A novel high-throughput method for molecular serotyping and serotype-specific quantification of *Streptococcus pneumoniae* using a nanofluidic real time PCR system

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Abstract

Background: Serotype specific quantification data is essential for elucidating the complex epidemiology of *Streptococcus pneumoniae* and evaluating pneumococcal vaccine efficacy. Various PCR-based assays have been developed to circumvent the drawback of labor-intensive and time-consuming culture-based procedures for serotype determination and quantification of pneumococcus. Here we applied a nanofluidic real time PCR system to establish a novel assay. **Methods:** Twenty-nine primer pairs, 13 of which were newly designed, were selected for the assay to cover 50 serotypes including all currently available conjugate and polysaccharide vaccine serotypes. All the primer pairs were evaluated for their sensitivity, specificity, efficiency, repeatability, accuracy and reproducibility on the Fluidigm Biomark HD System, a nanofluidic real time PCR system by drawing standard curves with a serial dilution of purified DNA. We applied the assay to 52 nasopharyngeal swab samples from the patients with chest x-ray confirmed pneumonia to validate its accuracy. **Results:** Minimum detection levels of this novel assay on this nanofluidic real time PCR system were comparable to the conventional PCR based assays (between 30 and 300 copies/reaction). They were specific to their targets with good repeatability (standard deviation of copy number of 0.1), accuracy (within +/- 0.1 fold difference in $\log_{10}$ copy number), and reproducibility (standard deviation of copy number of 0.1). When artificially mixed DNA samples consisting of multiple serotypes in various ratios were tested, all the serotypes were detected proportionally, including a minor serotype of 1 in 1000 copies. In the nasopharyngeal samples, the PCR system detected all the culture positive samples and 22 out of 23 serotypes identified by the conventional method were matched with PCR results. **Conclusion:** This novel assay, which is able to differentially quantify 29 pneumococcus groups for 45 test samples in a single run, is applicable to a large-scale epidemiological study of pneumococcus. We believe that this assay will facilitate our understanding of the roles of serotype specific bacterial loads and implications of multiple serotype detections in pneumococcal diseases.

INTRODUCTION

Streptococcus pneumoniae (S. pneumoniae) is a leading cause of death in under-5 children in the world. It was responsible for about 6% of total deaths among the population in 2008 (WHO, 2012). It is a Gram-positive bacterium with a polysaccharide capsule, which enables the bacteria to evade the host defense mechanism. Antigenic structure of the capsule polysaccharide chain is determined by a series of glycosyltransferases encoded by the *cps* locus and characterizes at least 93 serotypes. The serotype distribution associated with invasive pneumococcal diseases varies according to the geographical regions, so does the efficacy of the pneumococcal conjugate vaccines (PCV) (Johnson *et al.*, 2010). After the introduction of heptavalent conjugate vaccine (PCV7), an increase in frequency of non-vaccine serotypes was observed and it has become a major global health concern (WHO, 2010; Weinberger *et al.*, 2011). Besides, different serotypes caused death in a different age group (Harboe *et al.*, 2009), and some serotypes were associated with fatal illness both in the young and the elderly (Weinberger *et al.*, 2010). Hence, to understand this complex epidemiology of pneumococcal diseases, it requires a large-scale surveillance in serotype specific manner.

Various PCR-based molecular methods have been developed to detect serotype specific nucleotide sequences within the *cps* locus of *S. pneumoniae* (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm). It has been revealed that a considerable proportion of people were colonized with more than one serotype (Kaltoft *et al.*, 2008), and in some cases of invasive pneumococcal diseases, multiple serotypes have been isolated (Chaves *et al.*, 2003; de Andrade *et al.*, 2004). Furthermore, accumulating data have shown that quantification of *S. pneumoniae* was also important, as the bacterial load was associated with the disease severity (Carrol *et al.*, 2007; Rello *et al.*, 2009). Therefore we believe that it is essential to do the surveillance of multiple serotypes in colonization and infections of pneumococci, and measure serotype specific bacterial loads both in a clinical and a large-scale epidemiology study. However, there had been no assay, which could accurately quantify *S. pneumoniae* at serotype level and detect a minor population of a subdominant serotype (present in multiple serotype) circulating in the field. To address these issues, we applied a nanofluidic real time PCR system and established a novel assay. This assay can identify and quantify 29 serotypes/serogroups (17 individual serotypes and 33 serotypes in 12 groups; in total 50 serotypes), including all vaccine serotypes. In a single run, 45 different clinical specimens can be tested with 29 serotype/serogroup-specific and one autolysin (*lytA*) primer pairs, equivalent to 1350 individual PCR in total, has made it a high-throughput assay. Following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments)

guidelines (Bustin *et al.*, 2006), here we report linear dynamic range, minimum level of detection (analytic sensitivity), correlation coefficient (r^2), efficiency, analytic specificity, repeatability (intra-assay variance), accuracy and reproducibility (inter-assay variance) of this novel assay. Then, we applied the assay to 52 nasopharyngeal swab samples from the patients with chest x-ray confirmed pneumonia to validate the accuracy of the assay by comparing the results of the assay with the results of the conventional serotyping.

