Streptococcus pneumoniae Type Determination by Multiplex Polymerase Chain Reaction

Ki Wook Yun1,2, Eun Young Cho1,2, Ki Bae Hong1, Eun Hwa Choi1,2 and Hoan Jong Lee1,2

1Department of Pediatrics, Seoul National University Children’s Hospital, Seoul; 2Department of Pediatrics, Seoul National University College of Medicine, Seoul, Korea

Received: 14 March 2011
Accepted: 25 May 2011

Address for Correspondence:
Eun Hwa Choi, MD
Department of Pediatrics, Seoul National University Children’s Hospital, 101 Daehak-ro, Jongno-gu, Seoul 110-769, Korea
Tel: +82.2-2072-3624, Fax: +82.2-766-7293
E-mail: eunchoi@snu.ac.kr

This study was supported by a grant (no. 0420100740) from the Seoul National University Research Fund.

INTRODUCTION

Streptococcus pneumoniae is the leading cause of invasive and noninvasive diseases in the pediatric population and places a high burden on the health care system worldwide, including in Korea (1). Although only a limited number among the 92 serotypes account for pneumococcal diseases, the serotype distribution can vary by patient age, geographic region, and time of surveillance (2). Currently, the licensure of the 7-valent pneumococcal conjugate vaccine (PCV7) heralds a new era in the control of pneumococcal diseases. Since the introduction of PCV7 in the United States, a decrease in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes has been observed in pediatric and nonpediatric age groups (3). Also in Korea, PCV7 brought about substantial reduction in carriage of vaccine serotypes and transmission of those in the community (4). On the other hand, extensive antibiotic use has resulted in increased pressure and promotion of several antibiotic-nonsusceptible non-PCV7 serotypes, mainly 19A (5). Thus, the combined pressure by antibiotic use and some replacement in nasopharyngeal carriage by PCV7 has resulted in increased carriage of and disease from some strains such as serotype 19A, 6C and a few others. Like this, the possibility of replacement of the vaccine types by nonvaccine types exists. Therefore, continuous monitoring on serotype changes of nasopharyngeal carriage and isolates from IPD is very important in making decisions on the national immunization strategy. A convenient and accurate method for serotype determination is needed for evaluation of vaccine efficacy and changes in pneumococcal epidemiology. These have led to renewed interest in developing accurate and efficient systems for pneumococcal serotyping.

Currently, serotype distribution is monitored by culture of the organism followed by serological determination of the capsular type by the standard capsular test (6). The high cost of antisera, subjectivity in interpretation, and technical expertise requirements are serious drawbacks of the system. The development of PCR-based serotyping systems has the potential to overcome some of the difficulties associated with serologic testing. Recently, the Centers for Disease Control and Prevention (CDC), United States, established a multiplex PCR protocol for the identification of pneumococcal serotypes that can be applied to clinical or research use (7). Furthermore, the recently recognized serotype 6D can only be detected by the molecular method (8). This molecular typing method can be used for establishment of clonal epidemiology of pneumococci (9).

We aimed to compare pneumococcal typing using multiplex PCR with the conventional quellung capsular reaction, and to detect new serotypes, 6C and 6D, by PCR and sequencing.
MATERIALS AND METHODS

Pneumococcal isolates
Hospital-wide surveillance has continued to monitor pneumococcal diseases as a part of routine clinical care at Seoul National University Children’s Hospital (SNUCH) since 1991. Each pneumococcal isolate was identified using standard microbiological tests, including colony morphology, hemolysis pattern, and optochin susceptibility. These isolates were all stored at -80°C until use.

Pneumococcal strains for this study included 77 isolates from the stock obtained during the period from 2006 to 2010. These strains were selected to represent 26 different serotypes that were relatively common in Korea, as recent epidemiologic studies had suggested (2, 10).

Serotyping by quellung reaction
Serotype was determined by the quellung reaction using antisera (Statens Serum Institute, Copenhagen, Denmark). Briefly, the stored clinical isolates were recovered by plating on 5% sheep blood agar plates (tryptic soy agar base supplemented with 5% sheep blood), and incubating in 5% CO₂ at 37°C overnight. A 1.5 μL aliquot of a suspension of the isolate in Mueller-Hinton broth and 1.5 μL antisera were mixed under a glass coverslip and examined for capsular swelling using a phase contrast microscope at × 1,000 magnification.

