JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 2990–2996 Vol. 34, No. 12
0095-1137/96/$04.00+0

Group-Specific Identification of Polioviruses by PCR Using Primers Containing Mixed-Base or Deoxyinosine Residues at Positions of Codon Degeneracy

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Received 1 May 1996/Returned for modification 23 July 1996/Accepted 28 August 1996

We have developed a method for differentiating polioviruses from nonpolio enteroviruses using PCR. A pair of panpoliovirus PCR primers were designed to match intervals encoding amino acid sequences within VP1 that are strongly conserved among polioviruses. The initiating primer hybridizes with codons of a 7-amino-acid sequence that has been found only in polioviruses; the second primer matches codons of a domain thought to interact with the cell receptor. The panpoliovirus PCR primers contain mixed-base and deoxyinosine residues to compensate for the high degeneracy of the targeted codons. All RNAs from 48 vaccine-related and 110 wild poliovirus isolates of all three serotypes served as efficient templates for amplification of the 79-bp product. None of the genomic sequences of 49 nonpolio enterovirus reference strains were amplified under equivalent reaction conditions. Sensitivities of poliovirus detection were as low as 100 fg (equivalent to ~25,000 genomic copies or 25 to 250 PFU) when the amplified products were visualized by ethidium bromide fluorescence. These degenerate PCR primers should aid in the detection of all polioviruses, including those wild poliovirus isolates for which genotype-specific reagents are unavailable.

Virologic surveillance is essential to the success of the World Health Organization initiative to eradicate poliomyelitis worldwide by the year 2000 (20, 54). Expanded surveillance for cases of acute flaccid paralysis has been complemented by the development of improved methods for poliovirus identification. Two independent approaches are currently employed for routine identification of polioviruses: (i) antigenic characterizations using cross-adsorbed antisera (52) or (ii) molecular characterizations using genotype-specific probes (10, 11) or PCR primers (55, 56). Serologic methods can generally differentiate vaccine-related isolates from wild polioviruses (52) but have a limited capacity to differentiate among wild poliovirus genotypes. The molecular diagnostic methods of probe hybridization and PCR give identifications that are in excellent agreement with results obtained by the more detailed analyses of genomic sequencing (11, 25, 55, 56) or restriction fragment length polymorphism assays of PCR-amplification products (2, 47).

Vaccine-related polioviruses are identified directly with Sabin-strain-specific nucleic acid probes (11) or PCR primers (55). Wild polioviruses can be identified indirectly by their nonreactivity with the Sabin-strain-specific molecular reagents. Direct identification of wild polioviruses is possible with wild-genotype-specific probes (10) and PCR primer sets (56). However, the current catalog of wild-genotype-specific molecular reagents does not cover all of the many different poliovirus genotypes still in circulation worldwide (25). Consequently, identification of wild polioviruses through the exclusive use of Sabin-strain-specific molecular reagents is dependent upon the accurate typing of virus isolates.

The standard methods for poliovirus typing with neutralizing antibodies are comparatively time-consuming and laborious (33). Poliovirus neutralizing antibodies are type specific (33), and no well-characterized poliovirus-specific group antigen has been described (13, 33, 34). Molecular reagents, in the form of nucleic acid probes (10, 11) and PCR primers (21, 40, 56) targeted to highly conserved nucleotide intervals within the 5′ untranslated region, have been developed for the reliable detection of nearly all members of the enterovirus group (including polioviruses). Other reagents have been described recently that permit the detection of some, but not all, polioviruses by PCR (1, 12). An alternate method to screen out nonpolio enteroviruses (NPEVs) is to isolate virus in recombinant murine cells expressing the human gene for the poliovirus receptor (17). This approach also permits detection of underlying polioviruses in virus mixtures, and, like our PCR assay, avoids the laborious virus neutralization and recultivation steps of standard methods (33).

We describe here the properties of a degenerate panpoliovirus (panPV) PCR primer set that can be used to distinguish polioviruses from NPEVs. The primer set was targeted near VP1 sequences encoding a domain thought to interact with the cell receptor. RNAs of all polioviruses tested (110 diverse wild isolates and 48 vaccine-related isolates), but none of the NPEVs (49 reference strains), served as efficient templates for amplification in PCR assays using the panPV PCR primer sets.