METHODS

Bacterial isolates for positive controls

Twenty one clinical isolates of *S. pneumoniae* serotypes 1, 3, 4, 6B, 7F, 9V, 10F, 10A, 11A, 14, 15B, 18C, 19F, 19A, 22F, 23F, 23A, 33F, 34, 35F and 35B were used; these were isolated from Japanese patients with invasive pneumococcal diseases such as pneumonia, meningitis, septicemia and otitis media as well as from healthy carriers. Seven additional isolates of serotypes 2, 5, 8, 9N, 12F, 17F, and 20 (ATCC®6302, ATCC®6305, ATCC®6308, ATCC®6309, ATCC®6312, ATCC®6317 and ATCC®6320 respectively) were purchased from American Type Culture Collection (Manassas, VA USA). Bacteria were cultured on trypticase soy agar with 5% sheep blood in 5% CO_2 atmosphere at 37°C overnight. All the isolates were confirmed as *S. pneumoniae* by optochin sensitivity test and bile solubility test according to the standard procedures (http://www.evaluations-standards.org.uk/), and the serotype was verified by latex agglutination test and Quellung reaction by pool antisera, and type and factor antisera respectively from Statens Serum Institut, Denmark.

DNA extraction and copy number determination

DNA was extracted from the pneumococcal isolates by Qiamp DNA mini kit (Qiagen KK Japan, Tokyo, Japan) following the manufacture's protocol. DNA concentration was determined by NanoDrop spectrophotometer (Thermo scientific, Waltham, MA USA). Average molecular mass of the genome DNA of *S. pneumoniae* was calculated from the whole genome nucleotide sequence data of 9 serotypes in the Genbank (serotypes 2, 3, 4, 6B, 11A, 14, 19A, 19F and 23F; GenBank accession # CP000410.1, FQ312027.1, AE005672.3, CP002176.1, CP002121.1, CP001033.1, CP000936.1, CP001015.1 and FM211187.1) and was used to obtain copy number values in the standard DNA samples.

Primer design and the conventional real-time PCR condition

Thirteen primer pairs targeted for serotype-specific sequence in the *cps* locus (Bentley *et al.*, 2006; Mavroidi *et al.*, 2007) were newly designed by Primer 3 software (http://frodo.wi.mit.edu/) and their specificity was checked by Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Remaining primers, which amplify their respective targets at the same condition of PCR, were adopted from the previous publications and were also checked for their specificity in our sample set. All the primers sequences were checked again *in silico* for their

representative targets by BLASTN (http://www.ncbi.nlm.nih.gov/Blast), by which the each primer showed 100% identity exclusively to its specific target sequence with significant E-value. Sequences and target sites of the primers are listed in Table 1. All the 30 primer pairs were tested for their sensitivity and specificity first in Light Cycler 480 real time PCR system (Roche) with 384-well heat block, at the final concentration of 0.4µM of each. Real time PCR was set up with 2x concentrate of SsoFast EvaGreen Supermix with Low ROX (BioRad, Hercules, CA USA) in which EvaGreen® (Biotium, Hayward, CA USA) was used as a detecting dye; a 10µl reaction consisted of 5.0µl of SsoFast EvaGreen Supermix with Low ROX, 0.4µl of each 10µM primer solution, 2µl of DNA sample and 2.2µl of deionized water. The thermal cycle condition was as follows: the initial incubation for enzyme activation at 98°C for 3 minutes (ramp rate 4.8°C/sec), 45 times of two-step cycle consisting of denaturation at 98°C for 30 seconds (ramp rate 4.8°C/sec) and annealing/extension at 59°C for 30 seconds (ramp rate 2.5°C/sec). Fluorescence signals were acquired at the end of each annealing/extension stage (59°C). After the completion of PCR, a melting curve analysis for the test of specific products of the PCR was performed from 65°C to 98°C continuously at the ramp rate of 0.11°C/sec. Minimum level of detection was determined as the lowest copy number of DNA in the triplets when all the three reactions were positive, this was done by 10-fold serial dilutions of the DNA samples from 3 million copies to 3 copies per reaction with each reaction in triplets; and from the Cq values of the DNA samples the standard curves were drawn for each serotype specific primers. Minimum level of detection, efficiency and correlation coefficient (r2) of each primer pairs were determined in the Light Cycler 480 real time PCR system (Supplementary Table 1). The autolysin (*lytA)* primers were used for the identification of *S. pneumoniae* and for quantification of total pneumococcus load (Table 1). The specificity of the *lytA* primers was verified by testing with all the available standard samples of *S. pneumoniae* and other bacterial species, *Streptococcus mitis*, *Neisseria meningitidis*, *Haemophilus influenzae* b and *Moraxella catarrhalis.* All the primer pairs, which first worked out on the conventional real time PCR system, were adopted into the nanofluidic real time PCR system.

Specific target amplification and the nanofluidic real time PCR system

Following the manufacturer's recommendation, specific target amplification (STA) was performed in Applied Biosystems Veriti 96 well thermal cycler (Life Technologies Japan, Tokyo, Japan) as the initial step for BioMark HD system (Fluidigm, South San Francisco, CA USA). In brief, STA pooled primer mix was prepared by mixing the forward and reverse primers for the same 30 amplicons as above and diluted with 10mM Tris HCl and 0.1 mM

EDTA (pH=8) so as to make the concentration of 200nM of each primer. 8.0 µL of STA reaction was prepared by adding 4.0 µL of TaqMan PreAmp Master Mix (Applied Biosystem, California, USA), 2.0 µL of STA pooled primer mix and 2.0 µL of DNA solution. The final concentration of each primer in the STA reaction was 50 nM. STA was performed with activation at 98°C for 10 min, and 14 times of two-step cycles: denaturation at 98°C for 15 seconds and annealing/extension at 59°C for 4 minutes. The STA product was diluted five times with 10mM Tris HCl and 0.1 mM EDTA (pH=8) before proceeding to the next step.

