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ONE ASSAY FOR ALL: A MICROARRAY TECHNOLOGY FOR IDENTIFICATION AND SEROTYPING OF *STREPTOCOCCUS PNEUMONIAE* IN CULTURE NEGATIVE QMPCR POSITIVE SERUM SPECIMENS

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ABSTRACT

Serotyping of *Streptococcus pneumoniae* is of importance for disease management and vaccine studies. Current serotyping methods require culturing which is often negative and cannot differentiate homologous serotypes. Serum provides a convenient and widely available source of pathogen detection, but the presence of host DNA hinder the detection of *S. pneumoniae*. This study aimed to evaluate a microarray method to detect and serotype *S. pneumoniae* from serum specimens. Unique serotype-specific probes were identified and added to the custom pneumococcal array and evaluated with 32 homologous reference strains, 8 Non- pneumococcal isolates and 454 serum samples. With a combination of Microbial DNA enrichment, the custom pneumococcal array identified serotype of homologous reference strains and serum samples accurately with an additional advantage of multiple serotypes and other respiratory bacterial pathogens detection in spiked samples. Our results signify the ability of microarray technology to identify and detect *S. pneumoniae* serotype from serum specimens.

INTRODUCTION:

Pneumonia is the leading cause of deaths worldwide, accounting for 15% of all deaths of children under 5 years old. Pneumonia killed an estimated 935000 children under the age of five in 2013.WHO estimated the high burden of pneumonia in Asia with most cases in India (43 million).[1] The proportion of pneumococcal pneumonia among all severe pneumonia was 15.8% whereas pneumococcal pneumonia deaths among all-cause pneumonia deaths were 29.5%.[2,3]

Identification and serotyping of pneumococcus have been an integral part of both pneumococcal research and clinical settings. All currently available vaccines against *Streptococcus pneumoniae* are based on selections of the over 90 different serotypes, which underlines the importance of serotyping for surveillance and vaccine efficacy monitoring. Precise serotyping of pneumococci is essential to better understand the pathogenicity and trends of drug resistance. It closely monitors the emergence of non-vaccine strains, replacement serotypes, and new serovars.[4]

The conventional culture-based and PCR methods such as Quellung, Pulse Field Gel Electrophoresis (PFGE), Restriction fragment length polymorphism (RFLP), sequential multiplex PCR, have significant limitations, like low capacity, may fail if prior antibiotics administered, cannot quantify serotype abundance levels, and have a limited number of targets.[5,6,7,8] Modern molecular methods such as quantitative multiplex PCR (qmPCR), Sequencing and Microarray developed based on capsular polysaccharide (CPS) and glycosyltransferase (GT) genes have given new life to solve the problem associated with pneumococcal detection. The limitation of these methods was their inability to distinguish homologous serotypes (serotypes which share sequence similarity with few bases pairs difference).[9,10,11,12,13,14]

A major limitation to our understanding of pneumococcal serotype distribution is that most clinical cases, remain culture negative due to the widespread use of antibiotics prior to admission to the hospital. Conventional serotyping assays cannot be performed directly on clinical samples since the growth of *S. pneumoniae* on culture media is required. Serum, on the other hand, provides a convenient source of true disease burden and pathogen genetic information. A barrier to the approach of detecting pneumococcal infection from serum is the overwhelming ratio of human to pathogen DNA in samples with low pathogen abundance, which is typical of most clinical specimens.[15,16] In our previous study, we successfully identified S. *pneumoniae* serogroup/ type from serum samples using our custom pneumococcal chip by enriching microbial DNA through Microbial DNA enrichment method.[17] The custom array detected the *S. pneumoniae* serogroup accurately but not the serotype of homologous strains and serum specimens. In Present study, we re-designed our custom array to identify homologous serotypes of *S. pneumoniae* from Serum specimens. Additional probes were included to detect other potential bacterial pathogens and quantify their relative abundance levels in spiked specimens.