MATERIALS AND METHODS

Viruses. Poliovirus isolates (Table 1) had been previously characterized by neutralization with hyperimmune equine sera and partial genomic sequencing (25). Vaccine-related isolates were also identified by PCR (55) and probe hybridization (11) assays using Sabin-strain-specific reagents. Human NPEVs, from a collection of prototypes maintained by the Centers for Disease Control and Prevention (Table 1), were identified by neutralization tests using type-specific hyperimmune sera. Viruses were propagated in HEp-2 (human larynx epidermoid carcinoma cell line, ATCC CCL23) or RD (human rhabdomyosarcoma cell line, ATCC CCL136) monolayers to produce high-titer inoculation stocks.

Oligonucleotide synthesis. Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as described previously (55). The degenerate primers used

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TABLE 1. Reactivities of poliovirus and NPEV templates in PCR assays with panPV primers

Isolate type	No. positive/no. tested
Vaccine-related poliovirus[a]	
Type 1	16/16
Type 2	16/16
Type 3	16/16
Wild poliovirus[b]	
Type 1	60/60
Type 2	20/20
Type 3	30/30
NPEV[c]	
Coxsackie A viruses	0/11
Coxsackie B viruses	0/6
Echoviruses	0/28
Enteroviruses	0/4

[a] Vaccine-related isolates tested include the following: PV1, 3875/MOG91, 0074/PER88, 5498/USA84, 2800/HON91, 0584/GUT91, 9825/USA89, 9703/ELS89, 9360/VEN89, 9240/HON89, 8315/MEX88, 8284/HON88, 8221/GUT87, 7245/COL86, 6529/CHI86, 6440/ARG85, 6258/MOR85; PV2, 0042/ELS90, 9364/GUT89, 7653/SOA86, 0636/ELS91, 9897/GUT90, 0078/PER89, 9819/BRA89, 9818/PER89, 9579/USA89, 8378/PER88, 8238/GUT87, 8018/GUT87, 7170/MEX86, 6700/HON86, 7837/PER84, 6886/GUT83; PV3, 6114/LAO94, 1063/USA91, 0131/MEX89, 0644/HON91, 0642/ELS91, 0405/GUT90, 0040/ELS90, 9896/GUT89, 9847/MEX89, 9442/NIC89, 9441/GUT89, 1339/CHN89, 0044/GUT89, 8774/TRT88, 6880/COL86, 7149/MEX84.

[b] Wild isolates tested, in addition to those shown in Fig. 3, include the following: PV1, 6306/TKM95, 6103/THA94, 5740/CHN93, 6118/NEP92, 6111/MMR92, 6015/CAM94, 6001/VTN94, 3940/THA92, 3706/MAA92, 3677/CYP92, 0004/TJK91, 18655/PAK91, 16838/TUR90, 11270/EGY91, 8649/IND91, 8645/IND91, 7362/PAK91, 7169/BUL91, 3643/CHN91, 2609/ETH91, 1184/ROM91, 0427/SSR91, 0124/CHN91, 0062/PER91, 6701/TUR90, 3727/AZE90, 2786/VTN90, 9475/ZAI89, 9366/SAA89, 2758/VTN89, 1338/CHN89, 0006/CHN89, 8771/OMA88, 5145/UZB88, 8425/ISR88, 1607/SOA88, 0955/SRL88, 8223/GUT87, 2662/COL87, 0941/SRL87, 0289/POR87, 7054/IND86, 6750/SEN86, 0285/INO86, 0109/CHN86, 9258/TUN85, 6224/ZIM85, 3638/CHN85; PV2, 8654/IND91, 7354/PAK91, 3869/IND91, 6876/COL86, 0302/YUG81, 2996/SWE77, 0290/TUR73, 0710/KEN71; PV3, 6112/MMR93, 6093/THA93, 6113/LAO92, 3904/SYR91, 8668/IND91, 7350/PAK91, 3838/EGY91, 15953/FRA90, 4076/ARM90, 2619/MOL90, 0010/TJK90, 0380/MEX90, 2732/UZB89, 9259/TUN88, 9035/BRA88, 0324/INO86, 8178/VEN87, 7840/PER86. Country abbreviations are as defined by the World Health Organization (11).

