Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children

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Capsular serotype surveillance of clinical isolates of *Streptococcus pneumoniae* is essential for evaluation of the potential impact of introducing multivalent capsular serotype-based vaccines in Latin America. Here, a previously described sequential multiplex PCR method was revised for optimal targeting of prevalent serotypes in Latin America. The revised protocol successfully serotyped 139/147 pneumococci (94.6 %) from Brazilian children, demonstrating a labour-efficient, accurate method requiring only conventional PCR capability.

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INTRODUCTION

Streptococcus pneumoniae causes up to 1 million deaths per year among children less than 5 years of age, mostly in developing countries (Hausdorff *et al.*, 2000; Garcia *et al.*, 2006). The antiphagocytic polysaccharide capsule of *S. pneumoniae* is an essential virulence factor, with 91 known types distributed among this normally commensal inhabitant of the nasopharynx (Park *et al.*, 2007). About 15 serotypes appear to cause the majority of invasive infections (Hausdorff *et al.*, 2000; Robinson *et al.*, 2001; Whitney *et al.*, 2003). In Latin America, 13 capsular types account for >85 % of invasive isolates (Di Fabio *et al.*, 2001; Garcia *et al.*, 2006; Laval *et al.*, 2006).

The introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) resulted in a dramatic decline in invasive disease caused by *S. pneumoniae* in children and a significant decline in the non-vaccinated population due to a pronounced herd effect (Whitney *et al.*, 2003, 2006; Ray *et al.*, 2006). Serotype surveillance will continue to be necessary in vaccinated and non-vaccinated populations for evaluation of the impact and suitability of current multivalent vaccines (Whitney *et al.*, 2003; Ghaffar *et al.*, 2004; Kyaw *et al.*, 2006). Studies of serotype distribution rely on conventional serotyping (the Quellung reaction) for direct determination of serotypes of *S. pneumoniae*. 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 (Brito *et al.*, 2003; Pai *et al.*, 2006). Sequential multiplex PCR was recently developed for deducing 29 serotypes or serogroups (Pai *et al.*, 2006), providing the capability to perform surveillance of serotypes causing the majority of disease for laboratories with basic DNA amplification and electrophoresis equipment.

The purpose of our study was to test a reformulation of the current sequential multiplex PCR procedure (Pai *et al.*, 2006), modified on the basis of past surveillance, to identify the most frequent serotypes of *S. pneumoniae* isolated in Latin America (Di Fabio *et al.*, 2001; Garcia *et al.*, 2006; Laval *et al.*, 2006). In the accompanying study (Morais *et al.*, 2007), we present the same strategy based on past serotype surveillance in Mozambique. It is important to demonstrate the flexibility of the general method by altering combinations of serotype specificities and to demonstrate that each serotype-specific primer pair is specific for a given serotype, regardless of strain diversity within different countries.

METHODS

Bacterial strains and culture conditions. A collection of 147 *S. pneumoniae* isolates was obtained from children less than 5 years old living in the city of Porto Alegre, Brazil. Most of the isolates (115/147; 78.2 %) were recovered from normally sterile fluids, comprising blood (74 isolates), cerebrospinal fluid (19 isolates) and pleural fluid (22 isolates). Twenty-seven different serotypes were represented in the

Abbreviation: CDC, Centers for Disease Control and Prevention.

collection: serotypes 14 (45 isolates), 6B (23 isolates), 19F (nine isolates), 18C (eight isolates), 1 (seven isolates), 3 (seven isolates), 9V (six isolates), 23F (six isolates) and 5 (five isolates), followed by serotypes 6A, 19F, 23B (four isolates each), 11A (three isolates), 8 and 16F (two isolates each), and serotypes 7C, 7F, 9N, 10A, 13, 15B, 18A, 24B, 28A, 29, 31 and 34 (one isolate each). Bacteria were cultured on trypticase soy agar with 5% sheep blood BBL (BD) in 5% CO₂ at 37 °C.

Optochin-susceptibility test. Optochin-susceptibility testing was performed as described previously (Arbique *et al.*, 2004) to identify α -haemolytic colonies as *S. pneumoniae*.

Bile solubility test. The tube bile solubility test was performed as described previously (Arbique *et al.*, 2004) to identify α -haemolytic colonies as *S. pneumoniae*.

Quellung serotype reaction. All serotype results were confirmed with sera prepared at the Centers for Disease Control and Prevention (CDC), using latex agglutination followed by the Quellung reaction.

DNA extraction and multiplex PCRs. DNA extraction and multiplex PCRs were performed as described previously (Pai *et al.*, 2006) with modifications: serotype 14 primers were redesigned to avoid the occurrence of cross-reactivity among isolates of serotypes 15B and 15C. The new type 14 primers were 14-F2 (5'-GAAATGTTACTTGGCGCAGGTGTCAGAATT-3') and 14-R2 (5'-GCCAATCTTCTTAGTCTCTCAGATGAAT-3'), which yielded an amplification product of 189 bp from serotype 14 pneumococci. Type 9N specificity was added to the typing scheme, as 9N is a common serotype among clinical pneumococcal isolates of this region. Primers targeting 9N (9N/L-F: 5'-GAACTGAATAAGTCAGATTTAATCAGC-3'; and 9N/L-R: 5'-ACCAAGATCTGACGGGCTAATCAAT-3') also detect the rarely occurring 9L serotype, yielding a product of 516 bp. The primer concentrations for serotypes 14 and 9N/L were 1.0 and 0.5 μ M, respectively, and in all reactions of the Latin America scheme, a concentration of 3.5 mM MgCl₂ was used.

