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Sequential Triplex Real-Time PCR Assay for Detecting 21 Pneumococcal Capsular Serotypes That Account for a High Global Disease Burden

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We developed and validated a real-time PCR assay consisting of 7 triplexed reactions to identify 11 individual serotypes plus 10 small serogroups representing the majority of disease-causing isolates of *Streptococcus pneumoniae*. This assay targets the 13 serotypes included within the 13-valent conjugate vaccine and 8 additional key serotypes or serogroups. Advantages over other serotyping assays are described. The assay will be expanded to 40 serotypes/serogroups. We will provide periodic updates at our protocol website.

The pneumococcal capsular serotype is an essential parameter for vaccine-related disease surveillance. Conventional serotyping is difficult and not applicable for culture-negative clinical specimens. Conventional PCR assays targeting serotype-specific genes (1–4) are useful for serotyping isolates and clinical specimens (5–12); however, real-time PCR is faster and more sensitive (13–17). Here we describe a triplexed real-time multiplexed PCR (rmPCR) assay that provides advantages over previously described assays.

Twenty-one oligonucleotide sets targeting 21 serotypes/serogroups (Table 1) were designed using published *cps* sequences, Primer Express version 3.0 (Applied Biosystems, Foster City, CA), and Beacon Designer (Premier Biosoft International, Palo Alto, CA). Probes were 5′ labeled with 6-carboxyfluorescein (FAM), hexachloro-6-carboxyfluorescein (HEX), 6-carboxy-X-rhodamine (ROX), or indodicarbocyanine (CY5). Black hole quencher 1 or 2 was placed either at the 3′ end of the probe or internally on a thymidine base. If internally quenched, the 3′ end was capped with a phosphate group to prevent probe extension. Due to issues pertaining to sensitivity, specificity, and annealing temperature, it was necessary for five probes to contain locked nucleic acids. Primers/probes were synthesized at the CDC Biotechnology Core Facility.

The 21 serogroups/serotypes were grouped into seven triplex reactions in four different regional schemes (Table 2). Reaction mixtures contained 5 μl of DNA, primers/probes, 12.5 μl Invitrogen-Platinum Quantitative PCR SuperMix-UDG master mix, 1.5 μl $MgCl_2$ (50 nM), and water for a final 25-μl volume. Amplification in the Stratagene Mx3005P employed a temperature of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Primer and probe concentrations were formulated to obtain the highest DNA dilution yielding a cycle threshold (C_T) value of ≤35.

Assay validation employed 967 pneumococcal strains representing 78 capsular serotypes and 5 capsule-deficient strains lacking type-specific biosynthetic genes (Table 3). Of these, 803 were collected through invasive pneumococcal disease surveillance in the United States (http://www.cdc.gov/abcs/index.html). In addition, 169 isolates from Brazil, India, Kenya, Mongolia, Mozambique, Nepal, Peru, and Thailand were included. Forty-three isolates of 15 related species, which included *Streptococcus pseudopneumoniae* (10), *Streptococcus gordonii* (6), *Streptococcus mitis* (4), *Streptococcus oralis* (3), *Streptococcus cristatus* (2), *Streptococcus sanguinis* (2), *Streptococcus parasanguinis* (3), *Streptococcus salivarius* (3), *Streptococcus vestibularis* (3), *Streptococcus infantis* (1), *Streptococcus australis* (1), *Streptococcus intestinalis* (1), *Streptococcus peroris* (1), *Streptococcus sinensis* (1), and *Streptococcus oligofermentans* (1), were tested. Finally, 11 strains of undetermined species within the Mitis group (based upon 16S rRNA gene sequences and DNA reassociation data [18]) were tested. DNA was extracted using the Qiagen DNA minikit (Qiagen Inc., Valencia, CA) (18). A loopful of bacteria from a blood agar plate after overnight growth was resuspended in lysis buffer containing 0.04 g/ml lysozyme and 75 U/ml of mutanolysin and incubated for 1 h at 37°C. The remaining extraction procedure was performed by following the kit manufacturer's instructions. Serial dilutions of DNAs were prepared in PCR-grade water to obtain C_T values in the range of 20 to 30. Specific amplification for serogroups/serotypes within each triplex reaction was assessed against all strains, with no cross-reactivity observed between serogroups/serotypes in monoplex or triplex reactions. No amplification was observed for any assay when testing capsule-deficient pneumococci and nonpneumococcal strains.

