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Research Note

Evaluation of PCR-based approach for serotype determination of *Streptococcus pneumoniae*

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Abstract. Determination of *Streptococcus pneumoniae* serotypes is essential for epidemiological surveillance. Therefore accurate, reliable and cost effective serotyping method is crucial. In this study, we determined the serotypes of 41 pneumococcal isolates recovered from human anterior nares by multiplex Polymerase Chain Reaction (PCR) utilizing published primers. The data was then compared with conventional serology using latex agglutination (LA) and the Quellung reaction. Based on the PCR-approach, 8 different serogroups/serotypes were detected with one isolate classified as non-typeable (*cpsA*-negative). In reference to the serology-based data, the results were in agreement except for one isolate. For the latter isolate, the LA and Quellung tests failed to show a reaction but the PCR-approach and sequencing identified the isolate as serogroup 15B/C. Based on this experimental setting, we found that the PCR-approach for pneumococcal serotypes determination is reliable to serve as the alternative for determining the pneumococcal serotyping.

Streptococcus pneumoniae (pneumococci) plays an important role worldwide in causing otitis media, sinusitis and life threatening diseases such as pneumonia, sepsis and meningitis, particularly among young children and the elderly (Bogaert, 2004; Mitchell & Mitchell, 2010; Le *et al.*, 2011). The capsular polysaccharide (cps) of this bacterium contributes to virulence and also differentiates pneumococcus into more than 90 distinct capsular (Kadioglu *et al.*, 2008). Nevertheless only 20 serotypes are commonly associated with disease and their distribution varies in different continents (Brito *et al.*, 2003). It is important to determine the prevalence of serotypes to facilitate the management of pneumococcal disease, as some serotypes have the potential for decreased antibiotic susceptibility. Furthermore, there is a necessity to monitor pneumococcal serotype distribution after the introduction of pneumococcal conjugate vaccines (PCVs) which are based on limited serotype valency.

The conventional approach for identifying pneumococcal serotypes is by

serological determination of the capsular types using latex agglutination (LA) and Quellung reaction tests. Both tests rely on *in situ* immunoprecipitation between pneumococcal capsular polysaccharide and its homologous antibody. The LA method is more-straight forward to undertake than the Quellung reaction although the latter remains the gold standard method (Konradsen *et al.*, 2005). Nevertheless, the antisera panels for LA and Quellung testings are very costly for developing countries. In addition, their application is also highly dependent on human expertise for interpretation and is relatively time-consuming (Brito *et al.*, 2003; Konradsen *et al.*, 2005; Rivera-Olivero *et al.*, 2009; Miernyk *et al.*, 2011). PCR-based serotyping method has been recently developed to specifically determine the capsular type-specific DNA sequences of pneumococci which offer a simple, sensitive and economical approach (Brito *et al.*, 2003; Pai *et al.*, 2006; Dias *et al.*, 2007; Carvalho *et al.*, 2010). As accurate serotyping is important for surveillance and vaccination strategy, evaluation of PCR-based approaches in reference to serology is needed in order to establish the reliability of the data.

In this study, capsular serotypes for a total of 41 pneumococcal isolates from human anterior nares were determined by sets of primers in multiplex PCR and compared to the serology-testing using the LA and Quellung reaction tests, as determined previously (Yatim *et al.*, 2012). Published primers recommended by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm) were selected to include common serotypes in Asia and Malaysia which are 6A/B, 19A, 19F, 23F, 1, 7A/F, 14, 15/BC, 11A, 20, 7C, 23A, 23B, 9V/A, 18A/B/C, 17F, 2, 4, and 9N/L (O'Brien *et al.*, 2009; Le *et al.*, 2011; Rohani *et al.*, 2011; Jauneikaite *et al.*, 2012). These serotypes also include all the serotypes of the isolates as previously determined by serology-based methods. Two of the isolates were non-typeable (NT) and also included for re-evaluation by PCR.

All isolates were re-identified as *S. pneumoniae* by standard bacteriological methods comprising colony morphology, susceptibility to ethylhydrocuprein (optochin) disc, catalase test and bile solubility. Pneumococcal genomic DNA was extracted using commercial kit GeneAll® Exgene™ (GeneAll Biotechnology Co. Ltd., Korea).

As for PCR, the primers were sorted into 5 multiplex sets named as A, B, C, D and E respectively as shown in Table 1. Each set contain four primer pairs targeting four different serotypes/serogroups and a pair of primers targeting a conserved region of the capsular polysaccharide synthesis gene (*cpsA*). The gene is located at the *cps* locus in pneumococci and serves as the internal positive control. The arrangement of primer pairs in the respective multiplex sets (A to E) was to allow differentiation of PCR-product based on DNA-band size and technical feasibility of the multiplex PCR in the respective single reactions. It is also to consider the prevalent serotypes in this region whereby the more common serotypes will be first targeted followed by the less ones. Thus, when the multiplex sets are tested sequentially from A to E in local isolates of unknown serotypes, the possibility of getting amplification is higher due to high-prevalence-serotypes-associated primer in the earlier multiplex sets. However, all the multiplex sets were tested against all isolates in this study for validation purposes.

