same strain of wild poliovirus into a southern Alberta community with a similar religious affiliation. Viral culture of stool samples from consenting individuals in the community resulted in viral isolates which typed as poliovirus type 3. Sequencing of amplicons generated from both the 5′ nontranslated region and the VP1/2A portion of the genomes from representative poliovirus isolates indicated a greater than 99% genetic similarity to the strain from The Netherlands. The results of this study show that the utilization of PCR-based diagnostics offers an important molecular tool for the concise and rapid surveillance of possible cases of wild poliovirus importation into communities with individuals at risk for infection.

The introduction of an inactivated poliovirus vaccine in 1955 and an oral poliovirus vaccine in the early 1960s appears to have resulted in the elimination of indigenous wild poliovirus native to North America (11, 21). Indeed, there have been no recorded cases of paralytic poliomyelitis resulting from wild poliovirus in the last 2 years in the entire Western Hemisphere (4). Although much progress has been made in the global eradication of wild polioviruses, it is believed that significant numbers of wild strains continue to circulate in parts of Asia and Africa (11). Thus, countries with highly effective immunization programs in place are vulnerable to cases of poliomyelitis by imported wild strains if there exist closed communities which remain unvaccinated. This was the case in 1978 when an epidemic of poliomyelitis occurred in certain communities in The Netherlands which had remained unvaccinated because of religious beliefs (1, 2). Contact between these Dutch religious groups and Canadian communities of similar religious affiliation led to the importation of the type 1 wild poliovirus into Canada (8, 9, 23). The importation of this viral strain into communities largely unvaccinated for poliovirus resulted in 11 confirmed cases of poliomyelitis in several provinces during the spring and summer of 1978. By 1979 this wild poliovirus strain had spread from Canadian communities to an Amish population in the United States, resulting in a further 10 paralytic cases in four states (19).

Between September 1992 and February 1993 an outbreak of poliomyelitis involving 71 patients occurred in The Netherlands (16). As in 1978, the outbreak involved communities within The Netherlands which belong to orthodox reformed churches and refuse vaccination on religious grounds. Because of presumed contact between an affiliated religious community in southern Alberta, a community that reported the only case of poliomyelitis in the province of Alberta in 1978 (23), and affected communities in The Netherlands, a poliovirus surveillance was initiated during the winter and spring of 1993 to look for evidence of poliovirus importation (3). This communication describes the isolation and identification of a wild poliovirus type 3 strain determined to have been imported from The Netherlands into this small rural community. This report details how wild poliovirus isolates were rapidly distinguished from vaccine-related strains and further identified as being the same strain of virus responsible for the poliomyelitis outbreak in The Netherlands in 1992 and 1993 by a combination of PCR amplification and direct amplicon sequencing (15).

MATERIALS AND METHODS

Viral isolation and typing. Stool specimens were collected from 45 individuals ranging in age from 5 to 39 years, with a mean age of 16, in six households in a small southern Alberta community during January and February of 1993. A questionnaire was administered to each participant in the study. Included on the questionnaire were questions regarding recent illnesses, past immunizations, occupation, school attended, source of residential water, and method of residential sewage disposal. Also included were questions regarding travel destinations since October 1992 and contact with visitors from The Netherlands, also since October 1992. Stool filtrates were prepared and inoculated onto conventional tube cultures of primary rhesus monkey kidney cells (Whittaker Bioproducts). Cell cultures were monitored for the appearance of cytopathic effects characteristic of enteroviruses. Viral isolates were passaged in rhabdomyosarcoma and buffalo green monkey kidney cells for use in serotyping. The serotyping was carried out by an agarose diffusion neutralization method involving the use of intersecting pools of antisera according to the Lim-Benyesh-Melnick schema for identification (13).

RNA isolation and RT-PCR. Extraction of viral RNA from the southern Alberta isolates and subsequent cDNA synthesis were performed as previously described (7). To a 20-μl cDNA reaction mixture was added 80 μl of a solution containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, and 0.1% Triton X-100), 50 pmol of each primer, and 1 U of *Taq* DNA polymerase (Perkin-Elmer, Rexdale, Ontario, Canada). The amplification was

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TABLE 1. Poliovirus type 3 isolates obtained from 21 members of four different families[a]

