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Identification of Vaccine-Related Polioviruses by Hybridization with Specific RNA Probes

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We developed RNA probes for the identification of poliovirus isolates by blot hybridization. Two sets of vaccine strain-specific probes were prepared. They complemented variable genomic domains within (i) the 5′-untranslated region and (ii) the amino-terminal codons of VP1. An enterovirus group probe (EV/5UT) matching highly conserved 5′-untranslated region sequences was used to estimate the quantities of poliovirus (or enterovirus) RNA in the samples. Poliovirus sequences amplified from Sabin strain virion RNA templates by PCR were inserted into the pUC18 plasmid vector. The antisense PCR primer for each probe set contained sequences encoding a T7 promoter. Hybrids were detected by a sensitive nonisotopic method. RNA probes were labeled by incorporation of digoxigenin-uridylate into the transcripts. The binding of probe to immobilized poliovirus RNAs was visualized by hydrolysis of the chemiluminescent substrate 4-methoxy-4-(3-phosphate-phenyl)-spiro-(1,2-dioxetane-3,2′-adamantane) catalyzed by alkaline phosphatase conjugated to anti-digoxigenin (Fab) fragments. The specificities of the probes were evaluated with a panel of poliovirus isolates that had previously been characterized by sequence analysis. The RNAs of vaccine-related isolates hybridized with the appropriate probe sets. Wild polioviruses representing a broad spectrum of contemporary genotypes were recognized by the inabilities of their genomes to form stable hybrids with the Sabin strain-specific probes.

Polioviruses have been identified by the molecular methods of RNase T_1 oligonucleotide fingerprinting (23, 32, 34), partial genomic sequencing (21, 40, 41), and hybridization with synthetic oligodeoxynucleotide probes (10). More recently, the high degree of sensitivity of in vitro amplification by PCR has been used to identify polioviruses either by amplification with genotype-specific primers (59, 60) or by restriction fragment length polymorphism analysis of the products amplified with broadly reacting primers (1). The molecular methods are complemented by techniques that measure differences in the neutralizing antigens of vaccine-related and wild polioviruses detected with cross-absorbed polyclonal antibodies (15, 54) or panels of monoclonal antibodies (9, 11, 36). While serologic methods have been the accepted standards in viral diagnostics, molecular approaches, with their greater specificities, sensitivities, and speed (10, 59), have become increasingly accessible with the development of improved nonisotopic detection systems (20).

Among the available molecular methods, nucleic acid probe hybridization appears to have the widest diagnostic potential. We have previously described synthetic oligodeoxynucleotide probes (21 to 27 nucleotides in length) that specifically hybridize to the RNAs of Sabin strain-related isolates or various genotypes of wild polioviruses (10). However, the widespread use of synthetic oligodeoxynucleotide probes in poliovirus diagnostics is countered by several important limitations. First, oligodeoxynucleotides must be prepared by organic synthesis by technologies not readily available in most countries where polio is endemic. Second, the genomic intervals sampled by the synthetic probes are short, potentially decreasing diagnostic accuracy by increasing the chances for localized sequence similarities at the probe-binding sites to yield cross-hybridizations among otherwise dissimilar poliovirus genomes. Third, oligodeoxynucleotides form hybrids having only moderate thermal stabilities, such that stringencies are controllable over comparatively narrow temperature ranges. Finally, oligodeoxynucleotide probes are restricted in the number of sites available for reporter molecules.

To overcome these limitations, we prepared sets of poliovirus genotype-specific RNA probes, transcribed from recombinant DNA plasmids (30), which can be used to identify representatives of specific poliovirus genotypes. We describe here the properties of RNA probes that specifically recognize the genomes of Sabin strain-related polioviruses and which have been adapted to a sensitive enzyme-linked immunosorbent assay (ELISA) that uses either chemiluminescent or histochemical substrates for the detection of hybrids (16, 50).

MATERIALS AND METHODS

Viruses. The poliovirus isolates (Table 1) were characterized previously by neutralization with hyperimmune equine sera and partial genomic sequencing (21, 41). In addition, three sets of poliovirus reference strains were examined: (i) the Sabin oral polio vaccine strains LSc 2ab (Sabin 1), P712 Ch 2ab (Sabin 2), and Leon 12 a_1b (Sabin 3) (obtained at passage levels of Sabin Original+2 [46]), (ii) strains Mahoney, MEF-1, and Saukett of the inactivated polio vaccine (48), and (iii) the original serotype reference strains Brunhilde, Lansing, and Leon (4). Viruses were propagated in RD (human rhabdomyosarcoma) cell monolayers to produce high-titer inoculation stocks.

Oligonucleotide synthesis. Before selection of the genomic intervals to be amplified for preparation of antisense RNA probes, the thermodynamic stabilities and potential secondary structures of candidate probes were predicted with the aid of the OLIGO 4.0 computer program (National Biosciences, Plymouth, Minn. [13]).

Synthetic oligodeoxynucleotide primers (Table 2) were prepared, purified, and analyzed as described previously (59). The PCR primers contained sequences for three-nucleotide spacers at their 5′ ends, single *Eco*RI or tandem *Hin*dIII recognition sites, T7 promoter sequences (antisense primers only), and intervals (18 to 23 nucleotides in length) specifically matching poliovirus RNA templates (Fig. 1).

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TABLE 1. Polioviruses characterized by probe hybridization