[c] The 49 NPEVs analyzed included the reference strains (33) of coxsackie A virus types 3 to 6, 8 to 10, 12, 14, 21, and 24; coxsackie B virus types 1 to 6; echovirus types 3 to 9, 11 to 21, 24 to 27, and 29 to 34; and enterovirus types 68 to 71.

for amplifying poliovirus sequences were panPV PCR-1 (A, 2935 to 2954) 5′-TTIAIIGC(A/G)TGICC(A/G)TT(A/G)TT-3′, and panPV PCR-2 (S, 2875 to 2894) 5′-CITAITCI(A/C)GITT(C/T)GA(C/T)ATG-3′. Numbers in parentheses indicate the genomic intervals matching the primers (A, antisense or antigenome polarity; S, sense or genome polarity) (Fig. 2), according to the numbering system of Toyoda et al. (50). Deoxyinosine residues are indicated by the letter I. Primer positions having equimolar amounts of two different nucleotides are enclosed in parentheses.

PCR amplification and analysis. In vitro amplification by PCR was performed by modification of previously described procedures (55, 56). Cultures of virus-infected cells (multiplicity of infection, >25 PFU per cell; maintained in Eagle's minimal essential medium without serum) were harvested after development of extensive cytopathology (usually 18 h postinfection) and frozen-thawed three times to release virions. The suspensions were clarified by centrifugation (800 × *g*, 2 min), and 1 μl of each supernatant (equivalent to ~500 infected cells) was added to the amplification reaction mixtures (50 μl), which contained 50 mM Tris-HCl (pH 8.3); 70 mM KCl; 5 mM $MgCl_2$; 10 mM dithiothreitol; 80 pmol each of primer sets panPV PCR-1 and panPV PCR-2 (equivalent to 10 pmol of each discrete primer per set); 100 μM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, N.J.); 5 U of placental RNase inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); 1.25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim); and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Reaction mixtures (without RNase inhibitor, reverse transcriptase, and *Taq* DNA polymerase) were overlaid with mineral oil, heated for 5 min at 95°C to release virion RNA, and chilled on ice. The enzymes were then added, and the samples were incubated at 42°C for 30 min, followed by 30 cycles of programmed amplification (denaturation, 94°C, 1 min; annealing, 42°C, 1 min; extension, 60°C, 1 min) in a DNA thermal cycler (Perkin-Elmer). Conditions for polyacrylamide gel electrophoresis and detection

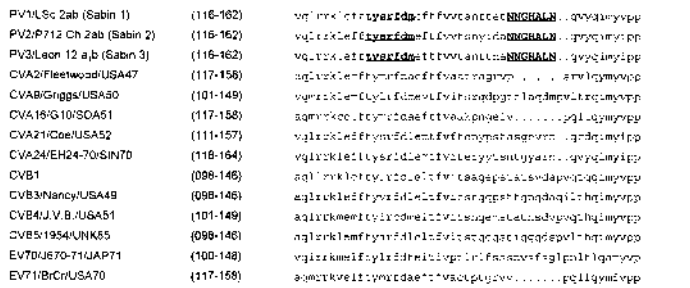

FIG. 1. Alignment of corresponding amino acid residues within the VP1 proteins of reference strains of 14 human enteroviruses. Poliovirus amino acid sequences whose codons match the degenerate panPV-specific primers are underlined. Primer panPV PCR-1 (antisense) complements codons of a VP1 sequence (NNGHALN). Primer panPV PCR-2 (sense) matches a sequence (TYSRFDM). Abbreviations for virus groups are followed by serotype number: PV, poliovirus; CVA, coxsackievirus A; CVB, coxsackievirus B; EV, echovirus (type 22) or enterovirus (types 70 and 71). Sequence alignments were adapted from the work of Palmenberg (41) and the following reports: PV1, -2, and -3 (50); CVA2 (44); CVA9 (7); CVA16 (44); CVA21 (19); CVA24 (48); CVB1 (23); CVB3 (27); CVB4 (24); CVB5 (57); EV70 (46); EV71 (5). Country abbreviations are as defined by the World Health Organization (11).

of amplified products by ethidium bromide staining were as described previously (55).

Nucleic acid sequence analysis. Partial VP1 sequences of wild poliovirus isolates were determined in cycle sequencing reactions (36) containing fluorescent dye-labeled dideoxynucleotide chain terminators (Applied Biosystems, Foster City, Calif.). Amplification products were further characterized in similar reactions, with panPV PCR-1 and panPV PCR-2 as primers. Nucleotide sequences were determined with the aid of an automated sequenator (Applied Biosystems, Model 373A).

Sequence relationships among poliovirus genomes were compared by the CLUSTAL computer program (16) from version 7 of the PC/Gene nucleic acid analysis package (IntelliGenetics, Mountain View, Calif.). Thermal stabilities of primer-template hybrids were estimated by using the OLIGO 5.0 program (National Biosciences, Plymouth, Minn. [14]).

