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A NOVEL APPROACH FOR SEROTYPING *S. PNEUMONIAE* USING AN AUTOMATED MICROARRAY ASSAY WITH AUGMENTED UTILITY

Microbiology

Vandana Govindan Msc. Biotechnology, Research scholar (Ph.D.), Central research laboratory, Kempegowda Institute of Medical Sciences, Bangalore-560004

Avid Hussain Shaik Msc. Microbiology, Research Scholar (Ph.D.), Central research laboratory, Kempegowda Institute of Medical sciences, Bangalore-560004

Dr. K L Ravikumar* MD, Microbiology, Chief- Central research laboratory, Kempegowda Institute of Medical Sciences, Bangalore-560004 *Corresponding Author

ABSTRACT

Streptococcus pneumoniae serotype detection is essential to track the local, global spread of pneumococci, estimate disease burden and epidemiology of infection. With quellung reaction, the gold standard test, cross reactions have been reported and unencapsulated strains produce false-negative results. It is time-consuming, subject to interpretation bias, needing an inventory of expensive antisera. Current molecular methods have limitations like amplicon contamination leading to inaccurate identification, inability to discriminate homologous types, false positive results for strains that do not express capsule and is available only in few research centers. The Microarray assay presented here is robust, easy to handle and could identify all known 90 serotypes. Out of 53 isolates representing different serotypes, all were identified accurately and invariably producing results concordant with the conventional serotyping technique. The assay will be useful for the evaluation of serotype prevalence changes after new conjugate vaccines introduction.

KEYWORDS

S. pneumoniae, Serotyping, Microarray

Introduction:

Streptococcus pneumoniae is a human pathogen of major importance causing pneumonia, bacteraemia, and bacterial meningitis with high rates of mortality and long-term morbidity.1An estimated 14.5 million cases of serious pneumococcal disease occur each year in children aged <5 years worldwide. 21% (0.28 million) of the 1.34 million deaths in India is due to pneumonia and 25-40% of these deaths are due to *S. pneumoniae.*[2]

The ability of pneumococci to cause disease is directly related to the production of capsule, a polysaccharide structure external to the cell wall that provides resistance to phagocytosis and promotes evasion of the host immune system by the bacteria. [3] Based on its structural diversity, *S. pneumoniae* is divided into 94 different serotypes.4 To optimize the development of future conjugate vaccines and to evaluate their efficacy, it is necessary to understand the serogroup-specific epidemiology of pneumococci and their associated disease types.5

The "gold standard" for typing of pneumococcus is the capsular reaction test (Neufeld test). As the method is labor intensive, expensive, requires a certain level of experience, it has been restricted for use in specialized reference or research laboratories. [6,7] Several alternative methods to the capsular reaction test, such as coagglutination, counter immune electrophoresis, enzyme-linked immunosorbent assay, Ouchterlony test have been described which are semi quantitative, cross reactive and a number of isolates cannot be typed. Current molecular approaches, such as the PFGE, BOX PCR, VNTR, AFLP and MLST although used, suffer from limitations. These methodologies are technically demanding, and lack inter-laboratory reproducibility.

Automated Microarray assay which is robust and could identify precise serotypes is potentially quicker and easier than established tests. It will be useful in clinical settings for the evaluation of serotype prevalence changes. 8 In this study we describe a rapid, reproducible, user friendly Microarray method to type pneumococci in one step and compare the efficiency of the assay with the standard quellung test.

Materials and Methods:

Bacterial Strains:

A total of 53 isolates (32 Invasive and 21 Nasopharyngeal), retrieved from storage of central research laboratory, KIMS Hospital and research centre were tested.

Conventional Serotyping:

Conventional serotyping was performed by the standard capsular reaction test by using the chessboard system and specific antisera. Briefly, a pure bacterial culture suspension was mixed with group-specific and type-specific antisera (SSI), and the reaction was considered positive when swelling of the capsule or agglutination occurred, as seen under the phase-contrast microscope.[9]

Molecular Serotyping:

i. Probe selection

All capsular Operon is flanked by Dex B and Ali A gene. The region between dex B and the first type specific gene contains the upstream common genes cps A, cpsB, cpsC, cpsD. The proteins encoded by these regions are unique to a given serotype or serogroup and consists of glycosyl transferases, polymerases, transporters and enzymes necessary for the synthesis of nucleotide sugars unique to a given capsule.10 The sequences of the probes for 90 serotypes imprinted on to the microarray chip (8 X 15K) formats were retrieved from Sangers and GitHub. Chips imprinted with the synthesized probes were imported from Agilent Technologies, Santa Clara, U.S.