Sample premix and assay premix were prepared in the wells of 96-well plate according to the manufacturer's protocol (available at http://www.fluidigm.com/product-documents.html), and 5 µL of each was transferred to the inlet of the primed 48.48 Dynamic Array IFC chip (Fluidigm, California, USA). The chip was set to the IFC Controller MX (Fluidigm, California, USA). The 6.25 nl reaction chambers were filled up with PCR reactions containing 4.5 µM each of specific primers and 40-fold diluted STA products containing negligible concentrations of carried over STA primers (1.25nM each) in 1x SsoFast EvaGreen Supermix with Low ROX. Nanofluidic real time PCR was carried out on the Fluidigm Biomark HD System with the thermal cycle condition as follows: the initial incubation for enzyme activation at 98°C for 3 minutes (ramp rate 2°C/sec), 40 times of two-step cycle that consists of denaturation at 98°C for 30 seconds (ramp rate 2°C/sec) and annealing/extension at 59°C for 30 seconds (ramp rate 2°C/sec). Fluorescence signals were acquired at the end of each annealing/extension stage (59°C). After the completion of PCR, a melting curve analysis for the test of specific products was performed from 65°C to 98°C continuously at the ramp rate of 0.11°C/sec. Quantitative analysis was carried out by using the crossing point (Cq) values of the PCR reactions with proper melting curves (Spurgeon *et al.*, 2008).

Standard curve and quantification in the nanofluidic real time PCR system

Standard curves for real time PCR were drawn with three independent serial 10-fold dilutions from 3×10^6 copies to 3 copies per 2 µl (the volume used in a single assay), which were made in the background of λ phage DNA (Takara Bio, Otsu, Japan) at 1 ng/µl of constant concentration. The linear standard curves were constructed by using Real-time PCR Analysis software implemented in the detection system. For the quantification of test samples, two positive controls (mixture of multiple different isolates at known concentrations) and one negative

control (λ DNA alone) were included to each run. The serotype-specific copy number of unknown samples was determined by analyzing the value of Cq in the equation of the standard curve of the serotype.

Determination of linear dynamic range, correlation coefficient (r^2), efficiency, analytical sensitivity, analytical specificity, repeatability, accuracy and reproducibility in the nonofluidic real time PCR system

Linear dynamic range was tested from 3 copies to 3 million copies in the standard curves. The coefficient of correlation (r^2) of each standard curve was determined. Amplification efficiency (e) of a given primer pair was calculated by $e = 10^{-1/m} - 1$, where m was the slope of the standard curve. Analytical sensitivity (lower limit of detection) was determined as the minimum number of copies of DNA that could be measured by the primers in the ten-fold serial dilutions of the purified DNA sample. Analytical specificity of each primer pair was assessed by crosschecking each with 28 available pneumococcal isolates as well as four non-pneumococcal bacteria in a single run of nanofluidic real time PCR system and melting curve analysis of their amplification products. Intra-assay variation (repeatability) was assessed by replicating the same samples for 4 times for vaccine serotypes and 2 times for non-vaccine serotypes in the same assay run, and presented as standard deviation (SD) for copy number of the samples. Accuracy of each primer pair was calculated as the ratio of difference between the observed copy number (in log base 10) and the expected copy number (in log base 10) to the expected copy number (in log base 10). Inter-assay variation (reproducibility) was computed as the SD of copy number (in log base 10) in different runs of the assay.

Artificial DNA mixture of multiple serotypes for assessment of detection and quantification of minor serotypes in the nanofluidc real time PCR system

To confirm the effectiveness of quantitative detection of multiple serotypes in a single specimen by the nanofluidic real time PCR system, we prepared the mixed DNA samples of two different serotypes to imitate those of multi-serotype co-colonization at different contribution of minor population at 1:10, 1:100 and 1:1000 ratios (Table 4).

Application of the assay to clinical specimens

Fifty two nasopharyngeal swab samples from children with chest X-ray confirmed pneumonia collected in a government referral hospital in central Vietnam were examined for the presence of pneumococcus and serotype determination by conventional microbiological/serological methods, previously (Vu *et al.*, 2011). DNA were

extracted from these nasopharyngeal swab samples and used to verify the practical usefulness of the nanofluidic real time PCR system.

RESULTS

Linear dynamic range, correlation, sensitivity, specificity and efficiency of the assays in the nanofluidic real time PCR system

The amplification signals remained specific to the target at least until cycle 25 in the nanofluidic real time PCR system which were confirmed by melting curve analysis. Linear dynamic range was 4 to 5 orders of magnitude for all the primer pairs. Within these dynamic ranges, the correlation coefficients (r^2) of the assays were 0.99 except for the assay of serotype 3, which was 0.98. The minimum levels of detection of all primer pairs were between 30 and 300 copies of the genome per 2 µl of DNA sample (Table 2). All the primer pairs were specific to their own targets as shown in Table 3. Efficiency of the primers ranged from 80 to 110%, except for the assays for serotypes 22F/22A and 33F/33A/37 that had 71 and 72% respectively (Table 2). Collectively, all the assay designs were successful to amplify the intended targets with a high sensitivity, specificity and linearity on the nanofluidic real time PCR system.