RESULTS

Selection of primer binding sites. The most variable protein among polioviruses (18, 29, 50) and NPEVs (41) is the capsid protein VP1. Alignment of VP1 amino acid sequences of representative human enteroviruses reveals an interval of high variability (Fig. 1), corresponding to VP1 amino acid residues 140 to 152 of poliovirus type 1. Within this interval is a 7-amino-acid sequence, NNGHALN, which has been found only in polioviruses. We reasoned that oligodeoxynucleotide primers complementary to nucleotides encoding the NNGHALN sequence might hybridize to poliovirus templates with very high specificities.

Comparison of the VP1 sequences of 40 independent wild poliovirus isolates (Fig. 2) showed that the amino acid sequence NNGHALN was strongly conserved. Only two isolates (PV3/5376/PHL93 and PV3/0780/OMA91) had sequences that differed from the consensus, and each had the same substitution to produce the sequence NSGHALN (Fig. 2). Contrasting with the strong amino acid sequence conservation of this interval, the corresponding codons were highly degenerate (average difference = 18%), as 21 of the possible 25 codons for NNGHALN were found to be used. To complement all possible codon combinations for NNGHALN, our antisense initiating primer (panPV PCR-1) contained either mixed-base residues or deoxyinosine residues at degenerate codon positions. Deoxyinosine residues, which can pair with all four bases (32, 39), were incorporated into the primers (at positions 3, 6, and 12) to match the three template positions having a possible fourfold degeneracy and (at primer position 5) one template position having twofold degeneracy. Three other template po-

