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Serotyping and Multiple Carriage Detection of *S. pneumoniae* in Nasopharyngeal Samples-Multiplex PCR Fluorescent Assisted Fragment Analysis Typing

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Abstract

Background: Continuous monitoring of serotypes is essential for epidemiological surveillance and long-term vaccine impact studies. With the challenges of Quellung serotyping, PCR-based serotype prediction is increasingly being used for large-scale epidemiological studies. This study aimed to serotype *S. pneumoniae* directly from nasopharyngeal (NP) swabs using a high-throughput PCR-based molecular assay.

Methods: In the first step, *S. pneumoniae* identification was done with quantitative multiplex real-time PCR (qmPCR) using pneumolysin (ply), autolysin (lytA) and pneumococcal surface adhesin A (psaA) primers. Subsequent amplification of cps loci was performed in a single multiplex PCR reaction with 40 fluorescent labeled primers (www.cdc.gov) for qmPCR positive samples. cpsA gene common to all pneumococcal serotypes is used as internal control. Serotyping was achieved by sizing the PCR products on ABI PRISM 3130xl Genetic Analyzer. Sixty eight standard strains (SSI, Denmark), twenty five invasive isolates, 108 nasopharyngeal isolates of *S. pneumoniae* and 325 qmPCR positive nasopharyngeal swabs were analyzed with conventional Quellung reaction and multiplex PCR - Fluorescent assisted fragment analysis (mPCR-FAF).

Results: mPCR-FAF assay could accurately identify serotype/s of standard strains and *S. pneumoniae* isolates. The results showed 100% concordance with quellung test. The assay was able to deduce serotype/s of qmPCR positive nasopharyngeal swabs. Multiple serotype carriage was observed in thirty six nasopharyngeal swabs.

Conclusion: The study confirms the usefulness of mPCR-FAF typing for serotyping of *S. pneumoniae* from culture and culture negative samples. An added advantage was the detection and typing of multiple serotypes. The use of this simple and high throughput protocol in pneumococcal surveillance can increase the accuracy of identification.

Keywords: *Serotyping; S. pneumoniae; Nasopharyngeal Samples; PCR Fluorescent Assisted Fragment*

Abbreviations

qmPCR: Quantitative Multiplex PCR; PCR: Polymerase Chain Reaction; SSI: Staten Serum Institute; mPCR-FAF: Multiplex PCR - Fluorescence Assisted Fragment Analysis; DNA: Deoxy Ribo Nucleic Acid; PCV13: Pneumococcal Conjugate Vaccine 13; CFU: Colony Forming Unit; STGG: Skim Milk, Tryptone, Glucose, and Glycerol; CDC: Centers for Disease Control and Prevention; CPS: Capsular Polysaccharide; STG: Serotype/Group; NT: Non Typeable

Introduction

Streptococcus pneumoniae, is a part of the normal bacterial flora of the human upper respiratory tract, but can occasionally infiltrate sterile sites of the body progressing to disease. The youngest and the elderly are those most prone to invasive pneumococcal infections, such as severe blood infection, meningitis and pneumonia. An estimated 1.6 million deaths associated with *S. pneumoniae* are reported every year worldwide, mostly affecting children under five years [1].

The pneumococcal polysaccharide capsule has long been recognized as the most important virulence factor of *S. pneumoniae* [2]. To date more than 93 pneumococcal types have been identified, each with unique structural differences in capsular polysaccharides [3]. Serotypes associated with invasive disease and nasopharyngeal carriage vary depending on clinical presentations and severities [4]. Widespread use of pneumococcal vaccines, has led to replacement with non-vaccine serotypes [5]. Continuous monitoring of serotypes is therefore, essential for epidemiological surveillance and long-term vaccine impact studies [6-10]. In addition, it aids in understanding pathogenicity of the organism and emergence of non-vaccine strains [11,12].

Carriage of pneumococci without symptoms in the nose of young children is common and plays a key role in pneumococcal transmission within the community [22]. Although a single serotype usually predominates at any given time, there is ample evidence for the carriage of multiple serotypes [23]. Multiple carriage is reported to promote genetic recombination, characterized by the acquisition of genetic elements from other microbes through transformation, transduction or conjugative transfer [24]. Given the pneumococcus is highly transformable and undergoes genetic recombination through horizontal gene transfer [25], recombination at the CPS locus may result in a change in serotype (capsule switching) [26] which could lead to vaccine escape [27]. Therefore, predicting genetic recombination and capsule switching in the context with vaccine-escape are necessary to understand long-term public health of pneumococcal vaccination [28].