A total of 377 cerebrospinal fluid (CSF) samples and 104 blood culture broth (BLB) samples were obtained in accordance with the CDC Institutional Review Board, including 256 CSF samples obtained from Turkish meningitis surveillance (our unpublished data). The remaining specimens, including culture-negative BLB specimens that had Gram stain and/or latex test results consistent

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TABLE 1 Primer and probe information for the real-time multiplex PCR serotyping assay[a]

GenBank accession no. (gene)	Coordinates	Primer/probe ID	Sequence (5′–3′)	Probe dye	Probe special chemistry	Quencher (3′)	nM
CR931632 (*wzy*)	9875–10035	1-F	TTTCATCCCTATGTGTGGTATAG				300
		1-R	GCTTTAGAAGGTAGAGTTAACAAC				300
		1-Probe	TGCCAAAGCCAGCCAT	FAM	LNA[b]	BHQ1	100
CR931633 (*wzy*)	10342–10452	2-F	TGTTATCCCATATAAGAACCGAGTGT				300
		2-R	AAAATTACCCCAAAAGCTATCCAA				300
		2-Probe	TTGCAATT"T"CAATTTTTTTGCCCCAATCTC	FAM/HEX[c]	"T"[d] = BHQ1	BHQ1	200
CR931634 (*galU*)	8564–8648	3-F	CCACTAAAGCTTTGGCAAAAGAAA				300
		3-R	CCCGAACGTAAAGCTTCTTCA				300
		3-Probe	TTGTAGACCGCCCCACAA"T"TCATTTTGT	HEX	"T"[d] = BHQ1	BHQ1	200
CR931635 (*wzy*)	10521–10734	4-F	GCTTCTGCTGTAACTGTTGTGC				300
		4-R	CACCACCATAGTAACCAAAGTTCC				300
		4-Probe	TTCCACAAAAGAAGAGCCTACAGGTAACCCCA	ROX/CY5[e]		BHQ2	100
CR931637 (wzy)	7001–7082	5-F	CATGATTTATGCCCTCTTGCAA				300
		5-R	GACAGTATAAGAAAAAGCAAGGGCTAA				300
		5-Probe	TCTTCTTCTCA"T"CGTTTCCGCATGCTTTT	FAM/HEX[c]	"T"[d] = BHQ1	BHQ1	200
CR931639 (*wciP*)	8796–8900	6A/6B/6C/6D-F	GTT TGCACTAGAGTATGGGAAGG				200
		6A/6B/6C/6D-R	TAGCCTTTCTGAAAACATTTAGCG				200
		6A/6B/6C/6D-Probe	TGTTCTGCCC"T"GAGCAACTGGTCTTGTATC	FAM	"T"[d] = BHQ1	BHQ1	200
EF538714 (*wciN*)	7102–7250	6C/6D-F	TTGGGATGATTGGTCGTATTAG				200
		6C/6D-R	CTCTTCAATTAGTTCTTCAGTTCG				200
		6C/6D-Probe	CCACGCAATTCGCCATC	FAM	LNA[b]	BHQ1	100
CR931643 (*wzy*)	14101–14204	7F/7A-F	ATGAAGGCTTTGGTTTGACAGG				200
		7F/7A-R	ATTCTCGCCATCAATTGCATATTC				200
		7F/7A-Probe	ACACCACTATAGGCTGTTGAGACTAACGCACA	ROX/CY5[e]		BHQ2	100
CR931648 (*wzx*)	11767–11920	9V/9A-F	AGGTATCCTATATACTGCTTTAGG				300
		9V/9A-R	CGAATCTGCCAATATCTGAAAG				300
		9V/9A-Probe	ACACATTGACAACCGCT	HEX	LNA[b]	BHQ1	100
CR931653 (*wzy*)	12015–12121	11A/11D-F	AAATGGTTTGGATATGGTTTGTTTGG				300
		11A/11D-R	AGTGCTAACTGTAAAACTTGATTATGAG				300
		11A/11D-Probe	ATTCCAACTTCTCCCAATTTCTGCCACGG	ROX/CY5[e]		BHQ2	100
CR931660 (*wzx*)	15066–15145	12F/12A/12B/44/46-F	GCACCCACGGGTAAATATTCTAC				300
		12F/12A/12B/44/46-R	CAACTAAGAACCAAGGATCCACAG				300
		12F/12A/12B/44/46-Probe	TGCCCACCAACACCAGGTCCAGGT	ROX/CY5[e]		BHQ2	200
CR931662 (*wzy*)	7920–8007	14-F	AGAGTGTATGAGGAATCC				300
		14-R	ATATATCTACTGTAGAGGGAAT				300
		14-Probe	CGCCAAGTAACA"T"TTCCATTCCATT	FAM	"T"[d] = BHQ1	BHQ1	100

