Development of nanofluidic real-time PCR system for molecular serotyping of pneumococcus and its applications

Bhim Gopal Dhoubhadel, MBBS, MTM
PhD Candidate

Supervisor
Prof Koya Ariyoshi, MD, PhD

Advisors
Prof Michio Yasunami, MD, PhD
Dr Lay Myint Yoshida MBBS, MMedSc, PhD

Department of Clinical Medicine
Institute of Tropical Medicine (NEKKEN),
Graduate School of Biomedical Sciences, Nagasaki University
Japan

2014

Table of contents

Acknowledgements

I am very grateful to Prof Ariyoshi for his kind supervision and guidance for my PhD. His insightful, focused and visionary teachings have always guided my learning and work. I am very grateful to Prof Yasunami. He has made lots of effort to make my project successful. His care and guidance have made my life easier during my PhD. I am very grateful to Dr Lay. He has guided and helped me throughout my work. I appreciate Dr Morimoto and Dr Suzuki for their help. Dr Suzuki has always been helpful for my statistical analysis. I am grateful to Mr Watanabe for pneumococcal isolates. I am grateful to my wife Hisako for managing life during this course.

Thank you very much!

Abstract

Streptococcus pneumoniae (Pneumococcus) is a major cause of life threatening diseases including pneumonia, sepsis and meningitis. It causes about 500 thousands deaths per year in under-5 children. It is a Gram-positive bacterium with polysaccharide capsule. The capsule determines its serotype and the protective immunity is serotype specific. There are 94 serotypes and till today only 13-valent conjugate vaccine (PCV13) is available for immunization in children. Serotyping by conventional method (culture and serotyping) is expensive, time consuming and insensitive. As prevalence of serotypes of pneumococcus varies both geographically and temporally, continuous surveillance is necessary, both in pre and post vaccine period. Non-vaccine serotypes have emerged in the USA after introduction of 7-valent vaccine (PCV7) in the year 2000. Though the presence of multiple serotype colonization has been reported, its role in a clinical outcome and emergence of non-vaccine serotype has not been clear.

Although various PCR based methods have been developed to replace the conventional method of serotyping, a high throughput sensitive and quantitative method was in need to meet the demand of epidemiological survey and to detect the minor population of the bacteria in the multiple serotype colonization. We have developed a nanofluidic real-time PCR system, which can detect 50 serotypes in 29 groups, including all serotypes of the 13-valent-conjugate vaccine (PCV13) and the 23-valent-polysaccharide

vaccine (PPV). The lower limit of detection was 30-300 copies per reaction. Minor population in the multiple serotypes, 1 in 1000 was easily detected. We have validated this assay with the conventional culture and serotyping.

We applied this assay in nasopharyngeal samples from ARI (Acute Respiratory Infection) cases and healthy children in Vietnam. We found a positive correlation of serotypes/serogroups specific bacterial load with serotype/serogroup prevalence. This implied that higher bacterial load of a serotype in the nasopharynx might be an attributable factor for higher transmission of the serotype among host so that it could maintain higher prevalence. Another finding was the association of multiple serotype of co-colonization with acute respiratory infections and dominance of one serotype over other in multiple serotype co-colonizations. This showed that the link of multiple serotypes with increased pathogenicity and competition among serotypes when they were present at the same time in the nasopharynx.

Chapter 1

Introduction

Streptococcus pneumoniae is a common cause of death in under-5 children in world [1]. This Gram-positive bacterium is encapsulated with the polysaccharide capsule, which is the major virulent factor for evading the host immune response. The capsule also determines the serotypes of pneumococcus. As protective immunity is serotype specific and distribution of pneumococcal serotypes vary both geographically and temporally, the efficacy of pneumococcal conjugate vaccines (PCV7, PCV10, PCV13) is not consistent in all over the world [2]. Moreover, there is emergence of non-vaccine serotypes of pneumococcus in places where the conjugated vaccines have been introduced, which has been a global concern [3].

Conventionally identification of serotypes of pneumococcus is done by bacterial culture and serotyping, which is expensive, labour-intensive and less sensitive especially when antibiotics are taken by the patients prior to sample collection. To overcome these drawbacks of the conventional method of serotyping, various molecular methods have been developed and assays based on real-time PCR are promising as they are more sensitive than multiplex PCRs and provides quantitative data [4-6]. The sensitivity of the assay to detect minor population of subdominant serotypes in multiple serotypes have become important as multiple serotypes have been thought to be involved in vaccine serotype replacement, carriage detection and

pneumonia diagnosis [7]. In addition, the assay should provide the quantitative data of serotypes, as they would be helpful to understand the complex epidemiology of multiple serotypes. To meet these technical challenges and be applicable in a large-scale epidemiological study, a highly sensitive, specific and high-throughput molecular assay was in need.

The antigenic structure of capsule polysaccharide chain of the pneumococcus is determined by a series of glycosyltransferases encoded by the *cps* locus, which characterizes at lease 93 serotypes [8,9]. 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). Recently, the Center for Disease Control and Prevention in the USA has developed a seven-triplexed real-time PCR system for identification of serotypes of 13-valent conjugate vaccine (PCV13) [10]. This system is probe-based system, which is more expensive than dye based system and lacked the description of quantitative detection of serotypes.

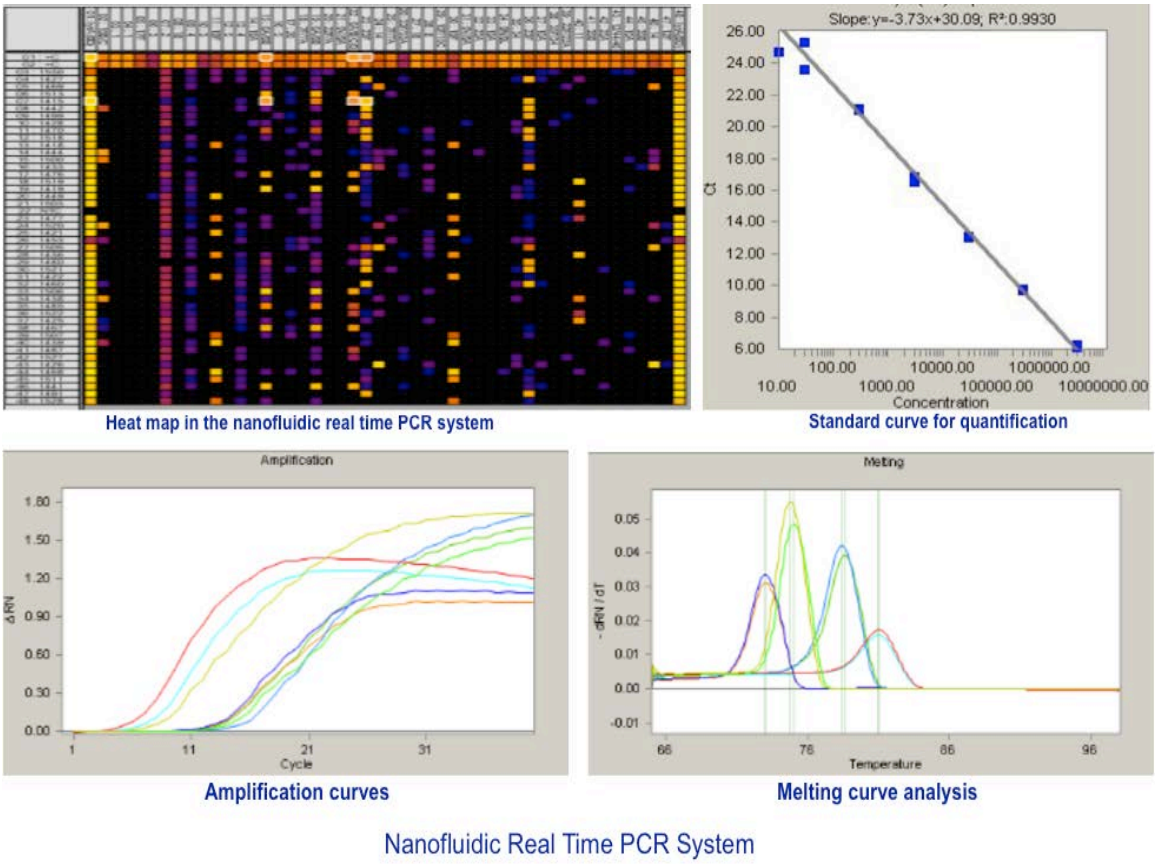
To meet the need of epidemiological studies at affordable price, we have developed nanofluidic real-time PCR system for identification and quantification of serotypes. In this system, we have used 29 primers pairs, which can identify and quantify 50 serotypes (in 29 groups), including all vaccines serotypes of not only conjugate vaccines but also polysaccharide vaccine (PPV23). Primers were collected from Centers for Disease Control (CDC) and previous publication [11], and 14 new primers were designed so

that all primers amplified the target in same temperature and condition. All the primers were tested for sensitivity, specificity, repeatability, accuracy and reproducibility according to Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines [12]. The assay was validated with conventional culture and serotyping by applying in nasopharyngeal samples from the children with pneumonia confirmed by chest X-ray. The assay was sensitive to detect with the minimum detection level of 30-300 copies of DNA, specific to the target and can test 45 samples at a run (high-throughput).

After developing and validating the assay, we have applied it to the nasopharyngeal samples of acute respiratory infections (ARI) cases and healthy children in Vietnam. We have found 2 major findings: a positive correlation of serotype/serogroup bacterial load with prevalence of serotypes/serogroups and association of multiple serotype co-colonization with acute respiratory infections (ARI) in children. To our knowledge, both of these findings are novel in the epidemiology of pneumococcal serotypes.

In conclusion, we have developed and validated a highly sensitive, specific and quantitative real-time PCR assay based on nanofluidic platform. With the help of the assay, we could decipher the relationship between the serotype specific bacterial load with its prevalence and the association of multiple serotypes with a clinical outcome (ARI).

Figure 1. The nanofluidic real-time PCR system for identification and quantification of pneumococcal serotypes.