Table 1: Probe details:

Probes per sequence	9
Length of probe	60 bp
Probe Orientation	Sense / Antisense
No. of Serotypes covered in specific probes	90
No. of genes having Specific probes	1187
No. of genes having Cross-hyb probes	471
No. of genes having no probes designed	22
Total No. of Specific Probes	15150

ii. DNA extraction

The isolates stored in STGG were recultured onto blood agar plates and incubated overnight at 37°C in 5% CO2. Genomic DNA extraction of the isolates was performed using the QIAamp minikit; Qiagen, Hilden, Germany following the manufacturers protocol.

iii. Microarray serotyping

Serotyping was performed on the DNA extracts using BµG@S SP-CPS v1.4.0 Microarray. Oligonucleotide Array – Based CGH for Genomic DNA Analysis by Direct method protocol from Agilent Technologies was followed. The data was analysed using the customized software developed inhouse. The signal and background intensities of each spot were quantified using Agilent Feature extraction software. The in-house model used Microsoft excel for data analysis taking into consideration the dependencies between serotypes induced by genes they have in common, and by homologous genes.

Results:

I. Serotyping results of Invasive isolates by Quellung

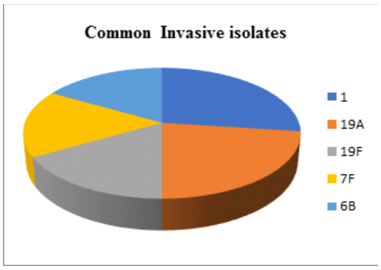

Fig 1: Illustrates the serotype distribution of the invasive isolates tested during the study.

A total of 32 invasive isolates were typed using quellung. Serotype 1 was the most common serotype followed by 19A,19F,7F and 6B.

ii. Serotyping results of Nasopharyngeal isolates by Quellung

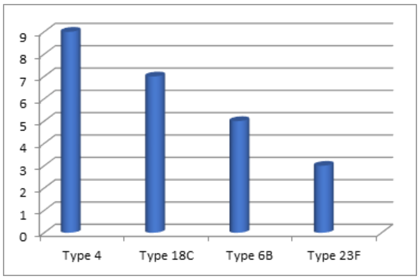

Fig 2: Illustrates the serotype distribution of the Nasopharyngeal isolates tested during the study.

A total of 21 Nasopharyngeal isolates were typed using quellung. Serotype 4 was the most common serotype followed by 18C,6B, and 23F.

Microarray Results:

The Microarray results were in 100% correlation with the Quellung test. All the isolates were accurately identified using the chip.

Discussion:

Serotype determination of Streptococcus pneumoniae provides invaluable insights to analyze clonal spread during epidemics, infection route, and assess evolutionary changes following vaccination. With the introduction of pneumococcal vaccine in the National immunization programme, 2.1 million children will be vaccinated in the first year – 2017.11 Consequently serotype epidemiology becomes essential since serotype replacement is a concern.

The capacity to detect pneumococcal serotypes is limited in large part of the developing world. The current reference standard for serotyping pneumococci is culture followed by a capsular reaction test. Only a few laboratories in the country have a comprehensive, set of typing antisera corresponding to the currently known [92] capsular serotypes. Accurate use of this methodology is particularly challenging and it cannot predict the carriage of multiple serotypes and emerging serovars[12.]

Several molecular typing methods currently used can address many of these issues and has the potential to provide further information. Conventional and sequential PCR based typing assays though employed by many groups, cannot quantify PCR products, detect multiple serotype in a single platform, and differentiate amplicons of similar size .[13,14]

Microarray is a rapid method with high throughput, sensitive in detecting low levels of cDNA per spot. Target-probe hybridization can reduce the detection of non-specific sequences in amplified products. In addition, hybridization increases the use of multiple probes for detection of wide range of serotypes which would decrease false negative results [15]. The assay has the flexibility of incorporating additional primers for the characterization of emerging serotypes. An added advantage of this method is that raw data from experiments can be analysed upon the addition of new entries to the serotyping database. The microarray method features good discriminatory power, reproducibility, and portability, making it suitable for epidemiological studies.