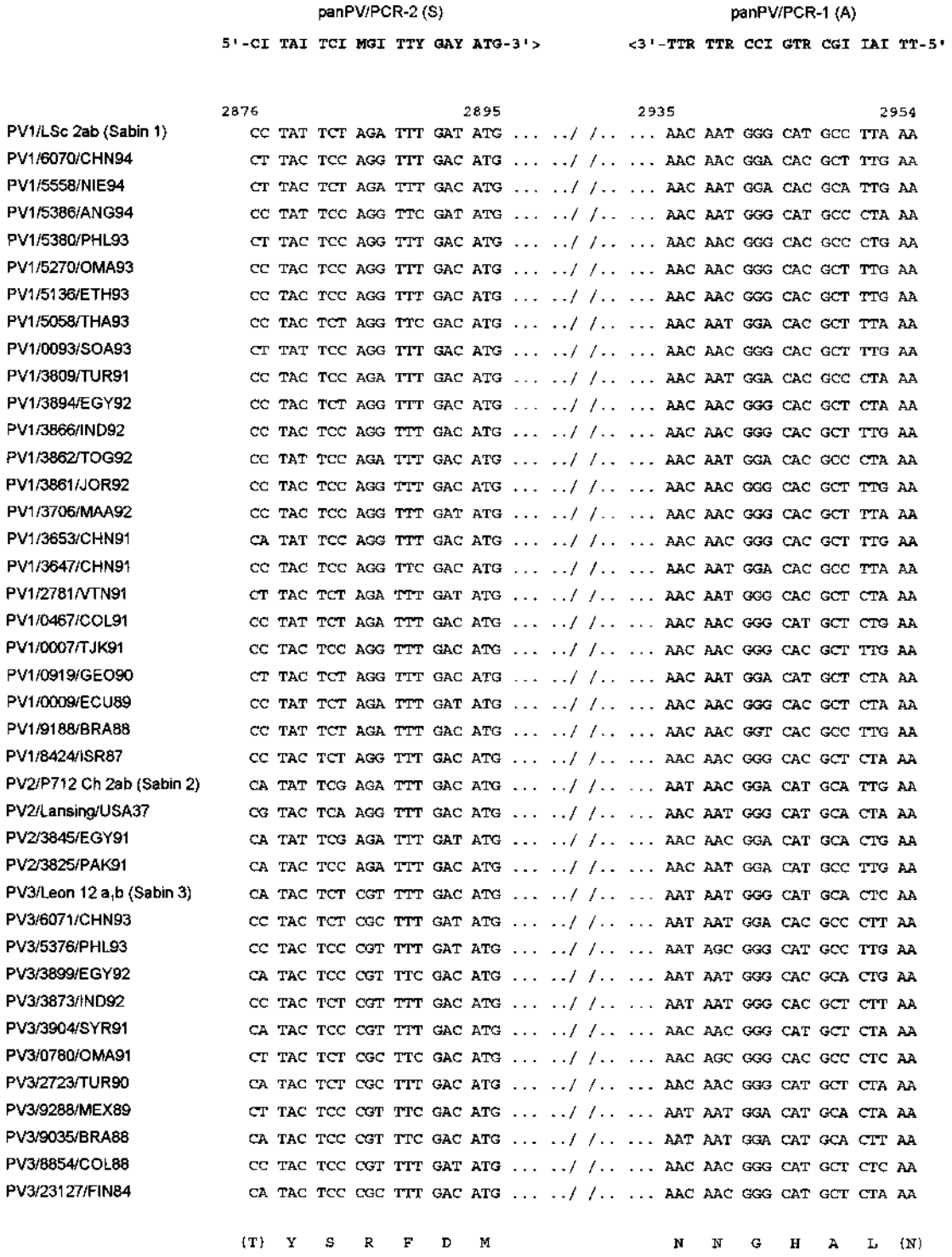

FIG. 2. Alignment of genomic sequences binding primers panPV PCR-1 and panPV PCR-2 within the VP1 regions of 40 polioviruses, representing 11 type 1 genotypes, 3 type 2 genotypes, and 11 type 3 genotypes. The deduced amino acid sequences are shown below the aligned nucleotides. Abbreviations for nucleotides follow International Union of Biochemistry (38) nomenclature: I, inosine; M, adenine or cytosine; R, adenine or guanine; Y, cytosine or thymine. Poliovirus VP1 sequences were from this study except for those of Sabin 1, Sabin 2, and Sabin 3 (50); PV3/9288/MEX89 (56); and PV3/23127/FIN84 (18).

sitions with twofold degeneracy were complemented (at primer positions 9, 15, and 18) by twofold mixed residues (Fig. 2).

A second degenerate primer (sense; panPV PCR-2) (Fig. 2) was designed to match nucleotides encoding a 7-amino-acid sequence, TYSRFDM, corresponding to VP1 residues 126 to 132 of poliovirus type 1, which was found to be absolutely conserved among the 40 poliovirus isolates compared (Fig. 1 and 2). Again, because of the high codon degeneracy within

this interval (21 of the 24 possible synonymous codons were observed), the primer set was designed to contain deoxyinosine residues at positions 6, 9, and 12 and base mixtures at positions 3, 10, and 15 (Fig. 2). Primer panPV PCR-2 (at positions 6 to 17) is complementary to positions 7 to 21 of the nondegenerate antisense poliovirus PCR primer UC1 of Balanant et al. (2). Because sequences similar to TYSRFDM have been found in the corresponding VP1 intervals of several NPEV strains (Fig. 1), it was important to the specificity of panPV PCR-2 that the 3′-donor sequences of the primer matched the codon for the terminal methionine residue, which among the reported NPEV VP1 sequences has been found only in coxsackievirus A9 (Fig. 1). The 3′-donor ends of both panPV PCR-1 (at positions 19 and 20) and panPV PCR-2 (at positions 18 to 20) were designed to match conserved nondegenerate codon positions (Fig. 1 and 2), to optimize efficiency of amplification by PCR (28).

The predicted chain length of the amplification product is 79 bp (Fig. 2). The primer binding sites were closely spaced along the templates to maximize diagnostic sensitivities by minimizing the lengths of the cDNA transcripts required to initiate the chain reactions (55).

Optimization of PCR conditions with degenerate primers. The incubation temperatures generally used for in vitro amplification by PCR (annealing, 55 to 60°C; extension, 65 to 72°C) are unsuitable for use with our degenerate primers because the presence of deoxyinosine residues results in low annealing temperatures with many poliovirus templates. The optimal annealing temperature was found to be approximately 42°C, near the temperature optimum for avian myeloblastosis virus reverse transcriptase. Annealing temperatures above (≥46°C) or below (≤38°C) the optimum frequently reduced the yield of the specific 79-bp amplicon and increased the generation of nonspecific amplification products (data not shown). The extension temperature was also decreased, to 60°C, still within the range for high *Taq* polymerase activity (8), to minimize dissociation of the primers from the templates. When these conditions were used, sequences of all poliovirus templates tested were efficiently amplified (see following sections), each reaction generating a 79-bp amplicon as the major product.

