Single-Step Multiplex PCR Assay for Determining 92 Pneumococcal Serotypes

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For pneumococcal disease surveillance, simple and cost-effective methods capable of determining all serotypes are needed. Combining a single-tube multiplex PCR with fluorescently labeled primers followed by amplicon analysis using automated fluorescent capillary electrophoresis, each serotype of 92 reference isolates and 297 recently collected clinical isolates was successfully determined.

Streptococcus pneumoniae is a major human pathogen responsible for a wide variety of infections, from colonization and mild disease, such as sinusitis or otitis, to more-severe and life-threatening infections, such as invasive pneumonia or meningitis. The capsular polysaccharide is considered a major virulence factor in pneumococcal disease, with the composition, order, and linkage of the monosaccharides that make up the capsule determining a specific antigenic response that classifies pneumococci in different serotypes (1). It has long been known that antibodies against capsular polysaccharides are serotype specific and protective (2), and pneumococcal conjugate vaccines (PCVs), which included the polysaccharides of a limited number of serotypes, were developed to prevent pneumococcal diseases in young children. The introduction of PCVs was accompanied by a decrease in the incidence of invasive pneumococcal diseases but also by a change in the distribution of circulating serotypes (3, 4).

Knowing which *S. pneumoniae* serotypes cause infection is crucial in the surveillance of pneumococcal disease. Surveillance studies usually comprise many isolates, so techniques are needed that can type a large number of isolates simply and accurately. Among the developed techniques, and following the description of genes encoding the pneumococcal capsule (the *cps* gene cluster) by the Sanger Institute (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/), are different PCR-based strategies. Those performed in a single PCR normally detect a limited number of serotypes (5–7), whereas those detecting more serotypes usually require 3 to 8 multiplex PCRs that are performed simultaneously (8) or sequentially (9–14), real-time multiplex PCR (15), or the combination of conventional and real-time PCR (16).

By use of multiplex PCR combined with fragment analysis using automated fluorescent capillary electrophoresis (FAF-mPCR), Lawrence et al. (17) detected 5 serotypes and 3 serogroups, whereas Selva et al. (18) identified 68 pneumococcal serotypes by multiplexing 40 pairs of primers in a unique reaction that was cost-effective in terms of reagent costs and labor time requirements. The primers used in the work of Selva et al. were those available on the CDC website (<http://www.cdc.gov/streplab/downloads/pcr-oligonucleotide-primers.pdf>).

In the present work using a similar strategy, we designed a single-tube multiplex PCR with 55 fluorescently labeled pairs of primers to identify 92 capsular serotypes described up to 2010 (19)

(see PCR mix preparation, amplification conditions, and readout in the supplemental material). The design of the primers for the multiplex PCR was performed so that the size of two consecutive amplicons representing two different serotypes differed by ~10 bp (range, 8 to 22 bp). Expected amplicons were arranged by size, from 84 to 650 bp, and forward primers were alternatively 5' end labeled with three different dyes (FAM, HEX, NED). Consequently, two consecutive amplicons labeled with the same fluorescent dye had a size difference of ~30 bp (range, 20 to 48 bp), easily distinguished by capillary electrophoresis (Table 1).

The reference isolates of the 92 serotypes tested gave 55 amplicons of the expected size, except for the reference isolate of serotype 6C (a well-characterized clinical isolate) that amplified the *wciNbeta* gene specific for serotypes 6C and 6D but not the common *wzy* gene of all serotypes of serogroup 6. Of the amplicons obtained with the 92 reference isolates, 31 identified a specific serotype, 20 serotypes of the same serogroup, and 4 serotypes of different serogroups.

The capsular serotypes of all the 297 pneumococcal clinical isolates were correctly deduced using FAF-mPCR. Of these, 187 (63%) were identified at the serotype level; of the remaining isolates, 66 (22.2%), 31 (10.5%), 4 (1.3%), and 9 (3%) gave an amplicon that comprised 2, 3, 4, or 5 serotypes, respectively (Table 2).