MATERIALS AND METHODS:

REFERENCE STRAINS

A total of 32 *S. pneumoniae* homologous reference strains and 8 Non-pneumococcal isolates such as *Streptococcus oralis, Moraxella catarrhalis, Neisseria meningitidis, Haemophilus influenzae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Mycoplasma pneumoniae* were sourced from Central Research Laboratory, KIMS Hospital. Pneumococcal reference Strains stored in skimmed milk, tryptone, glucose, and glycerol (STGG) media at –80 °C, were cultured on 5% sheep blood agar (Chromogen, Hyderabad) for 18–24 hrs at 37°C with 5% Co_2.

Serum Specimens

Culture negative qmPCR positive (N=408) and culture positive qmPCR positive (N=48) *S. pneumoniae* serum samples from children <5 years with IPD were sourced from Central Research Laboratory, KIMS Hospital, collected during 2014 to 2015 from India.

Ethics Statement

The study was conducted according to the guidelines and declaration of Helsinki. Written informed consent was obtained from each patient or legal guardian as applicable.

DNA extraction

Genomic DNA was extracted from reference strains, non-pneumococcal isolates and serum specimens using QiaAmp DNA mini kit protocol (Qiagen, Germany) following manufacturer's instructions. The extracted DNA was quantified using Nanodrop (Thermo fisher scientific, USA) and stored at -20şC until further analysis.

Custom Array Designing

In our previous study, the custom pneumococcal array was designed using suredesign software (Agilent, USA) and evaluated with 90 reference strains and 104 serum samples. The custom array comprises of 15167 oligonucleotide probes with multiple probes per serotype (16 - 575 probes) targeting 90 *S. pneumoniae* serotypes.[17] The custom array also contained species specific

probes for *Streptococcus pyogenes, Streptococcus mitis, Streptococcus oralis, Moraxella catarrhalis, Neisseria meningitidis, Haemophilus influenzae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Legionella pneumophila, Chlamydophila pneumoniae and Mycoplasma pneumoniae,* which reside in human nasopharynx along with *S. Pneumonia.* [17]

Designing of Unique Serotype-specific probes

In the present study, the homologous strain sequences were subjected to MUSCLE (multiple sequence comparison by log expectation) alignment software and identified unique sequences. (Table-1) MUSCLE is a public domain (https://www.ebi.ac.uk/Tools/ msa/muscle/), multiple sequence alignment software for protein and nucleotide sequences.[20] The unique sequences of Homologous groups/ types were further subjected to suredesign software (Agilent, USA) to design unique serotype specific probes and were added to custom pneumococcal array.

Microarray analysis of homologous reference strains and Serum samples

In the first step, the serum DNA (N=456) was subjected for NEBNext Microbiome DNA Enrichment Kit (New England BioLabs) as described in Shaik AH et al 2019 [17] and separated microbial DNA from host DNA. The enriched microbial DNA (N=456) (Figure-1) from serum and un-enriched DNA from homologous reference strains (N=32) (Table-2) was further subjected for microarray protocol as described in Shaik AH et al 2019 without any modifications. The samples were blinded throughout microarray experiment and data analysis.

Multiple serotype detection:

The redesigned custom array was validated for detection of multiple serotypes by pooling reference strains and spiking other commensals with different serotypes in 1:1 ratio and subjected for Agilent microarray protocol described previously. (Table-3)

Relative abundance levels quantification

The Image acquisition was performed by Agilent DNA scanner using Agilent Scan control software v.9.0. The data processing and signal intensity extraction were analysed using Agilent Feature extraction software v.3.0. Probes showing signal above 98 percentile or always above median were removed as a part of probe selection. Positive probes with 100x fold change with respect to the median of the negative probe distribution were removed for noise reduction. The signal intensities were determined by subtracting the local background values (as estimated by the Agilent platform) from the per-sample median. Each probe's three technical replicates were then averaged and a robust average (obtained removing values above the 95th percentile and below the 5th percentile) of all probes for the same serotype was considered. Species relative abundances were then estimated by relativizing this species average with respect to the sum of all averages.