References

1. WHO (2012) Measuring impact of Streptococcus pneumoniae and Haemophilus influenzae type b conjugate vaccination. Immunization, Vaccines and Biologicals WHO/IVB/12.08.
2. Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, et al. (2010) Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. PLoS Med 7.
3. WHO (2010) Changing epidemiology of pneumococcal serotypes after introduction of conjugate vaccine: July 2010 report. Weekly epidemilogical record No. 43: 434-436.
4. Azzari C, Moriondo M, Indolfi G, Cortimiglia M, Canessa C, et al. (2010) Realtime PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. PLoS One 5: e9282.
5. Kee C, Fatovich DM, Palladino S, Kay ID, Pryce TM, et al. (2010) Specificity of a quantitative real-time polymerase chain reaction assay for the detection of invasive pneumococcal disease: identifying streptococcus pneumoniae using quantitative polymerase chain reaction. Chest 137: 243-244.
6. Moore CE, Sengduangphachanh A, Thaojaikong T, Sirisouk J, Foster D, et al. (2010) Enhanced determination of Streptococcus pneumoniae serotypes associated with invasive disease in Laos by using a real-time polymerase chain reaction serotyping assay with cerebrospinal fluid. Am J Trop Med Hyg 83: 451-457.
7. Shak JR, Vidal JE, Klugman KP (2013) Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol 21: 129-135.
8. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, et al. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2: e31.
9. Mavroidi A, Aanensen DM, Godoy D, Skovsted IC, Kaltoft MS, et al. (2007) Genetic relatedness of the Streptococcus pneumoniae capsular biosynthetic loci. J Bacteriol 189: 7841-7855.
10. Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, et al. (2013)

Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 51: 647-652.

11. Kong F, Brown M, Sabananthan A, Zeng X, Gilbert GL (2006) Multiplex PCR-based reverse line blot hybridization assay to identify 23 Streptococcus pneumoniae polysaccharide vaccine serotypes. J Clin Microbiol 44: 1887-1891.
12. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622.

Chapter 2

Title:

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

Authors:

Bhim Gopal Dhoubhadel[1], Michio Yasunami[1§], Lay-Myint Yoshida[1], Hien Anh Thi Nguyen[2], Kiwao Watanabe[1], Motoi Suzuki[1], Konosuke Morimoto[1], Anh Duc Dang[2] and Koya Ariyoshi[1].

§ Corresponding author:

Michio Yasunami, MD, PhD

Department of Clinical Medicine, Institute of Tropical Medicine,

Nagasaki University

1-12-4 Sakamoto, Nagasaki 852-8523, Japan

E-mail: yasunami@nagasaki-u.ac.jp

Phone: +81-95-819-7857

Fax: +81-95-819-7843

Published on J Med Microbiology, 2014, 63, 528-539.

Affiliations:

[1]Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Japan

[2]Department of Bacteriology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam

Abstract

Serotype-specific quantification data are 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 labour-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. 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 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 patients with pneumonia confirmed by chest X-ray to validate its accuracy. Minimum detection levels of this novel assay using the nanofluidic real-time PCR system were comparable to the conventional PCR-based assays (between 30 and 300 copies per reaction). They were specific to their targets with good repeatability (SD of copy number of 0.1), accuracy (within ±0.1 fold difference in log10 copy number), and reproducibility (SD 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 one 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. We conclude that this novel assay, which is able to differentially quantify 29 pneumococcus groups for 45 test samples in a single run, is applicable to the 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 is a leading cause of death in children aged less than 5 years worldwide. It was responsible for ~6% of total deaths among the world population in 2008 [1]. It is a Gram-positive bacterium with a polysaccharide capsule, which enables the bacteria to evade the host defense mechanism. The 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, as does the efficacy of pneumococcal conjugate vaccines (PCV) [2]. After the introduction of the heptavalent conjugate vaccine (PCV7), an increase in the frequency of nonvaccine serotypes was observed and this has become a major global health concern [3,4]. In addition, different serotypes have caused death in a different age group [5], and some serotypes were associated with fatal illness both in the young and the elderly [6]. Hence, to understand this complex epidemiology of pneumococcal diseases requires large-scale surveillance in a 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 was revealed that a considerable proportion of individuals were colonized by more than one serotype [7] and in some cases of invasive pneumococcal diseases, multiple serotypes have been isolated [8,9]. Furthermore, accumulating data have

shown that quantification of S. pneumoniae was also important, as the bacterial load was associated with disease severity [10,11]. Therefore, we believe that it is essential to undertake surveillance of multiple serotypes in pneumococci colonization and infection, and measure serotype-specific bacterial loads both in clinical and a large-scale epidemiology studies. However, there had been no assay that could accurately quantify *S. pneumoniae* at the serotype level and detect a minor population of a subdominant serotype (present in multiple serotypes) circulating in the field. To address these issues, we applied a nanofluidic real-time PCR system and established a novel assay. This assay could 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 could be tested with 29 serotype/serogroup-specific and one autolysin (*lytA*) primer pairs, equivalent to 1350 individual PCRs in total; this has made it a high-throughput assay. Following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [12], here we report the 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. We also applied the assay to 52 nasopharyngeal swab samples from patients with pneumonia confirmed by chest X-ray to validate the accuracy of the assay by comparing the results of the assay with the results of conventional serotyping (Table S1, available with the online Supplementary Material).

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. 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.

DNA extraction and copy number determination

DNA was extracted from the pneumococcal isolates by QIAmp DNA mini kit (Qiagen) following the manufacture's protocol. DNA concentration was determined by NanoDrop spectrophotometer (Thermo scientific). The mean 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

numbers 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 [13,14] 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 amplified their respective targets at the same condition of PCR, were adopted from the previous publication 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), where each primer showed 100% identity exclusively to its specific target sequence with significant E-values. 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 LightCycler 480 real-time PCR system (Roche) with a 384-well heat block, at the final concentration of 0.4 μM of each. Real-time PCR was set up with 2x concentrate of SsoFast EvaGreen Supermix with Low ROX (BioRad) in which EvaGreen® (Biotium) was used as a detecting dye; a 10 μl reaction consisted of 5.0 μl of SsoFast EvaGreen Supermix with Low ROX, 0.4 μl of each 10 μM primer solution, 2 μl of DNA sample and 2.2 μl of deionized water. The thermal cycle conditions were: initial incubation for enzyme activation at 98°C for 3 min (ramp rate 4.8°C/s), 45 × two-step cycle [denaturation at 98°C for 30 s (ramp rate 4.8°C/s) and annealing/extension at

59°C for 30 s (ramp rate 2.5°C/s)]. Fluorescence signals were acquired at the end of each annealing/extension stage (59°C). After the completion of the 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/s. The 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 3×10^6 to 3 copies per reaction with each reaction in triplets and standard curves were drawn for each serotype-specific primer from the Cq values of the DNA samples. The minimum level of detection, efficiency and correlation coefficient (r^2) of each primer pairs were determined in the LightCycler 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 that 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) as the initial step for BioMark HD system (Fluidigm). 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 10 mM Tris HCl and 0.1 mM EDTA (pH 8) so as to make the concentration of 200 nM of each primer. Then, 8.0 μl STA reaction was prepared by adding 4.0 μl TaqMan PreAmp Master Mix (Applied Biosystem), 2.0 μl STA pooled primer mix and 2.0 μl 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 × two-step cycles (denaturation at 98°C for 15 s and annealing/extension at 59°C for 4 min). The STA product was diluted five times with 10 mM 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 plates according to the manufacturer's protocol (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). The chip was set to the IFC Controller MX (Fluidigm). The 6.25 nl reaction chambers were filled up with PCRs containing 4.5 μM of each of specific primers and 40-fold diluted STA products containing negligible concentrations of carried-over STA primers (1.25 nM each) in 1 x SsoFast EvaGreen Supermix with Low ROX. Nanofluidic real-time PCR was carried out on the Fluidigm Biomark HD System with the thermal cycle conditions of: initial incubation for enzyme activation at 98°C for 3 min (ramp rate 2°C/s) and 40 x two-step cycles [denaturation at 98°C for 30 s (ramp rate 2°C/s) and annealing/extension at 59°C for 30 s (ramp rate 2°C/s)]. Fluorescence signals were acquired at the end of each annealing/extension stage (59°C). After the completion of the 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/s. Quantitative analysis was carried out by using the crossing point (Cq) values of the PCRs with proper melting curves [15].

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 x 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) at a constant concentration of 1 ng/μl. 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 in 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 x 10^6 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 cross-checking 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 four times for vaccine serotypes and two times for non-vaccine serotypes in the same assay run, and presented as 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 $\log_{10}$) and the expected copy number (in $\log_{10}$) to the expected copy number (in $\log_{10}$). 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 nanofluidic 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 mixed DNA samples of two different serotypes to imitate those of multi-serotype co-colonization with different contributions of minor population at 1:10, 1:100 and 1:1000 ratios.

Application of the assay to clinical specimens

In total, 52 nasopharyngeal swab samples from children with pneumonia

confirmed by chest X-ray and collected in a government referral hospital in central Vietnam were examined for the presence of pneumococcus and serotype determination by conventional microbiological/serological methods, as described previously [16]. 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, as confirmed by melting curve analysis. The linear dynamic range was four to five 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 DNA sample (Table 2). All the primer pairs were specific to their own targets as shown in Table 3. Fig 1 illustrates the heat map output of the validation assay. 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 in amplifying 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

With regard to repeatability (intra-assay variation), the SD of the copy number (in $\log_{10}$) 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 <0.10 for all primer pairs (Table 2). With regard to accuracy, at a target concentration of 3×10^5 copies per reaction, the fold

difference in copy number (in $\log_{10}$) from the observed values was within +/- 0.10 for all primers pairs except for serotype 35B, which had + 0.12 (Table 2). With regard to reproducibility (inter-assay variation), the SD of the copy number (in $\log_{10}$) at the target concentrations of 3 x 10^5 copies per reaction between different runs was <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 $\log_{10}$ of +/-0.5; 0.38 ($\log_{10}$ of -0.42), 1.20 ($\log_{10}$ of 0.08) and 0.62 ($\log_{10}$ of -0.20) for the respective ratios (Table 4). Using 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 one 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 confirmed pneumonia cases that 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. The 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 the 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 [17-19], immunoblot [20] and immunoassay [21]. Multiplex PCR and real-time PCR have increased substantially the sensitivity of diagnosing pneumococcal diseases even in culture-negative cases [22] and identifying the presence of pneumococcus in the nasopharyngeal colonization [23,24]. Most multiplex PCR serotyping procedures use different sets of primers targeting for several serotypes/serogroups, and have been used in epidemiological studies [25-29], mainly because of their cost effectiveness. Real-time PCR is more sensitive than multiplex PCR [30], and has been used as clinical diagnostics too [31,32]. 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 [10,11,16]; this was also applied to the prediction of pneumonia [33]. 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* [34]. Among them, *lytA* has proved to be one of the most sensitive and specific targets, and is capable to differentiate *S. pneumoniae* from *S. pseudopneumoniae* [31,35-37]. 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 [10,30,38,39]. 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 only a few exceptional reports that describe assay performance in detail, having ~10 copies of the lower limit of detection, five orders of linear dynamic range and 0.3-1 cycles of reproducibility [40,41]. For serotype-specific assays, there have been no previous reports about the assay performance for comparison; we could achieve good performance, 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 an influence on the efficacy of pneumococcal vaccine [4,42]. In this nanofluidic real-time PCR system, the presence of minor serotype (in multiple serotype) could be identified precisely even down to as few as one in 1000 copies of background pneumococcal DNA, as demonstrated by mixed DNA samples in Table 4. This ability to detect multiple serotypes, even in a 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, equal to 1,350 PCRs, can be obtained by a single run in ~4 h including a STA step prior to the nanofluidic real-time PCR system. Furthermore, only 1/30 of PCR reagents are required in this assay compared to 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 for an efficient and cost-effective quantitative assay for large epidemiological studies of *S. pneumoniae*.