Bentley et al. [16]with Jason Hinds designed a microarray to determine *S. Pneumoniae* serotypes in nasopharyngeal specimens on the basis of serotype-specific oligonucleotides within the cps locus. The array could be used directly on nasopharyngeal specimens, detect multiple serotypes and determine their relative abundance. It could also identify novel serotypes and help to characterize non-typeable *S. pneumoniae* strains. In the study conducted by Kandasamy et al [17]microarray technique in addition to typeable pneumococci, detected non-typeable pneumococci and closely related Mitis-group Streptococci in Nasopharyngeal samples. In a Multi centric study conducted by Satzke et al microarray had the highest sensitivity (95.8%) and high PPV (93.7%) compared to other [20] alternate serotyping methods evaluated in the study. Wang et al.18 described microarray method using wzy and capA genes. Their method could identify 23 vaccine serotypes of *S. pneumoniae* and the other 20 closely related species. Yuka Tomita et al. 19 designed a DNA microarray to identify the 23 *S. pneumoniae* serotypes included in the 23-valent pneumococcal vaccine using GT genes in cps locus. They focused on the GT genes since GTs catalyze the transfer of the sugar moiety to an acceptor and generate a serotype-specific capsular polysaccharide.

An ideal microarray platform for the serotype prediction of Pneumococci should offer reliable, straight forward results without the need for sophisticated equipment and data management. The customized, automated microarray method described here allows fast and reliable differentiation of *S. pneumoniae.* For the assay, capsular serotype-specific genes present in the 20 Kb length CPS region of the 90 serotypes were targeted. It was designed with sufficient redundancy to allow accurate detection of the several variants and polymorphisms of known *S. pneumoniae* population, making it robust. The 100% correlation in the result demonstrated the ability of the assay to detect all serotypes accurately. The results were similar to the study conducted by Satzke et al [20] where microarray could detect all known serotypes from invasive and nasopharyngeal isolates.

To facilitate the preparation of optimally labelled nucleic acids, Cy3 and Cy5 are the fluorescent dyes frequently used in microarray analysis. These two dyes have favourable properties, including relatively high fluorescence quantum yields and a minimal spectral overlap, making them suitable for multiplex detection applications. 21 In our experiment, we modified and standardized the use of dual dye combination in place of monodye. This adaptation enabled us to process 16 samples at a time rather than the recommended 8 samples per chip format. It resulted in added advantage of easier data analysis and cost reduction.

It is implicit in the literature that homologous types which although not identical are similar to each other in sequence, are difficult to discriminate using DNA-based assays. 22 The vast majority of previously published nucleic acid serotyping methods do not discriminate such serotypes. In our protocol, these challenges were addressed by modifying the assay design, addition of specific probes and use of customized software. These changes resulted in accurate, specific identification of homologous types.

Conclusion:

Microarray with its ability to detect all the 90 pneumococcal serotypes uniquely is a novel platform that can be adopted for molecular epidemiological surveillance. Our technique provides the proof of concept of its utility.

References:

1. L M HallApplication of molecular typing to the epidemiology of *Streptococcus pneumoniae.* J Clin Pathol. 1998; 51: 270–274.
2. Watson, D. A. & Musher, D. M. A brief history of the pneumococcus in biomedical research. Semin. Respir. Infect. 1999; 14:198–208.
3. Levels and trends in child mortality. Reports 2014. Estimates developed by UN inter agency group for child mortality estimation.
4. Alonso DeVelasco, E., D. Merkus, S. Anderton, A. F. M. Verheul, E. F. Lizzio, R. van der Zee, W. van Eden, T. Hoffman, J. Verhoef, and H. Snippe. Synthetic peptides representing T-cell epitopes act as carriers in pneumococcal polysaccharide conjugate vaccines. Infect. Immun. 1995; 63:961–968.
5. Alonso DeVelasco, A. F. M. Verheul, A. M. P. Van Steijn, H. A. T. Dekker, R. G. Feldman, I. M. Fernandez et al. Epitope specificity of rabbit immunoglobulin G (IgG) elicited by pneumococcal type 23F synthetic oligosaccharide- and native

polysaccharide-protein conjugate vaccines: comparison with human anti-polysaccharide 23F IgG. Infect. Immun. 1994; 62:799– 808.

