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Enhanced detection and serotyping of *Streptococcus pneumoniae* using multiplex polymerase chain reaction

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Purpose: Methods for quick and reliable detection of *Streptococcus pneumoniae* are needed for the diagnosis of pneumococcal disease and vaccine studies. This study aimed to show that sequential multiplex polymerase chain reaction (PCR) is more efficient than conventional culture in achieving *S. pneumoniae*-positive results.

Methods: Nasopharyngeal (NP) secretions were obtained from 842 pediatric patients admitted with lower respiratory infections at Severance Children's Hospital in Korea between March 2009 and June 2010. For identification and serotype determination of pneumococci from the NP secretions, the secretions were evaluated via multiplex PCR technique with 35 serotype-specific primers arranged in 8 multiplex PCR sets and conventional bacteriological culture technique.

Results: Among the results for 793 samples that underwent both bacterial culture and PCR analysis for pneumococcal detection, 153 (19.3%) results obtained by PCR and 81 (10.2%) results obtained by conventional culture technique were positive for *S. pneumoniae*. The predominant serotypes observed, in order of decreasing frequency, were 19A (23%), 6A/B (16%), 19F (11%), 15B/C (5%), 15A (5%), and 11A (4%); further, 26% of the isolates were non-typeable.

Conclusion: As opposed to conventional bacteriological tests, PCR analysis can accurately and rapidly identify pneumococcal serotypes.

Key words: *Streptococcus pneumoniae*, Multiplex polymerase chain reaction, Culture

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Introduction

Streptococcus pneumoniae is a major pathogen, causing otitis media, sinusitis, pneumonia, bacteremia, and meningitis in children worldwide. It also offers some threat of mortality, mostly in developing countries[1,2]. The recent spread of antibiotic-resistant *S. pneumoniae* strains has caused considerable concern[3,4]. Pneumococcal conjugate vaccines (PCVs), used since 2000, have been shown to reduce the incidence of vaccine-serotype invasive pneumococcal disease (IPD) and nasopharyngeal (NP) colonization of pneumococcus by the vaccine serotypes[3,5-11]. In spite of the availability of these efficient vaccines, pneumococcal infections remain a global problem due to the emergence of resistance and replacement by nonvaccine-serotype strains[12,13].

Pneumococcal infections are traditionally diagnosed using bac-

terial culture combined with biochemical or immunochemical identification tests. However, conventional culture methods take at least 48 hours to obtain results, and antibiotic treatment prior to obtaining patient specimens or autolysis of pneumococci during transportation of samples may cause false-negative culture results. In addition, the current determination of serotypes by serological methods requires isolation of pneumococci, is time consuming and expensive, and the results are difficult to interpret. Rapid and precise methods of detection and serotyping of *S.pneumoniae* are needed to improve the diagnosis of IPD, but they are also essential for predicting the consequences of vaccinations on NP carriage. In this study we aimed to compare PCR with traditional culture isolation methods for detecting *S. pneumoniae* in NP aspirates and to improve the design of previously published protocols for sequential multiplex PCR-based serotyping of pneumococci according to the predicted distribution of pneumococcal serotypes in Korea.

Materials and methods

1. NP secretions

A total of 842 NP secretions were collected from children (younger than 5 years of age) hospitalized with lower respiratory infections at Severance Children's Hospital in Seoul, Korea, from March 2009 to June 2010. The NP aspirates obtained by suction were divided into two samples: one was used for bacterial culture and the other for multiplex PCR-based serotyping. Informed consent was obtained from the parents or guardians of the patients prior to the collection of samples. The study was approved by the institutional review board of Severance Hospital.

2. Bacterial culture

A portion of each of the NP specimens was cultured on blood agar plates for 48 hours at 37°C in an atmosphere enriched with 5% CO_2. Isolates were identifiedas *S. pneumoniaee* by typical colony morphology, alpha-hemolysis, negative catalase reaction, Gram-positive staining, optochin disk sensitivity, and bile solubility.

