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Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala

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Summary

The extensive nucleotide sequence heterogeneity among independent genotypes of wild polioviruses permits the systematic design of genotype-specific molecular reagents. We have prepared two sets of polymerase chain reaction (PCR) primer pairs specific for the genotype of wild poliovirus type 3 recently endemic to Mexico and Guatemala. Nucleotide sequences of a representative wild type 3 virus isolated in Mexico in 1989 differed from the corresponding Sabin 3 (Leon 12 a_1b) sequences at 167 of 900 positions within the VP1 region. From the sequence data, wild virus-specific primer pairs were designed to complement regions of high mismatch (> 33%) with Sabin 3 templates. Primer binding sites were spaced along the genome so that the predicted amplification products (142 bp and 163 bp) could be easily resolved electrophoretically from the products generated with our Sabin strain-specific primers (Sabin 1: 97 bp; Sabin 2: 71 bp; Sabin 3: 53 bp). RNAs of all wild type 3 poliovirus isolates from Mexico and Guatemala obtained over a 13-year period (1977–1990) served as efficient templates for amplification of the 142-bp

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and 163-bp products. Genomic templates derived from vaccine-related polioviruses and most heterologous wild polioviruses were inactive under equivalent reaction conditions. Amplifications generating a 114-bp product with a broadly reacting primer pair, matching highly conserved sequences in the 5'-noncoding region, provided a positive control for the presence in samples of poliovirus (or enterovirus) RNAs. Selective amplification of wild Mexico-Guatemala type 3 poliovirus sequences was obtained with either primer set in reactions containing large stoichiometric excesses (up to 10^6-fold) of vaccine-related RNAs. We have used wild genotype-specific PCR primer sets to facilitate identification of wild polioviruses present in both clinical and environmental samples.

Wild polioviruses; Polymerase chain reaction; In vitro amplification; Wild genotype detection

Introduction

In vitro amplification of variable virus sequences by the polymerase chain reaction (PCR) is an efficient, sensitive, and precise method for identifying poliovirus isolates. We recently reported the development of PCR primer and probe sets that permit the rapid identification of polioviruses related to the Sabin poliovaccine strains (Yang et al., 1991). These new reagents have facilitated the identification of polioviruses associated with cases occurring in the United States and other countries where the indigenous wild polioviruses have been eliminated (Nkowane et al., 1987, World Health Organization, 1990a).

The large majority of poliomyelitis cases worldwide (approximately 250,000 in 1989; World Health Organization, 1990a) are associated with wild polioviruses, among which many independent genotypes currently exist (Kew et al., 1990a). Poliovirus isolates may be recognized as wild by their nonreactivity with the Sabin strain-specific PCR primer and probe sets (Yang et al., 1991). This approach, while reliable, does not take full advantage of the speed, sensitivity, and specificity potentially available from PCR analyses (Saiki et al., 1985, 1988). A clear improvement upon the above approach is to use PCR primer and probe sets that permit the direct detection of wild polioviruses. Such reagents would not only simplify analyses, but also provide a clear, positive signal for the presence of wild poliovirus in the sample, even in specimens containing poliovirus or enterovirus mixtures.

We have prepared PCR primer and probe sets specific for each of the several wild poliovirus genotypes recently endemic to the American Region (Pan American Health Organization, 1990; Kew et al., 1990b; de Quadros et al., 1991; E. da Silva et al., in preparation). In this report, we describe the properties of PCR primer sets permitting the selective amplification of the sequences of the wild type 3 poliovirus genotype recently associated with paralytic cases in Mexico and Guatemala.

Materials and Methods

Viruses

Poliovirus isolates (Tables 1–3) had been previously characterized by neutralization with hyperimmune equine sera and partial genomic sequencing (Rico-Hesse et al., 1987; Kew et al , 1990a; De et al , in preparation). Vaccine-related strains were also positively identified by PCR using the Sabin strain-specific primer pairs (Yang et al., 1991). Human nonpolio enteroviruses (Table 4) were identified by neutralization with pools of immune sera (Melnick, 1990) followed by confirmation of serotype with monotypic neutralizing polyclonal antibodies. Viruses were propagated in HeLa or RD monolayers to produce high-titer inoculation stocks

Preparation of RNA

Freeze-thaw lysates of RD cell cultures infected with clinical isolates (80–400 μl per sample) were thoroughly mixed (4:1) with 5 × lysis buffer [250 mM Tris-HCl (pH 8.3), 350 mM KCl, 25 mM $MgCl_2$, 2.5% NP-40, 25 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories, Gaithersburg, MD)], and incubated on ice for 10 min. The mixtures were extracted three times with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1), followed by two chloroform extractions. Aqueous-phase extracts (1 to 2 μl) from infected cell cultures were used directly in the PCR reactions. Poliovirus RNAs used for sequence analysis and titrations were extracted from purified virions, and the RNA concentrations determined spectrophotometrically from the relationship A_{260} (1 mg/ml) = 22.0 (Rueckert, 1976).

Oligonucleotide synthesis

Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as described (Yang et al., 1991) The primers used for sequencing PV3/9288/MEX89 were:

Polio/2A	(A:3476–3495)	5′-AAGAGGTCTCTATTCCACAT-3′
9288/VP1-1	(A:3278–3297)	5′-CGCGGCGGTCTAGGGCACCA-3′
9288/VP1-2	(A:3137–3156)	5′-AGAGAGTCACCGATTTGGTC-3′
9288/VP1-3	(A 2978–2997)	5′-TGCCAGGTGTAATCGTCCCA-3
9288/VP1-4	(A:2747–2766)	5′-GTGGTTGGCTGTTCATTATC-3′
9288/VP1-5	(A.2609–2628)	5′-TTGGTGGCCCCAGTTTCAAC-3′

All isolates were sequenced using primer Polio/2A, previously described by Rico-Hesse et al. (1987) Sequences of the Mexico wild type 3 genotype PCR

primer pairs were:

9288/PCR-1	(A:3331–3352)	5′-CAGGGGGTGTAAGTCATCTTTG-3′
9288/PCR-2	(S:3190–3211)	5′-GCTAGCAATCCGGGTTGTTAAC-3′
9288/PCR-3	(A:2629–2650)	5′-TGTGTCAGATGGAACTAAGGGG-3′
9288/PCR-4	(S:2509–2530)	5′-AGGAGCATTGGCCCTCTCGCTT-3′

A new Sabin 3-specific PCR-1 primer was substituted for the one described by Yang et al. (1991) in order to generate a longer (53 bp) amplified product:

Sab3/PCR-1B	(A:2542–2560)	5′-AGTATCAGGTAAGCTATCC-3′

The numbers in parentheses indicate the genomic intervals matching the primers (A = antigenome polarity primer; S = sense or genome polarity primer; Fig. 1), following the numbering system of Stanway et al. (1984) for the Sabin 3 genome.

Sequences of a primer pair matching highly conserved sites within the 5′-noncoding regions of enterovirus genomes were:

EV/PCR-1	(A:539–565)	5′-ACACGGACACCCAAAGTAGTCGGTTCC-3′
EV/PCR-2	(S:452–476)	5′-TCCGGCCCCTGAATGCGGCTAATCC-3′

and the genomic intervals matching the primers were numbered according to the consensus system introduced by Toyoda et al. (1984).

Nucleic acid sequencing

Genomic RNA sequences were determined by extension of the above sequencing primers with avian myeloblastosis virus (AMV) reverse transcriptase in the presence of dideoxy chain termination inhibitors (Zimmern and Kaesberg, 1978; Biggin et al., 1983) as previously described (Rico-Hesse et al., 1987).