CR931663 (*wzy*)	7839–7968	15A/15F-F	AATTGCCTATAAACTCATTGAGATAG				200
		15A/15F-R	CCATAGGAAGGAAATAGTATTTGTTC				200
		15A/15F-Probe	CCCGCAACTCTGTCCT	FAM	LNA[b]	BHQ1	100
CR931668 (*wzy*)	12016–12214	16F-F	TAATGTTATGACCTTGGTAATCTTCCC				300
		16F-R	TCCCAAAGGATAATCAATAACTTTTAGAAG				300
		16F-Probe	AGCCATAAGTCT“T”CCAAATGCTTAACCGCT	HEX	“T”[d] = BHQ1	BHQ1	100
CR931673 (*wzy*)	12934–13081	18C/18A/18B//18F-F	TCGATGGCTAGAACAGATTTATGG				200
		18C/18A/18B/18F-R	CCATTGTCCCTGTAAGACCATTG				200
		18C/18A/18B/18F-Probe	AGGGAGTTGAATCAACCTATAATTTCGCCCC	HEX		BHQ1	100
CR931675 (*wzy*)	9492–9580	19A-F	CGCCTAGTCTAAATACCA				200
		19A-R	GAGGTCAACTATAATAGTAAGAG				200
		19A-Probe	TATCAATGAGCCGATCCGTCACTT	FAM		BHQ1	100
CR931678 (*wzy*)	11131–11350	19F-F	TGAGGTTAAGATTGCTGATCG				300
		19F-R	CACGAATGAGAACTCGAATAAAAG				300
		19F-Probe	CGCACTGTCAATTCACCTTC	ROX/CY5[e]	LNA[b]	BHQ2	100
CR931682 (*wcwV*)	11780–11868	22F/22A-F	TCTATTAAATAACCCATTGGAATTGAAACG				200
		22F/22A-R	TCGCAATTGAAGACCACATAAACTG				200
		22F/22A-Probe	TCCGTAAT“T”CGCTTATGGGCACATTCTCCA	HEX	“T”[d] = BHQ1	BHQ1	200
CR931683 (*wzy*)	8626–8711	23A-F	CTCCCCTCCATTACCCATTTGG				200
		23A-R	TGAAGAAAGTGCTGTTTGTGAACC				200
		23A-Probe	AGCTAGAAC“T”CCCACACTCCCTACTCCCA	ROX/CY5[e]	“T”[d] = BHQ2	BHQ2	100
CR931685 (*wzy*)	9049–9274	23F-F	GACAGCAACGACAATAGTCATCTC				300
		23F-R	TCCATCCCAACCTAACACACTTC				300
		23F-Probe	ATTGTGTCCA“T”AACCCTTCGTCGTATTTCCAAAG	ROX/CY5[e]	“T”[d] = BHQ2	BHQ2	200
CR931702 (*wzy*)	11778–11882	33F/33A/37-F	GGAACTGGTTCAGCAACTATACG				200
		33F/33A/37-R	GGTTCTAAGACCGTCTGAAATACC				200
		33F/33A/37-Probe	CCCCAAATAGGAC“T”TTTCTGCCATGCCAAA	HEX	“T”[d] = BHQ1	BHQ1	200

[a] Abbreviations: FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine; CY5, indodicarbocyanine; BHQ, black hole quencher; ID, identification; LNA, locked nucleic acid.