3. Multiplex PCR-based serotyping

Nucleic acid from each NP specimen was extracted using a QIAamp Genomic DNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The extracted DNA suspension was kept frozen at -70℃ until further use. Thirty-five serotype-specific primer

Table 1. Primers Used in This Study

Reaction	Primer set		Primer sequence (5'-3')	Product size (bp)
1	14	Forward	CTT GGC GCA GGT GTC AGA ATT CCC TCT AC	208
		Reverse	GCC AAA ATA CTG ACA AAG CTA GAA TAT AGC C	
	19A	Forward	GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	478
		Reverse	GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	
	19F	Forward	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	304
		Reverse	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	
	23F	Forward	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	384
		Reverse	CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	
2	6A/B	Forward	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
		Reverse	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
	9V	Forward	CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG	753
		Reverse	GTC CCA ATA CCA GTC CTT GCA ACA CAA G	
	15A	Forward	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	436
		Reverse	GAT CTA GTG AAC GTA CTA TTC CAA AC	
	15B/C	Forward	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	496
		Reverse	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C	
3	1	Forward	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280
		Reverse	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	
	3	Forward	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371
		Reverse	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
	10A	Forward	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	628
		Reverse	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	
	11A	Forward	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	463
		Reverse	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	

Table 1. Primers Used in This Study (Continued)

Reaction	Primer set		Primer sequence (5'-3')	Product size (bp)
4	4	Forward	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	430
		Reverse	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	
	5	Forward	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	362
		Reverse	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	
	7C	Forward	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	260
		Reverse	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC	
	17F	Forward	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	693
		Reverse	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC	
	Sg18	Forward	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	573
		Reverse	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC	
5	7F	Forward	CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG	826
		Reverse	CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	
	8	Forward	GAT GCC ATG AAT CAA GCA GTG GCT ATA AAT C	294
		Reverse	ATC CTC GTG TAT AAT TTC AGG TAT GCC ACC	
	12A	Forward	ACT CTT CCA AAT TCT TAT GCT TTT ATT GAT TC	656
		Reverse	ATG AAT GAG AAA AGG AAC TTA AAA TTC ATA GC	
	20	Forward	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	514
		Reverse	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC	
	23B	Forward	TTG TTA GTG GTA TTA AAT TGG GGA CTA CTA GG	216
		Reverse	ATA CCT ATC TGA AGT GTT ATT AAC CCA CCA AC	
6	31	Forward	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC	701
		Reverse	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC	
	33F	Forward	GAA GGC AAT CAA TGT GAT TGT GTC GCG	338
		Reverse	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C	
	35F	Forward	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A	517
		Reverse	GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC	
	38	Forward	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG	574
		Reverse	ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC	
7	2	Forward	GTC ATT GTT ACG ATT AGT TTC GAT AGT TGA GG	381
		Reverse	AAT TCA ATT CCT AAG TCC TCT TCC ATA AAC TC	
	29	Forward	ATT ATC TCG GAT CAA ACA ATT CTT TTG TAA AC	259
		Reverse	AAC GCT AAC ATT AAA ACT AGA ACG AGT AAA CC	
	34	Forward	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	408
		Reverse	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	
	35B	Forward	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	677
		Reverse	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G	
8	12F	Forward	GCA ACA AAC GGC GTG AAA GTA GTT G	376
		Reverse	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	
	16F	Forward	CTG TTC AGA TAG GCC ATT TAC AGC TTT AAA TC	988
		Reverse	CAT TCC TTT TGT ATA TAG TGC TAG TTC ATC C	
	22F	Forward	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	643
		Reverse	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	
	24A/F	Forward	TCT CAA CCA AGA TAC AGA TTT TGA TTT TAC TC	686
		Reverse	TAT AAA CCT TTA GTA AAC ACT CTG CTT GAT CG	
	45	Forward	GTT TAA TGG CTG ATG AAG TTA TTA TTG TTG	238
		Reverse	TTT ACC ATC AGT GAA ATT TTA TCT TTG TTC	
Positive control	*cpsA*	Forward	GCA GTA CAG CAG TTT GTT GGA CTG ACC	160
		Reverse	GAA TAT TTT CAT TAT CAG TCC CAG TC	