Specificities of panPV PCR primer sets. (i) Amplification of poliovirus templates. The specificities of the panPV PCR-1–panPV PCR-2 primer pair were first tested in amplification reactions containing RNAs of the Sabin vaccine reference strains and vaccine-related clinical isolates (Table 1). Efficient amplification was obtained with all 48 (16 of each serotype) templates. The electrophoretic mobilities of the major products were compatible with their having the predicted chain length of 79 bp (data not shown). Sequence analysis of the PCR products amplified from the templates of the three Sabin reference vaccine strains confirmed that the panPV PCR-1–panPV PCR-2 pair primed specific amplification of the targeted nucleotide interval (data not shown).

The corresponding primer binding sites differ by as much as 25% among the three Sabin strain templates (Fig. 2). Additional sequence variability at these sites may occasionally occur during replication of the poliovaccines in the intestine (31, 53). However, evolution of the poliovaccines is usually limited because vaccine virus spread is typically restricted to vaccine recipients and their immediate contacts (4).

A much wider range (up to 35%) (Fig. 2) of sequence variability is observed at the primer binding sites of wild poliovirus genomes. Moreover, additional codon combinations would be expected to arise through the extensive evolution of wild poliovirus genomes. To evaluate the specificities of the panPV PCR primer pair across wild poliovirus genotypes, we performed PCRs with RNA templates derived from the most diverse set of contemporary wild polioviruses in our collection (Fig. 3; Table 1). We used as our first measure of diversity the observed sequence differences at the primer binding sites (Fig. 2). However, because our database for these sequences is limited (especially for type 2 isolates), we sometimes used differences in VP1/2A sequences (nucleotide interval 3296 to 3445 [type 1]) (25) as an alternate measure of genotypic diversity. When sequences of both genomic intervals were available, either measure identified the most divergent polioviruses. Results of PCR assays containing the panPV PCR primers and templates from 12 of the most diverse wild polioviruses of each serotype are shown (Fig. 3); other wild polioviruses tested are listed (Table 1). A total of 110 wild poliovirus isolates were tested (60 of type 1, 20 of type 2, and 30 of type 3). The RNAs of all isolates were amplified efficiently; each reaction yielded as the major product the predicted 79-bp amplicon (Fig. 3; Table 1).

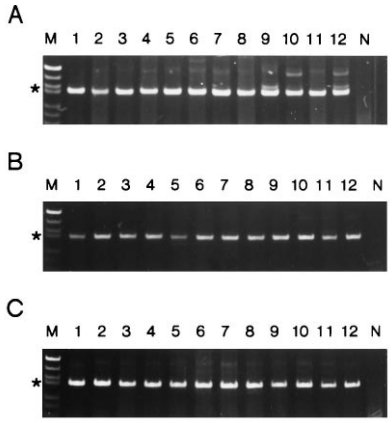

FIG. 3. Specific amplification of wild poliovirus genomes by PCR using the panPV PCR primer sets. Sources of templates for the products in each lane were as follows: (A) (PV1), 1, 6070/CHN94; 2, 5558/NIE94; 3, 5386/ANG94; 4, 5058/THA93; 5, 3894/EGY92; 6, 3866/IND92; 7, 3862/TOG92; 8, 3647/CHN91; 9, 2781/VTN91; 10, 0467/COL91; 11, 0919/GEO90; 12, 9188/BRA88; (B) (PV2), 1, 3874/IND92; 2, 3825/PAK91; 3, 3845/EGY91; 4, 2613/PAK89; 5, 0176/PER89; 6, 7079/IND86; 7, 1534/IND82; 8, 0301/CAE80; 9, 0298/EGY79; 10, 0298/ISR78; 11, MEF-1/EGY42; 12, Lansing/USA37; (C) (PV3), 1, 6071/CHN93; 2, 5376/PHL93; 3, 3899/EGY92; 4, 3873/IND92; 5, 0780/OMA91; 6, 3997/TKM90; 7, 2723/TUR90; 8, 9288/MEX89; 9, 9035/BRA88; 10, 8854/COL88; 11, 23127/FIN84; 12, Saukett/USA52. M, molecular weight marker. N, negative template sample. Asterisk, 79-bp band.

No correlation was found between the intensity of the 79-bp product band and the estimated stabilities (14) of primer binding to different poliovirus templates. For example, amplicon yields were similar for poliovirus templates predicted to form the most stable hybrids (PV3/0780/OMA91, midpoint temperature [T_m] [panPV PCR-1] ≈ 74°C; PV3/23127/FIN84, T_m [panPV PCR-2] ≈ 60°C) and the least stable hybrids (PV3/9288/MEX89, T_m [panPV PCR-1] ≈ 52°C; PV1/2781/VTN91, T_m [panPV PCR-2] ≈ 42°C) with the degenerate primers (Fig. 3).