Isolate type and isolate[a]	Sequence			Isolate	Sequence		
	Well[b]	Type[c]	Donor[d]		Well	Type[c,e]	Donor
Type 1 isolates							
PV1/0584/GUT91	1A	P1S	PC	PV1/5136/ETH93	3A	P1W	JA
PV1/0246/GUT90	1B	P1S	PC	PV1/3894/EGY92	3B	P1W	TN
PV1/9825/USA89	1C	P1S	MAP	PV1/3865/IND91	3C	P1W	AvL
PV1/9703/ELS89	1D	P1S	JRC	PV1/3862/TOG92	3D	P1W	AvL
PV1/9360/VEN89	1E	P1S	RS	PV1/3653/CHN91	3E	P1W	LBZ
PV1/9240/HON89	1F	P1S	PC	PV1/3643/CHN91	3F	P1W	LBZ
PV1/0074/PER88	1G	P1S	LB	PV1/1184/ROM91	3G	P1W	AvL
PV1/8316/MEX88	1H	P1S	JRG	PV1/0467/COL91	3H	P1W	JB
PV1/8315/MEX88	2A	P1S	JRG	PV1/3727/AZB90	4A	P1W	GYL
PV1/8284/HON88	2B	P1S	CMM	PV1/2786/VTN90	4B	P1W	PVT
PV1/8221/GUT87	2C	P1S	PC	PV1/9475/ZAI89	4C	P1W	AV
PV1/7245/COL86	2D	P1S	JB	PV1/9366/SAA89	4D	P1W	RS
PV1/6529/CHI86	2E	P1S	OP	PV1/1607/SOA88	4E	P1W	BDS
PV1/6440/ARG85	2F	P1S	AN	PV1/9026/BRA88	4F	P1W	EdS
PV1/6258/MOR85	2G	P1S	SN	PV1/0289/POR87	4G	P1W	AvL
PV1/5498/USA84	2H	P1S	MHH	PV1/8223/GUT87	4H	P1W	PC
Type 2 isolates							
PV2/0636/ELS91	5A	P2S	JRC	PV2/3874/IND92	7A	P2W	PM
PV2/0042/ELS90	5B	P2S	JRC	PV2/3849/PAK91	7B	P2W	AvL
PV2/9897/GUT90	5C	P2S	PC	PV2/3845/EGY91	7C	P2W	TN
PV2/0078/PER89	5D	P2S	LB	PV2/3819/UZB90	7D	P2W	GYL
PV2/9819/BRA89	5E	P2S	EdS	PV2/0176/PER89	7E	P2W	JB
PV2/9818/PER89	5F	P2S	LB	PV2/2613/PAK89	7F	P2W	TH
PV2/9579/USA89	5G	P2S	MAP	PV2/7079/IND86	7G	P2W	KD
PV2/9364/GUT89	5H	P2S	PC	PV2/6876/COL86	7H	P2W	JB
PV2/8378/PER88	6A	P2S	LB	PV2/1534/IND82	8A	P2W	TJJ
PV2/8238/GUT87	6B	P2S	PC	PV2/0301/CAM80	8B	P2W	AvL
PV2/8018/GUT87	6C	P2S	PC	PV2/0298/EGY79	8C	P2W	AvL
PV2/7653/SOA86	6D	P2S	BDS	PV2/6683/YUG78	8D	P2W	AvL
PV2/7170/MEX86	6E	P2S	MLZ	PV2/0295/ISR78	8E	P2W	AvL
PV2/6700/HON86	6F	P2S	CMM	PV2/2996/SWE77	8F	P2W	JHN
PV2/7837/PER84	6G	P2S	RML	PV2/0290/TUR73	8G	P2W	AvL
PV2/6886/GUT83	6H	P2S	JRC	PV2/0710/KEN71	8H	P2W	BN
Type 3 isolates							
PV3/1063/USA91	9A	P3S	MAP	PV3/3899/EGY92	11A	P3W	TN
PV3/0644/HON91	9B	P3S	JRC	PV3/3873/IND92	11B	P3W	AvL
PV3/0642/ELS91	9C	P3S	JRC	PV3/3904/SYR91	11C	P3W	TN
PV3/0405/GUT90	9D	P3S	PC	PV3/0672/OMA91	11D	P3W	RS
PV3/0040/ELS90	9E	P3S	JRC	PV3/4076/ARM90	11E	P3W	GYL
PV3/0131/MEX89	9F	P3S	JRG	PV3/3997/TRK90	11F	P3W	GYL
PV3/0044/GUT89	9G	P3S	PC	PV3/2723/TUR90	11G	P3W	AvL
PV3/9896/GUT89	9H	P3S	PC	PV3/2721/FRA90	11H	P3W	HK
PV3/9847/MEX89	10A	P3S	JRG	PV3/2619/RUS90	12A	P3W	GYL
PV3/9442/NIC89	10B	P3S	JRC	PV3/0380/MEX90	12B	P3W	JRG
PV3/9441/GUT89	10C	P3S	PC	PV3/9259/TUN88	12C	P3W	SA
PV3/8774/TRT88	10D	P3S	BH	PV3/9035/BRA88	12D	P3W	EdS
PV3/8239/GUT88	10E	P3S	PC	PV3/8854/COL88	12E	P3W	JB
PV3/8024/GUT87	10F	P3S	PC	PV3/0870/GEO87	12F	P3W	GYL
PV3/6880/COL86	10G	P3S	JB	PV3/0324/INO86	12G	P3W	AvL
PV3/7149/MEX84	10H	P3S	MLZ	PV3/7840/PER86	12H	P3W	LB

[a] Country abbreviations: ARG, Argentina; ARM, Armenia; AZB, Azerbaijan; BRA, Brazil; CAM, Cameroon; CHI, Chile; CHN, People's Republic of China; COL, Colombia; EGY, Egypt; ELS, El Salvador; ETH, Ethiopia; FRA, France; GEO, Georgia; GUT, Guatemala; HON, Honduras; IND, India; INO, Indonesia; ISR, Israel; KEN, Kenya; MEX, Mexico; MOR, Morocco; NEP, Nepal; NIC, Nicaragua; OMA, Oman; PAK, Pakistan; PER, Peru; POR, Portugal; ROM, Romania; RUS, Russia; SAA, Saudi Arabia; SOA, South Africa; SWE, Sweden; SYR, Syria; TOG, Togo; TRK, Turkmenistan; TRT, Trinidad and Tobago; TUN, Tunisia; TUR, Turkey; USA, United States; UZB, Uzbekistan; VEN, Venezuela; VTN, Vietnam; YUG, Yugoslavia; ZAI, Zaire.

[b] Numbering of sample wells (Fig. 2 and 3) differs from the standard microtiter format.

[c] Identified by partial genomic sequencing: P1S, Sabin 1-related; P1W, wild type 1 poliovirus; P2S, Sabin 2-related; P2W, wild type 2 poliovirus; P3S, Sabin 3-related; P3W, wild type 3 poliovirus.

[d] Donors: JA, J. Alexander; SA, S. Amine; LB, L. Belaúnde; JB, J. Boshell; PC, P. Cáceres; JRC, J. R. Cruz; EdS, E. da Silva; KD, K. Dave; MHH, M. H. Hatch; TH, T. Hovi; BH, B. Hull; TJJ, T. Jacob John; HK, H. Kopecka; GYL, G. Y. Lipskaya; RML, R. Méndez López; CMM, C. M. Mejía; PM, P. Minor; TN, T. Naguib; JHN, J. H. Nakano; SN, S. Nejmi; BN, B. Nottay; AN, A. Novillo; MJO, M. J. Oliveira; OP, O. Palacios; MAP, M. A. Pallansch; JRG, J. Ruiz Gómez; RS, R. Salas; BDS, B. D. Schoub; PVT, Phan Van Tu; AvL, A. van Loon; AV, A. Vernon; MLZ, M. L. Zárate; LBZ, L.-B. Zhang.

[e] Genomic sequence divergence among wild polioviruses estimated from comparisons of VP1/2A junction (90 nucleotides VP1 and 60 nucleotides 2A [21, 41]). Type 1 isolates included 11 independent genotypes (>15% nucleotide sequence divergence in VP1/2A interval); the average divergence was 22%. Isolates PV1/5136/ETH92 and PV1/3865/IND91 were the least divergent (7%); isolates PV1/1184/ROM91 and PV1/9366/SAA89 were the most divergent (32%). Type 2 isolates included eight independent genotypes; the average divergence was 21%. Isolates PV2/6683/YUG78 and PV2/2996/SWE77 were the least divergent (3%); isolates PV2/0295/ISR78 and PV2/0710/KEN71 were the most divergent (31%). Type 3 isolates included 13 independent genotypes; the average divergence was 22%. Isolates PV3/3899/EGY92 and PV3/9259/TUN88 were the least divergent (13%); isolates PV3/0380/MEX90 and PV3/0324/INO86 were the most divergent (31%).

