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Streptococcus pneumoniae Type Determination by Multiplex Polymerase Chain Reaction

Ki Wook Yun[1,2], Eun Young Cho[1,2], Ki Bae Hong[1], Eun Hwa Choi[1,2] and Hoan Jong Lee[1,2]

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

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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: +82.2-766-7283
E-mail: eunchoi@snu.ac.kr

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The purpose of this study was to develop pneumococcal typing by multiplex PCR and compare it with conventional serotyping by quellung reaction. Pneumococcal strains used in this study included 77 isolates from clinical specimens collected from children at Seoul National University Children's Hospital from 2006 to 2010. These strains were selected as they represented 26 different serotypes previously determined by quellung reaction. Molecular type was determined by 8 sequential multiplex PCR assays. Bacterial DNA extracted from cultured colonies was used as a template for PCR, and primers used in this study were based on *cps* operon sequences. Types 6A, 6B, 6C, and 6D were assigned based on the presence of *wciN*$_{\beta}$ and/or *wciP* genes in 2 simplex PCRs and sequencing. All 77 isolates were successfully typed by multiplex PCR assays. Determined types were as follows: 1, 3, 4, 5, 6A, 6B, 6C, 6D, 7C, 7F, 9V, 10A, 11A, 12F, 13, 14, 15A, 15B/15C, 19A, 19F, 20, 22F, 23A, 23F, 34, 35B, and 37. The results according to the PCR assays were in complete concordance with those determined by conventional quellung reaction. The multiplex PCR assay is highly reliable and potentially reduces reliance upon conventional serotyping.

Key Words: *Streptococcus pneumoniae*; Serotyping; Polymerase Chain Reaction/methods; Korea

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 out 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.

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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 antiserum (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% CO_2 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 1-r	CR931632	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	*wzy*	9935 10181	280	7
3-f 3-r	CR931634	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	*galU*	9020 9360	371	7
4-f 4-r	CR931635	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	*wzy*	9596 9995	430	7
5-f 5-r	CR931637	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	*wzy*	6123 6450	362	7
6A/6B/6C-f 6A/6B/6C-r	CR931639	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	*wciP*	8656 8877	250	7
7C/$_{(7B/40)}$-f 7C/$_{(7B/40)}$-r	CR931642	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC	*wcwL*	9438 9665	260	7
7F/7A-f 7F/7A-r	CR931643	CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	*wzy*	13356 14150	826	7
9N/9L-f 9N/9L-r	CR931647	GAA CTG AAT AAG TCA GAT TTA ATC AGC ACC AAG ATC TGA CGG GCT AAT CAA T	*wzx*	11948 12439	516	20
9V/9A-f 9V/9A-r	CR931648	GGG TTC AAA G TC AGA CAG TG A ATC TTA A CCA TGA ATG A AA TCA ACA TT G TCA GTA GC	*wzy*	9966 10753	816	11
10A-f 10A-r	CR931649	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	*wcrG*	12423 13020	628	7
11A/11D-f 11A/11D-r	CR931653	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	*wzy*	11640 12071	463	7
12F/$_{(12A/44/46)}$-f 12F/$_{(12A/44/46)}$-r	CR931660	GCA ACA AAC GGC GTG AAA GTA GTT G CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	*wzx*	14407 14753	376	7
13-f 13-r	CR931661	TAC TAA GGT AAT CTC TGG AAA TCG AAA GG CTC ATG CAT TTT ATT AAC CG C TTT TTG TTC	*wzx*	14005 14630	655	11
14-f 14-r	CR931662	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT GCC AAT ACT TCT TAG TCT CTC AGA TGA AT	*wzy*	7959 8119	189	20
15A/15F-f 15A/15F-r	CR931663	ATT AGT ACA GCT GCT GGA ATA TCT CTT C GAT CTA GTG AAC GTA CTA TTC CAA AC	*wzy*	7804 8212	434	7
15B/15C-f 15B/15C-r	CR931665	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C	*wzy*	7314 7779	496	7
18/$_{(18A/18B/18C/18F)}$-f 18/$_{(18A/18B/18C/18F)}$-r	CR931673	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC	*wzy*	12687 13230	573	7

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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, Mg^{2+} free), 2.0 μL of 2.5 mM dNTPs, 2.0 μL of $MgCl_2$, 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

Table 1. *(continued from the previous page)* Oligonucleotide primers used for pneumococcal typing by multiplex PCR