Repeatability, accuracy and reproducibility of the assays in the nanofluidic real time PCR system

Repeatability (intra-assay variation): The SD of the copy number (in log base 10) in the tests for replicated standard DNA in the same run was calculated for each primer pair at the target concentrations of 3×10^5 copies per reaction. It was less than 0.10 for all primer pairs (Table 2). Accuracy: When the target concentrations of 3×10^5 copies per reaction was estimated, the fold difference in copy number (in log base 10) from the observed values was within +/- 0.10 for all primers pairs except for serotype 35B, which had + 0.12 (Table 2). Reproducibility (inter-assay variation): The SD of copy number (in log base 10) at the target concentrations of 3×10^5 copies per reaction between different runs was less than 0.10 for all primers except for serotype 33F/33A/37 and 12F/12A/12B/44/46, which had 0.111 and 0.105 respectively (Table 2). Collectively, with a few exceptions, the indices for assay parameters fell within the range of excellent performance: repeatability of 0.10, accuracy of between - 0.10 to +0.10 and reproducibility of 0.10.

Multiple serotype detection in a single specimen

As a representative result of several trials, the recovery (the efficiency of quantitative detection of the minor populations in the presence of excessive amount of non-target DNA) of minor population at the ratios of 1:10,

1:100 and 1:1000 were within base 10 logarithm of +/-0.5; 0.38 (base 10 logarithm of -0.42), 1.20 (base 10 logarithm of 0.08) and 0.62 (base 10 logarithm of -0.20) for the respective ratios (Table 4). By similar experiments with artificially mixed DNA consisted of multiple serotypes in various ratios repeated several times, we could demonstrate that all the serotypes in the samples were detected almost proportionally, even if the minor components were as few as 1 in 1000 copies.

Comparison of the nanofluidic real time PCR system with conventional culture and serotyping methods in the clinical samples

Fifty-two nasopharyngeal swab samples from the chest x-ray confirmed pneumonia cases, which had been examined by conventional culture and serotyping methods were tested by the nanofluidic real time PCR system to demonstrate practical utility of the method (Supplementary table 1). PCR-positive rate was higher than culture-positive rate; 40 out of 52 were PCR-positive and all 23 culture-positive samples were PCR-positive also (Table 5). Serotyping results of nanofluidic PCR system for 23 culture-positive samples well matched with those of conventional serotyping method except for one sample (22/23=95.7%).

DISCUSSION

Bacterial culture and serological typing remain the gold standard methods for pneumococcal serotype identification, but they are expensive, labor-intensive and less sensitive especially if antibiotics were administrated prior to the sample collection. Various PCR and antibody based molecular methods have been developed to overcome the drawbacks of the conventional bacteriological and serological procedures for serotype identification and quantitative detection of *S. pneumoniae*. These molecular techniques include oligonucleotide probe array (Kong *et al.*, 2006; Wang *et al.*, 2007; Turner *et al.*, 2011), immunoblot (Bronsdon *et al.*, 2004), and immunoassay (Yu *et al.*, 2005). Multiplex PCR and real time PCR have substantially increased the sensitivity of diagnosing pneumococcal diseases even in culture-negative cases (Saha *et al.*, 2008) and identifying the presence of pneumococcus in the nasopharyngeal colonization (Azzari *et al.*, 2008; Antonio *et al.*, 2009). Most multiplex PCR serotyping procedures use different sets of primers targeting for several serotypes/serogroups, and have been used in epidemiological studies (Brito *et al.*, 2003; Lawrence *et al.*, 2003; Pai *et al.*, 2006; Dias *et al.*, 2007; Morais *et al.*, 2007), mainly because of their cost effectiveness. Real time PCR is more sensitive than multiplex PCR (Azzari *et al.*, 2010), and has been used as clinical diagnostics too (Kee *et al.*, 2010; Moore *et al.*, 2010). Real time PCR assays have a considerable advantage to multiplex PCR assays because they provide quantitative data. Obtaining quantitative data has been realized important as the nasopharyngeal bacterial load of pneumococcus is associated with severity of pneumonia and fatal outcome both in children and adults (Carrol *et al.*, 2007; Rello *et al.*, 2009; Vu *et al.*, 2011), and it was also applied to the prediction of pneumonia (Albrich *et al.*, 2012). Our novel assay, which is based on the nanofludic real time PCR system, has all characteristics of a rigorous real time PCR assay such as high sensitivity, specificity, accuracy, efficiency, repeatability and reproducibility, and also provides high throughput qualitative and quantitative data, even of minor populations of subdominant serotypes.

For the identification of *S. pneumoniae*, several genes are used as the target genes for quantitative PCR assays, such as autolysin (*lytA*), pneumolysin (*ply*), pneumococcal surface adhesin A (*psaA*) and a gene for hypothetical protein *Spn9802* (Suzuki *et al.*, 2005). Among them, *lytA* has shown one of the most sensitive and specific targets and is capable to differentiate *S. pneumoniae* from *S. pseudopneumoniae* (Carvalho Mda *et al.*, 2007; Kee *et al.*, 2010; Abdeldaim *et al.*, 2010; Greve *et al.*, 2012). Therefore, we adopted *lytA* primers for the confirmation of *S. pneumoniae* in this assay. We think *lytA* primers should be included when testing clinical samples along with the serotype specific primers, as it will estimate the total bacterial load of *S. pneumoniae* including serotypes not

covered by the assay. Although several studies of real time PCR for quantification of pneumococcal bacterial load have been published, most lacked the detail description on the assay performance (Corless *et al.*, 2001; Yang *et al.*, 2005; Carrol *et al.*, 2007; Azzari *et al.*, 2010). Regarding total bacterial load estimation, our quantitative assay has a comparable performance to the previously published assays in terms of sensitivity, specificity and reproducibility; there are few exceptional reports which described the assay performance in details, having around 10 copies of the lower limit of detection, 5 orders of linear dynamic range and 0.3 to 1 cycles of reproducibility (van Haeften *et al.*, 2003; Muñoz-Almagro *et al.*, 2011). For serotype-specific assays, there have been no previous reports about the assay performance to compare with; we could achieve good performance on them, which are comparable to the assay for the total bacterial load.