The optimal PCR condition in a 25 µl reaction comprised 5 µl of iDNA PCR master-mix (i-DNA Biotechnology, Singapore), 1.2 µl of DNA template (1.0 -10 ng), 1.0 µl (1 µM) of serotypes specific primer pairs and 0.5 µl (1 µM) of *cpsA* specific primer pair. The PCR was carried out in a BioRadMyCycler™ Thermal Cycler (BioRad, USA). Initial denaturation step was performed at 94°C for 4 min, and the 30 amplification cycles were performed with denaturation at 94°C for 45 s, annealing temperature at 54°C for 45 s and elongation temperature at 65°C for 2 min and 30 s, and finally completed with an elongation at 72°C for 2 min. The PCR product was electrophoresed on a 2% (w/v) agarose stained with GelRed™ (Biotium®, USA) for 1 hr at 90V. The bands were visualized and photographed under UV illumination. The 100 bp plus DNA ladder marker (Vivantis,

Table 1. List of oligonucleotide primers used for pneumococcal serotype-determination

Primer pair	Primer sequence (5'-3')	Target gene (GenBank accession No.)	Amplicon size (bp)	References*
Set A				
6A/B-f	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	*wciP* (CR 931639)	250	Pai *et al.*, 2006
6A/B-r	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA			
19A-f	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	*wzy* (CR 931675)	566	Pimenta *et al.*, 2009
19A-r	CAT AAT AGC TAC AAA TGA CTC ATC GCC			
19F-f	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	*wzy* (CR 931678)	304	Pai *et al.*, 2006
19F-r	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG			
23F-f	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	*wzy* (CR 931685)	384	Pai *et al.*, 2006
23F-r	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC			
Set B				
1-f	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	*wzy* (CR 931632)	280	Pai *et al.*, 2006
1-r	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C			
7A/F-f	TCC AAA CTA TTA CAG TGG GAA TTA CGG	*wzy* (CR 931643)	599	Carvalho *et al.*, 2010
7A/F-r	ATA GGA ATT GAG ATT GCC AAA GCG AC			
14-f	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT	*wzy* (CR 931662)	189	Dias *et al.*, 2007
14-r	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT			
15 B/C-f	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	*wzy* (CR 931665)	496	Pai *et al.*, 2006
15B/C-r	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C			
Set C				
11A-f	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	*wzy* (CR 931653)	463	Pai *et al.*, 2006
11A-r	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC			
20-f	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	*wci*L (CR 931679)	514	Pai *et al.*, 2006
20-r	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC			
7C-f	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	*wcw*L (CR 931642)	260	Pai *et al.*, 2006
7C-r	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC			
23A-f	TAT TCT AGC AAG TGA CGA AGA TGC G	*wzy* (CR 931683)	722	Carvalho *et al.*, 2010
23A-r	CCA ACA TGC TTA AAA ACG CTG CTT TAC			

Table 1. (continued)

Primer pair	Primer sequence (5'-3')	Target gene (GenBank accession No.)	Amplicon size (bp)	References*
Set D				
23B-f	CCA CAA TTA G CG CTA TAT TCA TTC AAT CG	*wzx* (CR 931684)	199	Carvalho *et al.*, 2010
23B-r	GTC CAC GCT GAA TAA AAT GAA GCT CCG			
9V/9A-f	GGG TTC AAA G TC AGA CAG TG A ATC TTA A	*wzy* (CR 931648)	816	Carvalho *et al.*, 2010
9V/9A-r	CCA TGA ATG A AA TCA ACA TT G TCA GTA GC			
18A/B/C-f	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	*wzy* (CR 931673)	573	Pai *et al.*, 2006
18A/B/C-r	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC			
17F-f	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	*wci*P (CR 931670)	693	Pai *et al.*, 2006
17F-r	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC			
Set E				
2-f	TAT CCC AGT TCA ATA TTT CTC CAC TAC ACC	*wzy* (CR 931633)	290	Carvalho *et al.*, 2010
2-r	ACA CAA AAT ATA GGC AGA GAG AGA CTA CT			
4-f	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	*wzy* (CR 931635)	430	Pai *et al.*, 2006
4-r	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G			
6C/D-f	CAT TTT AGT GAA GTT GGC GGT GGA GTT	*wci*Nbeta (EU714777.1)	727	Carvalho *et al.*, 2010
6C/D-r	AGC TTC GAA GCC CAT ACT CTT CAA TTA			
9N/L-f	GAA CTG AAT AAG TCA GAT TTA ATC AGC	*wzx* (CR 931647)	516	Dias *et al.*, 2007
9N/L-r	ACC AAG ATC TGA CGG GCT AAT CAA T			
Internal Positive Control				
cps A-f	GCA GTA CAG CAG TTT GTT GGA CTG ACC	*wzg* (CR931662)	160	Pai *et al.*, 2006
cps A-r	GAA TAT TTT CAT TAT CAG TCC CAG TC			