6. Lafong, A. C., and E. Crothers. Simple latex agglutination method for typing pneumococci. J. Clin. Pathol. 1988; 41:230–231.
7. Brito, D. A., M. Ramirez, and H. de Lencastre. Serotyping *Streptococcus pneumoniae* by multiplex PCR. J. Clin. Microbiol. 2003; 41:2378–2384.
8. Raymond F, Boucher N, Allary R, Robitaille L, Lefebvre B, Tremblay C, et al. Serotyping of *Streptococcus pneumoniae* Based on Capsular Genes Polymorphisms. PLoS ONE 8(9): e76197. journal. pone. 2013; 00761
9. Sorensen, U. B. Typing of pneumococci by using 12 pooled antisera. J. Clin. Microbiol. 1993; 31:2097-2100.
10. Janet Yother, Capsules. The Pneumococcus. 2004. ASM Press, Washington, D.C.
11. Anupam Sachdeva et al. Pneumococcal Conjugate Vaccine Introduction in India's Universal Immunization Program. Indian Paediatrics :2017; 445 Volume 54
12. Liu KH, Tong M, Xie ST and Yee NV. Genetic Programming Based Ensemble System for Microarray Data Classification. Comput Math Methods Med.2015;1-11
13. Malorny B, Bunge C, Guerra B, Prietz S, Helmuth R. Molecular characterisation of Salmonella strains by an Oligonucleotide multiprobe microarray. Mol Cell probes.2007;21(1):56-65.
14. Marimon JM, Monasterio A, Ercibengoa M, Pascual J, Prieto I, Simón L. Antibody microarray typing, a novel technique for Streptococcus pneumoniae serotyping. J Microbiol Methods. 2010;8(13):274-280.
15. Waggoner JJ, Abeynayake J, Sahoo MK, Gresh L, Tellez Y, Gonzalez K. Single-Reaction, Multiplex, Real-Time RT-PCR for the Detection, Quantitation, and Serotyping of Dengue Viruses. PLoS neglected tropical diseases. 2013;7(4).
16. Bentley SD, Aanensen DM, Mavroidi A et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. 2006; 2: e31.
17. Kandasamy R, Gurung M, Thapa A, Ndimah S, Adhikari N, Murdoch D et al. Multi-Serotype Pneumococcal Nasopharyngeal Carriage Prevalence in Vaccine Naïve Nepalese Children, Assessed Using Molecular Serotyping. https://doi.org/10.1371/journal.pone.0114286
18. Wang Q, Wang M, Kong F, Gilbert GL, Cao B, Wang L, Feng L. 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. 2007; 68:128–136.
19. Tomita Y, Okamoto A, Yamada K, Yagi T, Hasegawa Y, Ohta M. 2011. A new microarray system to detect *Streptococcus pneumoniae* serotypes. J Biomed Biotechnol. 2011:352736
20. Satzke C, Dunne EM, Porter BD, Klugman KP, Mulholland EK and Pneumocarriage project group. The Pneumo carriage project: A Multicenter Comparative Study to Identify the Best Serotyping Methods for Examining Pneumococcal Carriage in vaccine Evaluation Studies.PLoSMed.2015; 12: e1001903.
    Moreira BG
21. You Y, Owczarzy R . Cy3 and Cy5 dyes attached to oligonucleotide terminus stabilize DNA duplexes: predictive thermodynamic model. Biophys Chem. 2015; 198:36-44
22. Gonzalez TB, Rivera-Olivero IA, Sisco MC, Spadola E, Hermans PW, de Waard JH.
    PCR deduction of invasive and colonizing pneumococcal serotypes from Venezuela:
    a critical appraisal. The Journal of Infection in Developing Countries. 2014;8(4):469-473.