[b] Locked nucleic acid nucleotides are underlined.

[c] The probe for serotype 2 is labeled with FAM for the triplex reaction formulated for the Africa and Latin America regions and HEX for the triplex reaction formulated for the U.S. and Asia regions; the probe for serotype 5 is labeled with FAM for the triplex reaction formulated for the U.S. and Asia regions and HEX for the triplex reaction formulated for the Africa and Latin America regions.

[d] “T,” black hole quencher placed internally on the thymidine base.

[e] Probe is labeled with CY5 if ROX is used as a reference dye.

TABLE 2 Triplexed assays for 21 common pneumococcal serotypes or serogroups designed for current serotype distributions within 4 different geographic regions[a]

Reaction no.	Serotype distribution tested in each region scheme			
	United States	Africa	Latin America	Asia
1	3, 7F/7A, 19A	**1, 5, 23F**	14, 18C/18B/18A/18F, 19F	14, 18C/18B/18A/18F, 19F
2	6C/6D, 12F/12A/12B/44/46, 22F/22A	4, 6A/6B/6C/6D, 9V/9A	4, 6A/6B/6C/6D, 9V/9A	**2, 5, 23F**
3	15A/15F, 23A, 33F/33A/37	14, 18C/18A/18B/18F, 19F	**1, 5, 23F**	4, 6A/6B/6C/6D, 9V/9A
4	**1, 11A/11D, 16F**	3, 7F/7A, 19A	3, 7F/7A, 19A	3, 7F/7A, 19A
5	4, 6A/6B/6C/6D, 9V/9A	6C/6D, 12F/12A/12B/44/46, 22F/22A	6C/6D, 12F/12A/12B/44/46, 22F/22A	6C/6D, 12F/12A/12B/44/46), 22F/22A
6	14, 18C/18B/18A/18F, 19F	15A/15F, 23A, 33F/33A/37	15A/15F, 23A, 33F/33A/37	**1, 11A/11D, 16F**
7	**2, 5, 23F**	**2, 11A/11D, 16F**	**2, 11A/11D, 16F**	15A/15F, 23A, 33F/33A/37

[a] The U.S. distribution was determined from post-PCV7 Active Bacterial Core surveillance data. The other schemes relied upon our own relatively limited sampling of these regions. Assays indicated in bold indicate serotypes assayed within different triplex reactions used within more than one region scheme.

with pneumococcal diagnosis, were from invasive bacterial disease surveillance at the National Institute for Communicable Diseases in South Africa (http://www.nicd.ac.za). For clinical specimens, 200 μl of specimen or 50 μl of BLB was added to 100 μl of Tris-EDTA buffer containing 0.04 g/ml lysozyme and 75 U/ml mutanolysin (Sigma Chemical Co.), and the mixture was incubated for 1 h at 37°C. DNA extraction was performed by following Qiagen DNA minikit instructions. DNA extracted from BLB was diluted to 1:1,000 to avoid PCR inhibition often observed from specimens with extremely high pneumococcal DNA concentrations (our unpublished data). DNA extracts with real-time PCR *lytA* assay (18) (www.cdc.gov/ncidod/biotech/strep/protocols.htm) C_T values of ≤30 were subjected to both conventional multiplex PCR (cmPCR) (1) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm) and rmPCR serotyping. Positive *lytA* results were obtained for 104/377 (27.6%) CSF samples and 100/104 (96.1%) BLB samples (Table 4). Specimens with *lytA* C_T values of >30 were subjected to rmPCR and retested using individual monoplex real-time PCRs. As expected, rmPCR was negative for the serotypes/serogroups 7C/7B/40, 8, 9N/9L, 15B/15C, 17F, 21, 23B, 35B, 35F/47F, and 38F/25A/25F (not included in the rmPCR assay), which were cmPCR positive. Serotypes targeted by both rmPCR and cmPCR yielded identical positive results verified by monoplex real-time PCR. Positive real-time PCR serotyping reactions (single or triplexed) generally resulted in C_T values that were approximately the same as the predetermined *lytA* C_T values; however, variation of up to 3 C_T values was observed for rmPCR in 13 (12.5%) CSF samples in a comparison with *lytA* C_T values. Thirty randomly selected *lytA*-negative extracts were rmPCR negative (data not shown).