Isolate code	Poliovirus type 3 determination by:		
	5′ NTR amplification and sequencing	VP1 differential PCR	VP1/2A amplification and sequencing
Family 1			
3221	+		
3223	+	+	+
3225	+		
3226			
3418			
3419			
3420	+		
3421			
3422			
3742			
4854	+		
Family 2			
3562	+	+	+
3565			
3566	+		
3568	+		+
Family 3			
3743	+		
3744	+	+	+
3746			
3747			
3748			
Family 4			
4855	+		+

[a] PCR and/or amplicon characterization of particular isolates is indicated by the symbol +.

carried out in 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of primer annealing at 45°C, and 1 min of elongation at 72°C. For amplifications involving the NET primer set, the annealing temperatures were 58 and 50°C in separate experiments. At the end of the 35 cycles, the reaction mixture was left at 72°C for an additional 15 min to ensure that all DNA was double stranded. Amplifications were carried out in a Perkin-Elmer 9600 thermal cycler. Reverse transcription (RT)-PCR of wild poliovirus type 3 isolated from index cases of poliomyelitis during the 1992–1993 epidemic in The Netherlands was performed as previously described (14).

Primers. Primers for PCR and sequencing of the 5′ nontranslated region (NTR) were selected from highly conserved parts of the 5′ NTR of the enterovirus genome and are specific for genomic regions previously utilized for enteroviral amplification (5, 25). Primer EH, 5′-GGAAGCTTCAAGCACTTCTGTT TCCCCGG-3′, corresponds to nucleotide positions 164 to 184 of the Sabin 3 strain (22). This primer has been modified from primer 1, described by Zoll et al. (25), to include a restriction site for the *Hin*dIII restriction enzyme at the 5′ end. Antisense primer EB, 5′-GGGGATCCCACCGGATGGCCAATCCA-3′, is complementary to nucleotides 627 to 644 of the Sabin 3 sequence and has been modified from primer E1, described by Chapman et al. (5), to include a restriction site for *Bam*HI at its 5′ end. PCR amplification of the poliovirus genome upstream and downstream of the VP1/2A junction was carried out with the primers M3s, 5′-GTCAATGATCACAACCC-3′ (sense) (14), and Polio 2A, 5′-AGAGGTCTCTATTCCACAT-3′ (antisense) (18). The 290-nucleotide region targeted for amplification by the M3s and 2A primers is located in the VP1/2A region between nucleotide positions 3235 and 3527 (22). The M3s primer was designed to react broadly with circulating polioviruses and anneals between positions 3235 and 3251 of the 3′ end of the VP1 coding region. Primer 2A is complementary to nucleotides between positions 3508 and 3527 at the 5′ end of $2A^{pro}$.

The NET3 and NET4 primers were designed to specifically amplify the VP1/2A junction region of the type 3 poliovirus from the outbreak in The Netherlands. The design of these primers was based upon sequence alignments of isolates from the epidemic in The Netherlands with other known poliovirus type 3 strains, using the PCGene version 6.6 program. The NET3 primer, 5′-T ACAAGGATGGTTTGGCG-3′, is specific for nucleotides between positions 3358 and 3375 of VP1. The NET4 primer, 5′-CTCACTGCGTTCTGAAGG-3′, is complementary to nucleotides between positions 3483 and 3500 in the 5′ region of the genome encoding the 2A protease.

Sequencing of PCR products. PCR products generated from Canadian isolates were separated on agarose gels and purified by microcentrifugation through blotting paper as previously described (6). Direct double-stranded sequencing of amplicons was then carried out by the dideoxy chain termination method (20) with the sets of primers involved in the amplification procedures. Automated sequencing of amplicons generated from the RNA of isolates from the outbreak in The Netherlands (index cases) was performed as previously described by Mulders et al. (14).

RESULTS

Isolation and typing of enterovirus isolates. Stool specimens from 21 of the 45 individuals yielded enterovirus-like isolates in primary rhesus monkey kidney cell cultures following 3 days of incubation at 37°C. These 21 isolates came from residents in four of the six households (Table 1). Serotyping of the isolates with the Lim-Benyesh-Melnick antiserum pools established all 21 isolates as type 3 poliovirus. None of the 21 stool-positive individuals from the January survey had a previous history of immunization against poliovirus. Seventeen (71%) of these individuals had visited and/or had contact with individuals from The Netherlands.