Recently, the Centers for Disease Control and Prevention in the USA has developed a seven-triplexed real-time PCR system for identification of serotypes of PCV13 [43]. 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 sensitivity and specificity comparable to the probe-based system used by the Centers for Disease Control and Prevention.

One of the limitations is the initial cost of purchasing the nanofluidic equipment. The current price is about five to 10 times higher than a conventional real-time PCR machine. Another limitation, which is common to all PCR-based methods, is that 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). A similar overlap 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 reactions because of cross-reaction between different serotypes and free of false negative reactions 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 at the 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 detailed performance data presented according to the MIQE guidelines.

Acknowledgements

This work was supported in part by the Special Coordination Funds for Promoting Science and Technology, Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and Global COE Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The nucleotide sequences of 13 new primer pairs (Table 1, “New”) were submitted to Japan Patent Office on 13 April 2013 (No. 2013-090703).

References

1. WHO (2012) Measuring impact of Streptococcus pneumoniae and Haemophilus influenzae type b conjugate vaccination. Immunization, Vaccines and Biologicals WHO/IVB/12.08.
2. Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, et al. (2010) Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. PLoS Med 7.
3. WHO (2010) Changing epidemiology of pneumococcal serotypes after introduction of conjugate vaccine: July 2010 report. Weekly epidemilogical record No. 43: 434-436.
4. Weinberger DM, Malley R, Lipsitch M (2011) Serotype replacement in disease after pneumococcal vaccination. Lancet 378: 1962-1973.
5. Harboe ZB, Thomsen RW, Riis A, Valentiner-Branth P, Christensen JJ, et al. (2009) Pneumococcal serotypes and mortality following invasive pneumococcal disease: a population-based cohort study. PLoS Med 6: e1000081.
6. Weinberger DM, Harboe ZB, Sanders EA, Ndiritu M, Klugman KP, et al. (2010) Risk of death due to pneumococcal pneumonia is a stable serotype-associated property: a meta-analysis. Clin Infect Dis 51: 692-699.
7. Kaltoft MS, Skov Sorensen UB, Slotved HC, Konradsen HB (2008) An easy method for detection of nasopharyngeal carriage of multiple Streptococcus pneumoniae serotypes. J Microbiol Methods 75: 540-544.
8. Chaves F, Campelo C, Sanz F, Otero JR (2003) Meningitis due to mixed infection with penicillin-resistant and penicillin-susceptible strains of Streptococcus pneumoniae. J Clin Microbiol 41: 512-513.
9. de Andrade AL, Pimenta FC, Laval CA, de Andrade JG, Guerra ML, et al. (2004) Invasive pneumococcal infection in a healthy infant caused by two different serotypes. J Clin Microbiol 42: 2345-2346.
10. Carrol ED, Guiver M, Nkhoma S, Mankhambo LA, Marsh J, et al. (2007) High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease. Pediatr Infect Dis J 26: 416-422.
11. Rello J, Lisboa T, Lujan M, Gallego M, Kee C, et al. (2009) Severity of pneumococcal pneumonia associated with genomic bacterial load. Chest 136: 832-840.
12. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE

guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622.

13. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, et al. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2: e31.
14. Mavroidi A, Aanensen DM, Godoy D, Skovsted IC, Kaltoft MS, et al. (2007) Genetic relatedness of the Streptococcus pneumoniae capsular biosynthetic loci. J Bacteriol 189: 7841-7855.
15. Spurgeon SL, Jones RC, Ramakrishnan R (2008) High throughput gene expression measurement with real-time PCR in a microfluidic dynamic array. PLoS One 3: e1662.
16. Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, et al. (2011) Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 30: 11-18.
17. Kong F, Brown M, Sabananthan A, Zeng X, Gilbert GL (2006) Multiplex PCR-based reverse line blot hybridization assay to identify 23 Streptococcus pneumoniae polysaccharide vaccine serotypes. J Clin Microbiol 44: 1887-1891.
18. Wang Q, Wang M, Kong F, Gilbert GL, Cao B, et al. (2007) Development of a DNA microarray to identify the Streptococcus pneumoniae serotypes contained in the 23-valent pneumococcal polysaccharide vaccine and closely related serotypes. J Microbiol Methods 68: 128-136.
19. Turner P, Hinds J, Turner C, Jankhot A, Gould K, et al. (2011) Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 49: 1784-1789.
20. Bronsdon MA, O'Brien KL, Facklam RR, Whitney CG, Schwartz B, et al. (2004) Immunoblot Method To Detect Streptococcus pneumoniae and Identify Multiple Serotypes from Nasopharyngeal Secretions. Journal of Clinical Microbiology 42: 1596-1600.
21. Yu J, Lin J, Benjamin WH, Jr., Waites KB, Lee CH, et al. (2005) Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. J Clin Microbiol 43: 156-162.
22. Saha SK, Darmstadt GL, Baqui AH, Hossain B, Islam M, et al. (2008) Identification of serotype in culture negative pneumococcal meningitis using sequential multiplex PCR: implication for surveillance and vaccine design.

PLoS One 3: e3576.

23. Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, et al. (2008) Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by Streptococcus pneumoniae in Italian children. J Med Microbiol 57: 1205-1212.
24. Antonio M, Hakeem I, Sankareh K, Cheung YB, Adegbola RA (2009) Evaluation of sequential multiplex PCR for direct detection of multiple serotypes of Streptococcus pneumoniae from nasopharyngeal secretions. J Med Microbiol 58: 296-302.
25. Brito DA, Ramirez M, de Lencastre H (2003) Serotyping Streptococcus pneumoniae by Multiplex PCR. Journal of Clinical Microbiology 41: 2378-2384.
26. Lawrence ER, Griffiths DB, Martin SA, George RC, Hall LMC (2003) Evaluation of Semiautomated Multiplex PCR Assay for Determination of Streptococcus pneumoniae Serotypes and Serogroups. Journal of Clinical Microbiology 41: 601-607.
27. Pai R, Gertz RE, Beall B (2006) Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. J Clin Microbiol 44: 124-131.
28. Dias CA, Teixeira LM, Carvalho Mda G, Beall B (2007) Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. J Med Microbiol 56: 1185-1188.
29. Morais L, Carvalho Mda G, Roca A, Flannery B, Mandomando I, et al. (2007) Sequential multiplex PCR for identifying pneumococcal capsular serotypes from South-Saharan African clinical isolates. J Med Microbiol 56: 1181-1184.
30. Azzari C, Moriondo M, Indolfi G, Cortimiglia M, Canessa C, et al. (2010) Realtime PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. PLoS One 5: e9282.
31. Kee C, Fatovich DM, Palladino S, Kay ID, Pryce TM, et al. (2010) Specificity of a quantitative real-time polymerase chain reaction assay for the detection of invasive pneumococcal disease: identifying streptococcus pneumoniae using quantitative polymerase chain reaction. Chest 137: 243-244.
32. Moore CE, Sengduangphachanh A, Thaojaikong T, Sirisouk J, Foster D, et al. (2010) Enhanced determination of Streptococcus pneumoniae serotypes associated with invasive disease in Laos by using a real-time polymerase

chain reaction serotyping assay with cerebrospinal fluid. Am J Trop Med Hyg 83: 451-457.

33. Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, et al. (2012) Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin Infect Dis 54: 601-609.
34. Suzuki N, Seki M, Nakano Y, Kiyoura Y, Maeno M, et al. (2005) Discrimination of Streptococcus pneumoniae from viridans group streptococci by genomic subtractive hybridization. J Clin Microbiol 43: 4528-4534.
35. Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, et al. (2007) Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol 45: 2460-2466.
36. Abdeldaim G, Herrmann B, Molling P, Holmberg H, Blomberg J, et al. (2010) Usefulness of real-time PCR for lytA, ply, and Spn9802 on plasma samples for the diagnosis of pneumococcal pneumonia. Clin Microbiol Infect 16: 1135-1141.
37. Greve T, Moller JK (2012) Accuracy of using the lytA gene to distinguish Streptococcus pneumoniae from related species. J Med Microbiol 61: 478-482.
38. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, et al. (2001) Simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 39: 1553-1558.
39. Yang S, Lin S, Khalil A, Gaydos C, Nuemberger E, et al. (2005) Quantitative PCR assay using sputum samples for rapid diagnosis of pneumococcal pneumonia in adult emergency department patients. J Clin Microbiol 43: 3221-3226.
40. van Haeften R, Palladino S, Kay I, Keil T, Heath C, et al. (2003) A quantitative LightCycler PCR to detect Streptococcus pneumoniae in blood and CSF. Diagn Microbiol Infect Dis 47: 407-414.
41. Munoz-Almagro C, Gala S, Selva L, Jordan I, Tarrago D, et al. (2011) DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema. Eur J Clin Microbiol Infect Dis 30: 327-335.
42. Rivera-Olivero IA, Blommaart M, Bogaert D, Hermans PW, de Waard JH (2009) Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol 58: 584-587.
43. Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, et al. (2013)

Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 51: 647-652.