Co-colonization of multiple serotypes of *S. pneumoniae* is another emerging epidemiological concern because it may have influence on the efficacy of pneumococcal vaccine (Rivera-Olivero *et al.*, 2009; Weinberger *et al.*, 2011). In this nanofluidic real time PCR system, the presence of minor serotype (in multiple serotype) could be exactly identified even though it was as few as 1 in 1000 copies of background pneumococcal DNA, as demonstrated by mixed DNA samples in Table 4. This ability to detect multiple serotypes, even in lower population density of pneumococci, is one of the novelties of the assay. This assay has a wide coverage of 50 prevalent serotypes in the field, including all currently available vaccine serotypes, and has a considerably high throughput. Quantitative data of 30 amplicons for 45 test samples, which are equal to 1,350 PCR reactions, can be obtained by a single run in approximately four hours including specific target amplification (STA) step prior to the nanofluidic real time PCR system. Furthermore, only 1/30 of PCR reagents are required in this assay compared to the conventional real time PCR, which results in a lower running cost, and makes our assay cost-effective. Considering all these characteristics of this novel assay, we think it has met the demand of an efficient and cost-effective quantitative assay for large epidemiological studies of *Streptococcus pneumoniae.*

Recently Centers for Disease Control and Prevention (CDC), USA has developed a seven-triplexed real time PCR system for identification of serotypes of PCV13 (Pimenta *et al.*, 2013). This system can identify 11 individual serotypes and 10 serogroups in 7 triplexed reactions using probe based chemistry. In our nanofluidic real time PCR system, we can identify 17 individual serotypes and 12 serogroups (33 serotypes) including all vaccine serotypes of

PCV13 and PPV23. We used EvaGreen®, a dsDNA binding dye, which is cheaper than the probe based chemistries, and obtained comparable sensitivity and specificity of the probe based system used by CDC.

One of the limitations is the initial cost of purchasing the nanofluidic equipment. The current price is about five to ten-times higher than a conventional real-time PCR machine. Another limitation, which is common to all PCR-based methods, is some serotypes could not be identified individually by single primer sets. We could not use primer specific for serotypes 6C/6D, instead we used primers for serotypes 6A/6B and serotypes 6A/6B/6C/6D in our assay (Table 3). Similar overlapping of specificity exists between assay #28, specific to serotype 34, and assay #29, specific to serotypes 34/35F. All the serotyping assays are practically free of false positive reaction because of cross-reaction between different serotypes and free of false negative reaction because of competition between primers, which may take place in conventional multiplex PCR assays.

In conclusion, we have developed a reliable assay based on the nanofluidic real time PCR system for molecular serotyping of pneumococcus, which can provide qualitative and quantitative data of 50 pneumococcal serotypes in 29 groups directly from 45 test samples in a single run. As it can quantify pneumococcal load in serotype/serogroup level, we expect it may help us to understand the role of serotype specific pneumococcal loads in the pathogenesis of the pneumococcal diseases, particularly when multiple serotypes are present. To the best of our knowledge, this is the first report of serotype/serogroup specific quantitative real time PCR assay of pneumococcus with the detail performance data presented according to the MIQE guidelines.

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Table 1. Primers for nanofluidic real time PCR serotyping.