TABLE 2. Primers used to amplify poliovirus sequences

Primer[a]	Sequence (5′ → 3′)[b]
EV/T7/5UT/1 (A; 578–599)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG TGATTGTCACCATAAGCAGCCA
EV/5UT/2 (S; 494–517)	ctc*GAATTC*AACCAGTGACTGGCTTGTCGTAAC
Sab1/T7/5UT/1 (A; 717–740)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG TGATACAATTGTCTGATTGAAATA
Sab1/5UT/2 (S; 641–663)	ctc*GAATTC*AAGTGAGATTCATTATCTATCTG
Sab2/T7/5UT/1 (A; 719–741)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG ATTGTTGTTTTATCCTCGTATTG
Sab2/5UT/2 (S; 642–662)	ctc*GAATTC*GTGTTGTGTCAGGTATACAAC
Sab3/T7/5UT/1 (A; 721–741)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG GTGACACTGAAATCCTGTCTT
Sab3/5UT/2 (S; 642–663)	ctc*GAATTC*GTGAATCAGATTAATTACTCCC
Sab1/T7/VP1/1 (A; 2556–2575)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG GGCTTCAGTGTTTGGGAGAG
Sab1/VP1/2 (S; 2482–2502)	ctc*GAATTC*GTTAGGTCAGATGCTTGAAAG
Sab2/T7/VP1/1 (A; 2558–2577)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG CGGCTTGTGTCCAGGCAGGC
Sab2/VP1/2 (S; 2484–2504)	ctc*GAATTC*AATTGGTGACATGATTGAAGG
Sab3/T7/VP1/1 (A; 2547–2566)	gcc*AAGCTTAAGCTT*TAATACGACTCACTATAGGG GGCCTTAGTATCAGGTAAGC
Sab3/VP1/2 (S; 2479–2502)	ctc*GAATTC*TATTGAAGATTTGATTTCTGAAGT

[a] Numbers in parentheses indicate the genomic intervals matching the primers. The EV/5UT primer set is numbered according to the position of matching sequences on the Sabin 2 template (52). The Sabin 1, (Sab1), Sabin 2 (Sab2), and Sabin 3 (Sab3) primer sets are numbered according to the absolute position of matching sequences on their respective templates (33, 51, 52). Primer polarities: A, antisense; S, sense.

[b] Primers contained sequences for spacers (lowercase letters), restriction enzyme recognition sites (italic letters; *Eco*RI, *GAATTC*; *Hin*dIII, *AAGCTT*), T7 promoter (underlined letters), and intervals matching poliovirus templates (capital letters).

Construction of recombinant plasmids. RNAs extracted from purified virions of each Sabin strain were templates for in vitro amplification by PCR (47, 59). Templates were matched with the corresponding primer sets for production of the six Sabin strain-specific amplicons. Sabin 2 RNA served as the template for amplification with the primer pair EV/T7/5UT/1 and EV/5UT/2. Amplicons were digested with *Eco*RI and *Hin*dIII, purified by polyacrylamide gel electrophoresis, and ligated into gel-purified pUC18 plasmids (61) that had been digested with *Eco*RI and *Hin*dIII (49). Plasmids were transformed into competent DH5α cells (GIBCO-BRL, Gaithersburg, Md.), and transformants were screened for inserts by colony hybridization with the corresponding ^{32}P-labeled primers as probes (49). Recombinant plasmids were further characterized by restriction analysis (49) and sequence determinations of the inserts. Purified plasmids were prepared in quantity by a modification of the alkaline lysis method of Birnboim and Doly (3).

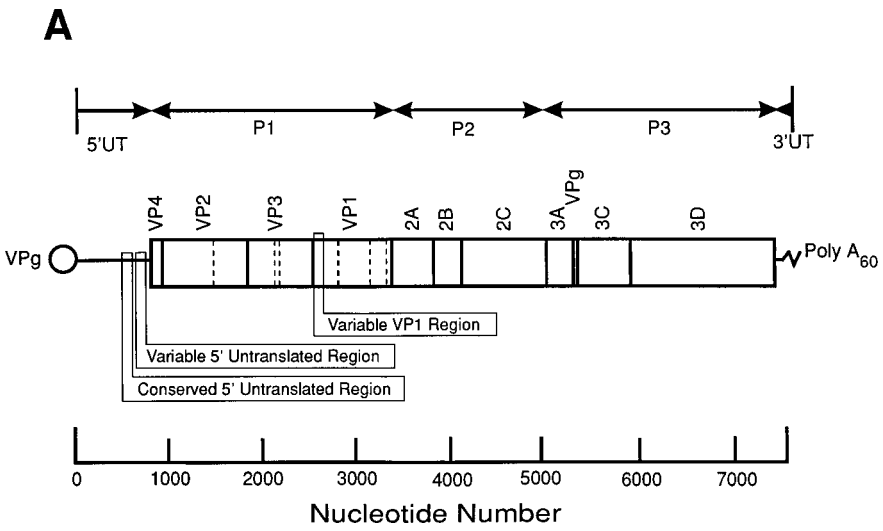
Preparation of labeled RNA transcripts. Digoxigenin (DIG)-labeled transcripts were prepared in 20-μl reaction mixtures containing 1 μg of purified plasmid DNA (linearized with *Eco*RI), DIG-RNA labeling mixture (1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, 0.35 mM DIG-11-UTP; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 10 U of placental RNase inhibitor, and 40 U of T7 RNA polymerase. After incubation at 37°C for 2 h, the reactions were terminated by heating at 65°C for 10 min. DIG-labeled transcripts are stable for at least 1 year upon storage at −20°C.

Radiolabeled transcripts were prepared similarly in 50-μl reaction mixtures except that UTP was present as [α-^{32}P]UTP (250 μCi, ~6 μM; DuPont NEN, Boston, Mass.) in 50 μM unlabeled UTP. The ^{32}P-labeled transcripts were analyzed by electrophoresis in formaldehyde-agarose gels (26), and each was found to contain a predominance of products having the expected chain length.