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	Kong (17)
3	2	*wzy*	CR931633	TATCCCAGTTCAATATTTCTCCACTACACC	ACACAAAATATAGGCAGAGAGAGACTACT	290 bp	CDC
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	Kong (17)
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	CDC
11	9V/9A	*wzy*	CR931648	GGTTCAAAGTCAGACAGTGAATCT	AAAAGAGGCTTTCAATTGTTGTT	122 bp	New
12	9N/9L	*wzy*	CR931647	TCAATGGCGACTTTATTTGC	AGTCTATTATCTCCTGTAGGGTGC	362 bp	Kong (17)
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	Kong (17)
17	14	*wzy*	X85787	CCTACTTCCAAAACAGTTTATGC	GTCCATAGCACCATACAAAAAGAC	129 bp	Kong (17)
18	15B/15C	*wzy*	CR931664	TAATAAGCGGATGATTGTAGCG	TATACTGATTAACTTTCCAGATGGG	352 bp	Kong (17)
19	17	*wzy*	CR931670	AGAGGGATTGTTGAAGGTATTC	AGTAGTCTCGCATTTCTATCATCC	308 bp	Kong (17)
20	18F/18A/18B/18C	*wzy*	AF316642	AATTGTTCTTTTCCTGTACTCAGTC	CGAACCATTGAAACTATCATCTG	271 bp	Kong (17)
21	19F	*wzy*	U09239	TCAGTATTTGCACTGGTTAATTC	AAGAACAAGGTTGTATATTTCCTTC	249 bp	Kong (17)
22	19A	*wzy*	AF094575	TGTATTTGCCCTTATTAATGTGC	AAGTGCAAGATTATGAATCTCTCTC	247 bp	Kong (17)
23	20	*wzy*	CR931679	CTTTATCAGGAATACGCCAATC	CTGTATAATAACGAGAACCAACG	300 bp	Kong (17)
24	22F/22A	*wzy*	CR931682	AGGATGCAGTAGATACCAGTGG	TATAAACGGAGGTTGTTGTCC	354 bp	Kong (17)
25	23F	*wzy*	AF057294	TGATAGTGAACTTGGGATTGTC	CTTTATCGGTAAGGTGGATAAG	245 bp	Kong (17)
26	23A	*wzy*	CR931683	CTGGGAATTGGCACTCTTCT	GCCGCAAAGAGATACGAAC	103 bp	New
27	33F/33A/37	*wzy*	CR931702	GAAGGCAATCAATGTGATTGTGTCGCG	CTTCAAAATGAAGATTATAGTACCCTTCTAC	338 bp	CDC
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; CDC, Centers for Disease Control and Prevention. (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	1	2	3	4	5	6A/6B/6C/6D	6A/6B	7F/7A	8	9V/9A	9N/9L	10A	10F/10C/33C	11A/11D/11E/11F	12F/12A/12B/44/4	14	15B/15C	17F	18F/18A/18B/18C	19F	19A	20	22F/22A	23F	23A	33F/33A/37	34	34/35F	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*

Mixed DNA Sample	Major serotype	Minor serotype	Observed ratio	Recovery of minor serotype†
Serotype 4: Serotype 5 = 1: 10				
	8.89×10^4 (copies/2μl)	3.44×10^3 (copies/2μl)	1: 0.0382	0.38 ($\log_{10}$ of -0.42)
Serotype 7F: Serotype 9V = 1: 100				
	9.58×10^4 (copies/2μl)	1.15×10^3 (copies/2μl)	1: 0.0120	1.20 ($\log_{10}$ of -0.08)
Serotype 10F: Serotype 11A = 1: 1000				
	7.23×10^4 (copies/2μl)	45.0 (copies/2μl)	1: 6.22×10^{-4}	0.62 ($\log_{10}$ of -0.20)

†Observed ratio of minor serotype/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 1.

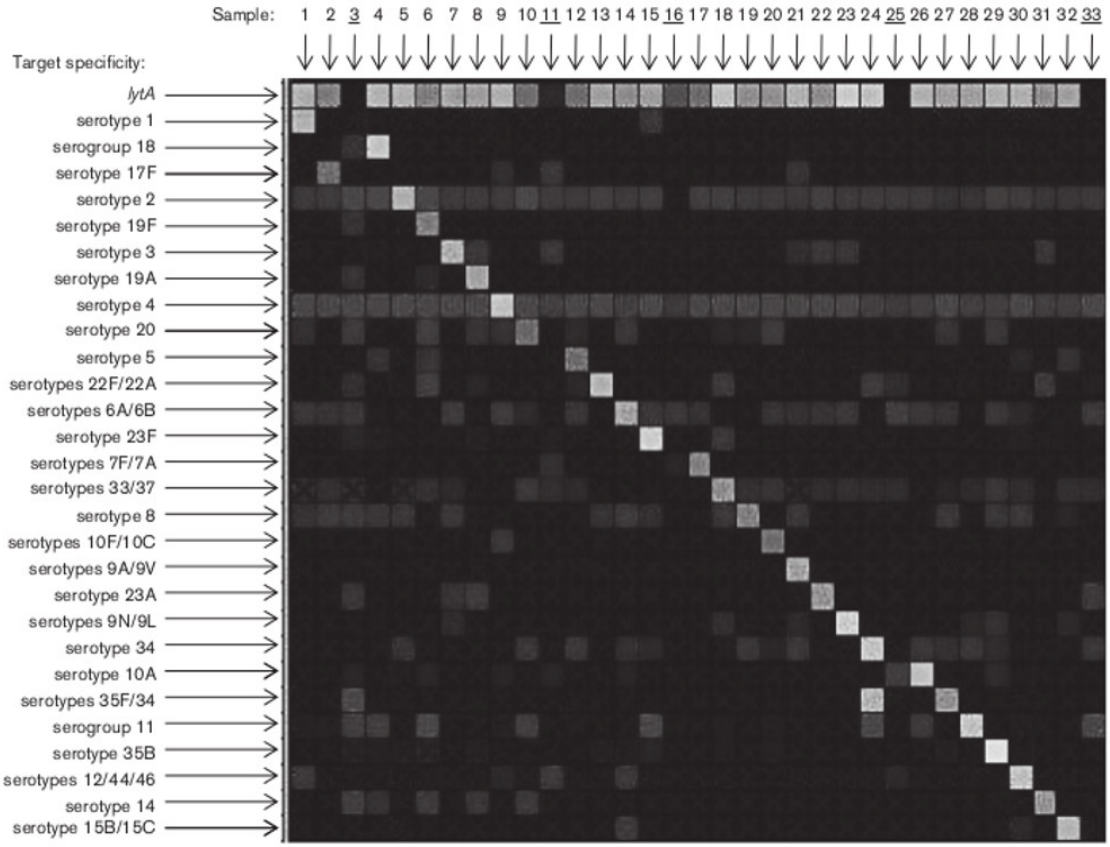


Figure 1. Cross validation of target specificity of the nanofluidic real-time PCR serotyping assay. Intensity of DNA amplification signals from the nanofluidic real-time PCR is shown as a heat map; the brighter rectangles of the checkerboard mean the stronger signals (the smaller Cq values). Each row 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.

Chapter 3

Title:

Bacterial Load of Pneumococcal Serotypes Correlates with Their Prevalence and Multiple Serotypes is Associated with Acute Respiratory Infections among Children Less Than 5 Years of Age

Authors:

Bhim Gopal Dhoubhadel[1], Michio Yasunami[1], Hien Anh Thi Nguyen[2], Motoi Suzuki[1], Huong Thi Thu Vu[2], Ai Thi Thuy Nguyen[3], Duc Anh Dang[2], Lay-Myint Yoshida[4§], and Koya Ariyoshi[1,5]

§ Corresponding author

Lay Myint Yoshida MBBS, MMedSc, PhD

Department of Pediatric Infectious Diseases

Institute of Tropical Medicine, Nagasaki University

1-12-4, Sakamoto, Nagasaki

852-8523, JAPAN

Phone: +81 (0) 95-819-7284

Fax: +81 (0) 95-819-7843

Published in PLoS One. 2014 Oct 31;9(10):e110777

Affiliations:

[1]Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Japan; [2]Department of Bacteriology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam; [3]Department of Microbiology, Khanh Hoa General Hospital, NhaTrang, Vietnam; [4]Department of Pediatric Infectious Diseases, Institute of Tropical Medicine, Nagasaki University, Japan

[5]Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Abstract

Background: Among pneumococcal serotypes, some serotypes are more prevalent in the nasopharynx than others; determining factors for higher prevalence remain to be fully explored. As non-vaccine serotypes have emerged after the introduction of 7-valent conjugate vaccines, study of serotype specific epidemiology is in need. When two or more serotypes co-colonize, they evolve rapidly to defend host's immune responses; however, a clear association of co-colonization with a clinical outcome is lacking.

Methods: Children less than 5 years old who were admitted to hospital due to acute respiratory infections (ARI) (n=595) and healthy children (n=350) were recruited. Carriage of pneumococcus was determined by culture and lytA PCR in the nasopharyngeal samples. Serotype/serogroup detection and its quantification were done by the nanofluidic real-time PCR system. Spearman's correlation and logistic regression were used to examine a correlation of serotype/serogroup specific bacterial load with its prevalence and an association of co-colonization with ARI respectively.

Results: Serotype/serogroup specific bacterial load was correlated with its prevalence, both in ARI cases (Spearman's rho = 0.44, n=186; P<0.0001) and healthy children (Spearman's rho = 0.41, n=115; P<0.0001). The prevalence of multiple serotypes was more common in ARI cases than in healthy children (18.5 % vs 7.1 %; aOR 2.92, 95% CI: 1.27-6.71; P=0.01). The dominant serotype in the co-colonization had a 2 log10 higher bacterial load than the subdominant serotype, both in ARI cases (P<0.001) and healthy children

($P<0.05$).

Conclusions: High bacterial load in the nasopharynx may help transmit pneumococci among hosts, and increase the chance of successful acquisition and colonization. Co-colonization of multiple serotypes of pneumococci is linked with ARI, which infers the interactions of multiple serotypes may increase their pathogenicity; however, they may compete for growth in number.

Introduction

Streptococcus pneumoniae (pneumococcus) is a major cause of life threatening diseases including pneumonia, sepsis and meningitis in children worldwide [1]. Pneumococcus has distinct polysaccharide capsule which characterizes its more than 90 serotypes. It colonizes in the nasopharynx, which is the precursor for pneumococcal diseases and the source for transmission among people [2]. The emergence of non-vaccine serotypes (19A, 35B) after the introduction of 7-valent pneumococcal conjugate vaccine (PCV7) has become a concern for future epidemiology of pneumococcal diseases, and it highlights the need of serotype specific study of this common pathogen [3,4].