Isolates were typed using the current scheme used in the USA (Pai *et al.*, 2006) and an alternative scheme targeting the most prevalent serotypes in Latin America, in which the 13 most prevalent serotypes in Latin America (Di Fabio *et al.*, 2001; Garcia *et al.*, 2006; Laval *et al.*, 2006) were included in the first three reactions. Primers for serotypes

5 and 9N/L were also included for a total of 30 serotypes distributed among six multiplex PCRs (Table 1).

RESULTS AND DISCUSSION

The six multiplex PCRs included the corresponding serotype controls from the CDC collection and the internal positive control *cpsA* (Fig. 1). PCR-deduced results from both schemes were concordant with conventional serotyping. Using the previously described scheme (Pai *et al.*, 2006), serotypes or serogroups were accurately predicted for 133/147 (90.5%) of the isolates (Table 1). The remaining 14 isolates were of serotypes not included in the scheme, including types 5 (five isolates), 23B (four isolates), 9N (one isolate), 13 (one isolate), 24B (one isolate), 28A (one isolate) and 29 (one isolate).

Using the first three reactions of the Latin America scheme, serotypes were accurately deduced for 131/147 (89.1%) of the isolates (Table 1). The remaining three reactions were used to deduce correctly the serotypes or serogroups from eight additional isolates, to give a final total of 139 (94.6%) of the 147 isolates. These results demonstrate the adaptability of this method for grouping alternative combinations of serotype-specific primer sets. In order to assure the specificity of PCR 3 for serotype 9N/9L, all six isolates of serotype 9V were subjected to PCR 3 without yielding an amplification product.

Eight isolates of serotypes 23B (four isolates) and 13, 24B, 28A and 29 (one isolate each) were not amplified, as these serotype specificities were not included. Only four of the eight non-typable isolates (of serotypes 23B, 28A and 29) were recovered from normally sterile sites. Therefore, 111/115 (96.5%) of the isolates obtained from a normally sterile fluid were typed accurately using this multiplex PCR approach.

Table 1. *S. pneumoniae* serotype determination using two different sequential PCR schemes formulated for the USA and for Latin America

Reaction	USA (Laval <i>et al.</i> , 2006)			Latin America (this study)		
	Serotypes in each reaction*	<i>n</i>	Cumulative score (%)	Serotypes in each reaction*	<i>n</i>	Cumulative score (%)
PCR 1	3, 6A/6B, 19A, 22F/(22A)	38	38 (25.8)	6A/6B, 9V/(9A), 14, 19F, 23F	93	93 (63.3)
PCR 2	4, 9V/(9A), 12F/(12A), 14	52	90 (61.2)	1, 4, 5, 18C/(18A, 18B, 18F), 19A	25	118 (80.3)
PCR 3	7F/(7A), 11A/(11D), 23F, 33F/(33A, 37)	10	100 (68.0)	3, 7F/(7A), 9N/9L, 10A, 11A/(11D)	13	131 (89.1)
PCR 4	16F, 18C/(18A, 18B, 18F), 19F, 35B	20	120 (81.6)	7C/(7B, 40), 12F/(12A), 15B/15C, 17F, 38	2	133 (90.1)
PCR 5	8, 15B/15C, 31, 38/(25F)	3	123 (83.7)	8, 20, 22F/(22A), 31, 34	4	137 (93.2)
PCR 6	1, 10A, 34, 35F/(47)	9	132 (89.8)	15A, 16F, 33F/(33A, 37), 35F/(47), 35B	2	139 (94.6)
PCR 7	7C/(7B, 40), 15A, 17F, 20	1	133 (90.5)	No reaction		

*Common serotype combinations are indicated by a slash mark. Rare serotypes are in parentheses.

Fig. 1. Latin America multiplex PCRs 1–6 for the determination of *S. pneumoniae* serotypes.

Serotypes 4, 12F, 15A, 17F, 20, 22F, 33F, 35B, 35F and 38 were included in the scheme, but were not found among these isolates. The ability to detect these serotypes could potentially become more important in the future for post-vaccine surveillance. For example, type 22F is among the most common serotypes from invasive pneumococci in the USA in the post-PCV7 period (Whitney *et al.*, 2006).

In summary, the multiplex PCR approach was successfully adapted to target serotypes most prevalent in Latin America and to identify serotypes from more than 90% of the isolates tested. This scheme can be used as an alternative to costly conventional serotyping in Latin American developing countries, allowing meaningful serotype surveillance by laboratories equipped only with basic PCR capability. The ability to determine prevalent pneumococcal capsular serotypes is currently important for the introduction and post-vaccine evaluation of new pneumococcal conjugate vaccines.

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