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Evaluation of Modified Sequential Multiplex PCR for *Streptococcus pneumoniae* Serotyping

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**Running title:** Modified SM-PCR for *S. pneumoniae*

**Key words:** *Streptococcus pneumoniae*, Serotype, sequential multiplex PCR.

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Summary

The aims of this study were to develop modified SM-PCR primer sets and to evaluate their ability and efficiency for serotype determination. We selected target serotypes for SM-PCR testing according to the prevalence of serotypes as reported in papers published in Asian countries. The modified SM-PCR consisted of six groups of PCRs, with each reaction being carried out using five pairs of primers. We evaluated the efficiency and performance of this modified multiplex PCR using 378 pneumococcal strains by comparing the findings with the results of the Quellung reaction. A total of 30 primer pairs were used for a consecutive set of six reactions. All results were concordant with those of the Quellung reaction, and there was no cross reactivity on multiplex PCR. We could identify the final serotypes of 370 isolates (97.9%). The coverage rates of modified SM-PCR were 42.6%, 65.8%, and 79.4% in Reactions 1, 2, and 3, respectively. The modified SM-PCR showed good performance in detecting pneumococcal serotypes and could be used as a helpful alternative to the Quellung reaction.
Introduction

*Streptococcus pneumoniae* is an important bacterial pathogen causing acute otitis media, pneumonia, and invasive pneumococcal diseases (IPD) including bacteremia and meningitis (1-4). It carries high rates of morbidity and mortality, with approximately 1.6 million deaths each year, especially in children, the elderly, and immunocompromised patients (5).

More than 93 streptococcal serotypes have been identified on the basis of antigenic differences in their capsular polysaccharides (6). Determination of serotype is essential because only a limited number of serotypes is associated with IPD: about 80% of IPD infections are caused by about 20 predominant serotypes (7, 8). The Quellung reaction (Statens Serum Institut, Copenhagen, Denmark) has been considered the gold standard for *S. pneumoniae* serotyping. However, it has limitations, particularly its high cost, labor intensiveness, and the need for a trained technician (9). Sequential multiplex PCR (SM-PCR) has proved to be sensitive and efficient for *S. pneumoniae* serotyping and has been used extensively in several countries as a replacement for the Quellung reaction (10, 11).

The U.S. Centers for Disease Control and Prevention (CDC) preserves the primers of each serotype for *S. pneumoniae*, and previous researchers have published several reports of the value of SM-PCR using these primers (10-12). However, the distribution of predominant serotypes has changed with time and is different by country and even region (13). The SM-PCR primer sets used in the U.S (11) and other Western countries (14) are not good choices in Asian counties.

The aims of this study were to develop a modified SM-PCR and to evaluate its ability and efficiency for serotype determination of *S. pneumoniae* isolates.

Materials and Methods
We selected target serotypes for SM-PCR according to the prevalence of various serotypes by reviewing reports of streptococcal infections published in Asian countries (15-23). The sequences of the primer sets were provided by the CDC (https://www.cdc.gov/streplab/pcr.html). The modified SM-PCR consisted of six groups of PCRs, and each reaction involved five pairs of primers. We especially considered two points when we developed this modified SM-PCR. The first point was the prevalence of a serotype. We allocated the prevalent serotype to the first reaction of the SM-PCR. The second was the size of the PCR product. We tried to ensure that the difference of product sizes was at least 100 bp for the same reaction to avoid ambiguous interpretations. When PCR product sizes were similar, we reassigned their combination by reflecting the prevalence of the serotype.

A total of 378 pneumococcal strains isolated from 2008 to 2014 that already had had their serotypes identified using the Quellung reaction were included in this study (15). The pneumococcal colonies were mixed with 200 μL of Tris-EDTA buffer solution (Sigma-Aldrich Co, St. Louis, MO, USA). The suspension was heated to 100°C for 10 min and promptly placed at -20°C for 5 min. Serotyping of pneumococcal isolates was determined by a modified sequential multiplex PCR developed in this study. The PCR conditions were as follows: 94°C for 5 min followed by 30 amplification cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, followed by 1 cycle at 72°C for 7 min. The amplification products were analyzed by electrophoresis in 2% agarose gels. The results of the modified SM-PCR were compared with the results of the Quellung reactions published previously (15).