TABLE 3 *Streptococcus pneumoniae* isolates used to validate the real-time multiplex serotyping PCR assay

S. pneumoniae serotype(s)	No. of isolates tested[a]
1, 3, 4, 5, 6C, 7F, 9V, 11A, 12F, 14, 15C, 16F, 18C, 19A, 19F, 22F, 23A, 23F, 33F	30
6A, 6B, 15A, 15B, 18A, 23B	20
18B	15
7C, 9A	14
38	13
2, 13, 37	11
21, 24F	10
11B, 28A, 35A	8
18F, 19B, 20, 31	7
7A, 15F, 35B, 39	6
8, 9L, 9N, 10A, 10F, 17F, 22A, 25F, 35F, nontypeable	5
33A, 34	4
24B, 28F, 46	3
6D, 12A, 24A, 25A, 35C, 47F	2
7B, 10C, 11C, 11D, 17A, 19C, 32F, 33C, 40, 41A, 42, 43, 44	1

[a] The number indicates the total number of isolates tested for each serotype in the row such that the entire number tested equals 967.

To determine the lower limit of detection (LLD), DNA was extracted from a suspension of overnight blood agar growth (in 0.85% saline) prepared at a density equivalent to a 0.5 McFarland standard (~1.5×10^8 CFU per ml), from which 10-fold serial dilutions were made. After vortexing, DNA was extracted from 200 μl of serial dilution suspensions (18). Real-time PCRs for each serotype/serogroup were performed in triplicate, with monoplex and triplex reactions run simultaneously. The LLD for each assay was the highest dilution that yielded a C_T value of ≤35. When tested in monoplex format, the assays for serotypes/serogroups 1, 6A/6B/6C/6D, 7F/7A, 9V/9A, 11A/11B, 12F/12A/12B/44/46, 15A/15F, 23A, and 23F reliably presented an LLD of ~7.5 cell genome equivalents per reaction. In monoplex format, the assays for serotypes/serogroups 2, 3, 4, 5, 6C/6D, 14, 16F, 18C/18A/18B/18F, 19A, 19F, 22F/22A, and 33F/33A/37 presented an LLD of ~15 cell genomes per reaction. Each of the 21 individual reactions presented an LLD of ~15 cell genome equivalents per reaction when tested in triplex format. When using thermocyclers that require master mix with ROX reference dye, used in combination with CY5 as a fluorescent dye, the LLD was ~150 genome equivalents per reaction for the serotype 4 assay. This discrepancy was not observed for master mix kits without ROX reference dye.

rmPCR offers advantages over cmPCR, including greater sensitivity and containment, in which amplification products are not potential contaminants for subsequent PCRs. Also, rmPCR offers more specificity in requiring hybridization to a probe in addition to amplification primers. Specificity is a concern, since related streptococcal strains carry homologs of pneumococcal capsular type-specific loci (19). Drawbacks of rmPCR relative to cmPCR include expense and limited multiplexing. A useful rmPCR assay (16) offers three 4-plex assays targeting the 13 serotypes included

TABLE 4 PCR serotyping results for *lytA*-positive CSF and blood culture broth (BLB) specimens with conventional and real-time mPCR