The prevalence of a serotype in the nasopharynx is inversely correlated with the invasiveness of the serotype [5]. Less invasive serotypes such as 6A, 6B, 19F, 23F tend to colonize more frequently and maintain the carriage for longer time; while the more invasive serotypes such as 1, 4, 5, 7F tend to colonize less frequently and maintain colonization for less duration [6,7]. The pneumococcal capsule, which is a major virulent factor, determines the serotype. It is found the thickness of the capsule positively correlates with the prevalence of the serotype/serogroup [8]. Furthermore, the capacity to grow of a serotype is correlated with its prevalence in an in vitro study [9]; however, it is unknown whether similar relationship exists in the natural niche in humans.

Co-colonization of multiple pneumococcal serotypes in the nasopharynx affects vaccine serotype replacement, carriage detection and pneumonia

diagnosis [10]. Accurate determination of co-colonization and its role in pathogenesis are difficult to establish by using conventional serotyping method because of its low sensitivity and tedious laboratory work; therefore, a highly sensitive and specific molecular method is in need to detect multiple serotypes accurately and efficiently [11,12]. Although varied prevalence rates of co-colonization have been reported from different parts of the world [11-15], an epidemiological evidence of their association with a clinical outcome is lacking. Some epidemiological models have demonstrated that the serotypes compete among themselves for acquisition and persistence of colonization in the nasopharynx [16,17]; however, it is yet to be demonstrated quantitatively in humans.

In this study, we applied a highly sensitive and specific novel nanofluidic real-time PCR system to identify serotypes/serogroups and quantify their bacterial load in nasopharyngeal samples of hospitalized acute respiratory infections (ARI) cases and healthy children. We aimed to assess the correlation of bacterial load of specific serotypes/serogroups with the prevalence and compare the prevalence of co-colonization of multiple serotypes between these two groups of children.

Methods

Study design

Acute respiratory infections (ARI) cases, as defined by World Health Organization (WHO), were recruited in the Department of Pediatrics, Khanh Hoa General Hospital, Nha Trang City, Vietnam from 07/04/2008 to 31/03/2009. The hospital has 750 beds, and is the only hospital that provides inpatient care for sick children in Nha Trang City. The participants were children under 5 years old from the study area, who were admitted to the pediatric ward during the study period with acute respiratory infections (ARI) defined by cough and/or difficulty in breathing (WHO). We also included healthy children to compare bacterial load and serotype distribution with ARI cases. Healthy children, who did not have fever, signs of respiratory infections or history of antibiotic intake in the month preceding the day of enrollment, were recruited from two communes in the study area. They were selected randomly during January 2008 from the under 5 years old children using the census data. Informed written consent was taken from the parents of the hospital admitted ARI cases and the healthy children. Details of the study site were described elsewhere [18].

Sample collection, storage and DNA extraction

A nasopharyngeal sample (NPS), (about 100 microliter in volume) was collected flexible dacron-tipped aluminum-shafted swabs (Copan, Brescia, Italy) from each participant according to the WHO protocol [19]. The samples were taken from ARI cases at the time of admission before they were treated with antibiotics. The samples were divided into two aliquots. One aliquot was

sub-cultured onto 5% sheep blood agar and incubated overnight with 5% CO2 at 37°C. Alpha hemolytic colonies with morphology suggestive of S. pneumoniae and positive Optochin test were considered potential S. pneumoniae isolates. The other aliquot was stored at -80°C. At a later stage DNA was extracted, following the protocol of QIAGEN kit for the Gram-positive bacteria, directly from the nasopharyngeal samples of the children from whom potential S. pneumoniae had been cultured. DNA was stored at -80°C, except for transportation to Nagasaki on dry ice, till used for the lytA PCR and the nanofluidic real-time PCR system.

Identification of pneumococcus, its serotypes, quantification of bacterial load and definition of co-colonization of multiple serotypes

The DNA extract of the NPS from children with a potential S. pneumoniae culture positive isolate was analyzed by a lytA PCR that targeted the autolysin gene using the Light cycler II (Roche). Carriage of pneumococcus is defined as having positive lytA PCR NPS. The lytA positive samples were subjected to the nanofluidic real-time PCR system for molecular serotyping. The nanofluidic real-time PCR system can identify 50 serotypes in 29 groups, and it can detect minor population of multiple serotypes in co-colonization of pneumococci with the minimum level of detection of 30 to 300 copies. Total and specific serotype/serogroup bacterial loads were quantified using the standard curves. Details of the nanofluidic real-time PCR system has been described elsewhere [20]. Samples positive for lytA and negative for all serotype/serogroup primer-pairs were grouped as non-typeable (NT). Co-colonization with multiple serotypes was defined as the presence of two or

more serotypes/serogroups in a sample. In co-colonization the first serotype and second serotype were defined according to their bacterial loads; a serotype with the highest load was assigned as first serotype, with the second highest load as second serotype.

Statistical analysis

The data of pneumococcal loads, DNA copies, were changed into log10 scale. To compare the groups, Wilcoxon rank sum test was used for the continuous variables and Chi-square or Fisher exact test was used for categorical variables. Logistic regression was used to test the effects of pneumococcal load and co-colonization as risk factors for hospitalized children due to ARI. Odds ratios were adjusted for age, sex and daycare attendance. To test the correlation of bacterial load and prevalence of individual serotypes, Spearman's correlation was used. Analyses were performed using Stata v12.1 (StataCorp, College Station, Taxes, USA).

Ethical approval

This study was approved by all the concerned research review boards: Nagasaki University Institutional Review Board Nagasaki, Japan; the National Institute of Hygiene and Epidemiology Scientific Review Committee, Hanoi and the Khanh Hoa Provincial Health Service Ethical Review Board, Nha Trang, Vietnam.

Results

Basic characteristics of ARI cases and healthy children

Among the hospital admitted ARI cases, 88.6 % (527/595) were children less than 2 years old, proportion of male was 62% (369/595), and the median age was 10 months. Almost half, 45.5% of ARI cases had history of antibiotic use before admission. The proportion of chest X-ray confirmed pneumonia among them was 22.8% (136/595). Among healthy children, 55.4% (194/350) were under 2 years of old, proportion of male was 52.6% (184/350), and the median age was 19 months.

Pneumococcal colonization and serotype distribution

Pneumococcal carriage rate was 32.6 % (194/595) in ARI cases and 40% (140/350) in healthy children (figure 1). The proportion of typeable serotypes in ARI cases and healthy children were 95.9% (186/194) and 82.1% (115/140) respectively. Thirteen serotypes/serogroups of pneumococcus were detected in ARI cases and healthy children (figure 2). Serotypes/serogroups 19F, 6A/6B, 23F and 6C/6D were more prevalent in hospitalized ARI cases while serotypes/serogroups 14, 6A/6B, 19F, 15B/15C, 11 and non-typeables (NT) were common in healthy children. The proportion of serotypes/serogroups covered by pneumococcal conjugate vaccines: 7-valent (PCV7), 10-valent (PCV10) and 13-valent (PCV13) were approximately equal to one another, which was about 74% in ARI cases and 55% in healthy children (this was an approximate estimation as a serogroup was treated as a serotype as required).

Pneumococcal bacterial load

Higher bacterial load was associated with hospitalization due to ARI. The median bacterial load (total) was 6.61 log10/ml in ARI cases and 4.36 log10/ml in healthy children (OR=9.96, 95%CI: 6.39-15.52; P<0.0001 and aOR=9.07, 95%CI: 5.69-14.4; P<0.0001). There was no difference in median bacterial load between males and females both in ARI cases (6.62 log10 in male Vs. 6.59 log10 in female; p-value: 0.76) and healthy children (4.36 log10 in male Vs. 4.32 log10 in female; p-value: 0.86). Bacterial loads of specific serotypes/serogroups were higher in ARI cases than healthy children in all detected serotypes (Figure 3). Serotypes 14, 19F, 23F, 6A/6B had higher median bacterial load than other serotypes in ARI cases while serotypes 14, 19F, 23A, 23F had higher load in healthy children. The bacterial load of serotype 6C/6D was found to be lower than other common serotypes in ARI cases.

Serotype/serogroup specific bacterial load was significantly higher in vaccine serotypes. When we compared the bacterial load of vaccine specific serotypes and non-vaccine serotypes, the load was higher in vaccine serotypes both in ARI cases: 6.61 log10 in vaccine serotypes and 5.51 log10 in non-vaccine serotypes (P<0.0001), and in healthy children: 4.68 log10 in vaccine serotypes and 3.81 log10 in non-vaccine serotypes (P=0.0001).

Correlation between bacterial load of serotypes and their prevalence

Serotype/serogroup specific bacterial load was positively correlated with serotype/serogroup prevalence. Serotype/serogroup specific bacterial load of individual NPA samples from ARI cases and healthy children were plotted

against the prevalence of individual serotype/serogroup (i.e., proportion of the serotypes/serogroups in carriage positive samples). We found that the serotype/serogroup specific bacterial load had positive correlation with its prevalence, both in ARI cases (Spearman's rho = 0.44, n=186, P<0.0001) and healthy children (Spearman's rho = 0.41, n=115, P<0.0001) (Figure 4).

Co-colonization with multiple serotypes

Co-colonization of multiple serotypes of pneumococci was associated with ARI. Co-colonization of multiple-serotypes was detected in 18.5% (n=36/194) in ARI cases and 7.1% (n=10/140) in healthy children (OR 2.96, 95%CI 1.41-6.19; P=0.004). When adjusted for age, sex and daycare, the adjusted odds ratio (aOR) was 2.92 (95%CI 1.27-6.71; P=0.012). Co-colonization of serotypes 19F and 11, 19F and 15B/15C, 23F and 6A/6B occurred more frequently in ARI cases. We observed none of the serotypes involved in co-colonization were 19A or 35B (Figure 5). Co-colonization of pneumococcus occurred in 18.56% (18/97) of ARI cases when pre-hospital antibiotics had been used and 38.71% (12/31) when no antibiotics were used (P=0.002) [Unknown antibiotic status in 9.09% (6/66) of ARI cases]. The prevalence of co-colonization was found to be higher 32.5% (13/40) in age group of less than 6 months of age than 14.94% (23/154) in age group of 6 or more than 6 months of age in ARI cases (P=0.01). But, we did not find any such difference in healthy children among these age groups, 0% (0/22) versus 7.04% (10/142) (P=0.60).