Since the pneumococcal capsular genes were described, there have been multiple molecular approaches to determine pneumococcal serotypes. In our work, we proved that at least 92 serotypes can be accurately determined in a single-tube multiplex PCR after establishment of their specific sizes and the colors of their fluorescent peaks. The methodology described here has been proved by

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TABLE 1 Sequences of the 55 primer pairs included in the pneumococcal serotyping multiplex PCR

Serotype	Amplicon size (bp)	Forward primer	Forward primer sequence (5' to 3')	5' Label	Reverse primer	Reverse primer sequence (5' to 3')	No. of serotypes detected
14	84	Csp-14-fw	CCGTCCTTTTGTATGGTGCTATG	HEX	Csp-14-rv	TGAACAGCCCAATCCATCAATCAG	1
8	94	Csp-8-fw	ATTAGCTGCATACGCAAGAACC	NED	Csp-8-rv	GTTAATCCCCAAATAGGATCCC	1
7B, 7C, 40	101	Csp-7BC40-fw	TTATTTTTTGAAGAACATTAATACTC	FAM	Csp-7BC40-rv	AACAATCATCTCTATTTCGACC	3
9L, 9N	111	Csp-9LV-fw	CACTGTTGGCTATGTTAGCCTC	HEX	Csp-9LV-rv	CTCTCCACGTGGCCAAATATAC	2
21	122	Csp-21-fw	TTATGCTGGTTTAAATATCGCTCC	NED	Csp-21-rv	TAACAAATATGCCAAAACGTAGCC	1
34	130	Csp-34-fw	TGTAAGAGGAGATTATTTTCACCC	FAM	Csp-34-rv	GTCACAATAAAAACTGTACCTCC	1
25A, 25F, 38	140	Csp-25AF38-fw	CAACACAATGCTTATGTCAGCC	HEX	Csp-25AF38-rv	AACGCACCCCCAAAATAACTTTCC	3
10A, 10B, 10C, 10F	151	Csp-10-fw	CRATGAGGCTATATGTTGGAATAG	NED	Csp-10-rv	GTATTGAACYCATAGATAACAGAG	4
43	160	Csp-43-fw	GGAATAGTTTAGGATTGTACACC	FAM	Csp-43-rv	TAGAGTCTGCTAACTGTAATATCC	1
9A, 9V	172	Csp-9AV-fw	GATCAATGGCAACTATATTTACCC	HEX	Csp-9AV-rv	GATTCAGTGTCTGACTTTGAACC	2
22A, 22F	181	Csp-22-fw	ACGTATAGGACGTTTCTCAATCC	NED	Csp-22-rv	ATCCCGAAACCAAATTGCTATCC	2
3	191	Csp-3-fw	AGAAATGCTATCCGCGTTGGG	FAM	Csp-3-rv	TTGTACAGAGATTACGCTCAGG	1
15B, 15C	201	Csp-15BC-fw	CGGATGATTGTAGCGTTTATCC	HEX	Csp-15BC-rv	ACTGTAGATTGTGTTCTGATTCC	2
6A, 6B, 6C, 6D	211	Csp-6-fw	GAAGTAGAAAATCGTGTAAGTGG	NED	Csp-6-rv	TCCAACAATAAACCTTATAAGGG	4
18A, 18B, 18C, 18F	220	Csp-18-fw	AGTCTTACTAGACGTAATGAACC	FAM	Csp-18-rv	AAGATAAATTGACTAAGTCTCTCC	4
36	230	Csp-36-fw	TTCCGGATCTATTCAATTTCCCC	HEX	Csp-36-rv	CAATAACAGCCTCCGTTTACC	1
27	240	Csp-27-fw	AATGCCGACGATTAAATGCAGCC	NED	Csp-27-rv	CTAGCCATGCTGGATATTCCCC	1
29	251	Csp-29-fw	TGTGGCAAAAATTTCTTTAGCGG	FAM	Csp-29-rv	ATATACCCAGTAAACAGACAAGG	1
23F	260	Csp-23F-fw	TTCACAAGTGATAGTGAACCTGG	HEX	Csp-23F-rv	TATTAGCTTTATCGGTAAGGTGG	1
19A	269	Csp-19A-fw	ATTGGAAGTAGCTGAGGTTTGG	NED	Csp-19A-rv	TATCCAATTTAAAACAGCACGG	1
33C	278	Csp-33C-fw	CGGCAGGTATAAGTATTATCGG	FAM	Csp-33C-rv	CCTACACCTCTTATAAACGTTGG	1
20	292	Csp-20-fw	TATTGTCCGAAAAAGAGTGCGG	HEX	Csp-20-rv	TACTCAAAGATTGTGTGGTACGG	1
31	301	Csp-31-fw	TTTCAAGGATATGATAGTGGTGG	NED	Csp-31-rv	TAGCATTACAGATGTCACTAAGG	1
23A	314	Csp-23A-fw	CTAGGTTCGTATCTCTTTGCGG	FAM	Csp-23A-rv	TACCAAATGGGTAATGGAGGGG	1