5′ NTR sequence analysis of SA-3 isolates. To determine the genetic relationship of the different poliovirus isolates to each other and to the Sabin 3 vaccine strain, a set of enterovirus-specific PCR primers was used to amplify a portion of the 5′ NTR of at least one viral isolate from each family (Table 1). The resulting 500-bp amplicon was then directly sequenced, and more than 300 nucleotides from each amplicon were determined. All 11 southern Alberta (SA-3) isolates from which

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NET/SA-3      GCGTTGCGCTCAGCACTCTACCCC-GAGTGTAGCTTAGGCTGATGAGTCT
          280                 *     *          *   *
SABIN 3       GCGUUGCGCUCAGCACUCAACCCCGGAGUGUAGCUUGGGCCGAUGAGUCU

NET/SA-3      GGGCATTCCCCACTGGCGACAGTGGCCCAGGCTGCGTTGGCGGCCT--AC
          330   *  *                 * **      *         ***
SABIN 3       GGACAGUCCCCACUGGCGACAGUGGCUCCAGCUGCGCUGGCGGCCGCGAC

NET/SA-3      CCATAGCT-AACGCTATGGGACGCTAGTTGTGAACAAGGTGTGAAGAGCC
          380  ** *  **  *  * *                  *
SABIN 3       CUGUGGCCCAAAGCCACGGGACGCUAGUUGUGAACAGGGUGUGAAGAGCC

NET/SA-3      TATTGAGCTAC-TCAAGAGTCCTCCGGCCCCTGAATGCGGCTAATCTCAA
          430           * *     *                        ***
SABIN 3       UAUUGAGCUACAUGAAGAG-CCUCCGGCCCCUGAAUGCGGCUAAUUCUAA

NET/SA-3      CCACGGAGCAGGTGCCCTCAACCCAGGGGGTGGCCTGTCGTAACGCGCAA
          480    *       *** **      ** ***
SABIN 3       CCAUGGAGCAGGCAGCUGCAACCCAGCAGCCAGCCUGUCGUAACGCGCAA

                                                          1
NET/SA-3      GTCTGTGGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATCTTT
          530    *                                        **
SABIN 3       GUCCGUGGCGGAACCGACUACUUUGGGUGUCCGUGUUUCCUUUUAUUCUU

NET/SA-3      CATATGGCT
          580 * *
SABIN 3       GA-AUGGCU
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FIG. 1. 5′ NTR nucleotide sequence generated from SA-3 isolates (referred to in Table 1) and compared to the amplicon sequence of the strain from the outbreak in The Netherlands and the genomic sequence of the Sabin 3 strain. The numbering of nucleotides corresponds to that of Toyoda et al. (22). Asterisks indicate positions at which nucleotides differ between the strain from the outbreak in The Netherlands and the Sabin 3 strain. The number 1 refers to the single nucleotide position within amplicons from isolates that deviated from the strain from The Netherlands (isolates had a G instead of a T).