Dot blot hybridization. Cultures of virus-infected RD cells (multiplicity of infection, >25 PFU per cell; maintained in Eagle's minimum essential medium without serum) were harvested after the development of extensive cytopathology (usually 18 h postinfection) and frozen-thawed three times to release the virions. The suspensions were clarified by centrifugation (12,000 × *g*, 15 min), 500 μl of each supernatant (equivalent to ~2.5×10^5 infected cells) was mixed with 300 μl of 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]) and 200 μl of 37% formaldehyde solution, and the mixture was heated at 65°C for 15 min (57). Samples (100 to 200 μl) were filtered through positively charged nylon membranes (Boehringer Mannheim or Schleicher & Schuell, Keene, N.H.) by using a 96-well manifold (Schleicher & Schuell). RNA was bound to the filters by UV crosslinking (λ = 254 nm, 120 mJ/cm^2, 30 s; Stratagene, La Jolla, Calif.). Prehybridization was performed at 65°C for 2 h in hybridization buffer (6× SSC, 50% [vol/vol] formamide, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 2% [wt/vol] blocking reagent [solubilized casein; Boehringer Mannheim]; 1 ml/1-cm^2 filter). Hybridization was performed at 65°C for 18 to 24 h in fresh hybridization buffer (0.1 ml/1-cm^2 filter) containing 5 μl (~2 μg, ~60 fmol) of labeled probe. Following hybridization, filters were washed at 75°C for 15 min once in 2× SSC–0.1% SDS and again in 0.1× SSC–0.1% SDS.

Detection of hybrids by ELISA. Hybrids formed with DIG-labeled probes were detected by the method of Höltke and Kessler (16) as modified for the Boehringer Mannheim nonradioactive detection kit. Filters were equilibrated in buffer A (100 mM sodium maleate [pH 7.5], 150 mM NaCl) for 1 min at room temperature and were transferred to buffer B (buffer A containing 2% blocking reagent) for incubation with gentle shaking at room temperature for 1 to 2 h. Buffer B was replaced with fresh buffer B containing 150 mU of anti-DIG-(Fab)–alkaline phosphatase conjugate (Boehringer Mannheim) per ml, and the filters were gently shaken for 30 min. The filters were washed twice for 15 min in buffer A and were then equilibrated for 2 min in buffer C (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$). ELISA detection used either the chemiluminescent substrate 4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2′-adamantane) (AMPPD; Lumi-Phos 530, Boehringer Mannheim [50] or the histochemical substrates 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitroblue tetrazolium (NBT). For chemiluminescence detection, filters were placed on clean acetate sheets, Lumi-Phos 530 was applied onto each filter (~5 ml/100 cm^2 of filter), and a second acetate sheet was placed on top of the filters to distribute the solution uniformly. After 30 min at 37°C, the filters were exposed to X-ray film (Kodak XAR2) for 2 to 10 min. For histochemical detection, filters were incubated at room temperature in the dark for 30 min in 120 mM BCIP–90 mM NBT in buffer C (10 ml/100 cm^2 of filter) before the color reaction was visualized.

Determination of limits of Sabin strain RNA detection by hybridization. Serial half-log dilutions of purified Sabin strain RNAs (100 ng to 32 pg) were immobilized onto filters (57) and were hybridized to complementary RNA probes labeled with either ^{32}P or DIG as described above. After hybridization, filters were washed under standard conditions (75°C for 15 min once in 2× SSC–0.1% SDS and again in 0.1× SSC–0.1% SDS). Filters incubated with ^{32}P-labeled probes were then air dried and analyzed by autoradiography (1 h of exposure).



B

CONSERVED 5′-UNTRANSLATED REGION

```
            494                                                                                                  598
Sabin 1     AACCAGUGAUUGGCCUGUCGUAACGCGCAAGUCCGUGGCGGAACCGACUACUUUGGGUGUCCGUGUUUCCUUUUAUUUU*AUUGUGGCUGCUUAUGGUGACAAUCA

            494                                                                                                  599
Sabin 2     ---------C----U----------U-----------------------------------G----------CA----------

            497                                                                                                  601
Sabin 3     -C----CAGCCA-----------------------------------------------------------C-UGA*A----------
```

VARIABLE 5′-UNTRANSLATED REGION

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            639                                                                                                  741
Sabin 1     GAAAGUGA*GAUUCAUUAUCUAUCUGUUUGCUGGAUUCGCUCCAUUGAG*UGUGUUUACU*CUAAG*UA*C*AAUU**UCAACAGUUAUUUUC*A**AUCAGACAAUU*G*UAUCAU

            640                                                                                                  746
Sabin 2     --GU--UGU-*-CAGG---AC-A-------U----AC-A--GUG--*---C***------U--C-UU--A-C----AA----*-AAC-A-A-G-GG--A-A-----**CAA--CU-C

            642                                                                                                  741
Sabin 3     -UG-A-C------A----CUCCCU------U-----C-A----*C*--AAC--*--------*C**U--A--**--A--UG-*-A--G---*G-AG-CAG--U**--CAG-G---C
```

VARIABLE VP1 REGION

```
            2480                                                                                                 2575
Sabin 1     GGG UUA GGU CAG AUG CUU GAA AGC AUG AUU GAC AAC ACA GUC CGU GAA ACG GUG GGG GCG GCA ACG UCU AGA GAC GCU CUC CCA AAC ACU GAA GCC

            2482                                                                                                 2577
Sabin 2     --A A-U --- G-C --- A-- --- G-G GCC G-- --A GGG -UU ACU AAA A-U G-A U-- -UU C-C C-G --U --C -CC A-U AGC --G --U GGA CAC A-G C-G