Typing by multiplex PCR assay
All pneumococcal isolates used in the PCR assays were delinked with the results of serotyping by quellung reaction, and the researcher who performed the PCR was also blinded to these results. Extraction and purification of DNA from pneumococcal colonies were performed as described in the QIAamp Kit (QIAGEN GmbH, Hilden, Germany). Briefly, a sweep of cultures were sampled and resuspended in 100 μL distilled water in 1.7 mL microfuge tubes. These were heated at 100°C for 5 min. After 500 μL nucleic lysis solution was added to each tube, the samples were incubated at 65°C for 15 min. Next, 200 μL of protein

Table 1. Oligonucleotide primers used for pneumococcal typing by multiplex PCR

| Primers | GenBank accession no. | Primer sequence (5’-3’) | Gene | Nucleotide position | Product Size (bp) | Reference |
|----------|-----------------------|-------------------------|------|---------------------|------------------|-----------|
| 1-f      | CR931632              | CTC TAT AGA AGT GAG TAT ATA AAC TAT GGT TA | wzy | 9905                | 280              | 7         |
| 1-r      |                       | CCA AAA AAA ATA CTA ACA TTA CAA TAC TTA GCG TGG C |       | 10181               |                  |           |
| 3-f      | CR931634              | ATG GTS TGA TTT TTC CTC CTA GAT TGG AAA GTA G | galU | 9020                | 371              |    |
| 3-r      |                       | CTT CTC CAA TGG TTT ACC AAG TGG AAT AAC CAA C |       | 9360                |                  |           |
| 4-f      | CR931635              | CTG TTA GTC GTC GAT TGC CGT CSG AAT TGA TG | wzy | 9596                | 430              |    |
| 4-r      |                       | GCC ACC TCC TGT TAA AAT CCT ACC CCG ATT G |       | 9995                |                  |           |
| 5-f      | CR931637              | ATA CCT ACA CAA CTT CTG ATT AGT CCT CTG TG | wzy | 6123                | 362              |    |
| 5-r      |                       | GCT CAA TAA ACA TAA TAA CCT GGG AAA GTA TG |       | 6450                |                  |           |
| 6A/6B/6C-f | CR931639          | AAT TGG TAT TTT ATT CAT GCC TAT ATC TGG TTA GGG GAG ATA ATT ATT AAT GAG TAC GA | wcP | 8656                | 250              |    |
| 6A/6B/6C-r |                  |       |       | 8877                |                  |           |
| 7C-wzy-f | CR931642              | CTA TCG GTC TCA TCT ATT GTT AAA GTT TAC GAC GGA A | wcwL | 9438                | 260              |    |
| 7C-wzy-r |                       | GAA CAT AGA TGG TGA GAC ATC TCT TTG TGT AAT TCT |       | 9665                |                  |           |
| 7F/TA-f  | CR931643              | CCT AGC GGA GGA TAT AAA ATT ATT TTT GAG CAA ATA AAC CAC CAC TAT AGG CTG TTA GGA CTA AC | wzy | 13356               | 826              |    |
| 7F/TA-r  |                       |       |       | 14150               |                  |           |
| 9N/9L-f  | CR931647              | GAA CTG AAT AAG TCA GAT TTA ATC AGC ACC AAG ATG TGA CCG GCT AAT CAA T | wzx | 11948               | 516              | 20       |
| 9N/9L-r  |                       |       |       | 12439               |                  |           |
| 9V/9A-f  | CR931648              | GGG TTC AAA G TC AGA CAG TG A ATC TTA A | wzy | 9966                | 816              |    |
| 9V/9A-r  |                       | CCA TGA ATG A AA TCA TCA T GG GCA GTA GC |       | 10753               |                  |           |
| 10A-f    | CR931649              | GGT GTA GAT TCA CCA TTA GTA TGG TGA CAC GAC | wcG | 12423               | 628              |    |
| 10A-r    |                       | GAA TTT CTT TTA TAA GA TAT TGG GAT ATT TCT C |       | 13020               |                  |           |
| 11A/11D-f| CR931653              | GGA CAT GTT CAG GAG TTT ATC CCA TAT AAT G | wzy | 11640               | 463              |    |
| 11A/11D-r|                       | GAT GAG TGT A AT A TTA TCT CCA CTT CTC CC |       | 12071               |                  |           |
| 12F/14A/15B-f| CR931660 | GCA ACA AAC GGG TGT AAA GTA GGT G | wzx | 14407               | 376              |    |
| 12F/14A/15B-r|                   | CAA GAT GAA TAC CAC TAA CAC AAA GAC |       | 14753               |                  |           |
| 13-f     | CR931661              | TAC TAA GGT GAT ATT CTC TGG AAA TGA AGG | wzx | 14005               | 655              |    |
| 13-r     |                       | CTC ATG CAT TTT ATT AAC CCG C TTT TGG TCT |       | 14630               |                  |           |
| 14-f     | CR931662              | GAA ATG TTA CTT GCA GCA GGT GTC AGA ATT G | wzy | 7969                | 189              | 20       |
| 14-r     |                       | GCC AAT ACT TCG TAC TGT TGC ATG GAA TAT |       | 8119                |                  |           |
| 15A/15F-f| CR931663              | ATT AGT ACA GCT GGT GTA ATA TCT CTT C | wzy | 7804                | 434              |    |
| 15A/15F-r|                       | GAT CTA GTC AAT GTA CTG CAA CAC A |       | 8212                |                  |           |
| 15B/15C-f| CR931665              | TGT GAA TTT ATT TAA TGG TCT ACC TA CAT CGG CTT ATT AAT TGG AAT CTA GAC C | wzy | 7314                | 496              |    |
| 15B/15C-r|                       |       |       | 7779                |                  |           |
| 18/19/11D/15A/16-f| CR931673        | CTT AAT ACG TCT CAT TAT TCT TTT TTA AGG CC TTA TCT GAT AAC CAT ATC AGC AGC ATG TGA AAC | wzy | 12687               | 573              |    |
| 18/19/11D/15A/16-r|                   |       |       | 13230               |                  |           |