PCR amplification and analysis

In vitro amplification by PCR was modified from the methods described previously (Yang et al., 1991). Amplification reactions were carried out in 100 μl reaction mixtures containing RNA templates in 50 mM Tris-HCl (pH 8.3), 70 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, one or more PCR-1 primers (20 pmol each), one or more PCR-2 primers [10 pmol unlabeled primer plus 500,000 cpm ($\approx$ 100 fmol) of 5′-radiophosphorylated primer], 200 μM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.5% NP-40, 10 U placenta ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2.5 U AMV reverse transcriptase (Boehringer Mannheim) and 2.5 U of *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The reaction mixtures were overlaid with mineral oil, and incubated at 42°C for 30 min before 30 cycles of programmed amplification (denaturation: 94°C, 30 s; annealing: 60°C, 45 s; extension: 72°C, 1 min) in a DNA thermal cycler (Perkin Elmer-Cetus). Conditions for ^{32}P-radiolabeling of oligodeoxynucleotides, polyacrylamide gel electrophoresis, and detection of ampli-

fied products by ethidium bromide staining or autoradiography were as described (Yang et al., 1991).

Results

Design of genotype-specific PCR primer pairs

VP1 sequences of PV3/9288/MEX89

Poliomyelitis has remained endemic to Mexico throughout the poliovaccine period (Pan American Health Organization, 1990; de Quadros et al., 1991). Before the late 1980s, most cases in Mexico and Central America were associated with wild type 1 polioviruses (M.L. Zárate, personal communication; Cruz et al., 1987; Rico-Hesse et al., 1987). The last poliomyelitis cases in Mexico, Guatemala, and El Salvador known to be associated with wild poliovirus type 1 occurred in 1987 (unpublished results). Since that year, all wild isolates obtained from poliomyelitis cases in Mexico and Central America have been type 3. An important reservoir for endemic poliovirus transmission has been the state of Sinaloa (Cárdenas-Ayala et al., 1988), along the central Pacific coast of Mexico, where 14 cases have been reported since 1988 (Pan American Health Organization, 1990). Sequence comparisons within a 150-nucleotide interval of the VP1/2A region (Rico-Hesse et al., 1987; Kew et al., 1990a) showed that all recent case isolates from Mexico (1988–1990) and Guatemala (1990) are closely related to each other (mean divergence = 3.4%), suggesting the occurrence of a recent common progenitor infection (Pan American Health Organization, 1990; De et al., in preparation). A 1989 isolate from Sinaloa, PV3/9288/MEX89, shown by these sequence surveys to be representative of the main lineage recently endemic to Mexico, was selected as our reference strain for further sequence analysis.

The VP1 nucleotide sequences of PV3/9288/MEX89 (Fig. 1) were quite divergent from those of the Sabin 3 reference strain, Leon 12 a_1b (Stanway et al., 1984; Toyoda et al., 1984). Nucleotide substitutions occurred at 18.6% (167/900) of positions, involving nearly half (149/300) of the VP1 codons. Most (90%) of the mutations generated synonymous codons, as only 16 amino acid differences from the Sabin 3 VP1 polypeptide were found. Nine (56%) of the amino acid differences clustered within two VP1 domains: residues 1–30 at the amino terminus (5 substitutions), and the surface residues forming neutralizing antigenic site 3a (4 of 5 positions altered). The remaining 7 missense codons were not clustered within the VP1 primary sequence.

Aside from differences encoding amino acid changes, nucleotide base substitutions were widely distributed along the VP1 sequence. Seven short intervals (22 nucleotides each, spanning 7 codons and the degenerate third position of an adjacent codon) contained substantial (>30%) mismatches with the Sabin 3 template. 2479–2500 (7 mismatches), 2508–2530 (11 mismatches), 2593–2614 (7 mismatches), 2629–2650 (7 mismatches), 3082–3103 (8 mismatches), 3190–3211 (8 mismatches), and 3331–3352 (10 mismatches) (Fig. 1).

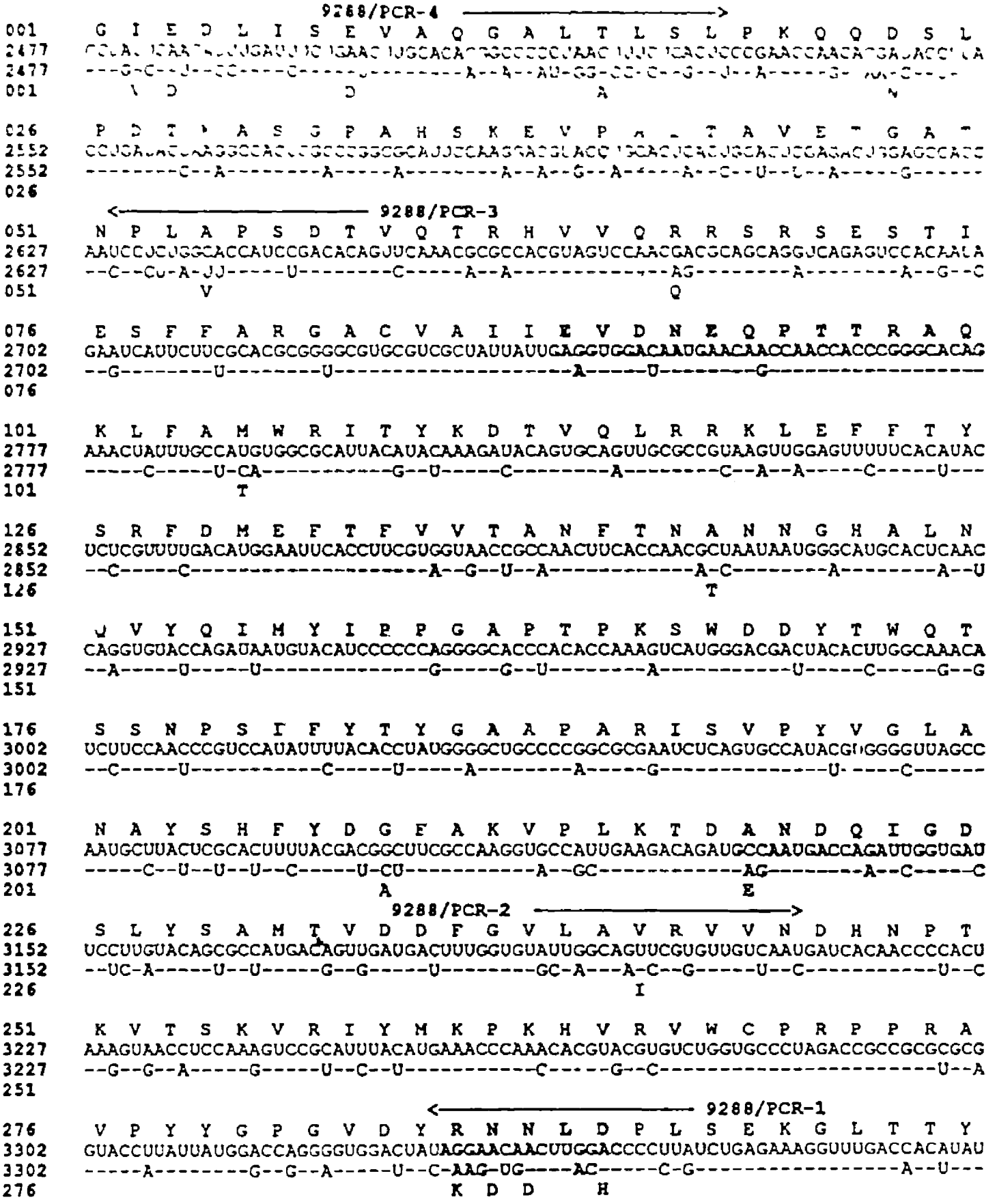

Fig 1 Comparison of the VP1 sequences of Sabin type 3 (Leon 12 a_1b) and PV3/9288/MEX89 Nucleotide (line 2) and deduced amino acid (line 1) sequences of the Sabin 3 reference are shown, nucleotide (line 3) and amino acid (line 4) differences in PV3/9288/MEX89 are indicated Sabin 3 nucleotide positions are numbered according to Stanway et al (1984), those of PV3/9288/MEX89 are numbered similarly for comparability VP1 amino acid residues are numbered 001 to 300 Sequences in bold letters identify amino acid residues that form neutralizing antigenic sites 1 (89–100), 2a (219–225) and 3a (286–290) (Minor et al , 1986b)