Primers	GenBank accession no.	Primer sequence (5′-3′)	Gene	Nucleotide position	Product Size (bp)	Reference
19A-f 19A-r	CR931675	GAG AGA TTC ATA ATC TTG CAC TTA GCC A CAT AAT AGC TAC AAA TGA CTC ATC GCC	*wzy*	9603 10142	566	30
19F-f 19F-r	CR931678	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	*wzy*	11135 11407	304	7
20-f 20-r	CR931679	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC	*wci*L	9567 10048	514	7
21-f 21-r	CR931680	CTA TGG TTA TTT CAA CTC AAT CGT CAC C GGC AAA CTC AGA CAT AGT ATA GCA TAG	wzx	13247 13412	192	11
22F/22A-f 22F/22A-r	CR931682	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	*wcw*V	11055 11666	643	7
23A-f 23A-r	CR931683	TAT TCT AGC AAG TGA CGA AGA TGC G CCA ACA TGC TTA AAA ACG CTG CTT TAC	*wzy*	7739 8434	722	11
23F-f 23F-r	CR931685	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC	*wzy*	8768 9119	384	7
24/(24A, 24B, 24F)-f 24/(24A, 24B, 24F)-r	CR931688	GCT CCC TGC TAT TGT AAT CTT TAA AGA G GTG TCT TTT ATT GAC TTT ATC ATA GGT CGG	*wzy*	11701 11770	99	11
33F/(33A/37)-f 33F/(33A/37)-r	CR931702	GAA GGC AAT CAA TGT GAT TGT GTC GCG CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C	*wzy*	11129 11436	338	7
34-f 34-r	CR931703	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	*wzy*	7350 7725	408	7
35B-f 35B-r	CR931705	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G	*wcr*H	10556 11199	677	7
35F/47F-f 35F/47F-r	CR931707	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC	*wzy*	7374 7858	517	7
38/25F-f 38/25F-r	CR931710	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC	*wzy*	13848 14392	574	7

This table was modified from reference 11.

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

PCR reaction	Total number of included serotypes	Serotypes				
Reaction 1	8	14	6A/6B /6C/6D	23F	19A	9V
Reaction 2	5	19F	3	15B/15C	18C	
Reaction 3	4	1	5	9N	7F	
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			

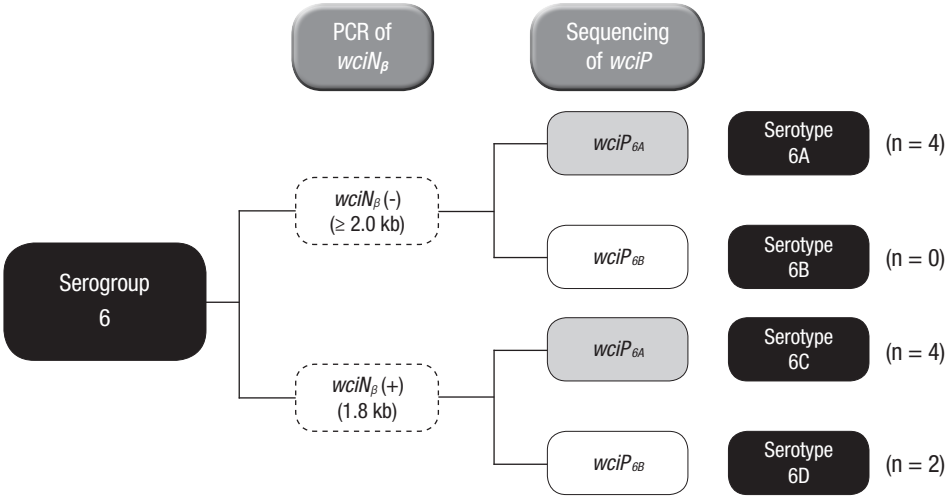

Fig. 1. Type determination of serogroup 6 based on the presence of *wciN*$_{\beta}$ and the sequencing results of the *wciP* genes.