Conventional pneumococcal serotyping relies on the gold standard "Quellung reaction" [13]. Requirement of viable pneumococci, high cost of antisera, technical expertise and subjectivity [14], restricts its use to a few central/ reference laboratories. Culture techniques do not reliably detect multiple serotypes present in nasopharynx if the second or third serotype is present in a much smaller proportion than the dominant type [29]. The World Health Organization (WHO) has highlighted the importance of developing more sensitive techniques for measuring carriage of multiple pneumococcal serotypes [31,32].

Molecular methods to deduce *S. pneumoniae* serotypes such as conventional sequential multiplex, real time multiplex PCR, sequencing and microarray are being used as they are highly sensitive compared with culture, can be applied to various specimen types and are not dependent on the growth of viable bacteria. The PCR-based assays detected serotypes in single or 3 - 8 multiplex PCRs or sequentially, real-time multiplex PCR, or the combination of conventional and real-time PCR. By use of multiplex PCR combined with fragment analysis using automated fluorescent capillary electrophoresis (FAF-mPCR), Lawrence., *et al.* [55] detected 5 serotypes and 3 serogroups, Selva., *et al.* [33] identified 68 serotypes in three different simultaneous multiplex PCR reactions and Marimon., *et al*. [54] could detect 92 serotypes in a single multiplex PCR assay. In this study, we aimed to evaluate the feasibility of using mPCR-FAF assay for serotyping pneumococci directly in nasopharyngeal (NP) swabs.

Materials and Methods

Bacterial isolates and swabs

Bacterial strains

Sixty eight reference strains of *S. pneumoniae* were obtained from SSI, Denmark. Twenty five Invasive and one hundred and eight NP isolates were sourced from Central Research Laboratory, KIMS Hospital, Bangalore (Table 1).

Sl. NO	Serogroup	Serotype	NCBI Accession No	mPCR-FAF Typing results
				Serotype
1	1	1	CR931632	1
2	2	2	CR931632	2
3	3	3	CR931634	3
4	4	4	CR931635	4
5	5	5	CR931637	5
6	GP6	6A	CR931638	GP6
7		6B	CR931639	GP6
8		6C	EF538714	GP6
9	GP7	7F	CR931643	7F/7A
10		7A	CR931640	7F/7A
11		7B	CR931641	7BC/40
12		7C	CR931642	7BC/41
13	8	8	CR931644	8
14	GP9	9A	CR931645	9V/A
15		9L	CR931646	9N/L
16		9N	CR931647	9N/L
17		9V	CR931648	9V/A
18	GP10	10F	CR931652	10F/10C/33C
19		10A	CR931649	10A
20		10C	CR931651	10F/10C/33C
21	GP11	11A	CR931653	11AD
22		11D	CR931656	11AD
23	GP12	12F	CR931660	12F/12A/12B/44/46
24		12A	CR931658	12F/12A/12B/44/46
25		12B	CR931659	12F/12A/12B/44/46
26	13	13	CR931661	13
27	14	14	CR931662	14
28	GP15	15F	CR931666	15A/15F
29		15A	CR931663	15A/15F
30		15B	CR931664	15B/15C
31		15C	CR931665	15B/15C
32	16	16F	CR931668	16F
33	17	17F	CR931670	17F

34	GP18	18F	CR931674	18GP
35		18A	CR931671	18GP
36		18B	CR931672	18GP
37		18C	CR931673	18GP
38	GP19	19F	CR931678	19F
39		19A	CR931675	19A
40	20	20	CR931679	20
41	21	21	CR931680	21
42	GP22	22F	CR931682	22FA
43		22A	CR931681	22FA
44	GP23	23F	CR931685	23F
45		23A	CR931683	23A
46		23B	CR931684	23B
47	GP24	24F	CR931688	24F/24A/24B
48		24A	CR931686	24F/24A/24B
49		24B	CR931687	24F/24A/24B
50	GP25	25F	CR931690	38/25F/25A
51		25A	CR931689	38/25F/25A
52	31	31	CR931695	31
53	GP33	33F	CR931702	33F/33A/37
54		33A	CR931698	33F/33A/37
55		33C	CR931700	10F/10C/33C
56	34	34	CR931703	34
57	GP35	35F	CR931707	35F/47F
58		35A	CR931704	35A/35C/42
59		35B	CR931705	35B
60		35C	CR931706	35A/35C/42
61	37	37	CR931709	33F/33A/37
62	38	38	CR931710	38/25F/25A
63	39	39	CR931711	39
64	40	40	CR931712	7BC/40
65	42	42	CR931715	35A/35C/42
66	44	44	CR931717	12F/12A/12B/44/46
67	46	46	CR931719	12F/12A/12B/44/46
68	47	47F	CR931721	35F/47F