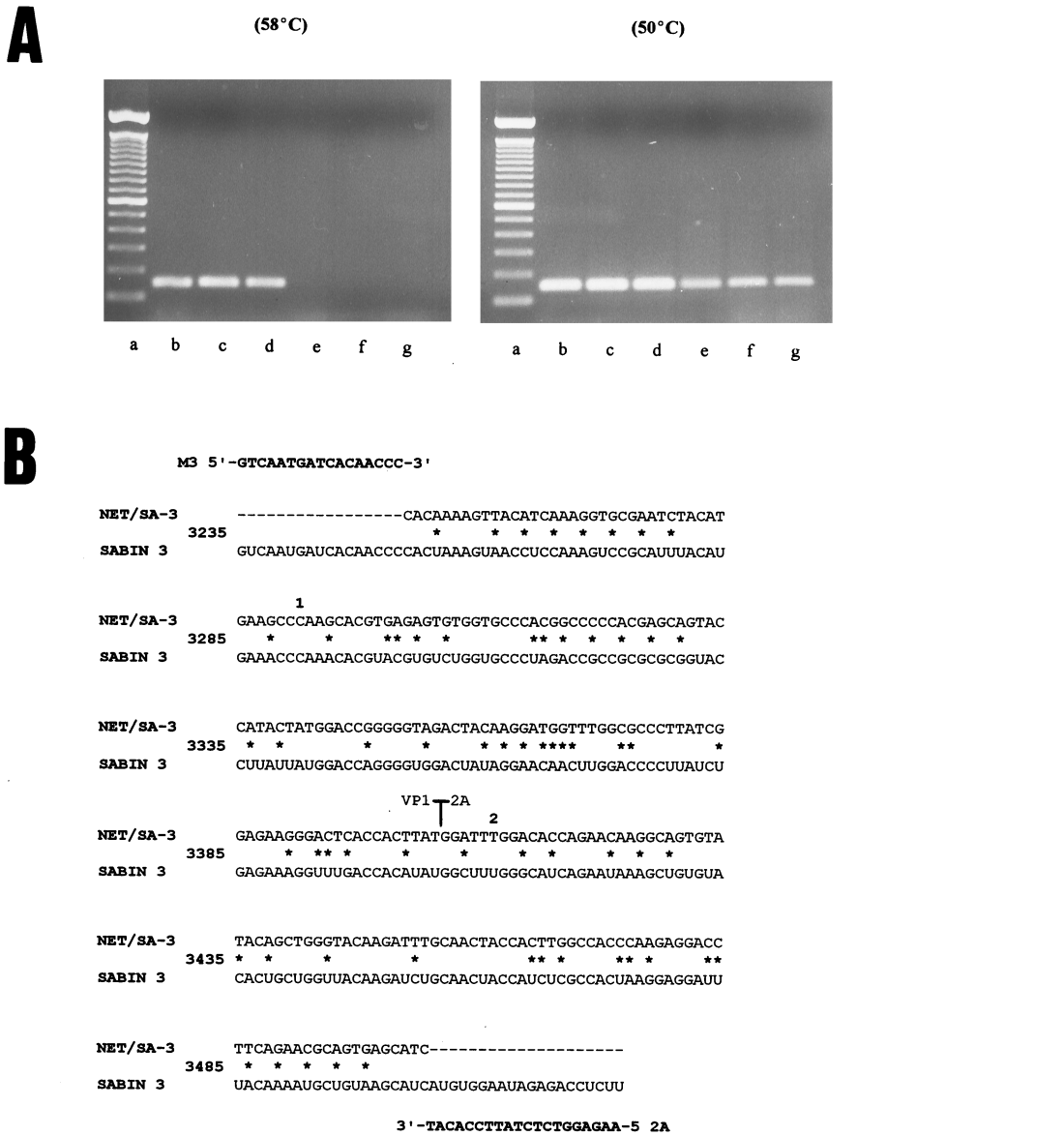

FIG. 2. (A) Specific amplification of the VP1 genomic region of SA-3 isolates by the NET3-NET4 primer set. Amplifications were carried out under annealing conditions of either 58 or 50°C. Lanes: a, 100-bp ladder (Gibco BRL, Burlington, Ontario, Canada); b, isolate 3223; c, isolate 3562; lane d, isolate 3744; e, Sabin 3 strain; f, Saukett 3 strain; g, Sabin 3 vaccine-related isolate. (B) Comparison of VP1/2A amplicon sequence generated from SA-3 isolates with the strain from the outbreak in The Netherlands and the genomic sequence of the Sabin 3 strain. The numbering of the first nucleotide in the sequence corresponds to that of Toyoda et al. (22). At the nucleotide position denoted by the number 1, isolate 3223 had a T instead of an A. The number 2 denotes a nucleotide position at which isolate 3562 differed from wild poliovirus consensus sequence by having a C instead of a T. Despite the difference in nucleotide composition, the amino acids encoded at these positions remain proline and phenylalanine, respectively.

5′ NTR amplicons were generated showed 100% sequence identity (Fig. 1). When compared to the 5′ NTR sequence of the Sabin 3 vaccine strain, a significant difference in sequence composition was found, with an observed sequence divergence of 14% between the isolates and the Sabin 3 virus (Fig. 1). The 5′ NTR sequence of the isolates was then compared to amplified 5′ NTR sequence that had been obtained for the poliovirus type 3 strain responsible for the 1992-1993 outbreak of poliomyelitis in The Netherlands. The sequence of the Canadian wild polioviruses matched that of the strain from The Netherlands by 99.7% (Fig. 1). The high degree of 5′ NTR sequence similarity between the SA-3 isolates and the outbreak strain provided preliminary evidence that an importation of wild poliovirus had occurred into the southern Alberta community.