One serotype/serogroup dominated the other serotype/serogroups in co-

colonization. In co-colonization, the serotype/serogroup specific bacterial load was 2.45 log10 higher in the dominant serotypes than the subdominant serotypes in ARI cases, while it was 2.04 log10 higher in healthy children (Figure 6). The dominant serotype was a vaccine serotype in 100% (10/10) of co-colonization in healthy children (P=0.003); while it was vaccine serotypes only in 72.22% (26/36) of co-colonization in ARI cases (P=0.76). The median total bacterial load was higher, 4.48 log10 versus 4.31 log10 (P=0.03), when co-colonization of multiple serotypes was present as compared to single serotype colonization in healthy children; however, no significant difference was found among them in ARI cases (6.65 log10 versus 6.58 log10, P=0.59).

Discussion

Our data suggest two major findings. First, a positive correlation of serotype/serogroup specific bacterial load with prevalence of serotypes/serogroups, which may help to understand why some serotypes of pneumococcus are successful for colonization and maintenance of the carriage for longer time. The second finding, an association of co-colonization of multiple serotypes with acute respiratory infections in children may infer a role of multiple serotypes of pneumococci in pathogenesis of ARI in children. Both of these findings are novel in pneumococcal pathogenesis and epidemiology in humans.

It is known that the prevalence of serotypes is inversely correlated with their serotype specific invasiveness [5]. It is found that some serotypes/serogroups: 1, 4, 5, 7F are more invasive but less prevalent in the nasopharynx than other serotypes/serogroups: 6A, 6B, 19F, 23F, which are more prevalent but less invasive [5]. Contributing factors for this inverse relationship of prevalence and invasiveness remains to be fully explained. It is found that colonizing serotypes: 19F, 6A, 6B, 23F have higher serotype specific rates of acquisition of colonization and longer duration of carriage than invasive serotypes: 1, 4, 5, 7F in children [6,7]. This shows that the colonizing serotypes are more capable to colonize and maintain the carriage than invasive serotypes. In this regard, our finding of positive correlation of serotype/serogroup specific bacterial load with prevalence of serotype/serogroup may explain that the higher numbers of bacteria in the nasopharynx may help them to transmit from one host to other, so that they have higher chance of colonization, and

their ability to grow in number may help to maintain the carriage for longer time against the normal mucosal clearance of the host.

Some in vitro studies show that serotype specific growth of pneumococcus is positively correlated with prevalence of the serotype [8,9]. Hathaway et al found that high carriage prevalence serotypes (6B, 9V, 19F, 23F) can produce their capsules that are less metabolically demanding, and they can grow even in nutritionally poor environment [9]. These in vitro findings match with our in vivo findings of high bacterial load of common serotypes/serogroups and their correlation with prevalence. Less prevalent but more invasive serotypes are characterized by poor growth, longer lag phase in bacterial growth [21], thinner capsule size and more prone to be killed by neutrophils than high prevalence serotypes [8].

We found the occurrence of co-colonization with multiple serotypes of pneumococcus was twice as common in hospitalized ARI cases as in healthy children. This is the first time to our knowledge that such an association of co-colonization of multiple serotypes of pneumococci with ARI is reported. Although prevalence of co-colonization has been reported to occur from 1.3% to 39% in children by using different methods in various settings [11-15], none has reported an association with a clinical outcome. Non-vaccine serotypes such as 19A and 35B, which have emerged after the introduction of PCV7 vaccine [3,4], were not detected in co-colonization both in ARI cases and healthy children in our study. Although the number of children with co-colonization was relatively small, our data discourage the “unmasking

phenomena" for emergence of non-vaccine serotypes [22]. The potential role of co-colonization in the emergence of 19A due to serotype replacement is partly explained by capsule switching phenomenon at the capsular locus by recombination [23,24]. As pneumococcus is highly recombinogenic and transformable bacteria, probability is high for genetic exchange when two or more pneumococcal serotypes inhabit at the same time in the nasopharynx [10]. This is true for evolution of not only vaccine-escape serotypes but also for the antibiotic resistant serotypes [24]. When multiple serotypes co-colonize, the genetic reservoir expands so called "Supragenome" will advance the microevolution of the pathogen and allow continued survival by evasion of serotype-specific immune response and adaptation by genetic change, which is demonstrated by the co-colonization of penicillin sensitive and penicillin resistance pneumococci at the same time [25]. Besides, it is found that in mouse model, pneumococci form a biofilm in the nasopharynx when multiple strains are present. The transformation efficiency becomes very high in the biofilm with multiple serotypes, so that the antibiotic resistance can easily be spread among the serotypes. Hence, co-colonization of multiple serotypes confers the development of supra-virulence and fitness in the pathogen for survival in the harsh environment of host [26].

We have further demonstrated the dominance of one serotype over the other in bacterial load when co-colonization is present. This difference in pneumococcal load among serotypes/serogroups in a co-colonization may suggest that there may be a competition among the serotypes for their growth due to limited nutrients and space. In mouse model, intra-species competition

among pneumococci is demonstrated and found mediated by bacteriocin [27,28]. Epidemiological models also suggest the existence of competition among serotypes for initiation and persistence of colonization in children [16,17]. A study with mathematical modeling shows that direct (physical) competition and indirect (antibody mediated) competition do exit among the serotypes of pneumococcus in co-colonization [29]. We considered the 100 fold higher bacterial load, which we found in the dominant serotype, is due to direct competition as naturally acquired immune response due to colonization lasts for a short duration in unvaccinated young children. This is consistent with the finding of the mathematical modeling [29].

This study has limitations. Due to logistic problems, we were unable to bring all nasopharyngeal samples to Nagasaki from Vietnam for DNA extraction and nanofluidic real-time PCR. First, we screened all the samples by culture, and only samples that grew alpha hemolytic colonies and Optochin sensitive isolates were brought to Nagasaki for molecular assays. It may have decreased the overall sensitivity of detection of pneumococcus and carriage rate. Minimum level of detection of the nanofluidic real-time PCR was 30-300 copies per reaction; other limitations of the nanofluidic real-time PCR have been described elsewhere [20].

In conclusion, this study showed a positive correlation of serotype/serogroup specific bacterial load with serotype/serogroup prevalence of pneumococcus in children. Higher bacterial load of a serotype in the nasopharynx might be an attributing factor for higher transmission of the serotype. The association of

multiple serotypes of pneumococcus with ARI showed its link with increased pathogenicity, and dominance of one serotype over the other might infer the competition among serotypes when multiple serotypes were present in the nasopharynx.

Acknowledgments

We are grateful to children and their parents who participated in this study. We would like to thank the staffs from Khanh Hoa Health Service and the medical staff in Khanh Hoa General Hospital who helped to carry out this research. BGD is grateful to the Government of Japan, Ministry of Education, Culture, Sports, Science, and Technology for Monbukagakusho (MEXT) scholarship. The study was funded by Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and Japan Science and Technology, Japan.

References:

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, et al. (2009) Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 374: 893-902.
2. Simell B, Auranen K, Kayhty H, Goldblatt D, Dagan R, et al. (2012) The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines 11: 841-855.
3. Weinberger DM, Malley R, Lipsitch M (2011) Serotype replacement in disease after pneumococcal vaccination. Lancet 378: 1962-1973.
4. Huang SS, Hinrichsen VL, Stevenson AE, Rifas-Shiman SL, Kleinman K, et al. (2009) Continued impact of pneumococcal conjugate vaccine on carriage in young children. Pediatrics 124: e1-11.
5. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis 190: 1203-1211.
6. Abdullahi O, Karani A, Tigoi CC, Mugo D, Kungu S, et al. (2012) Rates of acquisition and clearance of pneumococcal serotypes in the nasopharynges of children in Kilifi District, Kenya. J Infect Dis 206: 1020-1029.
7. Tigoi CC, Gatakaa H, Karani A, Mugo D, Kungu S, et al. (2012) Rates of acquisition of pneumococcal colonization and transmission probabilities, by serotype, among newborn infants in Kilifi District, Kenya. Clin Infect Dis 55: 180-188.
8. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, et al. (2009) Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 5: e1000476.
9. Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, et al. (2012) Capsule type of Streptococcus pneumoniae determines growth phenotype. PLoS Pathog 8: e1002574.
10. Shak JR, Vidal JE, Klugman KP (2013) Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol 21: 129-135.
11. Hare KM, Morris P, Smith-Vaughan H, Leach AJ (2008) Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs. Pediatr Infect Dis J 27: 178-180.
12. Huebner RE, Dagan R, Porath N, Wasas AD, Klugman KP (2000) Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes. Pediatr Infect Dis J 19: 1017-1020.
13. Hansman D, Morris S, Gregory M, McDonald B (1985) Pneumococcal carriage amongst Australian aborigines in Alice Springs, Northern Territory. J Hyg (Lond) 95: 677-684.
14. Brugger SD, Hathaway LJ, Muhlemann K (2009) Detection of Streptococcus

pneumoniae strain cocolonization in the nasopharynx. J Clin Microbiol 47: 1750-1756.
15. Ercibengoa M, Arostegi N, Marimon JM, Alonso M, Perez-Trallero E (2012) Dynamics of pneumococcal nasopharyngeal carriage in healthy children attending a day care center in northern Spain. Influence of detection techniques on the results. BMC Infect Dis 12: 69.
16. Lipsitch M, Abdullahi O, D'Amour A, Xie W, Weinberger DM, et al. (2012) Estimating rates of carriage acquisition and clearance and competitive ability for pneumococcal serotypes in Kenya with a Markov transition model. Epidemiology 23: 510-519.
17. Auranen K, Mehtala J, Tanskanen A, M SK (2010) Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. Am J Epidemiol 171: 169-176.
18. Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, et al. (2011) Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 30: 11-18.
19. O'Brien KL, Nohynek H (2003) Report from a WHO working group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. Pediatr Infect Dis J 22: 133-140.
20. Dhoubhadel BG, Yasunami M, Yoshida LM, Thi HA, Thi TH, et al. (2014) A novel high-throughput method for molecular serotyping and serotype-specific quantification of Streptococcus pneumoniae using a nanofluidic real-time PCR system. J Med Microbiol 63: 528-539.
21. Battig P, Hathaway LJ, Hofer S, Muhlemann K (2006) Serotype-specific invasiveness and colonization prevalence in Streptococcus pneumoniae correlate with the lag phase during in vitro growth. Microbes Infect 8: 2612-2617.
22. Azzari C, Resti M (2008) Reduction of carriage and transmission of Streptococcus pneumoniae: the beneficial "side effect" of pneumococcal conjugate vaccine. Clin Infect Dis 47: 997-999.
23. Brueggemann AB, Pai R, Crook DW, Beall B (2007) Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog 3: e168.
24. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, et al. (2011) Rapid pneumococcal evolution in response to clinical interventions. Science 331: 430-434.
25. Leung MH, Oriyo NM, Gillespie SH, Charalambous BM (2011) The adaptive potential during nasopharyngeal colonisation of Streptococcus pneumoniae. Infect Genet Evol 11: 1989-1995.
26. Marks LR, Reddinger RM, Hakansson AP (2012) High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae. MBio 3.