Results

A modified SM-PCR consisting of six consecutive reactions was developed for serotyping of *S. pneumoniae* isolates. A total of 30 primer pairs contributed to consecutive six SM-PCR reactions. The composition of the six reactions is as follows: Reaction 1 (35B, 11A/11D, 3,
6A/6B/6C/6D, and 24F/24A/24B), Reaction 2 (9V/9A, 10A, 19A, 15A/15F, 19F), Reaction 3 (22F/22A, 9N/9L, 34, 33F/33A/37, 14), Reaction 4 (16F, 13, 15B/15C, 23F, 1), Reaction 5 (23A, 20, 4, 12F/12A/12B/44/46, 35A/35C/42), and Reaction 6 (6C/6D, 18F/18A/18B/18C, 5, 7C/7B/40, 23B) (Figure 1). We checked the cross-reactivity and positivity of each primer set used in multiplex PCR employing streptococcal strains whose serotype had been confirmed by the Quellung reaction. Indeed, all results were concordant with those of the Quellung reaction, and there was no cross reactivity on multiplex PCR (Figure 2).

A total of 378 pneumococcal isolates were serotyped by the modified SM-PCR. We compared the performance of the modified SM-PCR with that of the previous Quellung reactions (15). A total of 97.9% of the isolates (370/378) yielded complete agreement between the two methods, and only 8 isolates (2.1%) were nontypeable by SM-PCR. Among these isolates, the serotypes of six isolates were not included in the modified SM-PCR. These were serotyped as 17F (N = 2), 7F/7A (N = 2), 8, 35F/47F by additional specific PCR primers. The serotypes of the two remaining isolates (serotypes 3 and 11) were not identifiable, although their serotypes were included in the modified SM-PCR.

We could identify the serotypes of 370 isolates (97.9%) in six consecutive reactions of modified SM-PCR. On the basis of the total of 378 isolate results, we analyzed the coverage rates of pneumococcal serotypes by comparison of SM-PCR reactions in the United States (11), Latin America (14), and this study. The final identification rates were 91.0%, 93.6%, and 97.9%, respectively (Table 1). The identification rates of Reactions 1, 2, and 3 of SM-PCR for United States were 40.2%, 48.4%, and 57.7%, respectively. The identification rates of Reactions 1, 2, and 3 of SM-PCR for Latin America were 31.2%, 43.1%, and 63.5%, respectively. In this study, the modified SM-PCR identified 42.6%, 65.8%, and 79.4% in Reactions 1, 2, and 3, respectively. The modified SM-PCR of this study reveals high coverage rates of pneumococcal serotypes.
Discussion

The serotype of *Streptococcus pneumoniae* is an important virulence factor, and a pneumococcal vaccine should include the most common serotypes involved in IPD (24). Pneumococcal conjugate vaccine (PCV) 7, PCV 10, and PCV 13 have been introduced gradually into Asian countries. The introduction of PCV 7 led to a reduction of IPD caused by PCV 7 serotypes; however, non-PCV7 serotypes such as 19A increased (25, 26). In Sweden, there was a report of an increase in non-PCV13 serotypes after the introduction of PCV 13 (27). The use of pneumococcal vaccine has constantly changed the distribution of serotypes; therefore, the monitoring of pneumococcal serotype is important for vaccine development and evaluation of vaccine efficacy (28).

The Quellung reaction is the gold standard for the serotyping of *S. pneumoniae*, but it is laborious, expensive, and hard to interpret. The molecular method based on genetic differences of polysaccharide capsule synthesis genes (*cps* locus) has been used as an alternative (11). SM-PCR is effective to identify pneumococcal serotypes because of its speed and cost-effectiveness (29). In the U.S., the CDC provides the information on SM-PCR primers on the Web. We can find the SM-PCR for the U.S. and Latin America; however, this is not adequate for Asian countries. A proper scheme of SM-PCR is needed there because there are significant differences in the serotype distribution in Asian countries (15). Kim et al. (16) reported that the major serotypes in 11 Asian countries between 2008 and 2009 were 19F, 23F, 19A, 14, 6B, and 3. A report from Thailand showed that the common serotypes were 6B, 19A, 14, 18C, and 6A between 2009 and 2012 (17). Serotypes 19F, 19A, 23F, and 6A in China were most common in 2013 (18). In Korea, serotypes 19F, 3, and 23F were most common between 1997 and 2012 (19), and serotypes 3, 35, 19A, and 6A were the most frequent from 2011 to 2014 (15). Similarly, the most common serotypes of *S. pneumoniae* in Japan from 2013 to 2015 were 3, 19A, and 22F (22).