(ii) Nonamplification of NPEV templates. The specificities of our primers were further tested in PCR mixtures containing panPV PCR-1–panPV PCR-2 and the RNA templates of 49 reference NPEV strains. None of the NPEV RNAs served as templates for amplification with our panPV PCR primer pair (Table 1).

To confirm that all NPEV samples contained RNA templates amplifiable by PCR, amplification reactions were per-

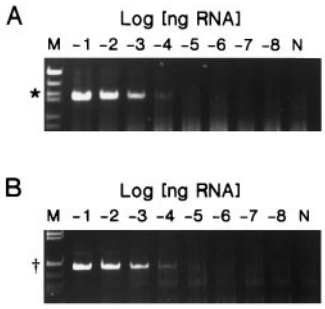

FIG. 4. Sensitivities of detection of poliovirus sequences in PCR assays containing the panPV PCR primer (A) and the EV/PCR primer (B) sets. Serial 10-fold dilutions of purified PV1/Sabin 1 virion RNA (100 pg to 10 ag) were added to reaction mixtures containing the corresponding primer pair sets. The average quantities of virion RNA present in the reaction mixtures at each dilution are indicated above the sample lanes. M, molecular weight marker. N, negative template sample. Asterisk, 79-bp band; dagger, 114-bp band.

formed in parallel with the primer pair EV/PCR-1–EV/PCR-2 (56). This pair targets highly conserved sequences within the 5′ untranslated regions of all enteroviruses (and polioviruses) except EV22 and EV23, which are members of a separate picornavirus group (22). As expected, the 114-bp amplification product was obtained with all NPEV templates except those of EV22 and EV23 (data not shown and Table 1). Separate PCR primers specific for EV22 and EV23 (31a) were used to confirm the presence of amplifiable templates of these NPEV types (Table 1).

Sensitivity of poliovirus RNA detection. To determine the limits of sensitivity for poliovirus detection with our panPV PCR primer pairs, serial 10-fold dilutions (10^{-1} to 10^{-8} ng; corresponding to 2.5×10^7 to 2.5 RNA molecules) of Sabin 1 RNA were used as templates for in vitro amplification reactions. Sabin 1 was selected because its template is predicted (14) to form comparatively weak hybrids with the degenerate panPV PCR primers (T_m [panPV PCR-1] $\approx$ 62°C; T_m [panPV PCR-2] $\approx$ 45°C). Sensitivities of detection of Sabin 1 RNA might be comparatively low if the stabilities of primer-template hybrids are important to the diagnostic sensitivities of our PCR assays. The limit of detection for Sabin 1 RNA with the panPV PCR primer set was ~100 fg (equivalent to ~25,000 poliovirus RNA molecules or 25 to 250 PFU [55], ~10% the yield from a single infected cultured cell [30]) when the amplicons were visualized by ethidium bromide fluorescence (Fig. 4). Interestingly, a similar detection limit was observed for the same template with the EV/PCR primer pair (56), which form more stable (T_m [EV/PCR-1] $\approx$ 79°C; T_m [EV/PCR-2] $\approx$ 85°C), perfectly matched hybrids with Sabin 1 RNA. Similar diagnostic sensitivities have been reported for PCR assays using other nondegenerate primer sets targeting conserved intervals in the 5′ untranslated region of enteroviruses (51).

DISCUSSION

Surveillance for wild polioviruses is an important component of the Global Poliomyelitis Eradication Initiative (20, 25, 54). Poliovirus surveillance is especially important for identifying remaining reservoirs of wild poliovirus circulation in regions that are approaching eradication (25), for certifying the eradication of indigenous wild genotypes in regions that have recently achieved polio-free status, and for detecting importation of wild polioviruses into regions that have not had indigenous wild poliovirus circulation for many years (37, 42, 54).