Selection of primer binding sites

Two separate primer pair sets were designed for the specific amplification of sequences of PV3/9288/MEX89 and related wild polioviruses Primer pairs 9288/PCR-1 + 9288/PCR-2 were designed to amplify VP1 nucleotides 3190 to 3352, yielding a 163-bp product. The second set, 9288/PCR-3 + 9288/PCR-4, amplifies VP1 nucleotides 2509 to 2650 to generate a 142-bp product (Fig. 1)

The primers matched four of the most variable genomic intervals within the VP1 region. They were generally targeted to clusters of codons having a high potential for silent genetic drift, having multiple mismatches with the Sabin 3 template, and having the potential for similar degrees of mismatch with other heterologous poliovirus templates. For example, primer 9288/PCR-2 mismatches the Sabin 3 template at 8 of 22 positions (Fig. 1) and spans an interval containing eight potentially degenerate codons (two sixfold, four fourfold, one threefold, and one twofold). Similarly, the genomic intervals covered by primers 9288/PCR-3 (containing eight potentially degenerate codons: two sixfold, four fourfold, and two twofold) and 9288/PCR-4 (containing seven potentially degenerate codons: four sixfold and three fourfold) have a high potential for silent codon drift. In contrast, 9288/PCR-1 complements a region capable of moderate silent drift (containing eight potentially degenerate codons: two sixfold, one fourfold, and five twofold), yet it mismatches the Sabin 3 template at 10 of 22 positions (Fig. 1). This high degree of mismatch occurs because 4 of the 7 codons fully complemented by 9288/PCR-1 encode amino acid differences between PV3/9288/MEX89 and Sabin 3.

All primers were designed with the 3 -terminal base matched to a third-position wobble base on the homologous template. Mismatches at the 3 -terminal base, especially those involving G:A and C:C, have the greatest potential to reduce amplification efficiency (Kwok et al., 1990). Four of the five PCR primers described here (including Sab3/PCR-1B, see below) contain either G or C at their 3 -termini. Only primer 9288/PCR-4 contained a 3′-terminal T base, which is essentially indiscriminate of mismatches (Kwok et al., 1990).

The wild genotype-specific PCR primer pairs were designed so that the amplification products (142 bp and 163 bp) could be easily resolved by electrophoresis from the products generated with our Sabin strain-specific PCR primer pairs (Sabin 1. 97 bp; Sabin 2: 71 bp; Sabin 3 53 bp, Yang et al , 1991) We generally use primer pairs that are closely spaced (<250 nucleotides) along the template because AMV reverse transcriptase has relatively low processivity (Berger et al , 1983). Diagnostic sensitivities are generally improved by reducing the lengths of the cDNA transcripts required to initiate the chain reactions

A modified Sabin 3-specific primer

Our original Sab3/PCR-1 primer yielded a 44-bp product when used with Sab3/PCR-2 (Yang et al 1991) Although the specificity of this primer set was high the 44-bp product corresponded with primer dimers (40–45 bp) produced with some primer combinations To overcome this problem, we introduced a new primer, Sab3/PCR-1B, that yields a longer product (53 bp) when coupled with

Sab3/PCR-2. The diagnostic specificity of the new primer pair is equivalent to that of the previous set.

A broadly reacting primer pair for enterovirus genomes

Highly conserved sequences within the 5'-noncoding regions of enterovirus genomes are targeted by the primer pair EV$_1$/PCR-1 + EV$_2$/PCR-2. Efficient amplification of the sequences of polioviruses (and most enteroviruses) is obtained with this pair (see below). PCR primers with similar specificities have been previously described by others (Hyypiä et al., 1989, Chapman et al., 1990, Olive et al., 1990, Rotbart, 1990). The generation of a specific 114-bp amplification product in reactions containing primers EV/PCR-1 + EV/PCR-2 confirms the presence of poliovirus (or enterovirus) RNA in the samples.

Specificity

Wild type 3 polioviruses from Mexico and Guatemala

All wild type 3 polioviruses isolated in Mexico and Guatemala since 1977 are members of a single genotype (defined as a group of polioviruses sharing $\geq 85\%$ base sequence homology within the VP1/2A region; Rico-Hesse et al., 1987), readily distinguishable by comparative nucleotide sequencing from wild polioviruses endemic to other regions (Kew et al., 1990a; Pöyry et al., 1990). Both sets of primer pairs (9288/PCR-1 + 9288/PCR-2; 9288/PCR-3 + 9288/PCR-4) were tested for their activity in amplification reaction mixtures containing genomic RNAs of wild type 3 poliovirus isolates of the Mexico-Guatemala genotype. The sequences of all isolates obtained during the period 1977–1990 were efficiently amplified with both primer sets (Fig. 2; Table 1). The chain lengths of the amplification products, estimated from their electrophoretic mobilities (Figs. 1 and 2), were in accord with the spacing of the primers along the templates (163 bp: 9288/PCR-1 + 9288/PCR-2, 142 bp: 9288/PCR-3 + 9288/PCR-4).

When the primer pair Sab3/PCR-1B + Sab3/PCR-2 was tested with the same templates from wild type 3 polioviruses, no specific amplification products were detected (Table 1). Thus type 3 isolates of the Mexico-Guatemala genotype may be recognized as wild by either of two properties: (1) the nonamplification of their templates with the Sabin strain-specific primer pairs (Yang et al., 1991), or (2) the specific amplification of their templates with the genotype-specific PCR primer sets to yield the characteristic 142-bp and 163-bp products.

The 114-bp group product was generated with all poliovirus templates when amplifications were performed with the primers EV/PCR-1 + EV/PCR-2 (Tables 1–3).

Vaccine-related isolates

Primer pairs 9288/PCR-1 + 9288/PCR-2 and 9288/PCR-3 + 9288/PCR-4 were further tested for their capacities to yield specific products with templates from vaccine-related poliovirus isolates. The wild genotype-specific primers were consistently negative for activity with templates from vaccine-related isolates (Fig. 3,