RESULTS:

Design probes to address homologous strains of *S. Pneumoniae*

A set of unique probes were designed and added to custom pneumococcal Array (Table-1)

Homologous Serotype	Unique probe ID	Unique probe sequence
6A and 6B	6A	GAACATGAAAGATGAAGCAATCAATGTACTGGATAAAGTTATGAAAAAGATTTTTTAAAAAGTTTTGCCCCTTTTTTGTCCTCTAAATACAAAAATAGCCCT
9A and 9V	9V	TTAATTTGAAGGGGAATGCCGTGATTGGCTAAGATTTGTTCGAGNGACATGATAGTAAGCATTCAAGTTCTCTTGTTTATCAAAATAAGCGCCTAGGATA
10F and 10C	10F probe1	GAGCAGGACAAAAGAGCCTCGTAAAAGGTATTGCAACTTGGTAATACCTTTTTGAGGTGCTTTTTGATAT
10F and 10C	10F probe 2	TCTAAATCAGGTTCGAGCGCTAAAGCTGAGAAGACATTCTCATACATTTATGAAACGGATCCTGACAACCTAAACTACTTGACAACTGGTAAGGCTGCGA
22A and 22F	22F	GCGGCGACTACTTTAGCTGCATGCTCTGGATCAGGTTCAAGCACTAAAGGTGAGAAGACATTCTCATACATTTATGAGACAGACCCTGATAACCTCAACTATTTG
32A and 32F	32A	GCGGCGACTACTTTAGCTGCATGCTCTGGATCAGGTTCAAGCACTAAAGGTGAGAAGACATTCTCATACATTTATGAGACAGACCCTGATAACCTCAACTATTTG
32A and 32F	32F	TGTCCAATGAAGAGCAAGACTTGACAGTAGAAGGAAAAGTCAAATCTGTCTTGATTGAAAACACCCTAGCTCAAGAAGTCTTTGAAAAACAAATCTTAGT
33A, 33B, 33F	33B	AGGTGTGGTTCTTTTTTTCGAGTGTAGCCCATAGCTTTGAGCGCATAGTGGATGGTAGTTGGATGACAGCCAAAGTCAGAAGCTATTTCAGTCAAATAAGCGTCTGGATTGTCAGTAAGATAGTTTTTAAGTCTATCTCTATCAACCTTTCTTG
33A, 33B, 33F	33A	TGAGTTAACGGGACTTTATTGGGCATGGAAGAATCTTGATTATAATTACTTAGGTTTAGTTCATTACCGT
12A,12F,44,46	12A	TAAATGGTATTACGTGAGATTTGGAAAACGTGTGATGCTTCTGTTATACTACCTGTTCGCTCACAATAAGAGAGAACTTTTTTACGAAAATCTATTGAATATGCCATAAAAAGATTATACCACATTGTGTACTAT
12A,12F,44,46	12A 46	TAATAGACTTGTTCGGTAACTGTAAAAAGTGTTATACTATTTTTATGGAAACAGTATACGACAAAGCACAAAAACTTAACTCAAAAAACTTCAAACTATTGATTGGTGTCAAAAAGGAAACCTTTCAACTCATGCTAGAACACCTGAATTCAGCCTATCAGATTCAGCAC
12A,12F,44,46	12A 44 46	GCGGCGACTACTTTAGCTGCATGCTCTGGATCAGGTTCAAGCACTAAAGGTGAGAAGACATTCTCATACATTTATGAGACAGACCCTGATAACCTCAACTATTTGACAACTGCTAAGGCTGCGACAGCAAATATTACCAGTAACGTGG
12A,12F,44,46	12F	AAGCCTTTCGAACTTTGGAAGATGTCATGAATCAACTCCAAGATGTTATACAAGGACTGG
11A, 11D, 18F	11A probe1	TTTTCCGTAGCGATTTGAAGATTATAAGCAGCCTTGAGTTGACCATTTTTCATATGATCTTCCTTCATCCGCATAAAAGTGGCATCTGGATCGGTTTTGGAAAAACTGTTAGGCCCTTCAACTGTCTCCTGATAGTTCTCATATTTTTCAGCACGTACTGAAAAATC
11A, 11D, 18F	18F probe1	ATTGTTAGAAATCGATTTGACTGTCCTGATCGATTTGTCATGTTCTTATTTCATTTTACTATATTTTTGGTTCGCGGGAAGTCTACTAAGATACTTA
11A, 11D, 18F	18F probe2	AGCCTTATTATCCAGTTAATAATGATCGTAATAATCATTTGTATAAATCATATAAAAAACTTGCTGATGAGCAAGGGAATGTTATCTTTGGTGGCCGCTTAGGACACTATCGTTATTACGATATGCACCAAGTAATTGGAG
11A, 11D, 18F	18F11A	AAAATGATGAAAAGTTCAAAACTATTTGCCCTTGCGGGCGTGACATTATTG
11A, 11D, 18F	11A probe2	GAGTCTGGAACAAAATAGTTCTCGACCACAAAAAAGCTAGAGATTTCCAATTGTGG
11A, 11B, 11C, 11D	11D	AGTGGATTGAAACTAGAATAGTGCACCTCTGCTTCTAAAACATTGTTAGAAATCGANNNTTTGACT
11A, 11B, 11C, 11D	11B	TAGTGCAGGCCTGACTTTTGCATCTGCTTTGCTTTTAGCTGCTTGCGACCAATCAGGTTCAGATACAAAAACTTACTCATCAACCTTTAGTGGAAATCCAACTACATTTAACTATCTATT