13	328	Csp-13-fw	TTAACAGGTAGATTACGACTTGG	HEX	Csp-13-rv	ATATCCCAAAAACAAATCGCTGG	1
19B, 19C	338	Csp-19BC-fw	TCAGTACGAATAGATGGAACACC	NED	Csp-19BC-rv	CCCAGTATCTAAATCCTAATCCC	2
11A, 11D, 11F	348	Csp-11ADF-fw	GGACATGTTCCAGGTGATTTCCC	FAM	Csp-11ADF-rv	TGCGCCAAATTTGGTATCGACC	3
32A, 32F	357	Csp-32-fw	CTTACAATGAGACGCTATTTTCC	HEX	Csp-32-rv	GTTATTACCATTGAATTCTGTTCCC	2
35A, 35C, 42	367	Csp-35AC42-fw	TCGTTACCTACTTTATTAATGCC	NED	Csp-35AC42-rv	AATAATTCCTAATACCATCTGCC	3
35F, 47F	375	Csp-35F47F-fw	TTGCTACAGTTTGTATGATCTCC	FAM	Csp-35F47F-rv	AGAATCCGTTTCATCATACAGCC	2
2	383	Csp-2-fw	GTTCAATATTTCTCCACTACACC	HEX	Csp-2-rv	CTAAGAGTTCCAATACGTTGACC	1
11B, 11C	392	Csp-11BC-fw	TAGAAATCGCAAGATAGCCTTCC	NED	Csp-11BC-rv	CTGATTATGAGCATAGTTGATCC	2
17F	402	Csp-17F-fw	TCTTGTCAAATACATACTTACCCC	FAM	Csp-17F-rv	GTAGTCTCGCATTTCTATCATCC	1
41A, 41F	413	Csp-41-fw	AGTTACTGGCCCTTTCTTATTCC	HEX	Csp-41-rv	AGGTAAAAAGTCATATCCATTCCC	2
47A	423	Csp-47A-fw	AATACATTGTACGCTCTTAACCCC	NED	Csp-47A-rv	CGAAGAATTAACCCACATAACC	1
4	433	Csp-4-fw	ATTCAGAGGCAGCTAGTTTCCAG	FAM	Csp-4-rv	CAGAAGCTACTGTTAGGCTGG	1
5	443	Csp-5-fw	TTATCTATTTTATCGCAGATCCC	HEX	Csp-5-rv	CTGCCGATAAAAAGATGATGCC	1
33A, 33F, 37	453	Csp-33AF37-fw	ATGTTAGATTAGATGGTTTGTCTGG	NED	Csp-33AF37-rv	ATTCAACACATAAACCGTTGGGG	3
45	463	Csp-45-fw	CAGATTGGTTTTTACATCACTCC	FAM	Csp-45-rv	ACATAACACGACTTTTAGTGACC	1
6C, 6D	473	Csp-6CD-fw	GTCGTGTAAAGTAGTATACAATCC	HEX	Csp-6CD-rv	ATAATCCTCTGGATTATCCACCC	2
35B	483	Csp-35B-fw	CATTAGTGTGCTATGTTGTTC	NED	Csp-35B-rv	GATTAGATAAATAAATACGCCCCC	1
7A, 7F	491	Csp-7AF-fw	TTGACTGCAAGTGTTTCAATGGG	FAM	Csp-7AF-rv	AAAGCACAAAATTTGGAAACGAGG	2
16A	501	Csp-16A-fw	GTTATGATAATGGTAACGCCTCC	HEX	Csp-16A-rv	CAGCCAATAAGTCATATACGCC	1
19F	523	Csp-19F-fw	TGTTCTTAGTAATGGATATACGGG	NED	Csp-19F-rv	AAAACCTTACCAGGATCTAATGG	1
48	532	Csp-48-fw	CCCTTTGATAGCTATAGTATCGG	FAM	Csp-48-rv	CCCTGGAATAGAAGTTTCTAGG	1
39	544	Csp-39-fw	TATGAGGTATCATTTAGCAGGGG	HEX	Csp-39-rv	ATCATCGAAATGGCACTAAAGG	1
1	555	Csp-1-fw	TTTGCTAGATGGTGAGTTGTATC	NED	Csp-1-rv	TTTGAAGATGCATGTGTAATCTC	1
24A, 24B, 24F	569	Csp-24-fw	TCATGCTTATGTTATGTGTTACGG	FAM	Csp-24-rv	GTGAGAGCTATATTTAGAATATGG	3
28A, 28F	578	Csp-28-fw	AGGTAGACTACCAATTTCAATTCC	HEX	Csp-28-rv	TACACCTGCTAATATCAATGTTCC	2
15A, 15F	598	Csp-15AF-fw	ATGAGAGGAAGATATATACGGG	NED	Csp-15AF-rv	AAATAAGTTGTCCATAGGAAGG	2
16F	606	Csp-16F-fw	ACTGCTTGATATATGCTTTATGG	FAM	Csp-16F-rv	TGATAGAGTGACAGACAATTTGG	1
17A	615	Csp-17A-fw	TTTACCCAAGATGGTTTCTAGG	HEX	Csp-17A-rv	AGCATACAAGTTTGGCGTATTGG	1
12A, 12B, 12F, 44, 46	627	Csp-124446-fw	CCTTTCTGATTCTGCTCCAGTTCC	NED	Csp-124446-rv	AGTTGAACCAACTCCCCATCC	5
33B, 33D	640	Csp-33BD-fw	ACAACAGCAATGTTGTTGTTACTC	FAM	Csp-33BD-rv	GAGAAGTAAGAGTTTTGTCTATCC	2
23B	650	Csp-23B-fw	TTGCATATGGATTAAATGGTGGG	HEX	Csp-23B-rv	ACCATTGCTGATAGAAGTAGAGG	1