Table 2. Serotyping results by multiplex PCR in comparison to serology-based methods

Serotypes	No. of isolates with serotypes determined by:	
	Multiplex PCR	Serology[a]
23F	9	9
19F	5	5
19A	5	5
6A/B	12 (6A), 4 (6B)	12 (6A), 4 (6B)
15B/C	3 (15B/C)[b]	2 (15C)
11A	1	1
23A	1	1
NT	1[1]	2[1,2]

[a]Yatim *et al.*, 2012, [b]sequencing equally matched serotypes 15B and 15C
[1]Isolate *N103SP39*, [2]Isolate *N22SP7*
No. of isolates = 41

Malaysia) was used for molecular weight reference. Confirmation of PCR-results was done by repeating the PCR-reaction for amplification with expected DNA-band size and sequencing of the product representatives generated by the respective primer pairs. Those that were denoted only at serogroup level (e.g. 6A/B, 7A/F, 15B/C, 9V/A, 18A/B/C, 6C/D and 9N/L) were fully sequenced to confirm the serotype. Sequence homology search was done in GenBank using Basic Local Alignment System Tool (BLAST) at www.ncbi.nlm.nih.gov/genbank.

Based on PCR and sequencing, 7 different serotypes and 1 serogroup were determined among 40 isolates, which were highly consistent with the serology-based data (Table 2). For two isolates categorised as NT in the serology-approach, one isolate was not detected for any of the targeted serotypes including the internal positive control gene (*cpsA*). This isolate could be of the other serotypes whereby the associated primers were not included in this study. But the failure in *cps*-amplification in this isolate could be due to the absence of the *cps* locus or *cps* sequence alterations (Brito *et al.*, 2003; Pai *et al.*, 2006; Miernyk *et al.*, 2011). This would result in encapsulated or capsular-mutated isolates, which could explain the no serology-reaction for this isolate to be classified as NT. Brito *et al.* (2003) in their study showed a consistency between *cps*-negative PCR and no serology-reaction in encapsulated pneumococcal (rough) isolates. For another NT isolate, it was determined as 15B/C by PCR and positive for the *cpsA*. Sequencing analysis showed an equal highest match with serotypes 15B and 15C in the GenBank. This is also true for the other two isolates determined as 15B/C by PCR but 15C by serology (Table 2). Pai *et al.* (2006) considered 15B and 15C as one serotype because they interconvert. The inconsistency between the serology and PCR in this isolate is not understood and could be due to technical or human mistake during the earlier work. This is not impossible as at a certain extent, serology-based method relies on human expertise in handling and interpreting the serotyping. Inconsistencies of serology-results among different laboratories have been reported by a study which evaluated the quality of serotyping for pneumococci throughout Europe but at a low rate (Kondradsen *et al.*, 2005).

This study evaluates the reliability of multiplex PCR-based method over the serology-approach for limited serotypes commonly found in Asian region. As far as the serotypes and isolates in this study are concerned, results are highly consistent with only one obvious discrepancy. This discrepancy highlights the potential weakness in the conventional serology, while in the PCR-approach, a good band resolution

of PCR-product, easier gel interpretation and further validation by sequencing may eliminate human error. The use of internal control targeting the conserved area of *cps* gene in the PCR may also infer the capsular status of isolates (Pai *et al*., 2006; Mierynk *et al.*, 2011). Carvalho *et al.* (2003) reported that NT isolates would represent approximately 10% of pneumococcal isolates from non-sterile sites of human body. In a study on pneumococcal carriage isolates from Alaskan community, the multiplex serotyping PCR-assay suggested that NT accounted for 12% of all samples obtained based on the *cps*-negative PCR (Mierynk *et al*., 2011). The prevalence of NT is of concern due to their potential increased capability in colonisation (Sa-Leao *et al*., 2006), and thus the need for accurate determination method such as PCR is also needed.

Finally, the use of multiplex PCR-approach is highly warranted for reliable detection of serotypes in a timely and economical manner. The multiplex system in this study was developed based on local serotype prevalence which may serve as a model for further assessment involving more isolates and serotypes.

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