Table 1: *mPCR-FAF typing results for 68 Pneumococcal reference strains.*

Nasopharyngeal (NP) swabs

S. pneumoniae qmPCR positive NP swabs (n = 325) stored in 1ml of STGG, collected from adults aged 25 - 85 yrs using nylon swabs (Copan, Germany) were sourced from Central Research laboratory, KIMS, Bangalore

Culture and characterization

S. pneumoniae was isolated by culturing on 5% Sheep Blood agar (Chromogen, Hyderabad) for 18 - 24 hrs at 37°C with 5% CO_2. The isolates were characterized by colony morphology, alpha hemolysis, bile solubility and optochin susceptibility.

Serotyping

Quellung reaction was performed using Pneumotest kit and type-specific antisera (SSI, Denmark) as recommended by the manufacturer.

mPCR-FAF Typing

DNA extraction

Genomic DNA was extracted from bacterial strains using QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's protocol.

DNA extraction from NP swab

The NP swabs stored in STGG at -80°C were thawed to room temperature (22°C) and vortexed for 15 seconds. Thereafter, 200 µ L of sample was subjected to automated total nucleic acid extraction on the QIAcube instrument (Qiagen, Hilden, Germany) using QIAamp® DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Total nucleic acid was eluted in 50 µ L elution buffer and stored at -20°C until processing.

Multiplex PCR -fluorescent assisted fragment analysis

Primers

Forty primer sets targeting seventy serotypes whose sequences are detailed in the CDC web page (http://www.cdc.gov/ncidod/biotech/strep/pcr.html) were used. Twenty two primer sets targeting non-homologous types, seventeen primer sets targeting forty eight homologous groups and primers specific to the *cpsA* (*wzg*) gene were used in the reaction. Forward primers sourced from Cgix India were labeled with 6-carboxyfluorescein (FAM), 2'-chloro-7'-Phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) and 2'-chloro-5'-fluoro-7',8'-benzo-1,4-dichloro-6-carboxyfluorescein (NED). Distinct fluorophores were used to obtain good discrimination of products (Table 2).

Sl.no	Serotype	Homologous (H)/ Non-homologous (NH)		Primers	Target gene	Size	Dye
1	1	NH	FP	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	wzy	280	FAM
			RP	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C			
2	2	NH	FP	TAT CCC AGT TCA ATA TTT CTC CAC TAC ACC	wzy	290	NED
			RP	ACA CAA AAT ATA GGC AGA GAG AGA CTA CT			
3	3	NH	FP	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	galU	371	FAM

			RP	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G			
4	4	NH	FP	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	wzy	430	FAM
			RP	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G			
5	5	NH	FP	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	wzy	362	HEX
			RP	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG			
6	6ABCD	H	FP	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	wciP	250	HEX
			RP	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA			
7	7BC/40	H	FP	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	wcw L	260	FAM
			RP	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC			
8	7FA	H	FP	TCC AAA CTA TTA CAG TGG GAA TTA CGG	wzy	599	FAM
			RP	ATA GGA ATT GAG ATT GCC AAA GCG AC			
9	8	NH	FP	GAA GAA ACG AAA CTG TCA GAG CAT TTA CAT	wzy	201	HEX
			RP	CTA TAG ATA CTA GTA GAG CTG TTC TAG TCT			
10	9NL	H	FP	GAA CTG AAT AAG TCA GAT TTA	wzx	516	HEX
				ATC AGC			
			RP	ACC AAG ATC TGA CGG GCT AAT CAA T			
11	9VA	H	FP	GGG TTC AAA G TC AGA CAG TG A ATC TTA A	wzy	816	FAM
			RP	CCA TGA ATG A AA TCA ACA TT G TCA GTA GC			
12	10A	NH	FP	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	wcr G	628	FAM