            2477                                                                                                 2566
Sabin 3     --U A-U -AA G-U U-- A-- *** UCU GAA G-- *** GCA CAG -G- GCC CU- --U U-- UCA CUC C-G -A- CAA CAG --U AGC U-A --U G-U --- A-G ---
```

FIG. 1. (A) Location along the poliovirus genome of sequences matching RNA probes. Untranslated regions are shown as lines; the region of the translated polyprotein is represented by the rectangle. Nomenclature for viral proteins follows that of Rueckert and Wimmer (44) except for the capsid proteins VP1, VP2, VP3, and VP4 (or 1D, 1B, 1C, and 1A, respectively). Dashed vertical lines indicate the locations of sequences encoding virion surface residues. The arrow indicates the interval at the VP1/2A junction routinely analyzed by comparative sequencing (21, 41). (B) Sabin strain genomic sequences (52) complementary to RNA probes. Sequences matching the PCR primers used to prepare the RNA probes are underlined. Nucleotide positions are numbered consecutively for each genome; asterisks indicate base deletions. The lengths (in nucleotides) of the intervals in the cloned probes matching the corresponding Sabin strain templates were as follows: EV/5UT, 105; Sab1/5UT, 100; Sab2/5UT, 100; Sab3/5UT, 100; Sab1/VP1, 94; Sab2/VP1, 94; Sab3/VP1, 88.

Filters incubated with DIG-labeled probes were processed for detection by ELISA with either AMPPD or BCIP-NBT substrates as described above.

Determination of hybridization kinetics. Purified, filter-bound RNAs (100 ng per dot) of each Sabin strain were hybridized under standard conditions (65°C in hybridization buffer; 5.0 ml per reaction mixture) to the complementary ^{32}P-labeled probes (2 μg, ~60 fmol, ~2.3 μCi; probe:target sequence stoichiometric ratio ≈ 60:1). Filters were cut into 3.2-cm^2 pieces that had two RNA dots each, and samples were taken at 2-h (from 0 to 12 h) or 4-h (from 12 to 24 h) intervals. Filters were washed immediately at room temperature in 2× SSC–0.1% SDS and were then stored moist at room temperature until all samples had been taken. Samples were then washed together at 75°C for 15 min in 0.1× SSC–0.1% SDS. Filters were air dried and analyzed by autoradiography (Kodak XAR2 film), and the label bound to each RNA dot was determined quantitatively by scintillation counting and electronic blot analysis (Betascope model 603; Betagen, Waltham, Mass.).

Measurement of the thermal stabilities of filter-bound RNA-RNA duplexes. The stabilities of the RNA-RNA duplexes formed between the RNA probes and the poliovirus templates were determined by a modification of the method of Jacobs et al. (19). Hybridization of ^{32}P-labeled probes to duplicate RNA dots was performed as described above. After hybridization, filters were washed once at room temperature in 2× SSC–0.1% SDS to remove nonhybridized probe. Filters were dispensed into separate scintillation vials for incubation (10 min with

shaking) in 2 ml of wash buffer preequilibrated to defined temperatures (over the range of 60 to 100°C). This step was repeated by the successive transfer of filters to vials containing wash buffer preequilibrated to the next higher temperature. Thermal elution curves were determined in three different wash buffers: (i) 6× SSC–50% formamide–0.1% SDS (hybridization buffer without blocking agent), (ii) 2× SSC–0.1% SDS (standard first wash buffer), and (iii) 0.1× SSC–0.1% SDS (standard second wash buffer). Another filter set was washed in 3.0 M tetramethylammonium chloride (TMAC; [pH 7.0])–0.1% SDS (19, 29). The amount of labeled probe eluted at each temperature was determined by scintillation counting and was compared with the amount of probe initially bound to the filters.

RESULTS

Design of RNA probes. We set out to design genotype-specific probes of sufficient length to ensure high specificities of hybridization assays and to confer high thermal stabilities to matched hybrids. We designed the probes to complement intervals that are highly variable across independent poliovirus genotypes but whose rates of evolution are moderate, thereby favoring efficient hybridization among closely related (≥90% sequence similarity) genomes. The longest contiguous stretches of high genomic variability reside in two intervals: (i) the nucleotides preceding the initiation codon for the poliovirus polyprotein (Sabin 1, positions 641 to 740; Sabin 2, positions 642 to 741; Sabin 3, positions 642 to 741 [Fig. 1] [40, 52]) and (ii) the first 30 to 32 codons of the VP1 region (Sabin 1, positions 2482 to 2575; Sabin 2, positions 2484 to 2577; Sabin 3, positions 2579 to 2566 [Fig. 1] [52]). In accord with these and other considerations (10, 59, 60), we prepared two sets of RNA probes (probes Sab1/5UT, Sab2/5UT, and Sab3/5UT and probes Sab1/VP1, Sab2/VP1, and Sab3/VP1) complementary to these variable regions of each Sabin strain genome.

We also prepared a group probe, EV/5UT, capable of forming stable hybrids with all poliovirus RNAs. The group probe matches a highly conserved interval (residues 494 to 599) within the 5′-untranslated region (5′-UTR) (Fig. 1) (52) that extends from the terminal loop of secondary structure domain D to the terminal loop of domain E (39). The RNA probe is predicted to have less self-complementarity (~27%) than that which occurs overall for the conserved 5′-UTR sequences (~44% [39]). Sabin 2 RNA was selected as the template for preparation of EV/5UT because its conserved 5′-UTR sequences are intermediate to those of Sabin 1 and Sabin 3 (Fig. 1) (52). Probe EV/5UT overlaps the sequences of other probes (10, 38, 43, 53) and PCR primers (7, 35, 42, 60) found to hybridize to the RNAs of nearly all human enteroviruses except echovirus types 22 and 23, which have atypical 5′-UTR sequences (8, 18). Therefore, the reactivity of a viral RNA with the group probe identifies the source virus as an enterovirus, but not necessarily as a poliovirus.

Specificities of RNA probes. (i) Reference poliovirus strains. The RNA probes were tested for hybridization with the genomic RNAs of the standard poliovirus reference strains (Table 3). The group probe, EV/5UT, hybridized efficiently with all poliovirus RNAs (Table 3; see also Fig. 2 and 3). Because the sequences targeted by EV/5UT are highly conserved, all poliovirus templates would be expected to form hybrids with nearly equivalent stabilities, so that the intensity of the hybridization signal with EV/5UT indicates the approximate quantity of poliovirus RNA applied to the filter.