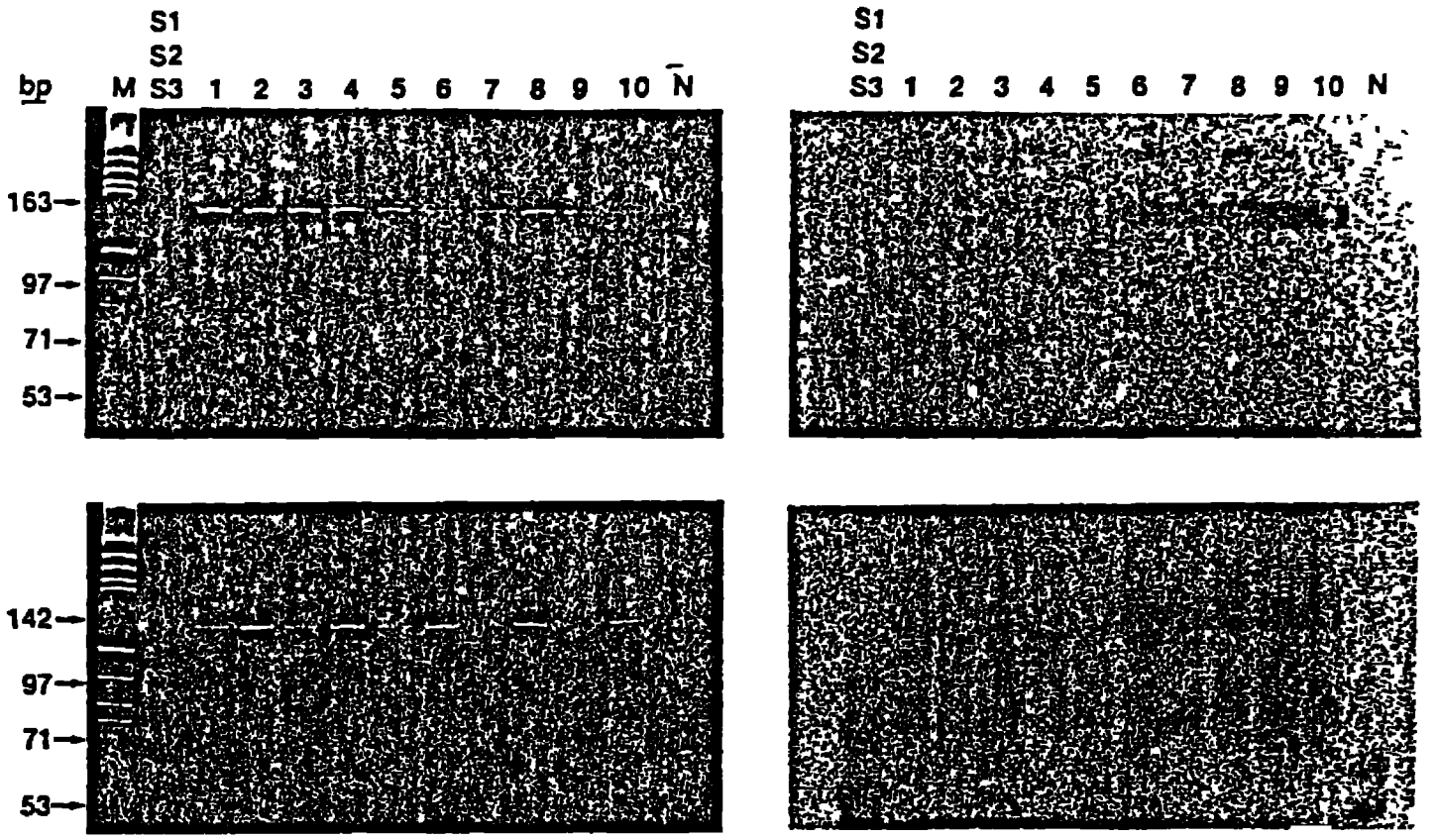

Fig. 2. Specific PCR amplification of the genomes of wild type 3 clinical isolates from Mexico and Guatemala (Table 1). Viral RNAs were extracted directly from clarified freeze-thaw lysates of infected cells and amplified in reaction mixtures containing 9288/PCR-1+9288/PCR-2 (upper panels) and 9288/PCR-3+9288/PCR-4 (lower panels). In the amplification reactions 9288/PCR-1 and 9288/PCR3 were unlabeled; 9288/PCR-2 and 9288/PCR-4 were 5′ end labeled with ^{32}P. After 30 amplification cycles, DNA products were separated by electrophoresis on 12.5% polyacrylamide gels and visualized by ethidium bromide fluorescence (left panels) or autoradiography of the dried gels (right panels). Sources of templates in reaction mixtures are given after each lane number: 1: 0380, 2: 0326, 3: 0248, 4: 0179, 5. 9849, 6: 9379, 7: 9288, 8 8697, 9. 6887, 10: 7134; M. molecular weight markers (Hae III digest of pBR322, base pair lengths: 587/540/504/458/434/267/234/213/192/184/124/123/104/89/64/57/51/21/18/11/8, Boehringer Mannheim); S1, S2, S3. reaction mixtures containing all three Sabin strain RNAs amplified with the Sabin 1 and 2 strain-specific PCR primers (Yang et al., 1991) and the modified Sabin 3 primer pair described in Materials and Methods; N: reaction mixtures lacking RNA.

Table 2). These observations follow from the multiple mismatches (at ≥ 6 of 22 nucleotide positions) of each primer for the corresponding sites on the vaccine strain templates (Toyoda et al, 1984).

The primer pair Sab3/PCR-1B + Sab3/PCR-2 supported the exclusive amplification of Sabin 3-related templates, yielding a 53-bp product (Table 2). The high degree of primer mismatch at homologous sites (Sab3/PCR-1B. 10 with Sabin 1, 9 with Sabin 2, Sab3/PCR-2: 10 with Sabin 1. 13 with Sabin 2; Toyoda et al, 1984, Yang et al, 1991) probably eliminates cross-amplifications with templates derived from the types 1 and 2 Sabin strains

Other wild poliovirus isolates

When the genomic RNAs of recent wild poliovirus isolates representing all known American genotypes were tested for amplification with primer pairs 9288/PCR-1 + 9288/PCR-2 and 9288/PCR-3 + 9288/PCR-4, no specific prod-

TABLE 1

Wild type 3 poliovirus[es] from Mexico and Guatemala characterized by PCR

Strain [a]	Origin	Amplification products [b]				Donor [c]
		114bp	53bp	142bp	163bp	
PV3/0380/MEX90	Tomatlan Jalisco	+	−	+	+	JRG
PV3/0379/MEX90	Cihuatlán Jalisco	+	−	+	+	JRG
PV3/0377/MEX90	Tecomán Colima	+	−	+	+	JRG
PV3/0327/MEX90	Tecomán Colima	+	−	+	+	JRG
PV3/0326/MEX90	Tecomán, Colima	+	−	+	+	JRG
PV3/0281/GUT90	San Juan Sacatepéquez	+	−	+	+	PC
PV3/0248/GUT90	Chimaltenango	+	−	+	+	PC
PV3/0179/GUT90	Totonicapán	+	−	+	+	PC
PV3/0174/MEX90	Navolato, Sinaloa	+	−	+	+	JRG
PV3/0111/MEX90	Guasave, Sinaloa	+	−	+	+	JRG
PV3/9933/MEX89	Hermosillo, Sonora	+	−	+	+	JRG
PV3/9932/MEX89	Hermosillo, Sonora	+	−	+	+	JRG
PV3/9931/MEX89	El Fuerte, Sinaloa	+	−	+	+	JRG
PV3/9849/MEX89	Hermosillo, Sonora	+	−	+	+	JRG
PV3/9846/MEX89	Guasave, Sinaloa	+	−	+	+	JRG
PV3/9379/MEX89	Culiacán, Sinaloa	+	−	+	+	JRG
PV3/9289/MEX89	Guasave, Sinaloa	+	−	+	+	JRG
PV3/9288/MEX89 [d]	Navolato, Sinaloa	+	−	+	+	JRG
PV3/9159/MEX89	Culiacán, Sinaloa	+	−	+	+	JRG
PV3/9160/MEX89	Navolato, Sinaloa	+	−	+	+	JRG
PV3/8923/MEX88	Acapulco, Guerrero	+	−	+	+	JRG
PV3/8698/MEX88	Zihuatenejo, Guerrero	+	−	+	+	JRG
PV3/8697/MEX88	Acapulco, Guerrero	+	−	+	+	JRG
PV3/7124/MEX87	—	+	−	+	+	MLZ
PV3/6887/GUT83	Escuintla	+	−	+	+	JRC
PV3/1565/USA80	Oregon (México, D.F) [f]	+	−	+	+	MHH
PV3/7134/MEX77	Nuevo Leon	+	−	+	+	MLZ

[a] All polioviruses were independently characterized by partial genomic sequencing.