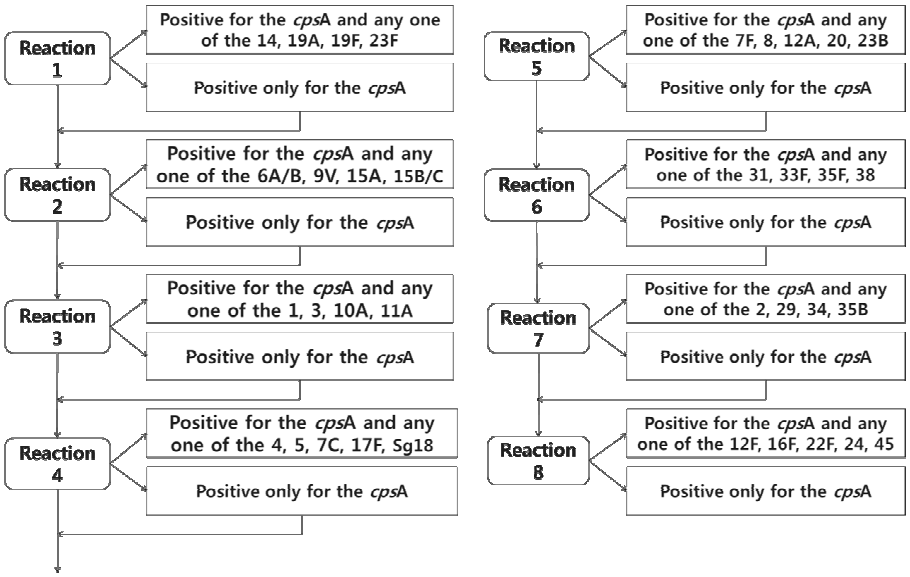

Fig. 1. Scheme of sequential multiplex polymerase chain reaction-based pneumococcal serotyping showing the 8 reactions and the serotypes identified in each reaction.

pairs were designed as described in published reports (Table 1)[14-16]. A primer pair targeting *cpsA* found in all 90 known pneumococcal serotypes was used as the positive control. The primers were grouped into eight sets for sequential testing (Fig. 1) based on the ranking of serotypes that are frequently detected in South Korea[17-19]. PCR for 35 serotypes were performedon all samples to check the status of co-colonization. PCR mixtures consisted of 12.5 μL 2× multiplex PCR Pre-Mix, 3.5 μL purified DNA, primers at concentration of 1 μm, and *cpsA*-f and *cpsA*-r primers at a concentration of 0.5 μm in a 25 μL reaction volume. Amplification conditions for all reactions were as follows: initial denaturation at 94℃ for 4 minutes; 30 cycles at 94 ℃ for 45 seconds, 54℃ for 45 seconds and 65℃ for 2 minutes 30 seconds; and a final extension cycle of 72℃ for 5 minutes. The PCR products were analyzed by gel electrophoresis on a 1.4% agarose gel at 120 volts for 45 minutes, stained with ethidium bromide, and visualized with ultraviolet transillumination.

Results

1. Comparison of PCR and bacterial culture results for identification of *S. pneumoniaee*

Among the 842 total cases, 793 underwent simultaneous bacterial culture and PCR analysis for pneumococcal isolation, while 49 were only analyzed by PCR. Bacterial culture yielded positive results in 10.2% (81/793) cases and PCR in 19.3% (153/793). Both methods revealed identical results in 85.6% (679/793) of tests. Among 14.4% (114/793) that showed different results, 11.7% (93/793) showed negative results with culture and positive results with PCR, and 2.6% (21/793) showed positive results with culture and negative results with PCR (Table 2).

Table 2. Comparison of the Results of Culture and Multiplex Polymerase Chain Reaction (PCR)

Streptococcus pneumoniae	Culture-positive	Culture-negative	Total
PCR-positive	60 (7.6)	93 (11.7)	153 (19.3)
PCR-negative	21 (2.6)	619 (78.1)	640 (80.7)
Total	81 (10.2)	712 (89.8)	793 (100)

Values are presented as number (%).

2. Distribution of pneumococcal serotypes

Among the total 842 specimens, 163 were positive by PCR analysis. In total, 163 PCR-positive samples and 4 co-colonization strains were analyzedfor serotyping. A total of 13 different *S. pneumoniae* serotypes could be identified, and 43 pneumococcal strains were non-typeable (26%). The most frequent serotypes were 19A (n=39, 23%) and 6A/B (n=27, 16%), followed by 19F (19, 11%), 15A (n=9, 5%), and 15B/C (n=8, 5%). These five serotypes together accounted for 61% of overall isolates. Among all isolates, 30.5% were the serotypes included in PCV7, and 69.5% were non-PCV7 vaccine types (Fig. 2).

Discussion

In this study, multiplex PCR was used to detect pneumococci and to determine pneumococcal serotypes among 842 total cases. Of the total samples, 793 underwent simultaneous bacterial culture and PCR analysis, with a concordance of 85.6%. The detection