			RP	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C			
13	10F/10C/ 33C	H	FP	GGA GTT TAT CGG TAG TGC TCA TTT TAG CA	wzx	248	FAM
			RP	CTA ACA AAT TCG CAA CAC GAG GCA ACA			
14	11AD	H	FP	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	wzy	463	FAM
			RP	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC			
15	12F/12A /12B/44 /46	H	FP	GCA ACA AAC GGC GTG AAA GTA GTT G	wzx	376	HEX
			RP	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC			
16	13	NH	FP	TAC TAA GGT AAT CTC TGG AAA TCG AAA GG	wzx	655	HEX
			RP	CTC ATG CAT TTT ATT AAC CG C TTT TTG TTC			
17	14	NH	FP	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT	wzy	189	FAM
			RP	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT			
18	15A/15F	H	FP	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	wzy	434	HEX
			RP	GAT CTA GTG AAC GTA CTA TTC CAA AC			
19	15B/15C	H	FP	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	wzy	496	HEX
			RP	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C			
20	16F	NH	FP	GAA TTT TTC AGG CGT GGG TGT	wzy	717	NED
				TAA AAG			
			RP	CAG CAT ATA GCA CCG CTA AGC AAA TA			
21	17F	NH	FP	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	wci P	693	HEX

			RP	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC			
22	18GP	H	FP	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	wzy	573	HEX
			RP	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC			
23	19A	NH	FP	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	wzy	566	FAM
			RP	CAT AAT AGC TAC AAA TGA CTC ATC GCC			
24	19F	NH	FP	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	wzy	304	FAM
			RP	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG			
25	20	NH	FP	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	wci L	514	FAM
			RP	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC			
26	21	NH	FP	CTA TGG TTA TTT CAA CTC AAT CGT CAC C	wzx	192	HEX
			RP	GGC AAA CTC AGA CAT AGT ATA GCA TAG			
27	22FA	H	FP	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	wcw V	643	FAM
			RP	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC			
28	23A	NH	FP	TAT TCT AGC AAG TGA CGA AGA TGC G	wzy	722	HEX
			RP	CCA ACA TGC TTA AAA ACG CTG CTT TAC			
29	23B	NH	FP	CCA CAA TTA G CG CTA TAT TCA TTC AAT CG	wzx	199	FAM
			RP	GTC CAC GCT GAA TAA AAT GAA GCT CCG			

30	23F	NH	FP	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	wzy	384	FAM
			RP	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC			
31	24F/24A /24B	H	FP	GCT CCC TGC TAT TGT AAT CTT TAA AGA G	wzy	99	FAM
			RP	GTG TCT TTT ATT GAC TTT ATC ATA GGT CGG			
32	31	NH	FP	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC	wzy	701	FAM
			RP	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC			
33	33F/33A /37	H	FP	GAA GGC AAT CAA TGT GAT TGT GTC GCG	wzy	338	FAM
			RP	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C			
34	34	NH	FP	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	wzy	408	HEX
			RP	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC			
35	35A/35C /42	H	FP	ATT ACG ACT CCT TAT GTG ACG CGC ATA	wzx	280	HEX
			RP	CCA ATC CCA AGA TAT ATG CAA CTA GGT T			
36	35B	NH	FP	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	wcr H	677	FAM
			RP	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G			
37	35F/47F	H	FP	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A	wzy	517	NED
			RP	GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC			
38	38/25F/ 25A	H	FP	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG	wzy	574	NED

			RP	ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC			
39	39	NH	FP	TCA TTG TAT TAA CCC TAT GCT TTA TTG GTG	wzy	98	HEX
			RP	GAG TAT CTC CAT TGT ATT GAA ATC TAC CAA			
40	cpsA	Internal control	FP	GCA GTA CAG CAG TTT GTT GGA CTG ACC	wzg	160	HEX
			RP	GAATATTTTCATTATCAGTCCCAG TC			

Table 2: *Primers used in mPCR-FAF typing assay.*

Multiplex PCR

Multiplex PCR reaction was performed as described by Marimon., *et al.* [54] with modifications. The reaction mix contained 1X Multiplex PCR master mix (Qiagen Multiplex PCR Plus Kit), 1X primer mix (2 µM each primer, 40 sets of fluorescent primers), ~ 50 ng Template DNA and the final reaction volume was made to 50 µl with milliQ water. Thermal cycling was carried out in GeneAmp PCR system 9700 (Applied Biosystems) with the conditions: 95°C for 5 minutes, followed by 40 amplification cycles of 95°C for 30s, 58°C for 90s, and 72°C for 90 s, and a final extension step at 68°C for 10 min as recommended for the use of Qiagen kit.