Assay	Specificity	Target gene	GenBank accession	Forward primer	Reverse primer	Amplicon size	Source†
Identification of *S. pneumoniae*							
1	*lytA*	*lytA*	AE005672	TCGTGCGTTTTAATTCCAGC	TGACGGACTACCGCCTTTAT	106 bp	New
Serotype determination							
2	1	*wzy*	Z83335	GGGACTTTAATTTTATGCAGTG	CACAATGGCTTTAGAAGGTAGAG	270 bp	1
3	2	*wzy*	CR931633	TATCCCAGTTCAATATTTCTCCACTACACC	ACACAAAATATAGGCAGAGAGAGACTACT	290 bp	2
4	3	*galU*	CR931634	GTGAATTTGTGGGCAAACG	ATTGCTTACCAAGTGCAATAACG	132 bp	New
5	4	*wzy*	CR931635	GCGGGTAGGATTTTAACAGG	TCCTGAACTAGCTGCCTCTG	120 bp	New
6	5	*wzy*	CR931637	CAACTTCTGATTATGCCTTTGTG	CAAAGCAATAGTGCTAATTAAACAAC	126 bp	New
7	6A/6B/6C/6D	*wzy*	AF316640	TCAACCTGCAGTAATTTTAACA	CTACTTTCTGAATTTCACGGATATAAAG	293 bp	1
8	6A/6B	*wciN*	HQ662200	TCTGGGAAGTGTTCTTCCATC	CACCCCATAGAGAATTTTACCC	124 bp	New
9	7F/7A	*wzy*	CR931643	CAAACTATTACAGTGGGAATTACGG	GGACCATACAATAAGCGCAAT	130 bp	New
10	8	*wzy*	CR931644	GAAGAAACGAAACTGTCAGAGCATTTACAT	CTATAGATACTAGTAGAGCTGTTCTAGTCT	201 bp	2
11	9V/9A	*wzy*	CR931648	GGTTCAAAGTCAGACAGTGAATCT	AAAAGAGGCTTTCAATTGTTGTT	122 bp	New
12	9N/9L	*wzy*	CR931647	TCAATGGCGACTTTATTTGC	AGTCTATTATCTCCTGTAGGGTGC	362 bp	1
13	10A	*wcrG*	CR931649	AGACGACAAGATTGCGACAG	AAGGTGATCCGCTACCACAT	121 bp	New
14	10F/10C/33C	*wzx*	CR931652	GATCTATGATCAGGCACTTAAGTTG	TTAACCGCTTTGTGTTCACC	120 bp	New
15	11A/11D/11E/11F	*wzx*	CR931653	GTTCAGGTGATTTCCCAATATAGTG	TCGTTAAAATCAACAGCAACTG	109 bp	New
16	12F/12A/12B/44/46	*wzy*	CR931660	TGAATATGGACGGTGGAG	AGCAAAGAAAGCCGAAAG	242 bp	1
17	14	*wzy*	X85787	CCTACTTCCAAAACAGTTTATGC	GTCCATAGCACCATACAAAAAGAC	129 bp	1
18	15B/15C	*wzy*	CR931664	TAATAAGCGGATGATTGTAGCG	TATACTGATTAACTTTCCAGATGGG	352 bp	1
19	17	*wzy*	CR931670	AGAGGGATTGTTGAAGGTATTC	AGTAGTCTCGCATTTCTATCATCC	308 bp	1
20	18F/18A/18B/18C	*wzy*	AF316642	AATTGTTCTTTTCCTGTACTCAGTC	CGAACCATTGAAACTATCATCTG	271 bp	1
21	19F	*wzy*	U09239	TCAGTATTTGCACTGGTTAATTC	AAGAACAAGGTTGTATATTTCCTTC	249 bp	1
22	19A	*wzy*	AF094575	TGTATTTGCCCTTATTAATGTGC	AAGTGCAAGATTATGAATCTCTCTC	247 bp	1
23	20	*wzy*	CR931679	CTTTATCAGGAATACGCCAATC	CTGTATAATAACGAGAACCAACG	300 bp	1
24	22F/22A	*wzy*	CR931682	AGGATGCAGTAGATACCAGTGG	TATAAACGGAGGTTGTTGTCC	354 bp	1
25	23F	*wzy*	AF057294	TGATAGTGAACTTGGGATTGTC	CTTTATCGGTAAGGTGGATAAG	245 bp	1
26	23A	*wzy*	CR931683	CTGGGAATTGGCACTCTTCT	GCCGCAAAGAGATACGAAC	103 bp	New
27	33F/33A/37	*wzy*	CR931702	GAAGGCAATCAATGTGATTGTGTCGCG	CTTCAAAATGAAGATTATAGTACCCTTCTAC	338 bp	2
28	34	*wzy*	CR931703	TGACTGGTAGTAGAGGGTCCTTG	GAGGAGCAACTGCCACTACA	147 bp	New
29	35F/34	*wcrO*	CR931707	TATTAGTATTTGTGAAGAAAATAACCTGACC	TATCAATATCGTCATCCCAAGG	107 bp	New
30	35B	*wcrH*	CR931705	TACGCTTTCCCAAGTTTCCT	CTCCGTACATTGGATGTTGG	144 bp	New

†New: newly designed in this study; 1: Kong, F., Brown, M., Sabananthan, A., Zeng, X., & Gilbert, G. L. (2006). Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. *J Clin Microbiol*. 44, 1887-1891; 2: Centers for Disease Control and Prevention, USA. (http://www.cdc.gov/ncidod/biotech/files/pcr-oligonucleotide-primers.pdf)

Table 2. Performance of the nanofluidic real time PCR serotyping.

Assay	Specificity	r^2	Efficiency (%)	Limit of detection (Copies)	Intra-assay variation (SD of Copy number)	Accuracy (Fold changes of Copy number)	Inter-assay variation (SD of Copy number)
1	lytA	0.99	94	30	0.009	0.039	0.023
2	1	0.99	108	30	0.016	-0.079	0.068
3	2	0.98	100	30	0.015	-0.066	0.058
4	3	0.99	90	300	0.040	-0.055	0.002
5	4	0.99	99	300	0.015	-0.052	0.017
6	5	0.99	90	300	0.024	-0.050	0.041
7	6A/6B/6C/6D	0.99	92	300	0.011	-0.016	0.088
8	6A/6B	0.99	93	300	0.010	-0.081	0.084
9	7F/7A	0.99	84	300	0.014	-0.051	0.036
10	8	0.99	94	300	0.002	-0.062	0.054
11	9V/9A	0.99	102	30	0.002	0.000	0.050
12	9N/9L	0.99	123	300	0.010	0.004	0.027
13	10A	0.99	101	300	0.038	-0.057	0.013
14	10F/10C/33C	0.99	94	300	0.013	-0.055	0.040
15	11A/11D/11E/11F	0.99	99	30	0.015	-0.083	0.087
16	12F/12A/12B/44/46	0.99	103	300	0.028	-0.004	0.105
17	14	0.99	108	300	0.046	-0.049	0.024
18	15B/15C	0.99	95	30	0.020	-0.082	0.082
19	17	0.99	94	30	0.014	-0.081	0.083
20	18F/18A/18B/18C	0.99	85	30	0.016	-0.069	0.037
21	19F	0.99	100	300	0.021	-0.088	0.088
22	19A	0.99	80	300	0.012	-0.061	0.034
23	20	0.99	80	300	0.013	-0.073	0.065
24	22F/22A	0.99	71	300	0.008	0.076	0.046
25	23F	0.99	93	300	0.018	-0.074	0.073
26	23A	0.99	86	300	0.022	-0.051	0.038
27	33F/33A/37	0.99	72	300	0.017	-0.079	0.111
28	34	0.99	92	30	0.020	-0.050	0.024
29	35F/34	0.99	108	300	0.011	-0.075	0.057
30	35B	0.99	88	300	0.004	0.127	0.060