(continued to the next page)
precipitation solution was added to each 600 μL sample, and then the samples were cooled on ice and centrifuged for 3 min at 13,200 rpm to pellet the cell debris. An aliquot of each supernatant containing extracted DNA was used as a template for PCR.

Thirty primer pairs were designed to target serotypes 1, 3, 4, 5, 6A/6B/6C, 7C, 7F, 9N, 9V, 10A, 11A, 12F, 13, 14, 15A, 15B/15C, 18C, 19A, 19F, 20, 21, 22F, 23A, 23F, 24F, 34, 35B, 35F, 37, and 38. The sequences for the type-specific primers were published by the CDC (USA) (11) and the primers were designed on the basis of cps loci containing the serotype-specific genes that serve as the basis for differentiation of pneumococci by PCR-based approaches (Table 1) (12). Eight multiplex reactions were sequentially performed as shown in Table 2. These primers were grouped together based on the frequency of serotype distributions among invasive pneumococci recovered in Korea (10), such that common serotypes were included in reactions 1 through 3.

The PCRs were performed in 25 μL volumes, with each reaction mixture containing the following: 2.5 μL of 10 Tris-HCl buffer (100 mM, pH 8.3, Mg²⁺ free), 2.0 μL of 2.5 mM dNTPs, 2.0 μL of MgCl₂, 0.25 μL of 5.0 U/μL Taq DNA polymerase (Takara Bio Inc., Japan), primers with pre-determined concentrations (Data not shown), and distilled water to a final volume of 20 μL. Finally, 5 μL of the DNA extract from the clinical specimens was added to each reaction mixture. Thermal cycling was performed in a PTC-200 Peltier Thermal Cycler DNA engine (MJ Research, Watertown, MA, USA) under the following conditions: 95°C for 15 min followed by 35 amplification cycles of 94°C for 30 sec, 63°C for 90 sec, and 72°C for 60 sec. Each PCR included distilled water as a negative control and DNA extracted from pneumococcal isolates from which serotypes were previously determined as the positive control. The samples were cooled on ice and centrifuged for 3 min at 13,200 rpm to pellet the cell debris. An aliquot of each supernatant containing extracted DNA was used as a template for PCR.