Sequence similarity between VP1/2A regions of SA-3 isolates and the strain from the outbreak in The Netherlands. To provide further evidence that the Canadian isolates were of the same strain as that of the wild poliovirus responsible for poliomyelitis in The Netherlands, a differential PCR amplification was carried out. In this case, RNAs extracted from three of the Canadian isolates (Table 1) and from Sabin 3 and Saukett V3B strains (Connaught Laboratories, Toronto, Ontario, Canada) were subjected to RT-PCR with the NET3-NET4 primer set (Fig. 2A). This primer set is designed to specifically amplify the VP1/2A genomic region of polioviruses

closely related to the wild poliovirus type 3 strain from The Netherlands. As Fig. 2A shows, only SA-3 isolate genomic material was amplified when these primers were used under high-stringency conditions (58°C annealing temperature). Only upon decreasing the annealing temperature to 50°C were the NET primers able to amplify the VP1 region of the Sabin 3 and Saukett V3B strains.

A 290-bp amplicon was also produced from the VP1/2A region of representative SA-3 isolates with the M3s-2A primer set (Table 1). Upon sequencing these VP1/2A amplicons, it was found that the different SA-3 isolates exhibited 100% sequence identity but showed a 22% divergence from the Sabin 3 strain (Fig. 2B). Sequence analysis of the 290-bp fragment also revealed 99% similarity to the sequence of the strain isolated from the index case of the epidemic in The Netherlands (Fig. 2B). The few differences in nucleotide sequence between a minority of SA-3 amplicons and the consensus sequence of the strain from The Netherlands were found to produce synonymous codons (Fig. 2B).

DISCUSSION

The usefulness of PCR-based procedures for the rapid identification of cases of viral importation is exemplified by this article. As an initial screening procedure, the use of panenteroviral primers to amplify and then sequence the 5′ NTR regions of viral genomes allowed us to rapidly obtain genetic information that distinguished the SA-3 isolates from Sabin 3-related strains. Comparison of this sequence with the strain from the outbreak in The Netherlands revealed a greater than 99% sequence similarity. The ability to use sequence from the conserved portion of the 5′ NTR to provide initial data on enteroviral strains responsible for outbreaks has been utilized previously (7). Close to 40% of the nucleotide positions within the conserved portion of the 5′ NTR can be variable, thus allowing strain-to-strain differentiation (7, 17). Indeed, when the 5′ NTR sequence generated from the SA-3 isolates was compared with other sequences in GenBank, the highest similarity score was obtained with the 5′ NTR of enterovirus type 70 (88%). An even higher sequence divergence (>12%) was found between the 5′ NTR sequence of the SA-3 isolates and 5′ NTR sequences available for various other poliovirus strains. Thus, one primer set used for routine detection of enteroviruses in clinical specimens can also serve as a molecular tool in the identification of clinically important strains through the utilization of standard amplicon sequencing procedures.

In addition to 5′ NTR sequence analysis, a molecular characterization of the VP1/2A portion of isolate genome was also carried out and comparisons were made to the strain from the outbreak in The Netherlands. Sequence analysis of the nucleotide composition proximal to the VP1/2A junction of the isolates showed a greater than 99% identity with the strain from The Netherlands. The sequence at the VP1/2A junction of polioviruses has been and continues to be utilized for determining genotypic relationships among poliovirus strains from different geographical regions (12, 18). The observation of identical sequence composition at two distinct regions of the poliovirus genome for both the SA-3 isolates and the strain from The Netherlands provides verification of importation of wild poliovirus into Canada. Prior to this importation of wild poliovirus, the National Centre for Enteroviruses and the Laboratory Centre for Disease Control, Health Canada, had no record of wild strains of poliovirus in Canada, imported or otherwise, since 1981.

The importation of wild poliovirus type 1 into Canada in 1978 resulted in 11 confirmed cases of poliomyelitis, of which 9 were paralytic (8). No cases of paralytic poliomyelitis were observed in the southern Alberta community under investigation in 1993. Paralysis rates are highest with poliovirus type 1; the observed frequency of paralysis with type 3 polioviruses appears to be lower, less than 1 case per 1,000 (10, 11), and this may have contributed to the lack of paralytic polio among the family members infected with the strain from The Netherlands.

The presence of isolated communities at high risk for wild-poliovirus-associated poliomyelitis will continue to pose problems to health care systems, even in countries with highly efficient immunization programs. Since the religious beliefs of certain communities will continue to impede efforts to establish 100% immunization, the long-term solution to this dilemma would be an eradication of wild poliovirus, as envisaged by the World Health Organization by the year 2000 (24). In the meantime, the cooperation of public health departments and diagnostic laboratories in gathering information necessary for up-to-date surveillance and detection of probable outbreaks will continue to be of major importance. As exemplified by this report, the advent of molecular tools such as PCR has made the surveillance process a much more rapid and informative procedure.

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