As expected, the Sabin strain RNA probes (Sab1/5UT, Sab2/5UT, and Sab3/5UT; Sab1/VP1, Sab2/VP1, and Sab3/VP1) recognized the corresponding reference polio vaccine strain RNAs (Table 3). In addition, the Sabin 1-specific RNA probes, Sab1/5UT and Sab1/VP1, hybridized to the RNA of PV1/Mahoney/USA42, the parent to Sabin 1 (46). The PV1/Mahoney/USA42 genome, which is >99% homologous to the Sabin 1 genome (33), mismatches Sab1/5UT at only two positions and Sab1/VP1 at only one position. Similarly, probes Sab3/5UT and Sab3/VP1 formed stable, completely matched (51) hybrids with the genome of the Sabin 3 parent, PV3/Leon/USA37 (46). Polio vaccine preparations may contain Leon 12 a_1b populations, in which most genomes contain a cytosine residue at position 2493 (55) (Fig. 1). However, variants containing a uridine residue at position 2493 (as occurs in PV3/Leon/USA37) are selected very rapidly during replication of oral polio vaccine in vaccinees (55), such that most vaccine-derived type 3 isolates predominantly contain templates that match probe Sab3/VP1 at position 2493.

TABLE 3. Reactivities of probes with reference poliovirus strains

Strain[a]	Reactivity of probe:						
	EV/5UT	5UT			VP1		
		Sab1	Sab2	Sab3	Sab1	Sab2	Sab3
PV1/Sabin 1	+	+	−	−	+	−	−
PV1/Brunhilde/USA39	+	−	−	−	+	−	−
PV1/Mahoney/USA42	+	+	−	−	+	−	−
PV2/Sabin 2	+	−	+	−	−	+	−
PV2/Lansing/USA37	+	−	−	−	−	−	−
PV2/MEF-1/EGY42	+	−	−	−	−	−	−
PV3/Sabin 3	+	−	−	+	−	−	+
PV3/Leon/USA37	+	−	−	+	−	−	+
PV3/Saukett/USA52	+	−	−	−	−	−	+

[a] The sources and genotypic characteristics of poliovirus reference strains are described by Kew et al. (21) and Yang et al. (59).

The patterns of probe reactivities with the RNAs of other reference strains (Table 3) were compatible with the sequence relationships between these viruses and the Sabin strains. For example, the capsid sequences of PV1/Brunhilde/USA39 are ~98% homologous to those of PV1/Mahoney/USA42 and Sabin 1, but flanking sequences are highly divergent (unpublished data). Consequently, RNA probe Sab1/VP1 (3 mismatches with the template) binds with PV1/Brunhilde/USA39 RNA, but probe Sab1/5UT (47 mismatches) does not (Table 3). The genome of PV3/Saukett/USA52, distantly related to PV3/Leon/USA37 (21), hybridized with probe Sab3/VP1 (11 mismatches) but not with Sab3/5UT (30 mismatches). Genomic RNAs of PV2/Lansing/USA37 and PV2/MEF–1/EGY42 mismatched both Sabin 2 RNA probes at multiple sites (Sab2/5UT; 43 and 59 mismatches, respectively; Sab2/VP1; 27 and 36 mismatches, respectively [25; unpublished data]).

(ii) Vaccine-related isolates. The polio vaccine strains frequently accumulate mutations during replication in the human intestine (22, 31, 32, 34, 55). However, unlike wild polioviruses, transmission of vaccine viruses is normally restricted to the immediate contacts of vaccinees (2), thereby limiting the extent of evolution by nucleotide substitution. Recombination is also an important mechanism for poliovirus evolution (5, 62). A large fraction of vaccine-derived isolates of serotypes 2 and 3 are intertypic recombinants, having one or more crossover sites in the noncapsid regions of their genomes (5, 14, 24, 27). When tested against vaccine-derived isolates that had been previously characterized by partial genomic sequencing (41, 59), both sets (Sab/5UT and Sab/VP1) of Sabin strain-specific probes were found to have the appropriate specificities. Figures 2 and 3 illustrate the typically strong hybridization signals obtained with the RNAs of vaccine-derived isolates (Table 1). Because the probes match sequences encoding the amino-

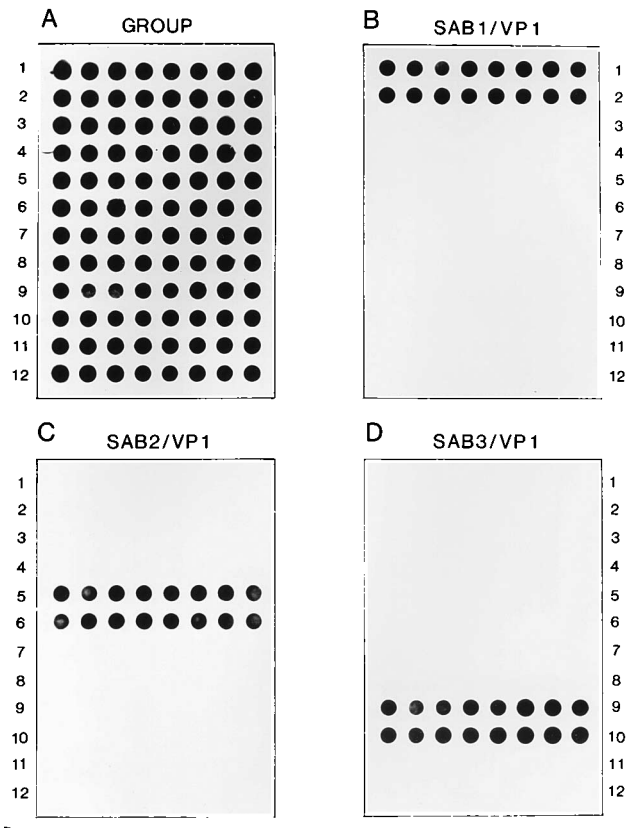

FIG. 2. Identification of poliovirus isolates by blot hybridization. Samples of heat-disrupted virions were applied to nylon filters at the positions indicated in Table 1 (A to H in Table 1 in the eight columns from left to right, respectively; 1 to 12, top to bottom, respectively). Filters were transferred to hybridization reaction mixtures containing DIG-labeled RNA probes EV/5UT (group; A), Sab1/VP1 (B), Sab2/VP1 (C), and Sab3/VP1 (D). Hybrids were detected by chemiluminescence as described in Materials and Methods.

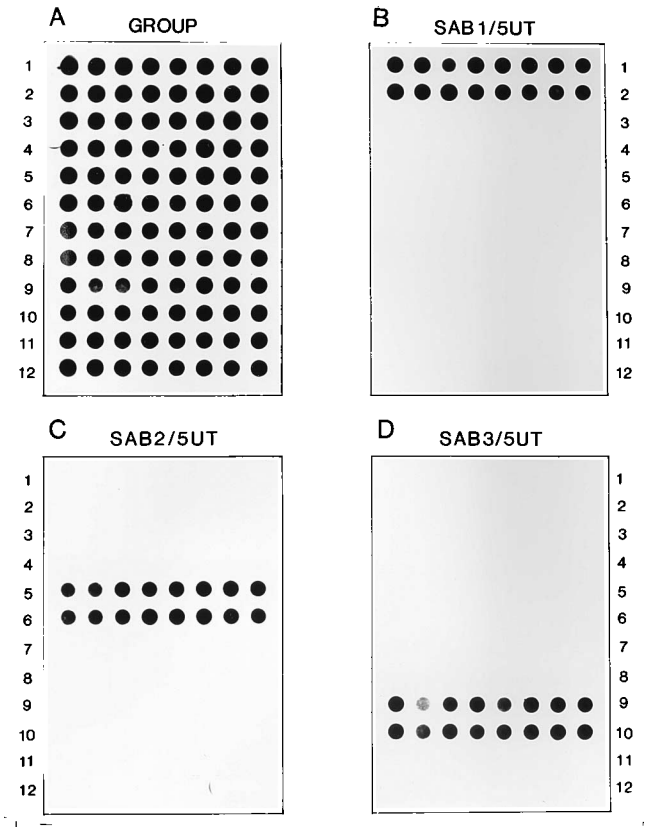

FIG. 3. Identification of poliovirus isolates by blot hybridization. Samples were applied to filters and analyzed as described in the legend to Fig. 2. Filters were transferred to hybridization reaction mixtures containing the DIG-labeled RNA probes EV/5UT (group; A), Sab1/5UT (B), Sab2/5UT (C), and Sab3/5UT (D).

terminal domains of VP1, all vaccine-related isolates, including intertypic recombinants, were correctly identified by serotype.

(iii) Wild poliovirus isolates. Wild polioviruses are genetically diverse. Within each serotype are numerous independent genotypes that are distinguished by the patterns of their nucleotide sequences (21, 41). The Sabin oral polio vaccine strains are derived from genotypes that were endemic to the United States in the 1930s through the 1950s (46). With one possible exception (see Discussion), the many wild poliovirus genotypes currently found worldwide appear to represent lineages that have been separated from each other for many decades (41). Sequence heterogeneity within the intervals targeted by our probes is particularly high, exceeding 45% (variable 5′-UTR) and 30% (variable VP1 region) within serotypes and 50% (both variable regions) across serotypes (10, 17, 25, 40, 52, 60). Nonetheless, the great diversity among wild poliovirus genomes raises the possibility that some wild isolates unrelated to the Sabin strains might be identified as vaccine-related because of cross-hybridization with our Sabin strain-specific probes. To evaluate whether our Sab/5UT and Sab/VP1 probes recognized vaccine-derived isolates only, we tested our probes for their reactivities with the RNAs of a diverse set of contemporary wild polioviruses representing all known major genotypes (Table 1). None of the wild poliovirus genomes hybridized to our Sabin strain-specific probes under the stringent standard hybridization conditions (Fig. 2 and 3).