| Primers   | GenBank accession no. | Primer sequence (5’–3’) | Gene | Nucleotide position | Product Size (bp) | Reference |
|-----------|-----------------------|-------------------------|------|---------------------|------------------|-----------|
| 19A-f     | CR931675              | GAG AGA TTC AIA ATG TGC CAC TTA GCC A   | wzy  | 9603                | 566              | 30        |
| 19A-r     |                       |                          |      |                     |                  |           |
| 19F-f     | CR931678              | GGT AAG AGT GCT GAT CTA TTA ATT GAT ATC C   | wzy  | 11135               | 304              | 7         |
| 19F-r     |                       |                          |      |                     |                  |           |
| 20-F     | CR931679              | GGA CAA GAG TTT TTC ACC TGA CAG CGA GAA G   | woL  | 9567                | 514              | 7         |
| 20-r     |                       |                          |      |                     |                  |           |
| 21-F     | CR931680              | CTA TGG TTA TTT CAA CTC AAT CGT CAC C   | wx   | 13247               | 192              | 11        |
| 21-r     |                       |                          |      |                     |                  |           |
| 22F/22A-f | CR931682              | GAG TAT AGC CAG ATT ATG GCA GTT TTA TGG TC   | woorV | 11055               | 643              | 7         |
| 22F/22A-r |                       |                          |      |                     |                  |           |
| 23A-f     | CR931683              | TAT TCT AGC AAG TGA TGA CAG TGC G   | wzy  | 7739                | 722              | 11        |
| 23A-r     |                       |                          |      |                     |                  |           |
| 23F-f     | CR931685              | GTA ACA GTT GCT GTA GAG GGA ATT GCC TTT TC   | wzy  | 8768                | 384              | 7         |
| 23F-r     |                       |                          |      |                     |                  |           |
| 24F/24A, 24B, 24F-f | CR931688          | GCT CCC TGC TAT TGT ATT CTT TAA AGA G   | wzy  | 11701               | 99               | 11        |
| 24F/24A, 24B, 24F-r |                       |                          |      |                     |                  |           |
| 33F/33D/34F-f | CR931702  | GAA GCC AAT CAA TGT GAT TGT TGC G   | wzy  | 11129               | 338              | 7         |
| 33F/33D/34F-r |                       |                          |      |                     |                  |           |
| 34-f     | CR931703              | GCT TTT GTA AGA GGA GAT TAT TTT TAC CAC ACC C   | wzy  | 7350                | 408              | 7         |
| 34-r     |                       |                          |      |                     |                  |           |
| 35B-f     | CR931705              | GAT AAG TCT GGT GTG GAG ACT TAA AAA GGA TG   | worH | 10556               | 677              | 7         |
| 35B-r     |                       |                          |      |                     |                  |           |
| 35F/347-f | CR931707              | GAA CAT AGT CGC TAT TGT ATT TTA TAA AGA A   | wzy  | 7374                | 517              | 7         |
| 35F/347-r |                       |                          |      |                     |                  |           |
| 38/25F-f | CR931710              | CAG TGT TTT ATC TCA CTG CAT ATG ATT TTA AGT TTT GTA AAG CTA AGC TAA CCA TCC   | wzy  | 13848               | 574              | 7         |
| 38/25F-r |                       |                          |      |                     |                  |           |

This table was modified from reference 11.

| PCR reaction | Total number of included serotypes | Serotypes |
|--------------|-----------------------------------|-----------|
| Reaction 1   | 8                                 | 6A/6B/6C/6D, 23F, 19A, 9V          |
| Reaction 2   | 5                                 | 19F, 3      |
| Reaction 3   | 4                                 | 1, 5      |
| Reaction 4   | 3                                 | 4, 20, 22F |
| Reaction 5   | 5                                 | 7C, 12F, 11A, 10A, 23A            |
| Reaction 6   | 5                                 | 21, 37, 15A, 35F, 13               |
| Reaction 7   | 2                                 | 38, 35B   |
| Reaction 8   | 2                                 | 24F, 34   |

DOI: 10.3346/jkms.2011.26.8.971

Table 2. Pneumococcal serotypes that are included in 8 multiplex PCRs

Table 1. Oligonucleotide primers used for pneumococcal typing by multiplex PCR

Table 2. Pneumococcal serotypes that are included in 8 multiplex PCRs
by the quellung reaction as a positive control.

Type determination of serogroup 6
To assign types to 6 serogroups, we screened 10 strains that were positive by PCR with 6A/6B/6C primer for the presence of either \(\text{wciN}_\beta\) or \(\text{wciP}\) genes using 2 simplex PCRs and subsequent sequencing analysis. First, the \(\text{wciN}\) gene was amplified with the forward primer (5106) 5’-TAC CAT GCA GGG TGG AAT GT-3’ and the reverse primer (3101) 5´-CCA TCC TTC GAG TAT TGC-3´, resulting in product sizes of 1.8 kb for types 6C or 6D for the \(\text{wciN}\) gene. Following this reaction, presence of an G or A at position 584, a characteristic of 6A and 6B \(\text{wciP}\) (\(\text{wciP}_{6A}\) and \(\text{wciP}_{6B}\)) respectively, was confirmed by sequencing analysis of the \(\text{wciP}\) gene using the forward primer 5´-AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG-3´ and the reverse primer 5´-TTA GCG GAG ATA ATT TAA AAT GAT GAC TA-3´ (11). Strains that carry \(\text{wciP}_{6A}\) genes are assigned as type 6A or 6C and those that carry \(\text{wciP}_{6B}\) are assigned as type 6B or 6D (Fig. 1).