We designed the modified SM-PCR to cover the predominant serotypes in Asian countries, and a total of 30 primer sets were included in six reactions. When we compared the results with those of the Quellung reaction, the results showed 97.9% concordance. Eight isolates were not
identified by the modified SM-PCR, and among these, the serotypes of six isolates were not included in the primer list. The two remaining isolates were not identified, although serotypes 3 and 11 were included in the modified SM-PCR. We suspect serotype 11 was missed in the modified SM-PCR because that test contains only 11A/11D whereas the Quellung reaction detects 11A/B/C/D/F.

The coverage rates of the modified SM-PCR were 79.4% and 97.9% for the third and the final reactions, respectively. This is a very high coverage rate compared with those of U.S. and Latin America. In this study, serotypes of 3, 35B, 19A, 19F, and 6A were common. Among these, serotype 3, 35B, and 6(A/B/C/D) were in reaction 1 and serotype 19A and 19F in reaction 2. SM-PCR of the U.S. contains serotype 3, 19A, 6(A/B/C/D) in reaction 1; however, serotypes 19F and 35B were located in reaction 4. SM-PCR for Latin America, serotypes 6(A/B/C/D), 19F/19A, and 35B are included in reaction 1, 2, and 6, respectively.

The modified SM-PCR showed good performance in identifying pneumococcal serotypes and can be used as an alternative to the Quellung reaction.

Conflict of interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

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References
Figure 1. Schematic approach to modified sequential multiplex PCR, indicating the six reactions and the serotypes identified in each reaction.

Figure 2. Six sequential multiplex PCR reactions for the determination of *S. pneumoniae* serotypes.

Table 1. Comparison of coverage in U.S., Latin America, and Asia (this study)
<table>
<thead>
<tr>
<th>Reaction</th>
<th>United States (Pai <em>et al.</em>, 2006)</th>
<th>Latin America (Dias <em>et al.</em>, 2007)</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serotypes in each reaction</td>
<td>Valid score (%)</td>
<td>Cumulative score (%)</td>
</tr>
<tr>
<td>1</td>
<td>19A, 3, 22F/22A, 6A/6B/6C/6D</td>
<td>40.2</td>
<td>40.2</td>
</tr>
<tr>
<td>2</td>
<td>4, 12F/12A, 14, 9V/9A</td>
<td>8.2</td>
<td>48.4</td>
</tr>
<tr>
<td>3</td>
<td>11A/11D, 7F/7A, 33F/33A/37, 23F</td>
<td>9.3</td>
<td>57.7</td>
</tr>
<tr>
<td>4</td>
<td>19F, 16F, 35B, 18F/18A/18B/18C</td>
<td>17.5</td>
<td>75.1</td>
</tr>
<tr>
<td>5</td>
<td>8, 15B/15C, 25F/38, 31</td>
<td>3.4</td>
<td>78.6</td>
</tr>
<tr>
<td>6</td>
<td>10A, 1, 35F/47F, 34</td>
<td>7.9</td>
<td>86.5</td>
</tr>
<tr>
<td>7</td>
<td>15A/15F, 7C/7B/40, 20, 17F</td>
<td>4.5</td>
<td>91.0</td>
</tr>
</tbody>
</table>

*Others*. These isolates were not identified by multiplex PCR reactions.
Figure 1. Schematic approach to modified sequential multiplex PCR, indicating the six reactions and the serotypes identified in each reaction.
Figure 2. Six sequential multiplex PCR reactions for the determination of *S. pneumoniae* serotypes.