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Rapid Group-, Serotype-, and Vaccine Strain-Specific Identification of Poliovirus Isolates by Real-Time Reverse Transcription-PCR Using Degenerate Primers and Probes Containing Deoxyinosine Residues▽‡

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We have adapted our previously described poliovirus diagnostic reverse transcription-PCR (RT-PCR) assays to a real-time RT-PCR (rRT-PCR) format. Our highly specific assays and rRT-PCR reagents are designed for use in the WHO Global Polio Laboratory Network for rapid and large-scale identification of poliovirus field isolates.

Four methods are currently in routine use in the WHO Global Polio Laboratory Network (GPLN) (3, 14, 15) for differentiation between vaccine-related and wild poliovirus isolates (intratypic differentiation): (i) the enzyme-linked immunosorbent assay, using highly specific cross-absorbed antisera (13); (ii) nucleic acid hybridization, using Sabin vaccine strain-specific RNA probes (4); (iii) reverse transcription-PCR (RT-PCR), using vaccine strain-specific primers (16); and (iv) RT-PCR followed by restriction fragment length polymorphism analysis (1, 13). To achieve the required specificities for binding to variable target sequences, our RT-PCR primers were designed to contain mixed-base or inosine residues at positions of codon degeneracy (8, 9). In the poliovirus diagnostic RT-PCR kits currently distributed throughout the GPLN, identifications are based upon the mobilities of amplicons in polyacrylamide gels (8, 9, 15, 16). This approach, while achieving the high levels of diagnostic accuracy and reliability required for global poliovirus surveillance, is especially laborious for GPLN laboratories with large workloads.

Development of real-time RT-PCR (rRT-PCR) has opened the way for more-rapid and -accurate diagnostic assays (2). We have adapted our previously described poliovirus diagnostic RT-PCR methods (7–9) to the real-time format with an emphasis on high template specificities rather than quantitative determination of template concentrations. These new assays were tested against both Sabin vaccine-related isolates and wild poliovirus isolates representing all currently circulating genotypes.

The enterovirus group-specific (panEV) primers used were essentially as described previously (10, 17). They target highly conserved sequences in the 5′ untranslated region, and the antisense polarity primer (PCR-1; 10 pmol per assay) and TaqMan probe (panEV probe; 5 pmol) each have only one mixed-base residue, while the sense polarity primer (PCR-2; 10 pmol) is nondegenerate.

To accommodate the wide variability and rapid evolution of poliovirus genomes, degenerate codon positions on the template were matched to mixed-base or deoxyinosine residues on both the primers and the TaqMan probes. Designing the degenerate TaqMan probes (a 15-pmol probe for each assay) was especially challenging because of the need to use longer sequences to obtain good hybrid stabilities while simultaneously compensating for the high level of degeneracy of sequences between primer binding sites. Although hybrid stabilities can be estimated by physicochemical calculations (12), development of the optimal primer and probe sets was a highly empirical process because variation within the target sequences was not predictable. The degenerate poliovirus group-specific (panPV; 80 pmol) and poliovirus serotype-specific (seroPV1, seroPV2, and seroPV3; 40 to 80 pmol) primer pairs are similar to those described previously (8, 9) (see Table S1 in the supplemental material). All contain numerous mixed-base and inosine-containing residues to compensate for the high levels of variability in capsid region target sequences within and across poliovirus serotypes. Although the panPV and seroPV sense primers were unchanged, three of the four antisense primers were modified from the original descriptions. The new panPV/PCR-1* primer target sequences were shifted 24 nucleotides downstream from those of the original panPV antisense primer (8) and encode the conserved amino acid residues (VYQIMYV). The new 103-bp amplicon contains the codons of an 8-amino-acid sequence (NNGHALNQ) that has been found only in polioviruses (9). This sequence includes the initiating primer sequences we described previously (8) and in this study was targeted by panPV probe 21A. The new seroPV1A* primer targets the same sequences as the original

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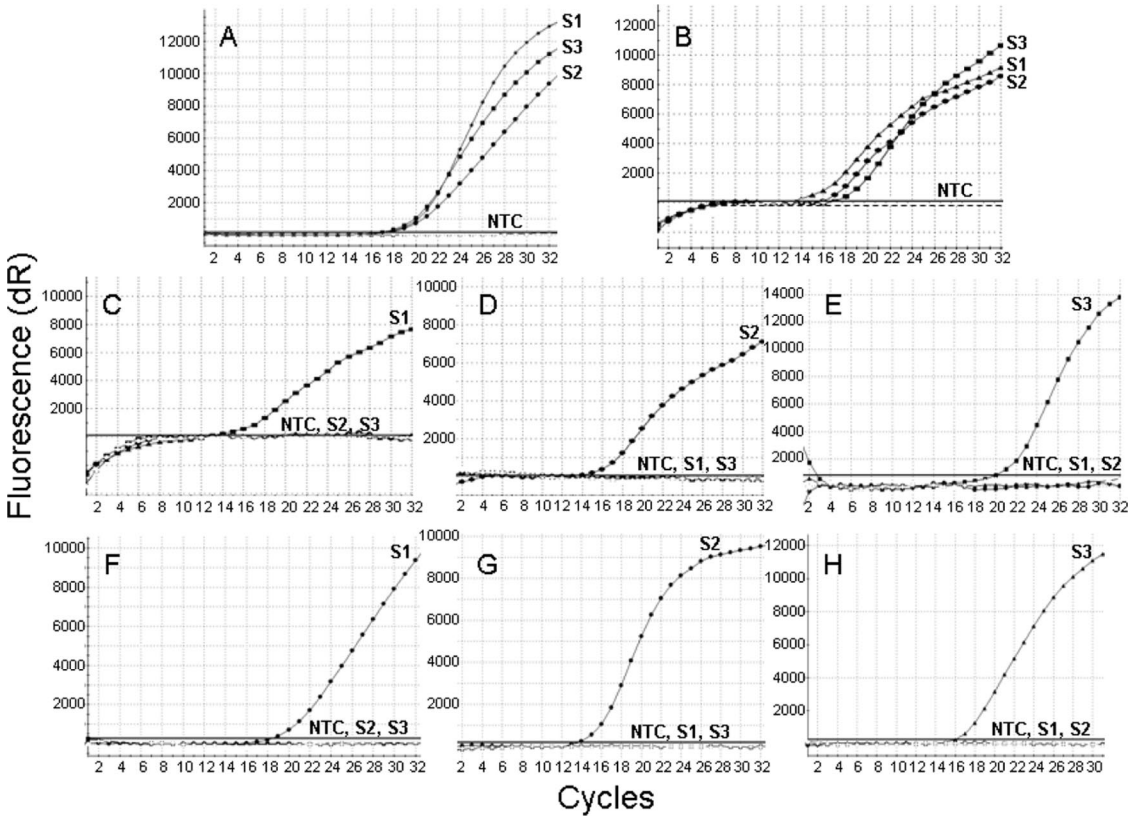