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 *wciN*$_{\beta}$ or *wciP* genes using 2 simplex PCRs and subsequent sequencing analysis. First, the *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 *wciN*$_{\beta}$ gene. Following this reaction, presence of an G or A at position 584, a characteristic of 6A and 6B *wciP* (*wciP*$_{6A}$ and *wciP*$_{6B}$) respectively, was confirmed by sequencing analysis of the *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 *wciP*$_{6A}$ genes are assigned as type 6A or 6C and those that carry *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 *wciN*$_{\beta}$ gene. Sequencing of the *wciP* gene showed *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 *wciP*$_{6A}$ gene in 4 strains (serotype 6C) and *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

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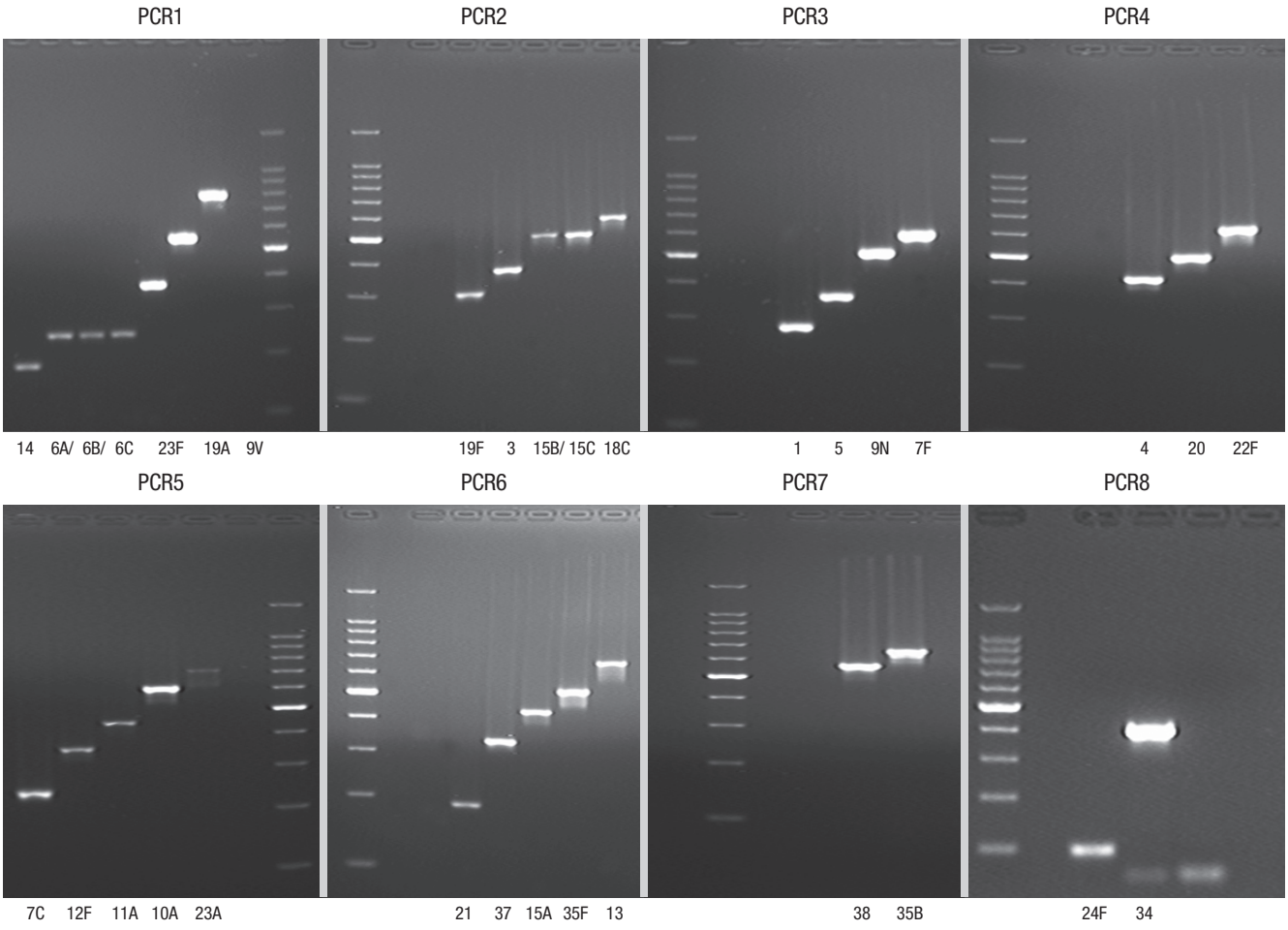

Fig. 2. Representative multiplex reactions. The serotypes determined by 8 different multiplex formulations are shown below the lanes, respectively.

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 polyssacharides (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 *cps* 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 *cps* 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-

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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.

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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 *wciN*$_{\beta}$ and/or *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.

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