[b] Amplification products of indicated chain length present (+) or absent (−) when reactions were performed in the presence of the following primer pairs:
114 bp. EV/PCR-1 + EV/PCR-2
53 bp: Sab3/PCR-1B + Sab3/PCR-2
142 bp: 9288/PCR-3 + 9288/PCR-4
163 bp: 9288/PCR-1 + 9288/PCR-2

[c] Donors: A. Alava, J. Boshell, P. Cáceres, J R. Cruz, K. Dave, S. Gu, M H. Hatch, J C. Hierholzer, T. Hovi, T. Jacob John, J. Kapsenberg, R. Méndez López, M.J. Oliveira, M A. Pallansch, J. Ruiz Gómez, R Salas, E. da Silva, A Vernon, M L. Zárate NIH: NIAID/NIH/USPHS.

[d] Reference isolate for design of 9288/PCR primer sets.

[e] City and state of origin unknown.

[f] Wild type 3 poliovirus isolated from United States citizen whose onset of paralysis occurred shortly after return from Mexico City

ucts were obtained (Fig. 4, Table 3). Moreover, no amplifications occurred when these templates were tested with the Sabin 3-specific primer pair, Sab3/PCR-1 B + Sab3/PCR-2 (Table 3), a result similar to our earlier observations with the

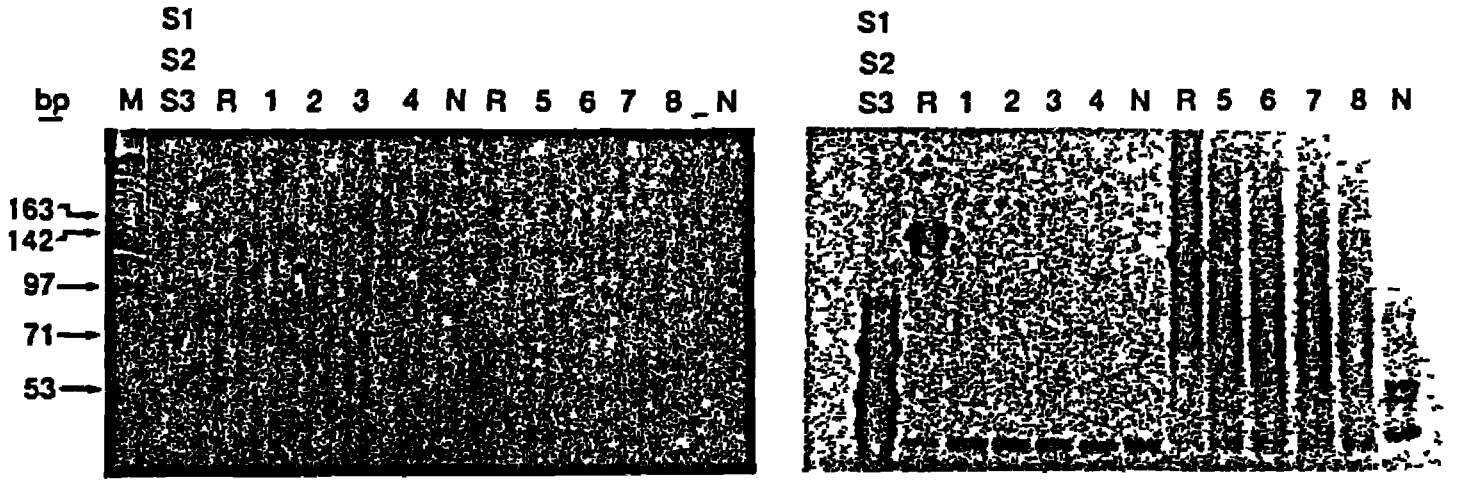

Fig 3. Nonamplification of the genomic sequences of vaccine-related isolates (Table 1) by the primer pairs 9288/PCR-1+9288/PCR-2 (lanes 1–4) and 9288/PCR-3+9288/PCR-4 (lanes 5–8) Experimental conditions were as described in Fig. 2. Products were visualized after polyacrylamide gel electrophoresis by ethidium bromide fluorescence (left panel) and autoradiography (right panel). Sources of templates in reaction mixtures are given after each lane number Sabin 1-related isolate. 1,5. 0246, Sabin 2-related isolate 2,6 9897; Sabin 3-related isolates. 3,7. 0131, 4,8 0044, M molecular weight markers; S1, S2, S3. reaction mixtures containing all three Sabin strain RNAs amplified with the Sabin 1 and 2 strain-specific PCR primers (Yang et al, 1991) and the modified Sabin 3 primer pair described in Materials and Methods, R: reaction mixtures containing 9288 RNA; N reaction mixtures lacking RNA.

other Sabin strain-specific PCR primer sets (Yang et al, 1991). The absence of amplification with these templates is attributable to the specificities of the primers, rather than to insufficiencies of RNA, because amplification with all templates occurred with the broadly reacting enterovirus primer pair, EV/PCR-1+EV/PCR-2 (Table 3).

Comparable results were obtained with representatives of independent wild genotypes from other regions (Fig. 4, Table 3). The exception was a 1983 isolate from Spain, PV3/V3/SPA83, whose RNA reacted with 9288/PCR-3+

TABLE 2

Vaccine-related polioviruses characterized by PCR

Strain [a]	Origin	Amplification products [b]				Donor [c]
		114bp	53bp	142bp	163bp	
PV1/0246/GUT90	Amatitlán	+	–	–	–	PC
PV1/8316/MEX88	México, D F	+	–	–	–	JRG
PV2/9897/GUT90	Chimaltenango	+	–	–	–	PC
PV2/9364/GUT89	San José Pinula	+	–	–	–	PC
PV3/0040/ELS90	Ilobasco, El Salvador	+	+	–	–	PC
PV3/0131/MEX89	Guasave Sinaloa	+	+	–	–	JRG
PV3 0044 GUT89	San Pedro Jocopilas	+	+	–	–	PC
PV3 9896 GUT89	Chimaltenango	+	+	–	–	PC
PV3 9447 MEX89	Culiacan Sinaloa	+	+	–	–	JRG
PV3 9441 GUT89	Amatitlan	+	+	–	–	PC