Sensitivities of detection. To determine the limits of sensitivity of our probe hybridization system to detect the presence of vaccine-related polioviruses in a sample, serial dilutions of purified Sabin strain RNAs (100 ng to 32 pg) were applied to filters and hybridized with EV/5UT and with the corresponding Sab/5UT and Sab/VP1 probes. Probes were labeled either with ^{32}P or DIG. Hybrids were detected by 60 min of direct autoradiography with the radiolabeled probes (Fig. 4A) or by ELISA by using the substrate AMPPD and 5 min of autoradiography (Fig. 4B) or by using the chromogenic substrates BCIP and NBT and visualization of a color reaction (data not shown). The limits of detection by autoradiography obtained with the ^{32}P-labeled probes were approximately 3 to 10 ng (1.3 to 4 fmol; equivalent to 7.9×10^8 to 2.5×10^9 RNA molecules) (Fig. 4A), while those obtained by ELISA and AMPPD chemiluminescence were 100 to 316 pg (40 to 126 amol; equivalent to 2.5×10^7 to 7.9×10^7 RNA molecules) (Fig. 4B). Comparable sensitivities were obtained with the DIG-ELISA system after 30 min of incubation with the substrates BCIP and NBT (data not shown). Sensitivities were about threefold lower with the Sab/5UT probe sets than with the Sab/VP1 probe set and the group probe, EV/5UT (Fig. 4). Under our conditions, detection by nonisotopic DIG-ELISA is approximately 30-fold more sensitive than detection by autoradiography with ^{32}P. The greater sensitivity of ELISA detection is not surprising because each probe-bound alkaline phosphatase conjugate can catalyze the hydrolysis of multiple substrate molecules, whereas each ^{32}P atom is limited to a single decay event and <0.15% of the

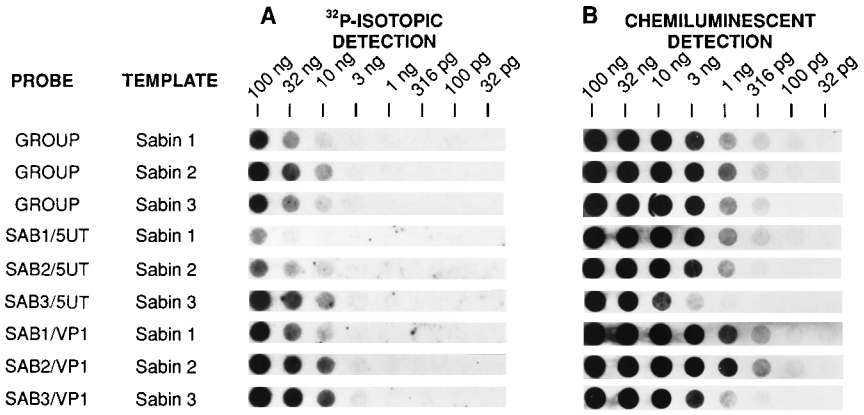

FIG. 4. Sensitivities of detection of homologous poliovirus templates with labeled RNA probes. Serial dilutions of Sabin strain RNAs (100 ng to 32 pg) were applied to nylon filters and were hybridized to the corresponding RNA probes (EV/5UT was hybridized to Sabin 1 RNA) labeled either with ^{32}P or DIG. After the filters were washed, hybrids were detected by exposure of filters to X-ray film for 1 h. (A) ^{32}P-labeled RNA probes; (B) DIG-labeled RNA probes.

^{32}P atoms incorporated into the radiolabeled probes decay during 1 h of autoradiography. The differences in signal yields for the two reporter systems are most evident when the amount of bound probe is limited by low concentrations of target sequence.

Kinetics of association. We measured the hybridization kinetics of each of our RNA probes to purified homologous templates in our dot blot format. Hybridization reactions and subsequent wash steps were performed under standard conditions with ^{32}P-labeled probes present in ~60-fold stoichiometric excess. The half-lives of association ($t_{1/2}$s) for the first-order reactions were calculated and compared with the values predicted theoretically (28) on the basis of the rates of hybridization in solution (56). The most rapid rates of association were obtained with probes EV/5UT and Sab2/VP1 ($t_{1/2}$ = 2.8 to 3.4 h), and the slowest rates were obtained with probes Sab1/5UT and Sab1/VP1 ($t_{1/2}$ = 6.1 to 6.3 h). The observed rates of association were about 6- to 12-fold lower than those calculated for optimal hybridization conditions (Table 4). Two factors may account for the differences from the calculated value. (i) Filter hybridization reactions are generally slower than the better-understood solution hybridization reactions (12), and (ii) the incubation of the hybridization reactions at a temperature approaching the temperature of irreversible denaturation (T_i) values for the hybrids decreased the net rates of duplex formation (see next section). For our standard hybridization times of 18 h, the most rapid associations would be 98 to 99% complete, while the slowest associations would be >85% complete. Hybridization times of >24 h are not recommended because of probe degradation and the elution of target RNA from the filters.

Stabilities of hybrids. The thermal stabilities of the duplexes formed between the RNA probes and the poliovirus templates were determined in each of the three buffers used in our hybridization assays (Table 5). Hybrids were most stable in 2× SSC–0.1% SDS (average T_i = 93°C), less stable in 0.1× SSC–0.1% SDS (average T_i = 80°C), and least stable in 6× SSC–50% formamide–0.1% SDS (average T_i = 78°C). Hybrids formed by EV/5UT and the Sab/VP1 set were generally stronger than those formed by the Sab/5UT set. The T_i values calculated from general relationships derived empirically (6, 19, 45) were in good agreement with our experimental values (Table 5). The widths of the thermal denaturation transitions (ΔT; 25 to 75% dissociation) in 6× SSC–50% formamide–0.1% SDS or 2× SSC–0.1% SDS varied between 10 and 21°C. The exceptionally broad transition for probe Sab2/VP1 is probably attributable to localized intervals forming highly stable duplexes (Fig. 2). Consistent with this view, much sharper denaturation transitions (e.g., Sab2/VP1 [ΔT = 8°C]) were obtained when filters were washed in 3.0 M TMAC (data not shown), a solvent in which A-T and G-C base pairs have nearly equivalent stabilities (19, 29). Denaturation transitions were also sharp (average ΔT = 6°C) in 0.1× SSC–0.1% SDS (Table 5).

Our routine hybridization and wash conditions were very stringent. The temperature of hybridization (65°C) was only 7 to 18°C below the T_i of the hybrid duplexes, and the temperature of the final wash in 0.1× SSC (75°C) was only 1°C above to 9°C below the T_is of the hybrids.

DISCUSSION

Nucleic acid probe hybridization offers several advantages as a method for the identification of poliovirus isolates. Identifications obtained by hybridization are generally accurate and

TABLE 4. Kinetics of RNA probe association to homologous templates

Probe	$t_{1/2}$ of association (h)[a]
EV/5UT	
Sabin 1	3.2
Sabin 2	2.8
Sabin 3	3.3
Sab/5UT	
Sabin 1	6.1
Sabin 2	5.0
Sabin 3	5.2
Sab/VP1	
Sabin 1	6.3
Sabin 2	3.4
Sabin 3	4.9

[a] Half-lives of association ($t_{1/2}$) under optimal hybridization conditions in solution can be calculated from the relationship (28, 56): $t_{1/2} = \ln 2(L^{1/2})/[(k_n)(c)] \approx 0.5$ h, where L is the chain length of the hybrid (in base pairs), k_n is the nucleation constant (3.5×10^5 s^{-1}), and c is the probe concentration (in moles liter^{-1}).

TABLE 5. Thermal stabilities of probe hybrids to poliovirus templates