The key to developing an assay specific for any group of viruses is to identify a structural feature unique to that group. In the case of polioviruses, virion surface determinants are unsuitable targets because they are type specific (33, 35). Internal capsid antigens and nonstructural protein antigens also appear to be unsuitable because they tend to be shared among enteroviruses (13, 34). However, polioviruses bind to a cell receptor that is distinct from those used by other enteroviruses (17). The canyon structure on the poliovirion surface that is postulated to bind the cell receptor is primarily formed from conserved intervals of VP1 and VP2. Genetic studies have suggested that VP1 residue Met-132, at the end of the TYS RFDM interval, whose codons are targeted by panPV PCR-2, interacts with the cell receptor (9). In contrast, the structural role of the highly conserved NNGHALN sequence, whose codons are targeted by panPV PCR-1, is unknown. It is tempting to speculate that this interval, found only in polioviruses and not exposed on the surface of intact virions, might also play a role in stabilizing interactions with the cell receptor, but direct evidence is lacking. We attribute much of the high template specificities of our panPV PCR primers to the base-pairing properties of panPV PCR-1, which targets a sequence unique to poliovirus capsids. The panPV PCR-2 primer probably contributes less to the overall specificity because the TYS RFDM interval is similar to sequences found in other enteroviruses.

The high degree of nucleotide sequence diversity among polioviruses presents a challenge to the systematic design of nucleic acid-based reagents. Genomic sequences that encode intervals of strong amino acid conservation can still be highly degenerate. In the present example, many different synonymous codon combinations could potentially occur within the primer-binding sequences (432 for panPV PCR-1 and 512 for panPV PCR-2, assuming usage only of the observed codons). If all degenerate codon combinations are permitted within both target sequences, our panPV PCR primers would have to match >200,000 different sequence combinations. To accommodate this wide variability, degenerate codon positions on the template were matched by mixed-base or deoxyinosine residues on the primers. Degenerate and deoxyinosine-containing probes and PCR primers have been used previously to target variable cellular (3, 39) and viral (6, 45) sequences. Some investigators, however, have reported unsatisfactory losses in PCR sensitivity and diagnostic specificity with degenerate primers (49). In our hands, PCR assays using the degenerate panPV PCR primers were positive for a very diverse sample of poliovirus genotypes, had excellent diagnostic specificities, and had template sensitivities comparable to those obtained with nondegenerate primers.

It is important that the poliovirus group-, serotype- (25a), and genotype-specific (10, 11, 55, 56) molecular reagents target capsid sequences. Polioviruses undergo frequent recombination in nature (15, 26, 58). Genetic exchange may place poliovirus capsid sequences into genetic backgrounds derived from other polioviruses (26, 58), or possibly NPEVs (15). Molecular reagents targeting capsid sequences should give identifications that correlate better with the most important biological properties of the virus (e.g., receptor specificity, serotype) than would reagents targeting other regions.

The availability of the panPV PCR primers should facilitate poliovirus surveillance by surmounting some of the technical problems encountered with current methods. Polioviruses present in isolates from paralytic cases can be efficiently detected in direct, rapid, and highly specific PCR assays and further characterized with wild-genotype-specific (10, 56) and Sabin-strain-specific (10, 11, 55) molecular reagents. Wild poliovirus isolates for which genotype-specific reagents are un-

available can be recognized by the nonreactivity of their RNAs with Sabin-strain-specific PCR primers (55) and probes (11).

Isolates containing virus mixtures are the most difficult to characterize by conventional techniques (33). In preliminary studies, underlying polioviruses in enterovirus mixtures could be detected with good sensitivities in our PCR assays. Several isolates that had been typed as NPEVs by the standard neutralization tests were found also to contain poliovirus, initially detected by our panPV PCR primers and confirmed by virus isolation. In regions of low polio endemicity, where a large proportion (up to 80%) of the isolates from paralytic cases are NPEVs (20, 42), it is particularly important that wild poliovirus infections are recognized. While the NPEVs can be the etiologic agents of the paralytic disease (33), they most often appear to be incidental isolates (42). The capacity to recognize mixed poliovirus-NPEV isolates will become increasingly important as poliovirus surveillance is intensified in areas where multiple enterovirus infections of children are common (43).

ACKNOWLEDGMENTS

We thank Silvia Peñaranda, Yvonne Stone, and Kathy Crane for preparing the poliovirus isolates and Edwin George and Melissa Olson of the Scientific Resources Program for preparing the synthetic oligodeoxynucleotide primers. We thank Galina Lipskaya (Moscow State University, Moscow, Russia) and Sharon Bloom for their collaboration in the development of the sequence database for wild polioviruses. Our thanks to the virologists and epidemiologists who contributed poliovirus samples for our studies. The cooperation and assistance of The Task Force for Child Survival and Development are appreciated.

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