Table 3. Reaction patterns of nanofluidic real time PCR serotyping of 28 *Streptococcus pneumoniae* isolates

template DNA	specificity of primers																														
	lytA	serotype 1	serotype 2	serotype 3	serotype 4	serotype 5	serotypes 6A/6B/6C/6D	serotypes 6A/6B	serotypes 7F/7A	serotype 8	serotypes 9V/9A	serotypes 9N/9L	serotype 10A	serotypes 10F/10C/33C	serotypes 11A/11D/11E/11F	serotypes 12F/12A/12B/44/46	serotype 14	serotypes 15B/15C	serotype 17F	serotypes 18F/18A/18B/18C	serotype 19F	serotype 19A	serotype 20	serotypes 22F/22A	serotype 23F	serotype 23A	serotypes 33F/33A/37	serotype 34	serotypes 34/35F	serotype 35B
Streptococcus pneumoniae																															
serotype 1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 2 (ATCC®6302)	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 3	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 4	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 5 (ATCC®6305)	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 6B	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 7F	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 8 (ATCC®6308)	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 9V	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 9N (ATCC®6309)	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 10F	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 10A	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 11A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 12F (ATCC®6312)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 14	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 15B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
serotype 17F (ATCC®6317)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
serotype 18C	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
serotype 19F	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
serotype 19A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
serotype 20 (ATCC®6320)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
serotype 22F	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
serotype 23F	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
serotype 23A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
serotype 33F	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
serotype 34	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
serotype 35F	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
serotype 35B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
other bacterial species																															
S. mitis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H. influenzae b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N. meningitidis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. catarrhalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Streptococcus pneumoniae strains except for seven of those obtained from American Type Culture Collection were local isolates in Japan. Reactivity +: positive reaction, -: negative reaction

Table 4. Quantitative detection of mixed DNA samples mimicking those of mixed co-infection/co-colonization with multiple serotypes of *S. pneumoniae*

Sample	Major serotype	Minor serotype	Observed ratio	Recovery of minor serotype†
Mixed DNA, Serotype 4: Serotype 5 = 1: 10^{-1}				
	8.89×10^4 (copies/2µl)	3.44×10^3 (copies/2µl)	1: 0.0382	0.38 (base 10 logarithm of -0.42)
Mixed DNA, Serotype 7F: Serotype 9V = 1: 10^{-2}				
	9.58×10^4 (copies/2µl)	1.15×10^3 (copies/2µl)	1: 0.0120	1.20 (base 10 logarithm of -0.08)
Mixed DNA, Serotype 10F: Serotype 11A = 1: 10^{-3}				
	7.23×10^4 (copies/2µl)	45.0 (copies/2µl)	1: 6.22×10^{-4}	0.62 (base 10 logarithm of -0.20)

†Recovery = observed ratio of minor serotype divided by expected ratio.