27. Lipsitch M, Dykes JK, Johnson SE, Ades EW, King J, et al. (2000) Competition among Streptococcus pneumoniae for intranasal colonization in a mouse model. Vaccine 18: 2895-2901.
28. Dawid S, Roche AM, Weiser JN (2007) The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo. Infect Immun 75: 443-451.
29. Zhang Y, Auranen K, Eichner M (2004) The influence of competition and vaccination on the coexistence of two pneumococcal serotypes. Epidemiology and Infection 132: 1073-1081.

Figure 1.

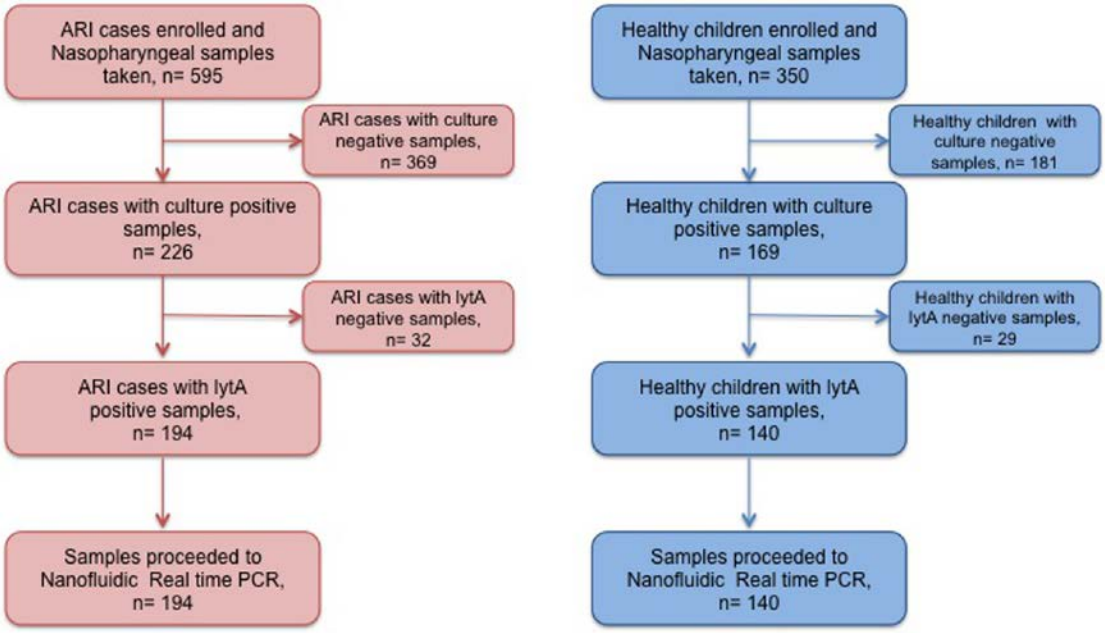


Figure 1. Participants recruited into the study, and their nasopharyngeal samples. Half of the aliquot of nasopharyngeal samples were cultured overnight in 5% sheep blood agar with 5% CO2 at 37°C. DNA was extracted directly from the other half of the aliquot of the nasopharyngeal samples which yielded alpha hemolytic colonies and positive Optochin test. DNA was subjected to lytA PCR to confirm pneumococcus; and lytA positive samples were then processed for molecular serotyping in the nanofluidic real-time PCR system.

Figure 2.

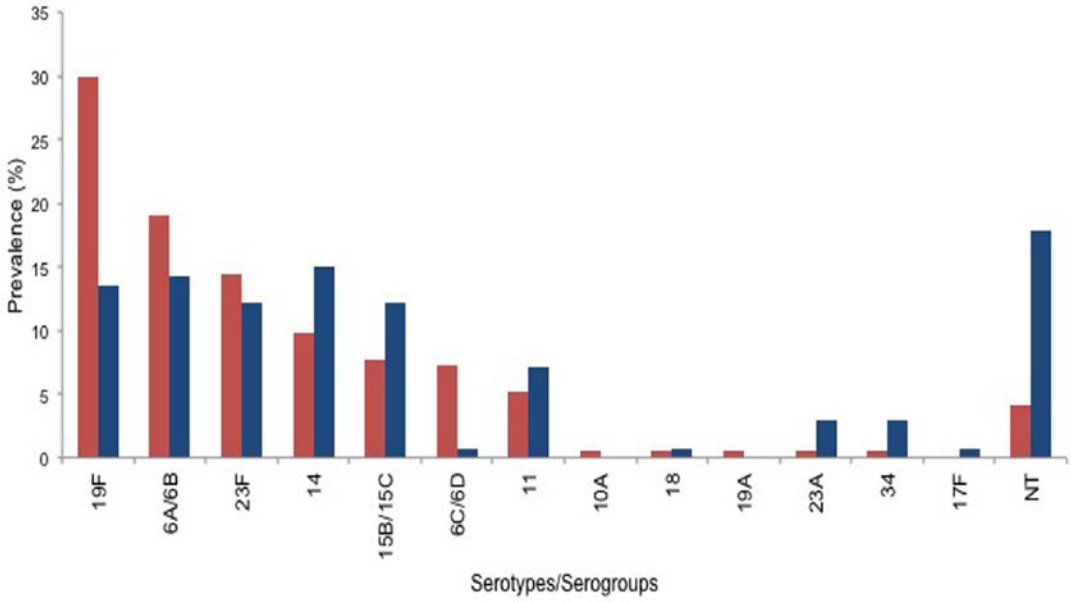


Figure 2. Distribution of serotypes/serogroups of pneumococcus in ARI cases and healthy children. Thirteen different serotypes/serogroups were detected; DNA samples which were positive for lytA (pneumococcus positive), but negative for tested 29 serotypes/serogroups were assigned as non-typeable (NT). Prevalence of each serotype/serogroup was calculated as proportion of total number of a serotype/serogroup to the total number of the lytA positive samples. Serotype/serogroup of ARI cases and healthy children were plotted in red and blue respectively. Proportion of serotype/serogroup covered by 13-valent conjugated vaccine (PCV13) was 74% in ARI cases and 55% in healthy children.

Figure 3.

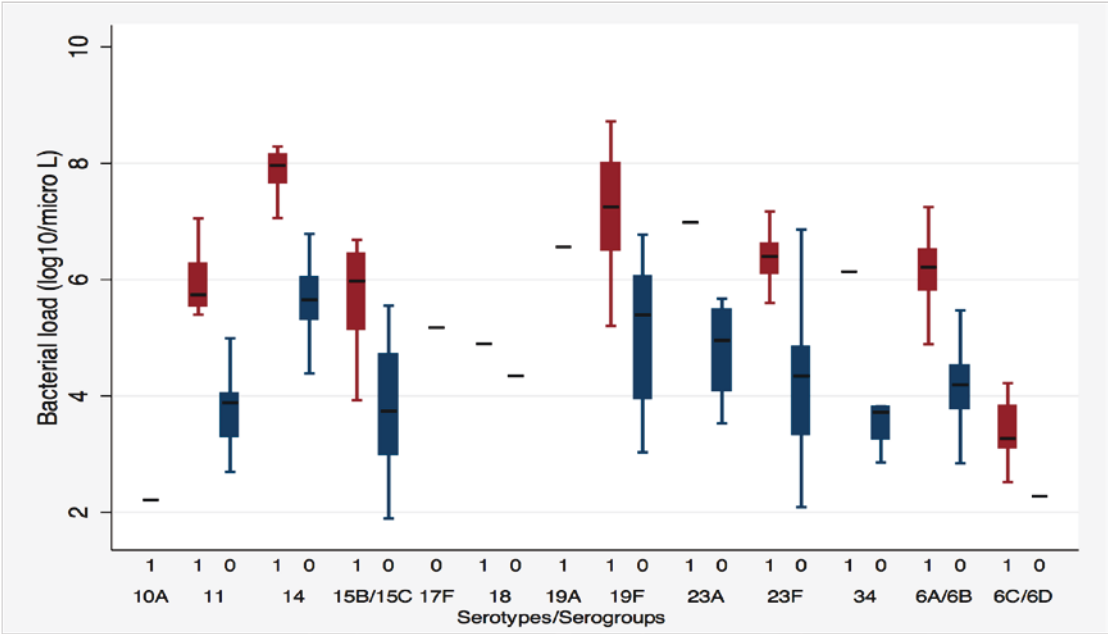


Figure 3. Bacterial load of specific serotypes/serogroups of pneumococcus in ARI cases and healthy children. Bacterial load of ARI cases (denoted by “1” in each serotype/serogroup) in red and healthy children (denoted by “0” in each serotype/serogroup) in blue, showed that the common serotypes 19F, 14, 23F, 6A/6B, 15B/15C had high bacterial load.

Figure 4.