[a-c] See footnotes to Table 1

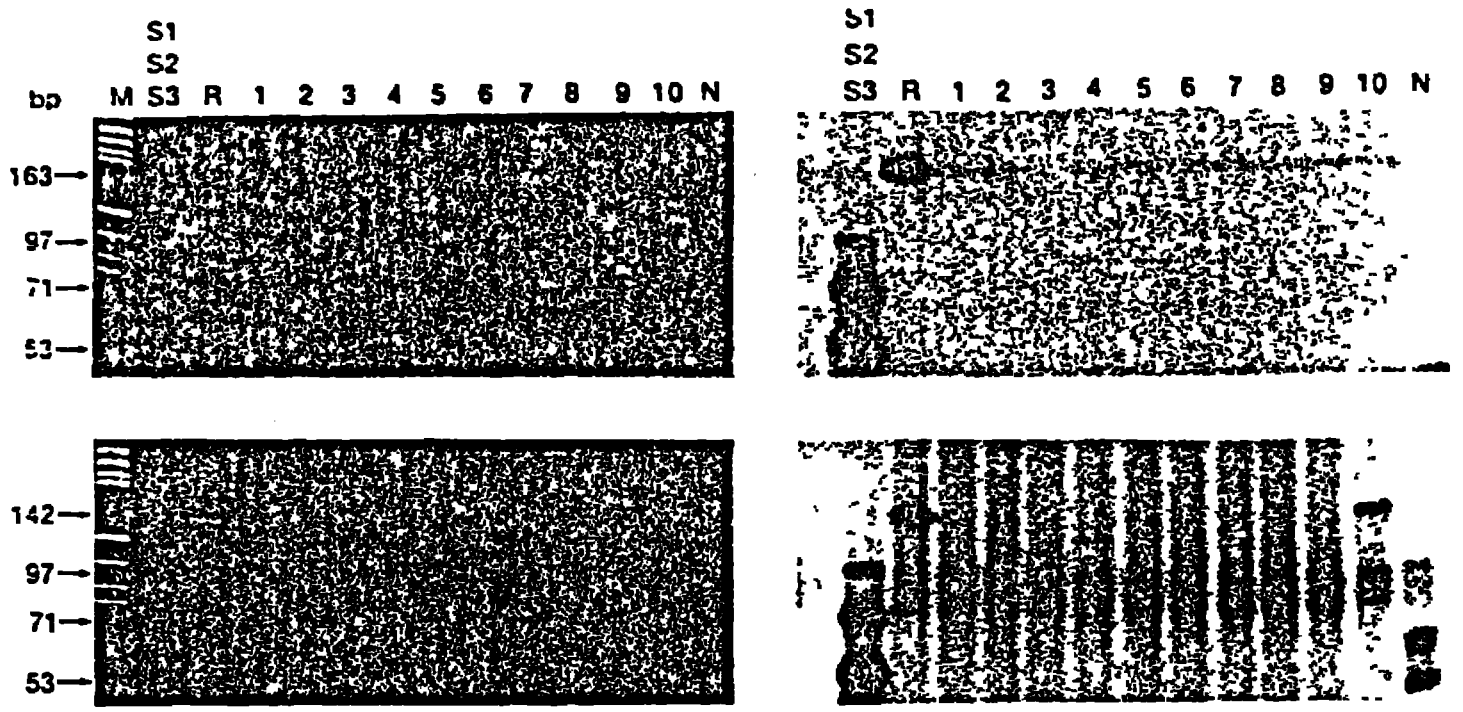

Fig. 4 Nonamplification of the genomic sequences of wild polioviruses unrelated to the type 3 genotype of Mexico and Guatemala (Table 2) in reactions containing 9288/PCR-1+9288/PCR-2 (upper panel) and 9288/PCR-3+9288/PCR-4 (lower panel). Experimental conditions were as described in Fig. 2. DNA products were separated by electrophoresis on 12.5% polyacrylamide gels and visualized by ethidium bromide fluorescence (left panels) or autoradiography (right panels). Sources of templates in reaction mixtures are given after each lane number: Type 1 isolates: 1: 9025, 2. 7166, Type 2 isolates: 3: 0176, 4: 7079; Type 3 isolates: 5: 9033, 6. 8854, 7: 8178, 8: 7840, 9: 7095, 10: V3; M. molecular weight markers, S1, S2, S3. reaction mixtures containing all three Sabin strain RNAs amplified with the Sabin 1 and 2 strain-specific PCR primers (Yang et al., 1991) and the modified Sabin 3 primer pair described in Materials and Methods; R: reaction mixtures containing 9288 RNA; N: reaction mixtures lacking RNA.

9288/PCR-4 to generate, at reduced yields, a 142-bp product (Fig. 4). This result was unexpected since the genomes of PV3/V3/SPA83 and PV3/9288/MEX89 differed by 24.6% within the interval amplified, and both primers mismatched the template at several positions (9288/PCR-3: 6 mismatches; 9288/PCR-4: 7 mismatches; data not shown). Restriction analysis of the product amplified from the PV3/V3/SPA83 template confirmed that the amplified sequences were derived from the PV3/V3/SPA83 VP1. In contrast, no amplification was observed with the primer pair 9288/PCR-1 + 9288/PCR-2 (Fig. 4). Under most surveillance conditions, such cross-amplifications would have little significance, because importation and spread of unrelated cross-amplifying strains is probably very rare (Kew et al, 1990a). However, if higher diagnostic specificities are required, both primer sets may be used in parallel, and only those isolates whose RNAs are amplified with both sets considered to be unambiguously identified as members of the Mexico-Guatemala type 3 genotype.

Nonpolio enteroviruses

Several serotypes of nonpolio enteroviruses (NPEV) are occasionally associated with cases of acute paralytic illness (Melnick, 1990, Hayward et al, 1989; Figueroa

TABLE 3

Heterologous wild polioviruses characterized by PCR

Strain [a]	Origin	Amplification products [b]				Donor [c]
		114bp	53bp	142bp	163bp	
PV1/0116/ECU90	Guayas, Ecuador	+	–	–	–	JB
PV1/9475/ZAI89	Kinshasa, Zaire	+	–	–	–	AV
PV1/9025/BRA88	Pernambuco, Brazil	+	–	–	–	EdS
PV1/7166/MEX86	México, Mexico	+	–	–	–	MLZ
PV1/7064/IND86	Bombay, India	+	–	–	–	KD
PV1/0109/CHN86	Nanning, China	+	–	–	–	SG
PV2/0176/PER89	Arequipa, Peru	+	–	–	–	EdS
PV2/7079/IND86	Bombay, India	+	–	–	–	KD
PV2/6876/COL86	Medellín, Colombia	+	–	–	–	JB
PV2/1534/IND82	Vellore, India	+	–	–	–	TJJ
PV3/9033/BRA88	Belém, Brazil	+	–	–	–	MJO
PV3/8854/COL88	Magdalena, Colombia	+	–	–	–	JB
PV3/8178/VEN88	Valencia, Venezuela	+	–	–	–	RS
PV3/7840/PER86	Lima, Peru	+	–	–	–	RML
PV3/7105/IND86	Aurangabad, India	+	–	–	–	KD
PV3/7095/IND86	Bombay, India	+	–	-	–	KD
PV3/6699/ECU86	Ecuador	+	–	–	–	AA
PV3/6184/FIN84	Vantaa, Finland	+	–	–	–	TH
PV3/V3/SPA83	Barcelona, Spain	+	–	+	–	JK
PV3/V1/EGY82	Egypt	+	–	–	–	JK

[a,b,c] See footnotes to Table 1.

et al., 1989). We tested the genomic templates of representatives of these NPEV serotypes for activity in PCR reactions separately containing four different PCR primer sets (9288/PCR-1 + 9288/PCR-2; 9288/PCR-3 + 9288/PCR-4; Sab3/PCR-1B + Sab3/PCR-2; EV/PCR-1 + EV/PCR-2). All NPEV templates except echovirus type 22 (EV22) yielded the predicted 114-bp products in reactions containing EV/PCR-1 + EV/PCR-2 (Fig. 5; Table 4). The 5′-noncoding sequences of EV22 (and EV23) are quite divergent from those of the major group of human enteroviruses (Coller et al., 1990; Hyypiä et al., 1992).

Amplification reaction mixtures containing poliovirus-specific primers did not yield detectable specific products with any of the NPEV templates (Table 4).

