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Modified sequential multiplex PCR for determining capsular serotypes of invasive pneumococci recovered from Seville

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Abstract

The heptavalent pneumococcal vaccine's introduction resulted in a decline in invasive disease caused by *Streptococcus pneumoniae*, but was accompanied by an increase in non-vaccine serotypes. We evaluated a modified scheme of the sequential multiplex PCRs adapted to the prevalence of serotypes in Seville (Spain) for determining capsular serotypes of *S. pneumoniae* invasive clinical isolates. In adults, the modified scheme allowed us to type 73% with the first three reactions, and 92% with two additional PCRs. In paediatric patients, it allowed us to type 73.5% with the first three reactions, and 90% with the two additional PCRs. The multiplex PCR approach was successfully adapted to target the serotypes most prevalent in Seville.

Keywords: Capsular polysaccharide, invasive infection, multiplex PCR, serotype, *Streptococcus pneumoniae*

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Streptococcus pneumoniae causes severe illnesses in the elderly and children. The immunochemistry of the capsular polysaccharide differentiates pneumococci into 91 distinct

serotypes [1], but only approximately 15 serotypes cause the majority of cases of invasive pneumococcal disease worldwide [2,3].

The introduction of the heptavalent pneumococcal vaccine resulted in a decline in invasive disease caused by *S. pneumoniae* in children and a significant decline in the non-vaccinated population, owing to a pronounced herd effect [4,5]. However, the overall decrease in the burden of pneumococcal invasive disease was accompanied by an increase in the number of cases caused by non-vaccine serotypes. The situation in countries where the vaccine is available but not universally administered, such as Italy [6], Spain and Portugal, is even more complex [7]. Depending on the fraction of vaccinated children, one might expect a variable magnitude of reduction in the infections caused by vaccine serotypes in the overall paediatric population, and also the existence and amplitude of indirect effects on infections in adults [7].

Serotype surveillance will continue to be necessary for evaluation of the impact and suitability of current and future multivalent vaccines [8]. Studies of serotype distribution rely on conventional serotyping (the Quellung reaction) for direct determination of serotypes. The expense and intrinsic technical difficulties of conventional serotyping limit its use to a few highly specialized laboratories. Multiplex PCR-based methods that specifically identify capsular serotype-specific sequences offer a simple and economical approach for the surveillance of pneumococcal disease [9]. In addition, the development of PCR-based assays for direct detection of select serotypes from clinical specimens could be a valuable aid in surveillance, particularly in situations where culture is insensitive [10–14]. Pai *et al.* [15] have developed a sequential multiplex PCR for deducing 28 serotypes, providing the capability to perform surveillance of serotypes with basic DNA amplification and electrophoresis equipment (the CDC has recently expanded the list of serotypes to 40; see http://www.cdc.gov/ncidod/biotech/files/pcr-serotyping-clinical-specimens-May2009.pdf). This approach has been used to identify the distribution of pneumococcal clinical isolate serotypes from several countries [16–18].

We evaluated a modified scheme of the CDC sequential multiplex PCRs for serotyping of *S. pneumoniae* invasive clinical isolates from adults and paediatric patients. In this method, the primer combinations used were adapted to the serotype distribution in Seville (Spain). The pneumococci were recovered at the Hospital Virgen Del Rocio (1031 beds, population coverage 875.331 habitants) in Seville. In Spain, from 1979 to 2007, different therapeutic approaches (antibiotics) and preventive measures (PCV7) were introduced [19]. The increased prevalence of antibiotic non-susceptibility and PCV7 serotypes that was associated with antibiotic consumption in the 1980s and 1990s was reversed in the 2000s, when vaccine dose distribution increased. At the same time, non-PCV7 serotypes became more prevalent, particularly serotypes 1 and 19A [19].

Two hundred and fifty-seven isolates obtained from 2004 to 2008 in Seville were tested [15], including 102 previously typed paediatric isolates, and 155 adult isolates, by serological determination of capsular type. All of the isolates were recovered from sterile fluids: blood (215 isolates), cerebrospinal fluid (30 isolates), pleural fluid (ten isolates), and synovial fluid (two isolates).

The samples were cultured on Columbia Blood agar in 5% CO_2 at 37°C, and the α-haemolytic colonies were tested for optochin susceptibility and bile solubility, in order to identify *S. pneumoniae*.

The published PCR protocol has seven sequential reactions. We modified the order of primers proposed in order to improve the efficiency of the original scheme for surveying our geographical area. The main changes were that primers for serotype 5 were included in PCR 3, and that serotype 1 was changed from PCR 6 to PCR 3. DNA extraction, PCRs and electrophoresis were performed as described previously [15]. To resolve the highly homologous serotypes 6A/C and 6B, we supplemented the multiplex assay with sequencing [20]. After this, we differentiated serotypes 6A and 6C with a PCR assay [21].

Concordance of the PCR with conventional serotyping was 91% in the paediatric isolates. Three of the five isolates for which serotypes could not be deduced were serotypes not included in the reaction scheme. The multiplex PCR typed 97.4% of adult isolates, and 93.1% of paediatric isolates. Two isolates (1.3%) in adults and five isolates (4.9%) in infants were not assigned serotypes. Two isolates in each group (1.3% and 2%) failed to amplify the positive control. Table 1 shows *S. pneumoniae* serotype determination using our sequential PCR schemes for adult and paediatric patients.

The first three reactions allowed us to type 73% of adult isolates, but this figure rose to 92% with two additional PCRs; using the original scheme, we could type 63% and 74.8%, respectively. In the paediatric group, the first three reactions allowed us to type 73.5% vs. 60%, and the two additional PCRs allowed us to type 90% vs. 72.5%. By sequencing and PCR, we identified seven of the 22 serotype 6A/B/C isolates as serotype 6A, 11 as serotype 6B, and three as serotype 6C. One isolate was not differentiated.

The CDC sequential multiplex PCRs for determining capsular serotypes of *S. pneumoniae* offers a simple and economical approach and a good alternative to conventional serotyping, and could be adapted to more efficiently detect