Automated fluorescence based capillary electrophoresis

Mixture of 1.0 µl of multiplex PCR product, 0.1 µl of sizing standard LIZ1200 (Applied Biosystems) and 9.0 µl of Hi-Di Formamide was denatured for 5 minutes at 95°C and rapidly cooled on ice before capillary electrophoresis (Manufacturer's protocol). Fluorescent fragment size analysis was performed on ABI 3730 XL genetic analyzer set up according to the manufacturer's instructions (Applied Biosystems). Data was collected with ABI3730 XL data collection software (version 3.0) and interpreted using GeneMapper software (version 4.0).

Optimization of mPCR-FAF primers for cross reactivity

Single PCR reactions were performed with serotype/s specific primer and respective reference strain (SSI, Denmark). Multiplex PCR reaction was performed with pools of different serotypes combination for checking the cross reactivity.

Blinded testing

Reference strains (n = 68) were coded and subjected to mPCR-FAF typing protocol.

Results

Reference strains

PCR amplification with serogroup/type primer was observed in sixty eight pneumococcal reference strains tested with mPCR-FAF typing protocol. Twenty two non-homologous strains were assigned to their respective serotype. Forty six strains were identified along with the homologous serotypes. Serotypes assigned by mPCR-FAF typing were concordant with quellung test results (Table 1). Blinded testing of the coded reference strains (n = 68) by mPCR-FAF and quellung typing methods provided similar results.

S. pneumoniae isolates

Twenty five Invasive and one hundred and eight nasopharyngeal pneumococcal isolates were evaluated with mPCR-FAF test and quellung reaction. mPCR-FAF typing data for the isolates were in concurrence with Quellung data (Table 3).

				mPCR-FAF typing	Quellung Test
Sl.no	Sample	Source	Clinical manifestation	Serotype	Serotype
1	CRL01	Blood	Bacteraemia	3	3
2	CRL02	Blood	Bacteraemia	1	1
3	CRL03	Blood	Bacteraemia	6AB	6B
4	CRL04	Blood	Bacteraemia	15BC	15B
5	CRL05	CSF	Meningitis	7FA	7F
6	CRL06	CSF	Meningitis	9VA	9V
7	CRL07	CSF	Meningitis	1	1
8	CRL08	Blood	Bacteraemia	8	8
9	CRL09	CSF	Meningitis	15BC	15B
10	CRL10	Blood	Bacteraemia	4	4
11	CRL11	Blood	Bacteraemia	1	1
12	CRL12	Blood	Bacteraemia	7BC/40	7B
13	CRL13	Blood	Bacteraemia	1	1
14	CRL14	Blood	Bacteraemia	10AF	10A
15	CRL15	CSF	Meningitis	1	1
16	CRL16	Blood	Bacteraemia	11AD	11A
17	CRL17	Blood	Bacteraemia	14	14
18	CRL18	Blood	Bacteraemia	14	14
19	CRL19	Blood	Bacteraemia	14	14
20	CRL20	Blood	Bacteraemia	20	20
21	CRL21	Blood	Bacteraemia	19F	19F
22	CRL22	Blood	Bacteraemia	15BC	15B
23	CRL23	Blood	Bacteraemia	1	1
24	CRL24	Blood	Bacteraemia	18GP	18C
25	CRL25	Plural Fluid	PF	19F	19F

Table 3: *Serotype data of S. pneumoniae invasive isolates.*

Nasopharyngeal swab

For the study, a total of 325 *S. pneumoniae* qmPCR positive NP swabs were subjected to culture and mPCR-FAF typing. Among 325 NP swabs, 108 were culture positive and 217 were culture negative. mPCR-FAF typing assay assigned serotype for 209 NP swabs and 8 samples were non-typeable. The mPCR-FAF results of 108 culture positive swabs were in complete agreement with quellung test result. Multiple serotype carriage was observed in 39 culture negative NP swabs (2 types in 33, 3 types in 6).