FIG. 1. Specificities of poliovirus TaqMan rRT-PCR primers and probes, demonstrated by using Sabin strain RNA templates (S1, Sabin 1; S2, Sabin 2; S3, Sabin 3; NTC, no-template control). Each assay (and fluorophore) was performed singly to demonstrate individual assay specificities: (A) panEV (FAM); (B) panPV (FAM); (C) seroPV1 (FAM); (D) seroPV2 (FAM); (E) seroPV3 (FAM); (F) Sab1 assay (CY5); (G) Sab2 assay (FAM); and (H) Sab3 assay (ROX). The no-template control was used to manually set the zero baseline fluorescence emissions. The efficiency of each assay was >90%, based on 10-fold dilutions of control RNAs (10 ng to 1 pg). Cycle threshold values of 30 or more were observed to approach the sensitivity limits of the real-time detection system; therefore, cycle threshold values of <30 were considered positive detections of the target template.

primer but with three base replacements, and the new seroPV3A* primer is two bases longer at the 5′ end and contains four base replacements (see Table S1 in the supplemental material). The panPV, panEV, and seroPV TaqMan probes all contained 6-carboxyfluorescein (FAM) at the 5′ ends and black hole quencher 1 at the 3′ ends.

The original Sabin strain primers (10 pmol each) were designed to generate amplicons with optimal resolution and spacing on polyacrylamide gels (16, 17), but the new primers and probes (S1-CY5, S2-FAM, and S3-ROX; 5 pmol each in a multiplex assay) were designed to target nonoverlapping Sabin strain-specific sequences (Fig. 1F through H). To maximize specificity to Sabin strain-related isolates, which usually show limited sequence variability, the Sabin primers and probes were nondegenerate.

The specificities of our rRT-PCR assays were tested against a large collection of poliovirus and other human enterovirus species C (HEV-C) isolates that had previously been characterized by sequencing of at least the ~900-nucleotide VP1 region. The panEV and panPV assays (Fig. 1A and B) were able to detect all 4,138 polioviruses tested (wild plus vaccine-related viruses: poliovirus type 1 [PV1] = 2,096 + 248; PV2 = 48 + 393; PV3 = 1,267 + 86). None of the poliovirus-specific assays amplified the HEV-C reference strains (CA1, CA11, CA13, CA15, CA16, CA17, CA18, CA19, CA20, CA21, CA22, and CA24; see Fig. S1 in the supplemental material) or HEV-C clinical isolates ($n > 100$). The panPV and seroPV rRT-PCR assays (Fig. 1B through E) must be run individually with the lower annealing and extension temperatures described previously (7–9), as spurious products are generated in multiplex reactions and specific products are obtained inconsistently at higher cycling temperatures because of the reduced stabilities of hybrids formed by the inosine-containing primers and probes. Molecular typing results from the serotype rRT-PCR assays were in complete agreement with the typing of wild poliovirus isolates determined by VP1 sequencing. Isolates tested included representatives of all known recent (1952 to 2008) wild poliovirus genotypes (5, 6, 18) spanning the widest known range of poliovirus genetic diversity and included nearly all of the wild PV1 ($n = 2,096$) and PV3 ($n = 1,267$) isolates from Africa (Nigeria and 12 other countries), the Middle East (Yemen and Saudi Arabia), and South Asia (Pakistan, Afghanistan, India, Nepal, Bangladesh, and Myanmar) sequenced by the CDC. The seroPV assays were also used to correctly type 727 vaccine-related isolates of all three serotypes, including 200 genetically divergent vaccine-derived poliovirus isolates from at least nine different vaccine-derived poliovirus emer-

gences (data not shown). No spurious cross-serotype amplifications were observed.

The sensitivities of our poliovirus rRT-PCR assays are approximately equivalent to those of our previously described standard RT-PCR assays (8, 9, 16). Sensitivities were maximized when the templates were RNAs extracted from infected cells. However, all assays also performed well with the template present in 0.5 μl of undiluted clarified lysate of infected RD cells. RD cells (ATCC CCL-136) are recommended because poliovirus titers are generally higher (~1 log) than in L20B cells (mouse L cells expressing the human receptor for poliovirus [11]).

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