Probe:template	6× SSC–50% formamide			2× SSC			0.1× SSC		
	T_i (obs) (°C)[a]	ΔT_i (°C)[b]	T_i (cal) (°C)[c]	T_i (obs) (°C)	ΔT (°C)	T_i (cal) (°C)	T_i (obs) (°C)	ΔT (°C)	T_i (cal) (°C)
EV/5UT:Sabin 1	78	11	81[d]	93	12	98[d]	83	4	76[d]
EV/5UT:Sabin 2	82	14	81	94	11	98	84	5	76
EV/5UT:Sabin 3	79	12	81[d]	92	11	98[d]	82	15	76[d]
Sab1/5UT:Sabin 1	78	10	74	88	14	91	77	4	70
Sab2/5UT:Sabin 2	72	12	74	92	12	91	74	6	70
Sab3/5UT:Sabin 3	73	11	76	93	12	93	75	6	72
Sab1/VP1:Sabin 1	83	13	82	96	12	99	84	8	78
Sab2/VP1:Sabin 2	79	19	81	92	21	98	83	4	76
Sab3/VP1:Sabin 3	74	10	79	93	12	96	82	4	74

[a] T_i (obs), observed T_i values.
[b] ΔT, width of thermal denaturation transition from 25 to 75% dissociation (29).
[c] T_i values were calculated [T_i (cal)] from the relationships $T_i = T_m + 10°C$ (19) and $T_m = 81.5°C + 16.6 (\log_{10} [Na^+]) + 0.41$ (% G+C) − 0.50 (% formamide) − $675/L$ (6, 45), where L is the chain length of the hybrid (in base pairs).
[d] T_i (cal) values were not adjusted for base mismatch.

unambiguous. The method requires comparatively simple equipment and can easily accommodate large numbers of samples. Moreover, reliable results can be obtained in 24 h or less. Different genomic regions having widely varying rates and patterns of sequence evolution can be precisely and systematically targeted by specific probes. Probe sequences can be incorporated into plasmid vectors for preparation in virtually unlimited quantities by standard bacteriologic procedures. Many different hybridization formats are available, and these differ in their sensitivities, levels of convenience, and speed (20).

Our RNA probes were designed to be used in conjunction with virus isolation and typing. Virus isolation in cell culture is essential for resolving virus mixtures that may exist in clinical specimens and for increasing virus yields to levels sufficient for achieving good hybridization signals. The hybridization method described here also requires virus typing for the identification of wild polioviruses. If an isolate is a poliovirus but its RNA does not hybridize to our Sabin strain-specific probes, the isolate is wild. Excellent correspondence is observed among identifications based on RNA probe hybridization, PCR (1, 59, 60), and genomic sequencing (41).

We tested the specificities of our probes against the broadest sampling of contemporary wild poliovirus isolates available to us (21). None of the isolates representing the diverse wild poliovirus genotypes cross-hybridized with the Sabin strain-specific probes under our standard conditions. However, at lower stringencies such cross-hybridizations are observed with RNAs of some type 1 polioviruses, most notably isolates of the genotype endemic to southern and western Asia. Hybridization of the genomic RNAs of this genotype with the Sab1/5UT and Sab1/VP1 probes is apparently reflective of a true evolutionary relationship with the Sabin type 1 strain, as indicated by extensive sequence similarities throughout the capsid region (21, 41; unpublished data). Because sequence similarities with the Sabin 1 genome can exceed 80% in the probe target regions, cross-hybridizations are eliminated only by high stringencies.

In optimizing for specificity, we accepted reductions in probe sensitivity and rates of hybridization. The maximal sensitivities for poliovirus RNAs (~40 amol) reported here are substantially lower than those reported for purified in vitro transcripts (30 fg; ~0.1 amol) by using DIG-labeled probes and prolonged (16 h) incubation of the BCIP-NBT histochemical detection reaction (16). Although the sensitivities of our ELISA-based detection systems can be increased with extended times for color development or autoradiography, such steps are usually not necessary because the quantities of poliovirus RNA in most cell culture isolate preparations are large (40 to 400 ng/ml).

For routine identifications, we use probe EV/5UT with the Sab/VP1 probe set. We generally prefer the Sab/VP1 probes, which form more stable hybrids, over the Sab/5UT set. The latter set is used primarily for confirmation of results. Sabin vaccine-derived isolates can be identified without virus typing, and the compositions of heterotypic vaccine-derived mixtures can be estimated from the relative intensities of hybridization signals. Homotypic mixtures of vaccine-derived and wild polioviruses, however, are more difficult to recognize. In most cases, wild polioviruses predominate in cell culture isolates, and their presence is indicated by a reduction in the Sab/VP1 and Sab/5UT signals relative to that of EV/5UT. A small proportion of homotypic mixtures may contain largely Sabin vaccine-derived viruses, such that underlying wild polioviruses might be overlooked without additional analyses.

To overcome this problem, we have developed RNA probes specific to different wild poliovirus genotypes (unpublished data). Isolates can therefore be screened directly for the presence of wild polioviruses, and the geographic and temporal distributions of indigenous wild genotypes can be monitored through straightforward, rapid methods.

We have also prepared an RNA probe specific for the type 2 reference strain, MEF-1. Poliovirus reference strains (usually Mahoney, MEF-1, and Saukett) are occasionally submitted as clinical isolates. From the specificities of our Sab/VP1 and Sab/5UT probe sets, Mahoney and Saukett would be recognized as Sabin vaccine related, but MEF-1 would be incorrectly identified as a wild isolate. To avoid the implementation of potentially unnecessary control measures that might follow the report of MEF-1 as a wild isolate, we have prepared an MEF-1/VP1 probe to supplement our Sab/VP1 set.

The reagents and methods described here have been distributed within a global network of laboratories supporting the Poliomyelitis Eradication Initiative (58). The RNA probes have replaced the synthetic oligodeoxynucleotide probes used earlier in the Americas (10, 37). The combined advantages of reliability, speed, technical simplicity, and safety have favored the implementation of RNA probe methodology for laboratory surveillance of wild polioviruses throughout the world.

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This paper is dedicated to the memory of our colleague, George Marchetti.

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