11A, 11B, 11C, 11D	11C	AGTAAACTCTTCATAATCCTTTTTATTTTTATGAAGATATTGTTTGAAAGATGTGAGTTTCCACGGATGGGTTTGTGGAGGGATATACTTGCGTCTTTCTTTTTTTGTTCTGGTTCTTGTTCAAAGTTTTTCGAATAGAGTTCATGATTTAGTAGCTCCTTTGTGTGATAGATTTTGTCA
9N and 9L	9N	GTTGTTTTCAAAAATCGTNNNAAGTCCCTTTTGTTAGTTAGTGTTTTTGCCTATATTTTCACACTATTTATTCAAACTGATATTTTTATTGTGCAACGGA
9N and 9L	9L	GCTGTTTTCAAAAATCGTAGAATGTTATTTTTGATANNNAGTGTTTTCGCTTATTGTTTAACCCTATTTGTTCAAACAGATATTTATATTATTCAAAGAA
15B and 15C	15B	ATTATAAGATTTGTGATTTTTAATAGGATTTCAGATTTAATAGGCTATCTAGCAACACCGTTGTGGTATTTATTTGCTATAT--TATATATATATATATATCTTTATTTTTCCAATAAAAGACTACTATTGACAAAACGATGGATTTCTA
15B and 15C	15C	ATTATAAGATTTGTGATTTTTAATAGGATTTCAGATTTAATAGGCTATCTAGCAACACCGTTGTGGTATTTATTTGCTATATTATATATATATATATATATCTTTATTTTTCCAATAAAAGACTACTATTGACAAAACGATGGATTTCTA

Table:1 Unique probe ID's and sequences of homologous serotypes

Homologous Reference strains results:

The redesigned custom pneumococcal array identified the homologous reference strains to their respective serotype accurately (Table-2).

S .No	Array no	Specimen	Dye	Homologous Serotype	Detected serotype
1	1_1	Reference Strain	Cy3	6A	6A
2	1_1	Reference Strain	Cy5	15C	15C
3	1_2	Reference Strain	Cy3	6B	6B
4	1_2	Reference Strain	Cy5	22A	22A
5	1_3	Reference Strain	Cy3	10C	10C
6	1_3	Reference Strain	Cy5	22F	22F
7	1_4	Reference Strain	Cy3	10F	10F
8	1_4	Reference Strain	Cy5	32A	32A
9	2_1	Reference Strain	Cy3	11A	11A
10	2_1	Reference Strain	Cy5	32F	32F
11	2_2	Reference Strain	Cy3	11B	11B
12	2_2	Reference Strain	Cy5	33A	33A
13	2_3	Reference Strain	Cy3	12A	12A
14	2_3	Reference Strain	Cy5	33B	33B
15	2_4	Reference Strain	Cy3	15B	15B
16	2_4	Reference Strain	Cy5	46	46
17	1_1	Reference Strain	Cy3	9A	9A
18	1_1	Reference Strain	Cy5	9V	9V
19	1_2	Reference Strain	Cy3	9N	9N
20	1_2	Reference Strain	Cy5	9L	9L
21	1_3	Reference Strain	Cy3	35A	35A
22	1_3	Reference Strain	Cy5	35B	35B
23	1_4	Reference Strain	Cy3	35C	35C
24	1_4	Reference Strain	Cy5	44	44
25	2_1	Reference Strain	Cy5	11D	11D
26	2_1	Reference Strain	Cy3	18C	18C
27	2_2	Reference Strain	Cy5	33F	33F
28	2_2	Reference Strain	Cy3	11C	11C
29	2_3	Reference Strain	Cy5	18F	18F
30	2_3	Reference Strain	Cy3	42	42
31	2_4	Reference Strain	Cy5	12F	12F
32	2_4	Reference Strain	Cy5	19F	19F