RESULTS

Optimization of multiplex PCR
Among a total of 54 serotypes that could be determined by 8 sequential multiplex PCRs with 30 primers, 34 serotypes (except serotypes 7A/7B, 9A/9L, 11D, 12A, 15F, 18A/18B/18F, 22A, 24A/24B, 25F, 33A/33F, 40, 44, 46, 47F) were available from the archived pneumococcal isolates for which serotypes were determined previously by the quellung reaction. A multiplex PCR system was optimized for these strains such that we were able to produce all the PCR products of each serotype-specific size (Fig. 2).

Types identified by multiplex PCR
Seventy-seven pneumococcal isolates were tested and all samples were successfully amplified by PCR. The types identified by the PCR assays were as follows: 1 (n = 1), 3 (n = 3), 4 (n = 1), 5 (n = 1), 6A/6B/6C/6D (n = 10), 7C (n = 1), 7F (n = 2), 9V (n = 4), 10A (n = 3), 11A (n = 4), 12F (n = 1), 13 (n = 3), 14 (n = 4), 15A/15B (n = 8), 15C (n = 4), 19A (n = 5), 19F (n = 3), 20 (n = 1), 22F (n = 3), 23A (n = 4), 23F (n = 4), 34 (n = 2), 35B (n = 4), and 37 (n = 1). Of the 77 total isolates, 28 were detected in reaction 1 (including serotypes 6A/6B/6C/6D, 9V, 14, 19A, 23F), 14 in reaction 2 (including serotypes 3, 15B/15C, 19F), and 4 in reaction 3 (including serotypes 1, 5, 7F). Therefore, 60% of all serotypes were determined within these 3 reactions.

Identification of 6C and 6D
Multiplex PCR reaction 1 identified 10 strains within serogroup 6. From them, 4 isolates of 6A or 6B and 6 isolates of 6C or 6D were delineated by the first simplex PCR using primers for the \(\text{wciN}\) gene. Sequencing of the \(\text{wciP}\) gene showed \(\text{wciP}_{6A}\) gene sequences for 4 strains within 6A or 6B subgroup, indicating these were all serotype 6A. Among the 6 strains within 6C or 6D subgroup, sequencing analysis revealed \(\text{wciP}_{6A}\) gene in 4 strains (serotype 6C) and \(\text{wciP}_{6B}\) gene in 2 strains (serotype 6D) (Fig. 1).

Comparison with the conventional quellung reaction
Serotypes determined by multiplex PCR were in complete concordance, with one exception, with those previously determined by conventional serotyping methods. One isolate was typed as 19A by PCR but previously assigned 19F by quellung reaction. When the conventional serotyping and PCR were repeated, the serotype was confirmed to be 19A.

DISCUSSION
This study aimed to develop a method for molecular serotyping of pneumococcus by multiplex PCR assays. We were able to identify 24 serotypes correctly out of 77 pneumococcal isolates for which serotypes were previously determined by the quellung reaction as a positive control.
reaction. In addition, the PCR assay identified one strain that was erroneously serotyped by the classic method. The findings in this study suggest that the performance of the multiplex PCR assay is excellent and it can be successfully used for pneumococcal serotyping.

Capsular serotype-specific polysaccharides are a major virulence factor in disease development following infection and pneumococcal impact on human health is various according to the capsular type (9). The PCVs provide significant serotype-specific protection against invasive disease; however, rates of IPD were still substantial. Serotype replacement is already eroding the efficacy of PCV7 in high-risk populations (13). The recent licensure of 13-valent pneumococcal conjugate vaccine (PCV13), which includes PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F, and 19A may reduce IPD caused by these 6 added serotypes. However, non-PCV13 serotypes may likely be associated with serotype replacement in the future (14). Therefore, continuous accurate monitoring for serotype in individual region is important.

Identification of pneumococcal serotypes is currently performed using large panels of expensive antisera in the quellung reaction, the traditional ‘gold standard’. Cross-reactions between serotypes and discrepancies between methods can occur, and some strains are non-serotypable (6). A rapid pneumococcal serotyping assay has been developed recently, (the ‘multibead assay’) based on the capacity of pneumococcal lysates to inhibit the ability of 24 different anti-capsule antibodies to bind to latex beads coated with 34 different polysaccharides (15). This assay is well suited for use in primary screening because of its high throughput and reproducible results, but access to serological reagents is quite limited. Also, serotype coverage is currently limited to only 24 serotypes of 92 (16).