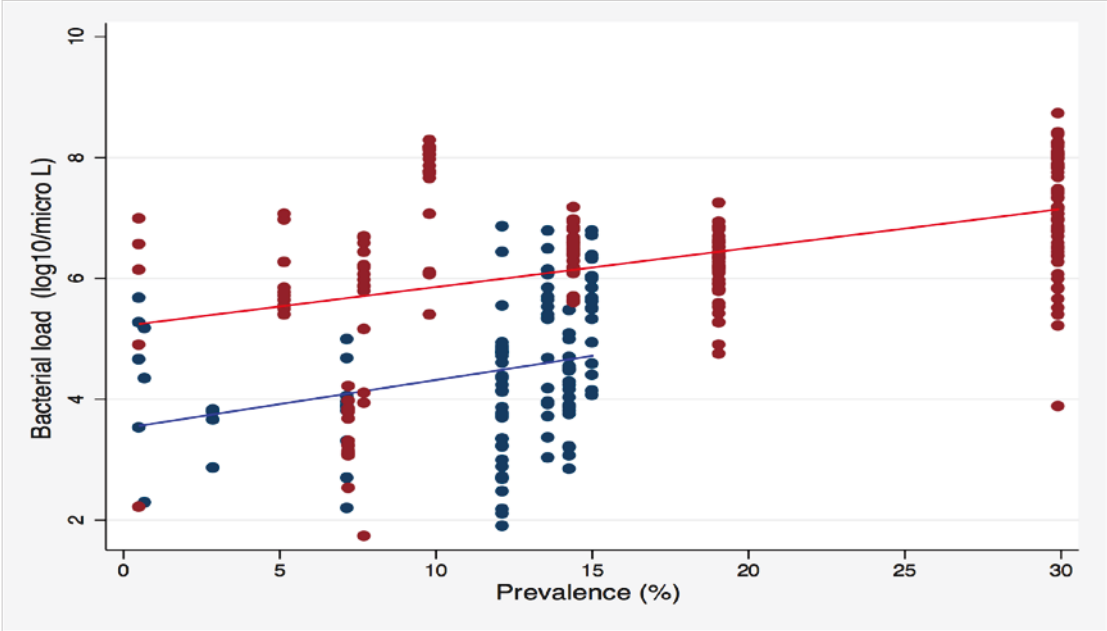


Figure 4. Relationship between bacterial load of specific serotypes/serogroups and their prevalence. Bacterial load of each of specific serotypes/serogroups was plotted against its prevalence. Both in ARI cases (red) and in healthy children (blue), a positive correlation was found. Each dot represents an ARI case or a healthy child in the plot, and the red and blue lines are the fitted values for ARI cases and healthy children respectively. Spearman's rho was 0.44 (n=186; P<0.0001) and 0.41 (n=115; P<0.0001) for ARI cases and healthy children respectively.

Figure 5.

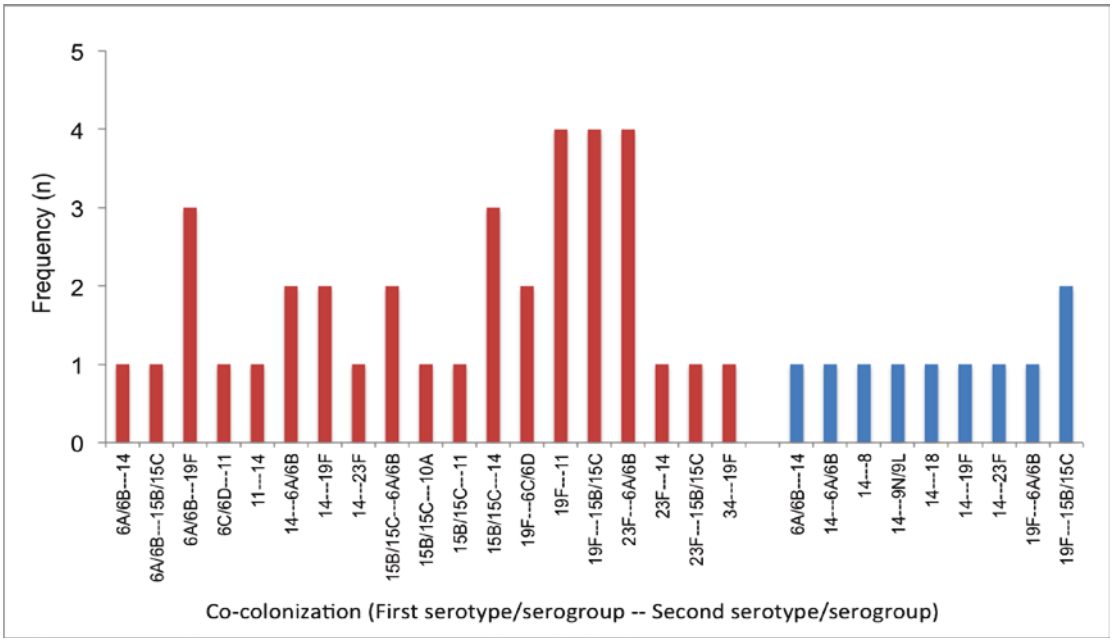


Figure 5. Distribution of co-colonization of multiple serotypes of pneumococcus. In ARI cases (red) co-colonization was detected in 36 samples out of 194 lytA positive samples (18.5 %), while in healthy children (blue) it was detected in 10 samples out of 140 (7.1%) lytA positive samples. The odds ratio, adjusted for age, sex and daycare, was 2.92 (95%CI 1.27-6.71; P=0.012). The serotypes/serogroups were positioned first and second according to their bacterial load.

Figure 6.

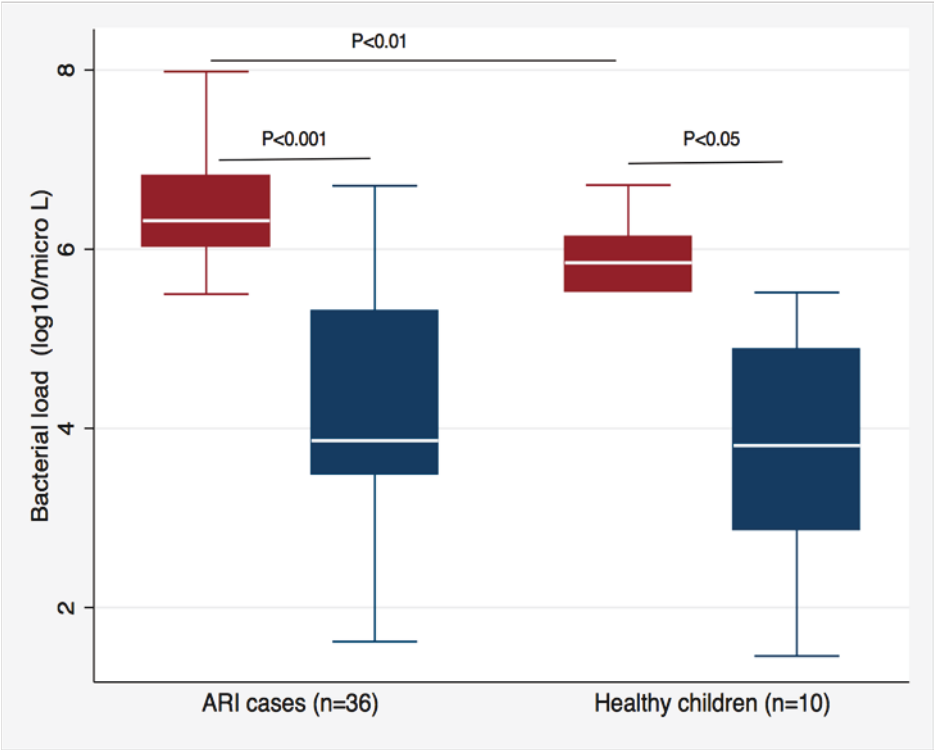


Figure 6. Dominance of one serotype/serogroup over the other in co-colonization of multiple serotypes/serogroups. Among two serotypes/serogroups present in a co-colonization, one serotype/serogroup (red) was found to be dominant by having 100 folds (2 log10) higher bacterial load than the other subdominant (blue) serotype/serogroup both in ARI cases and healthy children.

Chapter 4

Future plan and its rationale

Antibiotic resistance in Pneumococcus has dramatically increased in past three decades and now become a global health problem [1,2]. Although antibiotic resistance is determined by culture and sensitivity conventionally, it is less sensitive and tedious procedure; and it is also not available commonly in resource-limited countries. Pneumococcus has more than 90 serotypes, which have variable capacity to cause diseases and develop antibiotic resistance [3-5]. Moreover, the distributions of serotypes vary both geographically and temporally; therefore, to determine antibiotic resistance at serotype level is essential to treat and control this common pathogen effectively [3,4]. Scientists have developed molecular methods to determine the antibiotic resistance patterns in recent years [5-8]; however, these methods mostly target for one or two antibiotics and the serotype of the bacteria cannot be determined simultaneously. So, it takes more time, effort and resources to determine antibiotic resistance and individual serotypes, as we need to carry out different sets of experiments. Instead, we would like to develop a comprehensive assay, which determine both antibiotic resistance and serotypes of individual isolates simultaneously. This comprehensive assay will be more convenient, efficient and cost-effective than any other assay present at this time.

We would like to develop the comprehensive pneumococcal assay by including primers for antibiotic resistance genes in the present nanofluidic

real-time PCR system [9], so that we will be able to determine serotypes and antibiotic resistance simultaneously. This will give us a powerful tool to investigate both vaccine and antibiotic effectiveness in the control of this common pathogen. Besides, we hope we will discover many new insights in pneumococcal biology as we can explore the interrelationships of different antibiotic resistance genes, serotypes and their bacterial load simultaneously.

References:

1. Linares J, Ardanuy C, Pallares R, Fenoll A (2010) Changes in antimicrobial resistance, serotypes and genotypes in Streptococcus pneumoniae over a 30-year period. Clin Microbiol Infect 16: 402-410.
2. Lin TY, Shah NK, Brooks D, Garcia CS (2010) Summary of invasive pneumococcal disease burden among children in the Asia-Pacific region. Vaccine 28: 7589-7605.
3. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis 190: 1203-1211.
4. Imai S, Ito Y, Ishida T, Hirai T, Ito I, et al. (2009) High prevalence of multidrug-resistant Pneumococcal molecular epidemiology network clones among Streptococcus pneumoniae isolates from adult patients with community-acquired pneumonia in Japan. Clin Microbiol Infect 15: 1039-1045.
5. Ubukata K, Chiba N, Hasegawa K, Kobayashi R, Iwata S, et al. (2004) Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of Streptococcus pneumoniae strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob Agents Chemother 48: 1488-1494.
6. Page S, Vernel-Pauillac F, O'Connor O, Bremont S, Charavay F, et al. (2008) Real-time PCR detection of gyrA and parC mutations in Streptococcus pneumoniae. Antimicrob Agents Chemother 52: 4155-4158.
7. Bley C, van der Linden M, Reinert RR (2011) mef(A) is the predominant macrolide resistance determinant in Streptococcus pneumoniae and Streptococcus pyogenes in Germany. International Journal of Antimicrobial Agents 37: 425-431.
8. Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, et al. (2001) Evaluation of PCR primers to screen for Streptococcus pneumoniae isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. J Antimicrob Chemother 48: 915-918.
9. Dhoubhadel BG, Yasunami M, Yoshida LM, Nguyen Thi HA, Vu Thi TH, et al. (2014) A novel high-throughput method for molecular serotyping and serotype-specific quantification of Streptococcus pneumoniae using a nanofluidic real-time PCR system. J Med Microbiol.