Table-2: custom pneumococcal array results- Homologous reference strains

Microarray results for serum samples:

Enriched serum sample processing with Agilent Microarray protocol: The serum samples (#456) hybridized on custom pneumococcal chip were shown strong signal and serotype was identified accurately. (Figure-1). There was no signal observed for species specific probes, indicating absence of other pathogens (listed in custom array designing section) in tested serum samples.

Multiple serotype carriage detection and relative abundance levels quantification

The redesigned custom pneumococcal array identified spiked organisms and the serotypes of pooled reference strains accurately. Also determined relative abundance levels of each pooled serotype. (Table:3).

S. No	Serotype	Spiked serotype/organism	Multiple serotype detection by Microarray	Serotype relative abundance levels quantification
1	19F	1	19F and 1	19F (50%), 1 (50%)
2	6B	13	6B and 13	6B (50%), 13 (50%)
3	19A	3	19A and 3	19A (50%), 3 (50%)
4	18C	5	18C and 5	18C (50%), 5 (50%)
5	23F	13	23F and 13	23F (50%), 13 (50%)
6	6A	3	6A and 3	6A (50%), 3 (50%)
7	1	14	1 and 14	1 (50%), 14 (50%)
8	19A	23F	19A and 23F	19A (50%), 23F (50%)
9	19F	*Moraxella catarrhalis*	*19F and Moraxella catarrhalis*	*19F (50%), Moraxella catarrhalis (50%)*
10	6B	*Pseudomonas aeruginosa*	*6B and Pseudomonas aeruginosa*	*6B (50%), Pseudomonas aeruginosa (50%)*
11	19A	*Streptococcus oralis*	*19A and Streptococcus oralis*	*19A (50%), Streptococcus oralis (50%)*
12	18C	*Neisseria meningitidis*	*18C and Neisseria meningitidis*	*18C (50%), Neisseria meningitidis (50%)*
13	23F	*Klebsiella pneumoniae*	*23F and Klebsiella pneumoniae*	*23F (50%), Klebsiella pneumoniae (50%)*
14	6A	*Mycoplasma pneumoniae*	*6A and Mycoplasma pneumoniae*	*6A (50%), Mycoplasma pneumoniae (50%)*
15	1	*Haemophilus influenzae*	*1 and Haemophilus influenzae*	*1 (50%), Haemophilus influenzae (50%)*
16	19A	*Staphylococcus aureus*	*19A and Staphylococcus aureus*	*19A (50%), Staphylococcus aureus (50%)*

Table-3: Multiple serotype carriage and relative abundance levels quantification results