Molecular typing has the potential to improve discrimination and provide additional information. Although several molecular typing methods had been developed early in the 2000s, all required further evaluation and improvement (17, 18). Production of the capsule is largely controlled by capsular polysaccharide synthesis genes located at the cps locus, in which the first four genes are conserved in almost all serotypes, while the central parts of the loci contain the serotype-specific genes that serve as the basis for differentiation of pneumococci by PCR-based approaches (17). The cps gene clusters for at least 16 pneumococcal serotypes have been sequenced and serotype-specific
genes were identified earlier (19). The sequences of the cpx loci from all of the 92 known pneumococcal serotypes have been completed recently (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/), providing an opportunity to develop a simple sequence-based scheme for identifying the most commonly occurring serotypes. In 2006, the CDC (USA) recommended typing by PCR and developed a protocol for the same (7). On the basis of this protocol, two other groups developed modified PCR protocols to cover their own prevalent serotypes (20, 21). These studies determined that this method required minimal training and no prior experience with PCR for efficient and accurate serotype surveillance, and was far less time-consuming than classical quellung reaction-based serotyping.

Recent interest in pneumococcus has been focused on newly discovered pneumococcal serotypes, 6C and 6D (22, 23), and 6D prevalence has been higher than 6C since 1996 in Korea (8). In a recent study, factor 6d antiserum was validated for accurate serotyping of 6C and is now commercially available (24), but antiserum for the detection of 6D has not yet been developed. The detection of 6D is only possible by molecular methods using capsular DNA (25).

The PCR system used in this study is also well adapted for typing most prevalent serotypes of pneumococcus, including 6C and 6D, and required less work and skill for accurate typing. Additionally, we did not find evidence suggesting that the genetic variability between different strains of the same serotype could affect its serotype determination using a PCR-based approach, as suggested previously (26). There was only one case in which the results of the multiplex PCR scheme and original conventional serotyping were discrepant. In this instance, the PCR-based result proved to be accurate. We were able to reaffirm that subjectivity in interpretation of the quellung reaction can result in incorrect serotyping.

Major advantages of this molecular approach include the potential to type small numbers of isolates at moderate expense, prompt identification of predominant serotypes for case investigations, and a reduction in the number of isolates requiring transport to reference laboratories for serotyping. Since the first 3 reactions in this sequential multiplex scheme are designed to cover the predominant serotypes in Korea (2), they would theoretically detect -85% of all isolates obtained through the SNUCH surveillance (1991 to 2005) program. In reality, we were only able to determine types in 60% of the isolates within these 3 reactions. However, if test strains for the PCR assay were selected in exact proportion to our serotype epidemiology, the percentages should be greatly increased. In addition, this scheme allowed us to obtain results for all of the isolates within the PCV13 as well as the PCV7, except serotype 4 (included in reaction 4). The adaptability of this method to grouping alternative combinations of serotype-specific primer sets according to seroprevalence in individual countries could save cost and time.

A major limitation of pneumococcal serotype epidemiology is that most cases, including empyema and meningitis, remain culture-negative due to the widespread use of antibiotics prior to presenting at the hospital. Recently, several studies have revealed increased identification of pneumococcus and subsequent typing by multiplex PCR assay in IPDs. Application of molecular diagnosis improved detection rate of pneumococcus from 24% by culture to 71% by PCR and it also provided the opportunity to know the serotype distribution in pneumococcal empyema (27). Benefit of molecular typing method was shown in pneumococcal meningitis as well (28). Overall, molecular typing method can add further detail to the clinical profile and epidemiology of pneumococcal disease.

Additionally, the measurement of pneumococcal carriage in the nasopharyngeal reservoir is subject to potential confounders that include low-density and multiple-strain colonization. Detection of simultaneous carriage with more than one serotype using standard culture methods is severely limited (29), yet detection of all pneumococcal serotypes present in respiratory specimens is critical for the study of the epidemiology of pneumococcal colonization. If multiplex PCR typing could be done directly on clinical samples, more co-colonization may be detected.

Nevertheless, this PCR approach still has several problems. First, the amount of genetic variability that exists among different isolates expressing the same serotype is unknown. Also there is the potential to assign types to strains with defective cps operons, although such isolates are rarely recovered from invasive infections. Another weakness of the current PCR approach is the inability to determine all types due to the absence of some type specific-PCR primers. Furthermore, we are currently unable to resolve certain types from rarely occurring serotypes within the same serogroup because some primers are designed to detect multiple serotypes. Therefore, additional testing using the quellung reaction or sequencing is required for precise typing. However, because the sequences of the cpx loci from all of the known 92 pneumococcal serotypes have been recently completed, we have an opportunity to overcome most of these problems.