Selective amplification of wild poliovirus sequences in samples containing virus mixtures

Mixed poliovirus or NPEV infections are very common among children in countries where poliomyelitis is endemic (Parks et al., 1967). The frequency of mixed infections raises the concern that wild polioviruses in clinical specimens may be masked by high titers of other enteric viruses, especially vaccine-derived polioviruses. In principle, PCR offers an efficient systematic approach for the

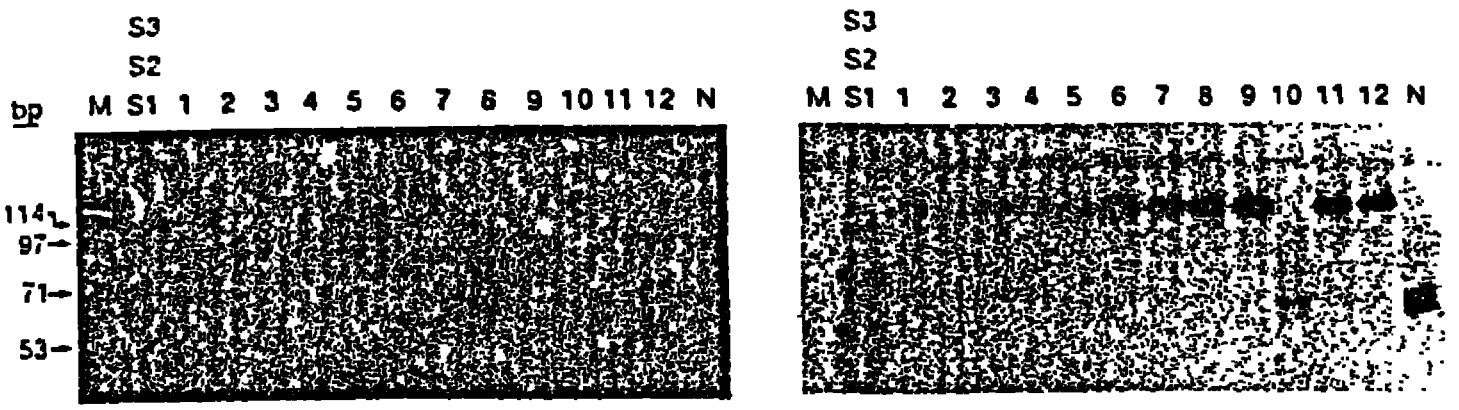

Fig. 5. Amplification of the genomic sequences of polioviruses and NPEV (Table 4) in reactions containing EV/PCR-1+EV/PCR-2. Experimental conditions were as described in Fig. 2. DNA products were separated by electrophoresis on 12.5% polyacrylamide gels and visualized by ethidium bromide fluorescence (left panel) or autoradiography (right panel). Sources of templates in reaction mixtures are given after each lane number: 1: Sab1, 2: Sab2, 3: Sab3, 4: CVA7, 5: CVA16, 6: CVB2, 7: CVB3, 8: CVB4, 9: EV11, 10: EV22, 11: EV70, 12: EV71; M: molecular weight markers; S1, S2, S3: reaction mixtures containing all three Sabin strain RNAs amplified with the Sabin 1 and 2 strain-specific PCR primers (Yang et al., 1991) and the modified Sabin 3 primer pair described in Materials and Methods; N: reaction mixtures lacking RNA.

selective detection of wild polioviruses in mixed populations. To evaluate the potential for differential amplification of wild poliovirus sequences by PCR, we mixed a constant quantity (1 pg; equivalent to ≈250,000 RNA molecules) of purified RNA from PV3/9288/MEX89 with varying amounts of Sabin 3 RNA in 10-fold concentration steps (1 pg to 1 μg; representing wild:vaccine template

TABLE 4

Nonpolio enteroviruses characterized by PCR

Strain [a]	Origin	Amplification products [b]				Donor [c]
		114bp	53bp	142bp	163bp	
CVA7/ABU/USSR	NIH Reference	+	−	−	−	NIH
CVA9/Griggs	NIH Reference	+	−	−	−	NIH
CVA16/G-10	Prototype	+	−	−	−	MAP
CVB2/Ohio-1	Prototype	+	−	−	−	MAP
CVB3/0101/USA90	Myocarditis case	+	−	−	−	MAP
CVB4/JVB	Prototype	+	−	−	−	MAP
CVB5/Faulkner	Prototype	+	−	−	−	MAP
EV2/Cornelis	Prototype	+	−	−	−	MAP
EV4/Pesascek	Prototype	+	−	−	−	MAP
EV6/D'Amori	Prototype	+	−	−	−	MAP
EV9/Hill	Prototype	+	−	−	−	MAP
EV11/Gregory	Prototype	+	−	−	−	MAP
EV22/6764/USA86	Paralytic case	−	−	−	−	MAP
EV30/Bastianni	Prototype	+	−	−	−	MAP
EV70/J670-71	Prototype	+	−	−	−	JCH
EV71/7423/USA87	Paralytic case	+	−	−	−	MAP

[a] CV = coxsackievirus; EV = echovirus or enterovirus (70, 71).

[b,c] See footnotes to Table 1.