other researchers to be useful for determining 68 pneumococcal serotypes (18). The limitation of this method is the need of a capillary electrophoresis system or a DNA sequencer capable of measuring the peaks of the amplicon of each serotype, which can

hinder the utility of the technique. Another limitation is the inability to distinguish between serotypes with similar *wzy* genes, although much fewer Quellung reactions are needed to determine the specific serotypes.

TABLE 2 Serotype distribution by the Quellung reaction and identification by FAF-mPCR of 297 *Streptococcus pneumoniae* clinical isolates

Serotype by Quellung	No. of isolates	Serotype by FAF-mPCR
1 ^a	17	1
3 ^a	43	3
4	2	4
6A ^a	2	6A/6B
6C	9	6C/6D
7F ^a	6	7F/7A
8	24	8
9L	5	9N/9L
9N	3	9N/9L
10A	4	10F/10A/10B/10C
11A	16	11F/11A/11D
12F	9	12F/12A/12B/44/46
13	2	13
14 ^a	14	14
15A	5	15F/15A
15B	9	15B/15C
16F	12	16F
17F	3	17F
19A ^a	21	19A
19F ^a	7	19F
20	1	20
21	3	21
22F	18	22F/22A
23A	12	23A
23B	6	23B
24B	1	24F/24A/24B
25A	1	25F/25A/38
38	4	25F/25A/38
31	13	31
33F	6	33F/33A/37
33A	3	33F/33A/37
33B	1	33B/33D
34	1	34
35B	5	35B
35F	8	35F/47
45	1	45

^a Serotypes included in the 13-valent pneumococcal conjugate vaccine.

On the other hand, the FAF-mPCR was faster, easier, and more objective than the Quellung reaction for pneumococcal serotyping. In addition, it was clearly cheaper, even excluding the personnel cost, which is obviously much higher in the Quellung reaction, because FAF-mPCR allowed the processing of batches of samples. The cost for one typing, adding the prices for one reaction of the 55 pairs of labeled primers (1.75 euros), multiplex PCR reactive isolates (1.25 euros), and capillary electrophoresis (1 euro), was ~4.5 euros for each isolate, including consumables (0.5 euros). No labor costs were included in this calculation. The cost of the Quellung reaction, excluding labor costs, was estimated at ~17 euros (\$21.25) (20). Another advantage, compared to other multiplex PCR techniques, is the possibility of amplifying all serotypes in a unique PCR, which opens the door to simpler detection methods, such as hybridization, for an easier and more user-friendly technology.

Multiple studies have been and are being conducted regarding the efficacy of the PCVs, for which determination of the serotypes causing disease is imperative (21, 22). Other studies have focused on the changes in serotypes carried by children (23). In the present

work, we observed that among 297 pneumococcal clinical isolates collected between 2013 and 2015, only 110 (37%) belonged to 13-valent PCV (PCV13) serotypes. In addition, as many as 36 different serotypes were detected. This heterogeneity in serotype distribution necessitates the development of simpler techniques that allow the serotyping of multiple isolates in an accurate and objective way in an era in which personnel costs have the greatest impact on the final price of the techniques.

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