We have attempted to develop a schematic approach to serotyping the isolates obtained through our surveillance program using 30 primer pairs divided among 8 multiplex PCRs as a modification of the protocol provided by the CDC (USA). Each multiplex reaction was designed to sequentially include the most frequently occurring serotypes, with 8 reactions progressively covering all the isolates identified in our center since 1991. For typing of serogroup 6, an additional 2-step simplex PCR and sequencing were included.

In conclusion, a multiplex PCR approach was successfully adapted to target the most prevalent serotypes in 3 reactions, and can be used as an alternative to costly conventional sero-
typing in Korea, allowing for meaningful serotype surveillance by laboratories equipped only with basic PCR capability. Overall, we have found this PCR approach to be highly reliable, with the potential to greatly reduce reliance upon conventional serotyping. These molecular typing methods will be essential for epidemiological studies that are needed to monitor serotype distribution and detect serotype switching, if any, among *S. pneumoniae* isolates before and after the introduction and widespread use of conjugate vaccines.

**REFERENCES**

1. Lee JH, Cho HK, Kim KH, Kim CH, Kim DS, Kim KN, Cha SH, Oh SH, Hur JK, Kang JH, Kim JH, Kim YK, Hong YI, Chung EH, Park SE, Choi YY, Kim JS, Kim HM, Choi EH, Lee HI. Etiology of invasive bacterial infections in immunocompetent children in Korea (1996-2005): a retrospective multicenter study. *J Korean Med Sci* 2011; 26: 174-83.

2. Kim SH, Song EK, Lee JH, Kim NH, Lee JA, Choi EH, Lee HJ. Changes of serotype distribution of Streptococcus pneumoniae isolates from children in Korea over a 15 year-period (1991-2005). *Korean J Pediatr Infect Dis* 2006; 13: 89-98.

3. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, Reingold A, Cieslak PR, Pilishvili T, Jackson D, Facklam RB, Jorgensen JH, Schuchat A. Decline in invasive pneumococcal disease after the introduction and widespread distribution and detect serotype switching, if any, among *S. pneumoniae* isolates before and after the introduction and widespread use of conjugate vaccines.

4. Kim KH, Hong JY, Lee H, Kwak KY, Nam CH, Lee SY, Oh E, Yu J, Nahm MH, Kang JH. Nasopharyngeal pneumococcal carriage of children attending day care centers in Korea: comparison between children immunized with 7-valent pneumococcal conjugate vaccine and non-immunized. *J Korean Med Sci* 2011; 26: 184-90.

5. Choi EH, Kim SH, Eun BW, Kim SJ, Kim NH, Lee JA, Lee HJ. Streptococcus pneumoniae serotype 19A in children, South Korea. *Emerg Infect Dis* 2008; 14: 275-81.

6. Sörensen UB. Typing of pneumococci by using 12 pooled antisera. *J Clin Microbiol* 1993; 31: 2097-100.

7. Pai B, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. *J Clin Microbiol* 2006; 44: 124-31.

8. Choi EH, Lee HJ, Cho EY, Oh CE, Eun BW, Lee J, Kim MJ. Prevalence and genetic structures of Streptococcus pneumoniae serotype 6D, South Korea. *Emerg Infect Dis* 2010; 16: 1751-3.

9. Sandgren A, Sjostrom K, Olsson-Liljequist B, Christensson B, Samuels A, Kronvall G, Henriques Normark B. Effect of clonal and serotype-specific properties on the invasive capacity of Streptococcus pneumoniae. *Infect Dis* 2004; 189: 785-96.

10. Song EK, Lee JH, Kim NH, Lee I, Kim DH, Park KW, Choi EH, Lee HJ. Epidemiology and clinical features of invasive pneumococcal infections in children. *Korean J Pediatr Infect Dis* 2005; 12: 140-8.

11. CDC. Oligonucleotide primers used for deducing 40 pneumococcal capsular serotypes. Available at http://www.cdc.gov/ncidod/biotech/files/pcr-oligonucleotide-primers-March2010.pdf [accessed on March 2010].

12. Kong F, Gilbert GL. Using cpsA-cpsB sequence polymorphisms and serotype-/group-specific PCR to predict 51 Streptococcus pneumoniae capsular serotypes. *J Med Microbiol* 2003; 52: 1047-58.

13. Singleton RJ, Hennessy TW, Bullow LR, Hammitt LL, Zalz T, Hurlburt DA, Butler JC, Rudolph K, Parkinson A. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 2007; 297: 1784-92.