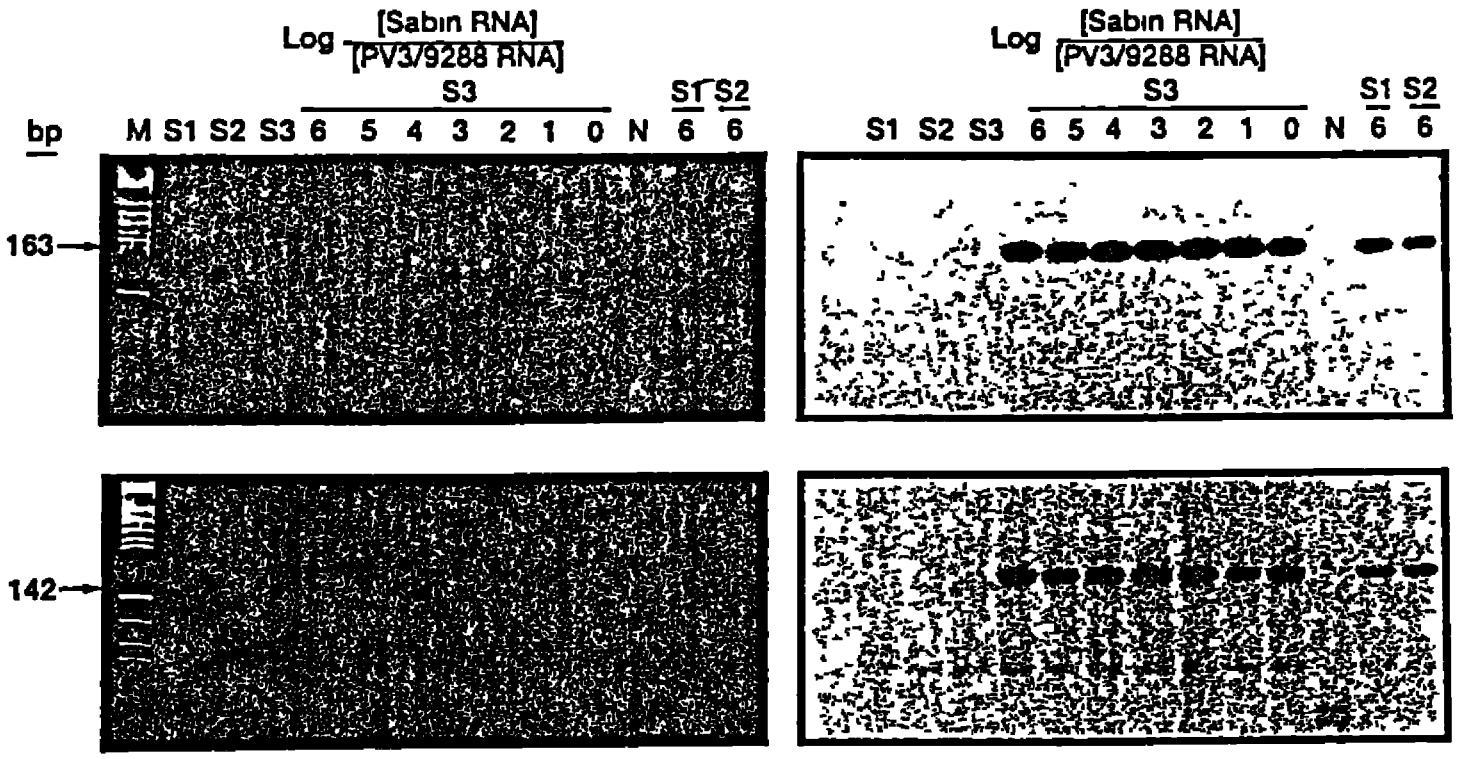

Fig 6. Selective detection of PV3/9288/MEX89 RNA in the presence of excess Sabin poliovaccine templates Serial 10-fold dilutions of purified Sabin 3 RNA (1 μg to 1 pg), or high template concentrations (1 μg) of Sabin 1 and 2 RNAs, were mixed with a constant amount of PV3/9288/MEX89 RNA (1 pg) and amplification reactions were performed using 9288/PCR-1+9288/PCR-2 (upper panels) and 9288/PCR-3+9288/PCR-4 (lower panels). The logarithm of the molar ratio of Sabin template to PV3/9288/MEX89 template is indicated above each sample lane. Amplifications and analyses of products were as described in Fig 2. Products were visualized after polyacrylamide gel electrophoresis by ethidium bromide fluorescence (left panels) or autoradiography of ^{32}P-labeled products (right panels).

ratios of 1:1 to 1:10^6). PV3/9288/MEX89 RNA was also mixed at a 1:10^6 ratio (1 pg:1 μg) with purified RNAs from Sabin 1 or Sabin 2. In reaction mixtures containing either of the two primer sets, 9288/PCR-1 + 9288/PCR-2 or 9288/PCR-3 + 9288/PCR-4, only the sequences of PV3/9288/MEX89 were amplified (Fig. 6). The presence of heterologous RNA templates at very high (10^6-fold) stoichiometric ratios did not impair the specific amplification of the wild poliovirus sequences

Discussion

The nucleotide sequences of contemporary genotypes of wild polioviruses are very heterogeneous (Rico-Hesse et al., 1987 Kew et al., 1990a) The extent of this diversity is indicated by comparisons of VP1 nucleotide sequences, where base substitution differences among separate genotypes of the same serotype typically exceed 20% (Toyoda et al 1984, Hughes et al., 1986, La Monica et al., 1986 da Silva et al., 1990b) As a result, it is generally possible to identify intervals within the VP1 region (or other capsid regions) that contain sequences characteristic of a

small number (possibly only one) of genotypes. Because poliovirus genomes evolve primarily by the accumulation of mutations to synonymous codons, regions most likely to contain genotype-specific sequences are those containing runs of highly degenerate codons. As shown in this study, unrelated polioviruses rarely share sequences in more than one variable region. Therefore, PCR identifications requiring efficient priming by two selective primers are highly specific for genotype. To avoid ambiguous identifications arising from recombination among different poliovirus genotypes, we favor primers that are targeted to capsid sequences (Yang et al., 1991), where genetic crossovers appear to be infrequent (Minor et al., 1986a, King, 1988; Kew et al., 1990b).

The development of the PCR primer sets described here illustrates our general approach for building a library of wild genotype-specific PCR primers. Development of these specific primers represents an especially rigorous test of this approach, because the sequences of the type 3 polioviruses from Mexico and Guatemala match those of Sabin 3 better than other wild poliovirus genotypes (Kew et al., 1990b; Pöyry et al., 1990). These similarities may reflect an evolutionary relationship, now very distant, between the contemporary Mexican and Guatemalan viruses and the wild parent to the Sabin 3 strain, PV3/Leon/USA37, isolated in Los Angeles in 1937 (Sabin and Boulger, 1973).

Most of our primers match codons of domains that are internalized in native virions (Hogle et al., 1985; Filman et al., 1989; Fricks and Hogle, 1990) and are probably under little or no pressure from immune selection (Minor et al., 1986a; Kinnunen et al., 1990). The chief exception is primer 9288/PCR-1, which complements codons of neutralizing antigenic site 3a (Minor et al., 1986b). However, the sequences of site 3a have been conserved among recent type 3 isolates from Mexico and Guatemala (De et al., in preparation). Furthermore, the variability of site 3a can be readily monitored by sequence analyses, using the broadly reacting primer Polio/2A, thereby permitting the rapid updating of primer 9288/PCR-1.

The methods described here are an improvement of our earlier procedures (Yang et al., 1991). Amplification reactions are performed in a single tube, thus reducing the potential for carryover of exogenous template (Kwok and Sninsky, 1989). Inclusion of the co-solvent NP-40 in the reactions improves specificity, enhancing the yield of the specific product bands with homologous templates while reducing the production of background bands with heterologous templates. Despite these refinements, we still obtained cross-amplification of a heterologous template with one primer set. Cross-amplifications may sometimes be suppressed by applying more stringent amplification conditions (e.g., shorter annealing times at higher temperatures). If the genetic heterogeneity among all members of a genotype is very limited, more stringent PCR conditions may be used without loss of diagnostic sensitivity. However, with more heterogeneous genotypes it is generally preferable to accept slightly reduced specificities in favor of greater sensitivities. Cross-amplifications among heterologous genotypes are usually of little practical importance, as the separate genotypes are geographically restricted (Rico-Hesse et al., 1987; Kew et al., 1990a). Thus, for most regional laboratories, it should be sufficient to prepare primers selective for the polioviruses commonly encountered

(e g , Sabin strains; indigenous wild viruses). Very high specificities are required of the Sabin-specific primer and probe sets (Yang et al , 1991), which are designed for use worldwide (following the global distribution of oral poliovaccine), and which must not support amplification of any template derived from a contemporary wild poliovirus.

Wild genotype-specific PCR primers are routinely used in the surveillance for wild polioviruses in the Americas. Because wild polioviruses are of the greatest epidemiologic importance, samples are immediately screened by PCR for the presence of wild virus sequences, which can be detected in < 8 h In addition to the primer sets described here, we have prepared additional sets that are specific for all other major genotypes recently endemic to the Americas (Brazil types 1 and 3; Colombia-Ecuador-Peru type 1; de Quadros et al., 1991; E. da Silva, C.F. Yang, L. De, and O. Kew, unpublished results).

The exceptional selectivity of in vitro amplification may open the way for development of a parallel surveillance system for wild poliovirus transmission based upon environmental sampling. The potential sensitivity of such a system is very high. In unimmunized communities, most ($> 99\%$) wild poliovirus infections are clinically inapparent (Melnick, 1990). The proportion of subclinical infections may be even higher in populations exposed to poliovaccine (Pöyry et al., 1988). Before the introduction of oral poliovaccine, transmission of polioviruses could readily be detected by sewage sampling (Melnick, 1947). The combination of sewage sampling and direct detection of wild poliovirus genomes by PCR may again permit recognition of virus circulation prior to the appearance of paralytic cases. Because the generation of amplification products of specific chain lengths may be the only evidence for the presence of wild polioviruses in environmental samples, at least two independent primer pairs should be used: one set for the initial screening and a second set for confirmation. The feasibility of this approach was recently demonstrated by the direct detection of wild poliovirus sequences in samples of wastewater from a community where a paralytic case had occurred a month earlier (G. Tambini et al., unpublished results). Additional studies are necessary to evaluate the potential role, within the global program to eradicate poliomyelitis (Pan American Health Organization, 1985; World Health Assembly, 1988), of environmental surveillance for wild poliovirus transmission

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