Discussion

Serotype surveillance of *S. pneumoniae* is critical to monitor pneumococcal vaccine efficacy, improve vaccine formulations, to know emerging serovars and changes in the sero-epidemiology. Presently, serotyping of pneumococci is dependent on isolation of the organism followed by serological determination by Quellung reaction [34]. The high cost of antisera, the requirements for technical skills and the complexity in interpretation of results are major hindrances of this procedure, thus further limiting its use in resource poor environments [35]. Because of difficulties in typing, our knowledge of pneumococcal serotype prevalence is limited to selected geographical areas and is deficient in a large part of the world. The areas with limited studies may have different serotype distribution harboring unusual serotypes [36].

The majority of epidemiological studies on pneumococcal disease are confined to Invasive pneumococcal diseases in Child populations. Given that pneumococcal disease is preceded by nasopharyngeal colonization [37], it is relevant to study the nasopharyngeal carriage patterns, however little data is available from developing and underdeveloped countries. Additionally, the measurement of pneumococcal carriage in the nasopharyngeal reservoir is subject to potential confounders that include low-density and multiple-strain colonization. Detection of simultaneous carriage with more than one serotype using standard culture methods is severely limited [30], yet detection of all pneumococcal serotypes present in respiratory specimens is critical for the study of the epidemiology of pneumococcal colonization [38].

With the limitations of conventional typing method, there is renewed interest in developing accurate and efficient systems for pneumococcal capsular typing [5]. An ideal serotyping system should not only be simple to perform but should also cover all pneumococcal serotypes [36]. The ability to test for all known serotypes is important, because the serotype prevalence not only differs due to vaccine-induced serotype shifts but also differs among different regions [39] or over time [40]. Though the existence of > 90 different capsular types makes it difficult to develop a typing scheme based on genetic approaches, the recent availability of sequence information of all serotypes (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/) [41] has provided an opportunity to develop a simple sequence-based scheme for typing *S. pneumoniae.*

In 2006, Centers for Disease Control and Prevention (CDC), United States, established a sequential multiplex PCR protocol for deducing 28 serotypes in seven sequential multiplex PCR scheme for the identification of pneumococcal serotypes that can be applied to clinical or research use [5]. On the basis of this method, several groups developed modified PCR protocols based on the regional serotype prevalence, to cover upto 35 serogroup/serotypes in 5 - 8 sequential multiplex PCR formats [13,21,42-44]. In 2012, Coskun-Ari., *et al.* [45] described a single multiplex PCR containing 12 primers, targeting PCV13-serogroup/serotypes. Serotype identification in these assays was performed on the basis of size-separation of PCR products on agarose gel. Lack of sensitivity and poor resolution of agarose gel limits its use to distinguish closely sized PCR products. Advances in PCR, capillary electrophoresis and laser-induced fluorescence allows the visualization of the PCR products to 1bp accuracy much below the visualization thresholds of conventional agarose/acrylamide gel [46]. In addition, the use of automated DNA sequencers has enabled a high-throughput analysis. With the use of three simultaneous multiplex PCR reactions and Fluorescence assisted fragment analysis in their assay, Selva*., et al.* [34] could analyze 70 pneumococcal serogroups/types. The primers used in the work of Selva et al were those available on the CDC website (http://www.cdc.gov/streplab/downloads/pcr-oligonucleotide-primers.pdf). Several research groups [47-51] have published their results using FAF assay. The constraint of the FAF protocol was the need to use minimum three different simultaneous multiplex PCR reactions. Marimon., *et al.* [54] developed a single-tube multiplex PCR assay with 55 fluorescently labeled pairs of primers to identify 92 capsular serotypes. Marimon., et al. designed their own set of primers and labeled with three different dyes (FAM, HEX, NED).

In the present study (mPCR-FAF Typing), with the use of 40 primer pairs (available on CDC website) in single Multiplex PCR plus reaction, 70 pneumococcal serogroups/types were identified using automated fluorescence assisted fragment analysis. The concentrations of the primers were adjusted to achieve similar levels of amplification of the products with the same amplification conditions and to avoid non-specific products in the reaction. The protocol was evaluated with reference strains, S. pneumoniae isolates and nasopharyngeal specimens. 100% concordance in the results of mPCR-FAF Typing and Quellung testing was observed for the reference strains and isolates. The added advantage of the assay lies in its ability to deduce serotype/s in culture negative qmPCR positive NP swabs and detect multiple carriage.