14. Pandya GA, McEllistrem MC, Venepally P, Holmes MH, Jarrah B, Sanka R, Liu J, Karamycheva SA, Bai Y, Fleischmann RD, Peterson SN. Monitoring the long-term molecular epidemiology of the pneumococcus and detection of potential ‘vaccine escape’ strains. *PLoS One* 2011; 6: e13590.

15. Yu J, Carvalho Mda G, Beall B, Nahm MH. A rapid pneumococcal serotyping system based on monoclonal antibodies and PCR. *J Med Microbiol* 2008; 57: 171-8.

16. Cho KY, Lee JA, Cho SE, Kim NH, Lee JA, Hong KS, Lee HJ, Kim KH. A study of serotyping of Streptococcus pneumoniae by multibead assay. *Korean J Pediatr* 2007; 50: 151-6.

17. Lawrence ER, Arias CA, Duke B, Beste D, Broughton K, Efstratiou A, George RC, Hall LM. Evaluation of serotype prediction by cpsA-cpsB gene polymorphism in Streptococcus pneumoniae. *J Clin Microbiol* 2000; 38: 1319-23.

18. Lawrence ER, Griffiths DB, Martin SA, George RC, Hall LM. Evaluation of semiautomated multiplex PCR assay for determination of Streptococcus pneumoniae serotypes and serogroups. *J Clin Microbiol* 2003; 41: 601-7.

19. Iang SM, Wang L, Reeves PR. Molecular characterization of Streptococcus pneumoniae type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. *Infect Immun* 2001; 69: 1244-55.

20. Dias CA, Teixeira LM, Carvalho Mda G, Beall B. Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. *J Med Microbiol* 2007; 56: 1185-8.

21. Morais L, Carvalho Mda G, Roca A, Flannery B, Mandomando I, Soria-Cabarrós M, Sigaque B, Alonso P, Beall B. Sequential multiplex PCR for identifying pneumococcal capsular serotypes from South-Saharan African clinical isolates. *J Med Microbiol* 2007; 56: 1181-4.

22. Park IH, Pritchard DG, Cartee R, Brandao A, Brandleine MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. *J Clin Microbiol* 2007; 45: 1225-33.

23. Jin P, Kong F, Xiao M, Oftadeh S, Zhou E, Liu C, Russell F, Gilbert GL. First report of putative Streptococcus pneumoniae serotype 6D among nasopharyngeal isolates from Fijian children. *J Infect Dis* 2009; 200: 1375-80.

24. Jacobs MR, Dagan R, Bajaksouzian S, Windau AR, Porat N. Validation of factor 6d antiserum for serotyping Streptococcus pneumoniae serotype 6C. *J Clin Microbiol* 2010; 48: 1456-7.

25. Batcher PE, Kim KH, Kang JH, Hong JY, Nahm MH. Identification of natural pneumococcal isolates expressing serotype 6D by genetic, biochemical and serological characterization. *Microbiology* 2010; 156: 555-60.

26. Yu J, Lin J, Benjamin WH Jr, Waites KB, Lee CH, Nahm MH. Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. *J Clin Microbiol* 2005; 43: 156-62.

27. Blaschke AJ, Heyrend C, Byington CL, Obando I, Vazquez-Barba I, Doby EH, Korgenski EK, Sheng X, Poritz MA, Daly JA, Mason EO, Pavía AT, Ampofo K. Molecular analysis improves pathogen identification and epidemiologic study of pediatric parapneumonic empyema. *Pediatr Infect Dis J* 2011; 30: 289-94.

28. Saha SK, Darmstadt GL, Baqui AH, Hossain B, Islam M, Foster D, Al-Emrani H, Naheed A, Arifeen SE, Luby SP, Santosham M, Crook D. Iden-
 AUTHOR SUMMARY

Streptococcus pneumoniae Type Determination by Multiplex Polymerase Chain Reaction

Ki Wook Yun, Eun Young Cho, Ki Bae Hong, Eun Hwa Choi and Hoan Jong Lee

In this study we develop pneumococcal typing by multiplex PCR and compare it with conventional serotyping by Quellung reaction. All isolates were successfully typed by multiplex PCR assays adapted to target the most prevalent serotypes in 3 reactions. In addition, type 6 was easily identified based on the presence of \( \text{wciN}_6 \) and/or \( \text{wciP} \) genes in 2 simplex PCRs and sequencing. The multiplex PCR assay was highly reliable, with the potential to greatly reduce reliance upon conventional serotyping.