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Multiplex PCR Method for Identifying Recombinant Vaccine-Related Polioviruses

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The recent discovery of recombinant circulating vaccine-derived poliovirus (recombinant cVDPV) has highlighted the need for enhanced global poliovirus surveillance to assure timely detection of any future cVDPV outbreaks. Six pairs of Sabin strain-specific recombinant primers were designed to permit rapid screening for VDPV recombinants by PCR.

The World Health Organization (WHO) is nearing its goal of worldwide eradication of circulating wild polioviruses (22, 23a). However, the recent appearance of outbreaks associated with circulating vaccine-derived poliovirus (cVDPV) in countries using oral poliovirus vaccine at low rates of coverage (8, 16, 16a, 25) underscores the critical importance of maintaining high rates of polio vaccine coverage in order to prevent the emergence of cVDPVs. The outbreaks also highlight the importance of maintaining sensitive poliovirus surveillance in order to detect any genetically divergent VDPVs (17). In response to the 2000 to 2001 cVDPV outbreak in Hispaniola (8), the WHO Global Polio Laboratory Network implemented additional guidelines for the testing of poliovirus isolates to screen for VDPVs (20). Poliovirus isolates are identified according to their genetic properties by probe hybridization (7), diagnostic PCR assays (11, 12, 24), or PCR-restriction fragment length polymorphism analysis (2). All isolates are also tested for antigenic change by using specific cross-absorbed sera in an enzyme-linked immunosorbent assay format or panels of monoclonal antibodies in neutralization tests (19).

All cVDPVs identified so far have been vaccine/nonvaccine recombinants having some or all of their noncapsid sequences derived from species C human enteroviruses (or wild polioviruses) (8, 16, 16a). The unusual recombinant properties of cVDPV genomes allowed us to develop an alternative approach to screen for cVDPVs. We designed Sabin strain recombinant (SAB-REC) primer sets, targeting sequences in the 2C and 3D regions, for use in a diagnostic PCR assay to detect recombination in the noncapsid regions of vaccine-related isolates. Nonrecombinants and vaccine/vaccine recombinants could be identified by their patterns of reactivity with the SAB-REC primers. Vaccine-related isolates that did not generate specific amplification products when the SAB-REC primers were used were likely to be vaccine/nonvaccine recombinants. We developed this indirect approach to screen for vaccine/nonvaccine recombinants because the high diversity of recombinant noncapsid sequences precluded the design of recombinant-specific PCR primers necessary for direct detection. Candidate vaccine/nonvaccine recombinants, which represent a small proportion of vaccine-related isolates, can be further characterized by sequencing.

The SAB1-REC-2C and SAB3-REC-2C primers amplified an interval at the 5′ half of the 2C region (nucleotides [nt] 4284 to 4482), yielding a 199-bp product, whereas the SAB2-REC-2C primers amplified an adjacent interval upstream (nt 4224 to 4412), yielding a 189-bp product (Table 1). The SAB1-, SAB2-, and SAB3-REC-3D primers targeted variable sequences near the middle of the 3D region (from approximately nt 6423 to 6850), yielding amplification products of 225, 226, and 228 bp, respectively (Table 1).

Mixtures for PCR assays with the Sabin recombinant primer sets (one SAB-REC serotype set per reaction tube) included the reference strains Sabin 1 (LSc 2ab), Sabin 2 (P712 ch 2ab), and Sabin 3 (Leon 12 a_1b), all cVDPV isolates from the outbreaks in Egypt (type 2; 1983 to 1993) (25), Hispaniola (type 1; 2000 to 2001) (8), the Philippines (type 1; 2001) (16a), and Madagascar (type 2; 2001) (16), and 2,096 vaccine-related isolates (771 type 1, 643 type 2, and 682 type 3) from cases of acute flaccid paralysis that occurred in the period from 2000 to 2003 in Africa, the Americas, Asia, and Europe. All RNA samples were amplified by reverse transcription-PCR (42°C for 30 min) followed by 30 cycles of amplification (95°C, 1 min; 50°C, 1 min; and 65°C, 1 min) in a DNA thermal cycler. Reverse transcription-PCR enzyme concentrations and conditions for electrophoresis in 10% polyacrylamide gels were as described previously (12).