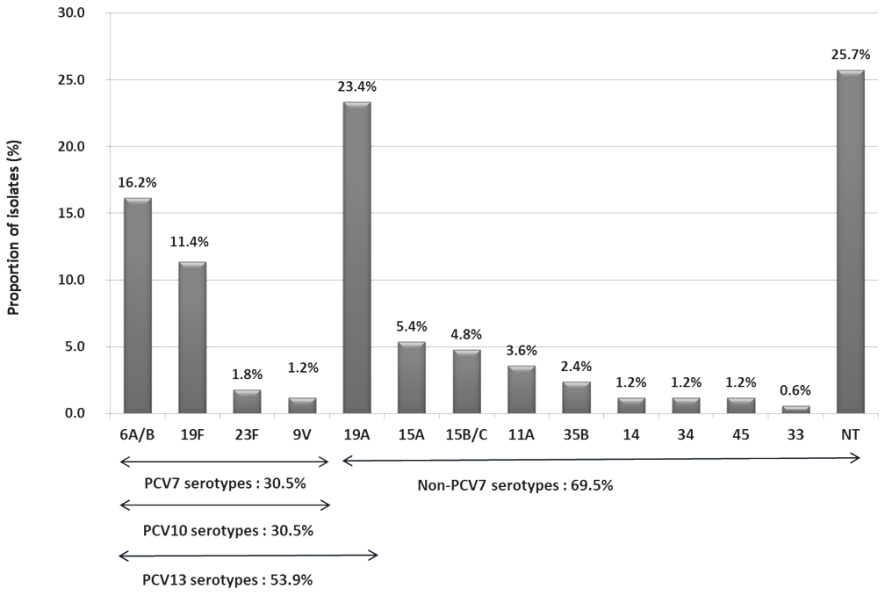

Fig. 2. Distribution of *Streptococcus pneumoniae* serotypes as a proportion of total serotypes. PCV7, 7-valent pneumococcal conjugate vaccine; NT, nontypeable (refers to the pneumococcal serotypes that we were unable to identify by using the 35 primer sets).

rate of pneumococcal strains by multiplex PCR (19.3%) was higher than that by bacterial culture (10.2%). PCR analysis based on the amplification of *cpsA* or other pneumococcal genes has been shown to be sensitive and efficient in detecting pneumococcus in clinical samples[16,20-23]. Virolainen et al.[20] found 18% of middle ear fluid specimens to be positive by culture and 30% by culture and PCR. Saha et al.[16] showed that sequential multiplex PCR of 127 culture-negative cerebrospinal fluid samples yielded serotypes for 51 additional cases. Our results are consistent with those of previous studies in this field. In the present study, although 2.6% of samples showed positive results in culture but negative by PCR, these are thought to be the result of operator error and will likely be reduced by increased operator experience.

In addition to detection of pneumococci, accurate serotype determination is important for vaccine development. Presently the choice of vaccine strains depends on serotype prevalence patterns. Although serological determination by a quelling reaction of pneumococcal cells with antipolysaccharide sera is the currently used method for pneumococcal serotyping[24,25], the high cost of antisera, the requirements for technical expertise, and subjectivity in interpretationof results are major shortcomings of the procedure[14,16]. Multiplex PCR has the potential to overcome these drawbacks and has been reported to have higher sensitivity and specificity than traditional quelling reaction methods[14,16,26,27].

Because the serotype distribution among NP carriage isolates varies by geographic region, time, and age-group, we designed primers and modified the sequential multiplex PCR scheme based on the distribution of serotypes that were frequently isolated in Korea[17-19]. Among the serotypes identified in our study, the most common serotype was 19A, followed by: 6A/B, 19F, 15B/C, 15A, 11A, and in a small minority 35B, 23F, 14, 9V, 45 and 33. In Korea, PCV7 has been available since November 2003 and PCV10 and PCV13 were introduced in May 2010. Thus, our results, obtained from March 2009 to June 2010, represent the impact of PCV7 on NP carriage of pneumococcus. Several studies conducted in Korea prior to the use of PCV7 demonstrated that the proportion of PCV7 serotypes in invasive and noninvasive isolates, including carried strains, was 54 to 72% and 57 to 83%, respectively[17-19,28,29]. Our study revealed that PCV7 serotypes decreased to 30.5% of carried serotypes over the course of this study period, indicating a replacement phenomenon in NP carriage after the introduction of PCV7.

As PCV10 or PCV13 have only recently been introduced, there will likely be changes in serotype distribution among pneumococcal carriage isolates in the future. Surveillance of pneumococcal serotypes should be continued in order to monitor changes in serotype distribution necessary for the pneumococcal vaccine policy. To that end, our multiplex PCR system can be used as an accurate and rapid way of isolating and serotyping *S. pneumoniae* compared to conventional microbiology culturing. However, our study had an important limitation; 26% of total isolates were non-typeable because only 35 primer sets for pneumococcal serotyping were used in the protocol. In the future, new serotype-specific primers will need to be added, so that more serotypes may be detected. This will enhance detection and serotyping of *S. pneumoniae* when using multiplex PCR.

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