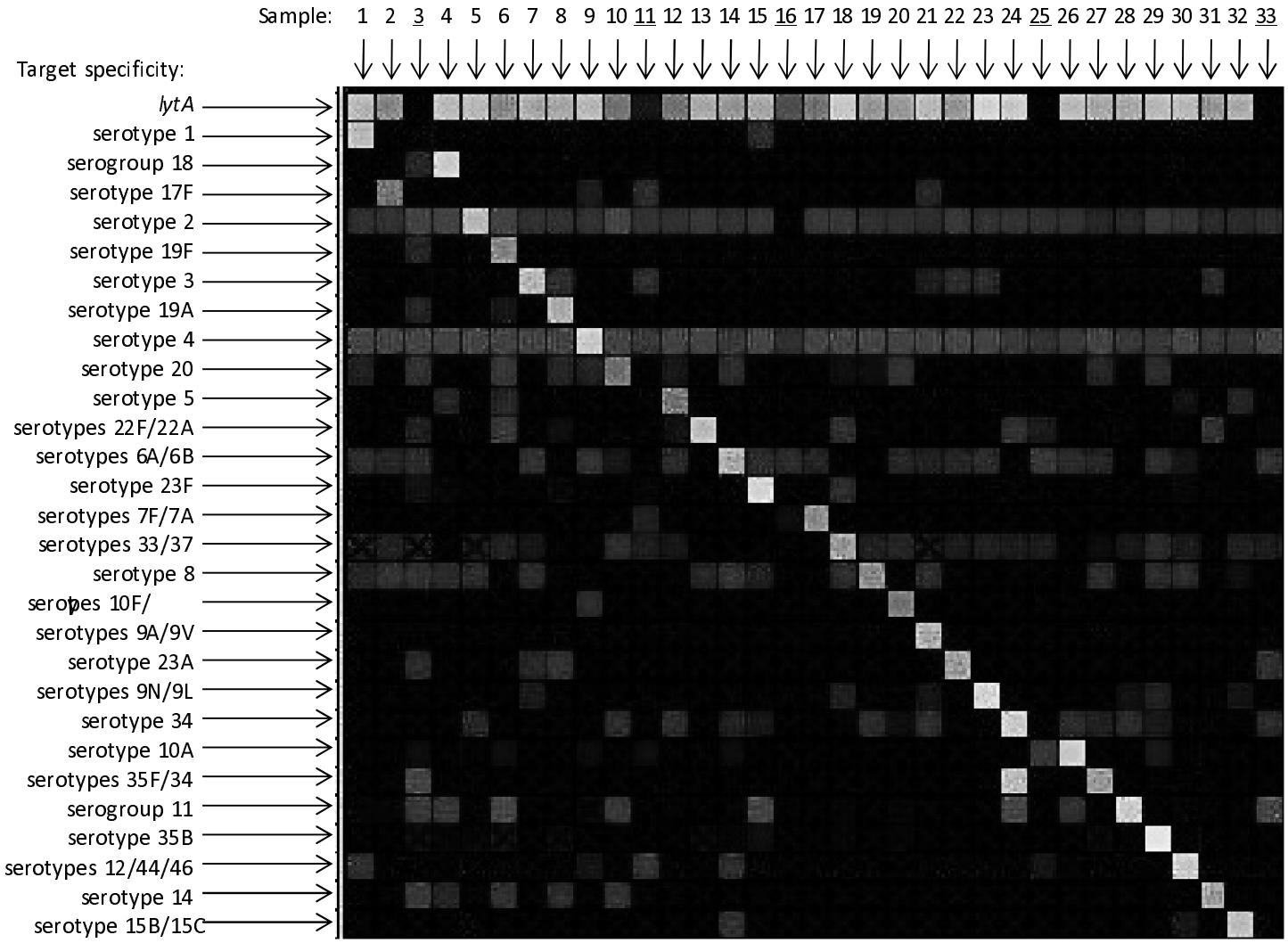
Table 5. Comparison of the results of conventional serotyping and the nanofluidic real time PCR system in nasopharyngeal samples

Serotype by the nanofluidic PCR system	Serotype by the conventional method								Culture negative	Total
	6†	6A	6B/6D	11	14	15	19F	23		
Serotypes 6A/6B	1	3	1	-	-	-	-	-	3	8
Serotypes 6C/6D	-	-	1	-	-	-	-	-	-	1
Serogroup 11	-	-	-	1	1*	-	-	-	-	2
Serotype 14	-	-	-	-	5	-	-	-	2	7
Serotypes 15B/15C	-	-	-	-	-	1	-	-	2	3
Serogroup 18	-	-	-	-	-	-	-	-	1	1
Serotype 19F	-	-	-	-	-	-	3	-	7	10
Serotype 23F	-	-	-	-	-	-	-	6	1	7
Non-typeable	-	-	-	-	-	-	-	-	1	1
PCR negative	-	-	-	-	-	-	-	-	12	12
Total		3	2	1	6	1	3	6	29	52

†Serotypes 6A/6B/6C/6D; serotyping reactions with factor sera were not certain.
*The result of PCR nanofluidic serotyping was confirmed by separate PCRs specific to serogroup 11 (positive) and serotype 14 (negative).

Figure Legends

Figure 1. Cross validation of target specificity of the nanofluidic real time PCR serotyping assay. Intensity of DNA amplification signals from the nanofluidic real time PCR is shown as a heat map; the brighter rectangles of the checkerboard mean the stronger signals (the smaller Cq values). Each row corresponds to a single target specificity and each column corresponds to a single DNA specimen. DNA specimens were from *Streptococcus pneumoniae* isolates except for 3, 11, 16, 25 and 33; 1: serotype 1, 2: serotype 17F (ATCC®6317), 3: *Streptococcus mitis*, 4: serotype 18C, 5: serotype 2 (ATCC®6302), 6: serotype 19F, 7: serotype 3, 8: serotype 19A, 9: serotype 4, 10: serotype 20 (ATCC®6320), 11: *Neisseria meningitidis*, 12: serotype 5 (ATCC®6305), 13: serotype 22F, 14: serotype 6B, 15: serotype 23F, 16: *Haemophilus influenzae* b, 17: serotype 7F, 18: serotype 33F, 19: serotype 8 (ATCC®6308), 20: serotype 10F, 21: serotype 9V, 22: serotype 23A, 23: serotype 9N (ATCC®6309), 24: serotype 34, 25: *Moraxella catarrhalis*, 26: serotype 10A, 27: serotype 35F, 28: serotype 11A, 29: serotype 35B, 30: serotype 12F (ATCC®6312), 31: serotype 14, 32: serotype 15B, and 33: no template control.


Sample: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Target specificity:
lytA
serotype 1
serogroup 18
serotype 17F
serotype 2
serotype 19F
serotype 3
serotype 19A
serotype 4
serotype 20
serotype 5
serotypes 22F/22A
serotypes 6A/6B
serotype 23F
serotypes 7F/7A
serotypes 33/37
serotype 8
serotypes 10F/
serotypes 9A/9V
serotype 23A
serotypes 9N/9L
serotype 34
serotype 10A
serotypes 35F/34
serogroup 11
serotype 35B
serotypes 12/44/46
serotype 14
serotype 15B/15C