The SAB-REC primers specifically amplified the sequences of the corresponding Sabin reference strain but not those of a diverse set of 52 contemporary wild poliovirus isolates representing all three serotypes (8, 9, 25). Sequences of 10 human enterovirus species C reference strains (4, 14), representing serotypes that appear to be frequent partners in noncapsid sequence recombination with polioviruses (4), were not amplified by the SAB-REC primer sets in our PCR assays. None of the cVDPV isolates from the outbreaks in Egypt (30 isolates), Hispaniola (31 isolates), the Philippines (4 isolates), and

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TABLE 1. Sabin recombinant primers

Primer	Sequence (5′→3′)	Position[a]	Predicted size (bp) of PCR product
SAB1-REC-2C-S[b]	TGTAACAAAACTTAGACAAC	4284–4303	199
SAB1-REC-2C-A[c]	TATGTAGTTGTTAATGGTATG	4482–4462	
SAB1-REC-3D-S	TAAGGAAATGCAAAAACTGC	6423–6442	226
SAB1-REC-3D-A	ATCGCACCCTACTGCTGA	6648–6631	
SAB2-REC-2C-S	CAAATTCATTAGTTGGTTGC	4224–4243	189
SAB2-REC-2C-A	TGGATAGATAGCCACCGC	4412–4395	
SAB2-REC-3D-S	AGGAAATGCGGAGACTCTTA	6425–6444	225
SAB2-REC-3D-A	GGATCACAACCAACTGCACT	6649–6630	
SAB3-REC-2C-S	TGTAACCAAATTGAAACAGT	4284–4303	199
SAB3-REC-2C-A	TATGTAATTATTAATGGTGTG	4482–4462	
SAB3-REC-3D-S	CAAAGAAATGCAAAGACTTT	6423–6442	228
SAB3-REC-3D-A	GGATCGCATCCAACTGCACT	6650–6631	

[a] Nucleotide positions are numbered according to the consensus system of Toyoda et al. (18).
[b] S, sense polarity.
[c] A, antisense polarity.

Madagascar (8 isolates) were amplified using the SAB-REC primers.

All of the 2,096 Sabin vaccine-related isolates tested with our SAB-REC primer sets had been previously identified by diagnostic PCR (24) and further characterized antigenically in enzyme-linked immunosorbent assays using cross-absorbed antisera (19). Only 2 of the 771 type 1 vaccine-related isolates were found to be recombinants (i.e., missing either or both of the 2C or 3D SAB1-REC bands). Sequence analysis confirmed that the two isolates were Sabin 1/Sabin 2/Sabin 1 double recombinants. A larger proportion (44 of 643; 6.8%) of the type 2 vaccine-related isolates were found to be recombinant. Of the 44 recombinants, 35 were vaccine/vaccine recombinants and only 9 (1.4% of all type 2 vaccine-related isolates) were vaccine/nonvaccine recombinants. Type 3 vaccine-related isolates had the highest proportion of recombinants (69 of 682; 10.1%): 61 were vaccine/vaccine recombinants which had patterns of reactivity with the SAB-REC primers that were fully consistent with the recombinant sequences of their noncapsid regions, and 8 were vaccine/nonvaccine recombinants with diverse noncapsid sequences. Genomic sequencing of 120 isolates (40 of each serotype) confirmed their identification as nonrecombinant by SAB-REC PCR.

The SAB-REC PCR assay described here provides a rapid, efficient method to screen for recombinants among large collections of vaccine-related isolates. Because the SAB-REC primers sample only part of the noncapsid sequences, we may have underestimated the number of vaccine/vaccine recombinants in our collection. Also, the SAB-REC primers should be used only with isolates that do not contain poliovirus mixtures, as the amplification patterns obtained from vaccine virus mixtures cannot be unambiguously interpreted. Because our PCR assay can be readily assimilated into the current diagnostic procedures of the WHO Global Polio Laboratory Network (1, 5, 20, 21), it offers a new, inexpensive, and broadly applicable approach for the prompt detection of candidate cVDPV isolates. Although the most definitive characterization of VDPV isolates is by genomic sequencing (3, 6, 8, 10, 13, 15, 16, 16a, 25) our screening method can be readily implemented in laboratories lacking their own sequencing facilities. Early detection of VDPVs, especially cVDPVs, will become increasingly important as the prevalence of wild polioviruses recedes and the only remaining source of poliovirus infection worldwide is oral poliovirus vaccine.

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