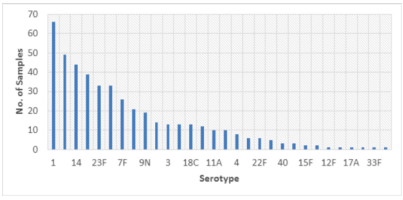

Figure 1: *S. pneumoniae* serotype distribution in 456 serum samples

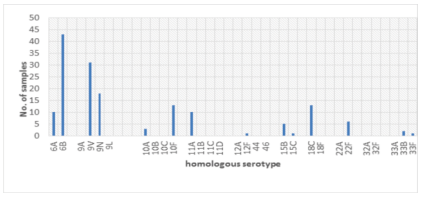

Figure-2: S. pneumoniae homologous Serotype distribution in 456 serum Samples

DISCUSSION

It is implicit in the literature that homologous serotypes/groups **which are similar to each other in sequence but differ in few bases** are difficult to discriminate using DNA-based assays.[13] Out of 90 *S. pneumoniae* serotypes, 29 serotypes shared close homology. They were 6A/6B, 9A/9V/9N/9L, 10A/10B/10C/10F, 11A/11B/11C/11D/18F, 15B/15C, 18C/18F, 22A/22F, 32A/32F, 33A/33B/33F and 12A/12F/44/46. The majority of previously published nucleic acid serotyping methods do not discriminate such serotypes.[11,13, 21-28] In this study, these challenges were addressed by designing unique serotype-specific probes (Table-1) with the help of MUSCLE alignment software and added these probes to custom pneumococcal array. The modification resulted in accurate, identification of homologous serotypes among reference strains (N=32) and serum specimens (N=456) tested in the study. Of the 456 serum samples, Serotype 1 was the most predominant serotype followed by 6B,14,19F and 23F. (Figure-1).
The Homologous serotypes 6A and 6B are most common and difficult to discriminate using DNA-based assays. Previously published nucleic acid serotyping methods failed to discriminate serotype 6A from 6B [11,13,21-28]. In the current study, serotype 6A and 6B were distinguished by identifying a unique sequence of 23bp (GAACATGAAAGATGAAGCAATCAATGTACTGGATAAAGTTAT GAAAAAGA) which resulted in accurate identification of 6A (N=10) and 6B (N=43). The serotype 6B was predominant followed by 9V, 9N and 18C among all homologous serotypes observed in the study. (Figure-2). Serum samples belonging to homologous serogroups 9 (N=49), 10 (N=16), 11 (N=10), 12A/12F/44/46 (N=1), 15 (N=6), 18 (N=13), 22 (N=6), and 33A/33B/33F (N=3) were discriminated accurately to 9V (N=31), 9A (N=0), 9L (N=0), 9N (N=18), 10A (N=3), 10B (N=0), 10C (N=0) and 10F (N=13), 11A (N=10), 11B (N=0), 11C (N=0) and 11D (N=0), 12F (N=1), 15B (N=5), and 15C (N=1), 18C (N=13), 22F (N=6), 33B (N=2), and 33F (N=1) respectively. Our results are in concordance with the recent study based on PCRSeqtyping by Nagaraj et al.[19]

Children are serially and simultaneously colonized in the nasopharynx by more than one *S. pneumoniae* serotypes. Studies about the impact of conjugate pneumococcal vaccines on NP colonization have shown a decrease in vaccine serotype (VT) colonization as well as an increase in nonvaccine serotype (NVT) colonization. The ability to distinguish and quantify between VT and NVT colonization is dependent on the sensitivity of the assay for detecting the carriage of multiple serotypes. Conventional quellung method does not reliably detect multiple serotypes if the second or third serotype is present in a much smaller proportion than the dominant type and is not practical for large pneumococcal vaccine field trials. Other methods, such as Immunoblot (IB) and mouse inoculation, are ooperator dependent, time-consuming, requires a culture step and is expensive. [29,30,31,32,33] In the present study, the redesigned custom pneumococcal array not only detected multiple serotypes from spiked samples and other bacterial pathogens (with 1:1 ratio) but also quantified relative abundance levels of each serotype in pooled samples (Table-3). However, future studies should explore other ratios perhaps 9:1 ratio to know the minor serotype and evaluate complex samples such as nasopharyngeal swabs and sputum. Studies from other groups have developed a low-density microarray which could detect limited numbers of bacterial pathogens.[21,22]

The current method is much more affordable, in terms of per-isolate or per-sample costs as two-colour hybridization approach was adopted for greater throughput (16 samples in single 8*15K array) than existing microarray methods. [21,22] The Microarray assay described herein is a reliable assay for the identification, serotyping, multiple serotype detection and relative abundance quantification of 90 *S. pneumoniae* serotypes from culture and serum specimens.

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