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...
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 in-
ability to distinguish between serotypes with similar
wzy genes, which can be detected although much fewer Quellung reactions are needed to determine
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Another advantage, compared to other 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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### REFERENCES


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