Specimen type (no. *lytA* positive/total no.) and C_T value	Serotype detection (no. of samples) by:	
	cmPCR[a,b]	rmPCR[b]
CSF (104/377)		
≤30	1 (8)	1 (8)
	19F (7)	19F (7)
	14 (6)	14 (6)
	4, 23F (4 each)	4, 23F (4 each)
	6A/6B, 8, 12F/12A/12B/44/46, 19A (3 each)	6A/6B, 12F/12A/12B/44/46, 19A (3 each)
	3, 5, 9N/9L, 38/25A/25F (2 each)	3, 5 (2 each)
	15A/15F, 15B/15C, 16F, 17F, 18C/18A/18C/18F, 21, 23B, 35B, 35F/47F (1)	15A/15F, 16F, 18C/18A/18C/18F (1 each)
	Nontypeable[c] (3)	Nontypeable[d] (16)
>30		6A/6B (6)
		18C/18A/18C/18F, 19F (3 each)
		5 (2)
		9V/9A, 12F/12A/12B/44/46, 14, 23F (1 each)
		Nontypeable[d] (25)
BLB (100/104)		
≤30	1 (19)	1 (19)
	4, 19A (13)	4, 19A (13 each)
	12F/12A/12B/44/46 (9)	12F/12A/12B/44/46 (9)
	6A/6B (8)	6A/6B (8)
	14 (6)	14 (6)
	3, 8 (4 each)	3 (4)
	5, 19F, 11A/11D (3 each)	5, 19F, 11A/11D (3 each)
	18C/18A/18B/18F, 23F, 38F/25A/25F (2 each)	18C/18A/18C/18F, 23F (2 each)
	7C/7B/40, 9N/9L, 9V/9A, 15B/15C, 35F/47F (1 each)	9V/9A (1)
	Nontypeable[c] (4 each)	Nontypeable[d] (14)

[a] Serotypes detected by cmPCR but not present in the real-time triplex reactions are underlined.
[b] These cmPCR and rmPCR reactions were all performed employing African schemes (described for the cmPCR assay at http://www.cdc.gov/ncidod/biotech/files/pcr-Africa-clinical-specimens.pdf).
[c] Nontypeable was defined by a reaction yielding no serotype/serogroup-specific band visible on agarose gel when tested for all 40 assays by conventional multiplex PCR.
[d] Nontypeable was defined by no C_T value for any of the 21 serotype/serogroup assays. The underlined samples in the preceding column, along with the nontypeable samples, were rmPCR nontypeable.

within the 13-valent conjugate vaccine PCV13. Although our assay employs only triplexed reactions, it includes the PCV13 types and 8 additional important serotypes/serogroups. Our assay provides better resolution of serogroup 6 through distinguishing 6A/6B from 6C/6D. This is important, since vaccination with the 7-valent conjugate vaccine does not protect against emergent serotype 6C (7, 20). Our assay includes serotype 2, which, although rare among U.S. disease isolates, is a significant cause of meningitis in Bangladesh (21) and Mongolia (our unpublished data). Another useful rmPCR assay (22) identifies 16 serotypes/serogroups, including PCV13 serotypes/serogroups and 3 additional targets. While it identifies serotype 8 and serogroup 15B/15C, not currently included in our rmPCR scheme, it does not identify serogroups 6C/6D, 11A/11D, 12F/12A/12B/44/46, 15A/15F, 22F/22A, and 33F/33A/37. Unlike our assay, it coidentifies 9N/9L with 9V/9A (our assay identifies only 9V/9A). Yet another useful real-time PCR serotyping scheme, which offers identification of 21 serogroups/serotypes (13) that overlap extensively with our assay but are only monoplexed, is available.

Our rmPCR assay appears best suited for regions where conjugate vaccines have not yet been implemented. For example, of serotyped invasive U.S. isolates collected during 1999 prior to PCV7 implementation, 92.8% (3,812/4,106) were among our rmPCR assay types (unpublished U.S. Active Bacterial Core surveillance data). For isolates collected during 2008, this fell to 79.3% (2,939/3,708), and the percentage declined further after implementation of PCV13, at which point sampling of 2011 and 2012 isolates shows that 74.2% (2,581/3,480) were covered by rmPCR. In contrast, our cmPCR assay (1) detects 40 serogroups/serotypes that encompass 99.9% (3,476/3,480) of this 2011-to-2012 sampling. Although our rmPCR assay is being expanded to all 40 cmPCR serotypes, it is useful in its current form. We will provide updates at http://www.cdc.gov/ncidod/biotech/strep/pcr.htm.

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