The limitation of the method was its inability to determine all known serotypes (92 serotypes) and differentiate homologous types due to the absence of type specific-PCR primers. As the sequences of the cps loci from all of the known 92 pneumococcal serotypes have been completed [41], we have the opportunity to develop and evaluate the new primers to address the issue. Failure in the reactions might be problematic in nasopharyngeal swabs which could be due to one or more of the following factors: i) insufficient PCR template concentration, ii) presence of residual inhibitory factors, and/or iii) uncommon serotypes for which primers are not yet designed and thus omitted from the multiplex PCR [52]. Recently, Carvalho., *et al.* have reported Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract specimens which may interfere in typing [53] directly from nasopharyngeal specimens, which needs to be addressed.

Conclusion

The study demonstrates the usefulness of mPCR-FAF protocol for detection and serotyping of *S. pneumoniae* from isolates and PCR positive culture negative specimens. The method is simple, less labor-intensive and detects multiple carriage.

Bibliography

1. Mobegi FM., *et al*. "Deciphering the distance to antibiotic resistance for the pneumococcus using genome sequencing data". *Scientific Reports* 7 (2017): 42808.

2. AlonsoDeVelasco E., *et al*. "Streptococcus pneumoniae: virulence factors, pathogenesis, and vaccines". *Microbiological Reviews* 59.4 (1995): 591-305.

3. Leung MH., *et al*. "Sequetyping: Serotyping Streptococcus pneumoniae by a Single PCR Sequencing Strategy". *Journal of Clinical Microbiology* 50.7 (2012): 2419-2427.

4. Alanee SRJ., *et al*. "Association of Serotypes of Streptococcus pneumoniae with Disease Severity and Outcome in Adults: An International Study". *Clinical Infectious Diseases* 45.1 (2007): 46-51.

5. Gladstone RA., *et al*. "Continued control of pneumococcal disease in the UK - the impact of vaccination". *Journal of Medical Microbiology* 60.1 (2011): 1-8.

6. Hausdorff WP., *et al*. "Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I". *Clinical Infectious Diseases* 30.1 (2000): 100-121.

7. Johnson HL., *et al*. "Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project". *PLoS Medicine* 7.10 (2010): e1000348.

8. Weinberger DM., *et al*. "Serotype replacement in disease after pneumococcal vaccination". *Lancet* 378.9807 (2011): 1962-1973.

9. Obaro SK., *et al*. "Carriage of pneumococci after pneumococcal vaccination". *Lancet* 348.9022 (1998): 271-272.

10. Habib M., *et al*. "Capsular Serotyping of Streptococcus pneumoniae using the Quellung Reaction". *Journal of Visualized Experiments* 84 (2014): e51208.

11. Pai R., *et al*. "Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates". *Journal of Clinical Microbiology* 44.1 (2006): 124-131.

12. Azzari C., *et al*. "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". *Journal of Medical Microbiology* 57.10 (2008): 1205-1212.

13. Jourdain S., *et al*. "Sequential multiplex PCR assay for determining capsular serotypes of colonizing S. pneumoniae". *BMC Infectious Diseases* 11 (2011): 100.

14. Richter SS., *et al*. "Evaluation of Pneumococcal Serotyping by Multiplex PCR and Quellung Reactions". *Journal of Clinical Microbiology* 51.12 (2013): 4193-4195.

15. Slinger R., *et al*. "Direct Streptococcus pneumoniae real-time PCR serotyping from pediatric parapneumonic effusions". *BMC Pediatrics* 14 (2014): 189.

16. Nagaraj G., *et al*. "Development of PCRSeqTyping-a novel molecular assay for typing of Streptococcus pneumoniae". *Pneumonia* 9 (2017): 8.

17. Yuka Tomita., *et al*. "A New Microarray System to Detect Streptococcus pneumoniae Serotypes". *Journal of Biomedicine and Biotechnology* (2011): 352736.

18. Ziane H., *et al*. "Capsular typing of Streptococcus pneumoniae isolated in an Algerian hospital using a new multiplex PCR-based scheme". *Journal of Microbiological Methods* 119 (2015): 243-246.

19. Miernyk K., *et al*. "Serotyping of Streptococcus pneumoniae Isolates from Nasopharyngeal Samples: Use of an Algorithm Combining Microbiologic, Serologic, and Sequential Multiplex PCR Techniques". *Journal of Clinical Microbiology* 49.9 (2011): 3209-3214.

20. Veeraraghavan B., *et al*. "Customized sequential multiplex PCR for accurate and early determination of invasive pneumococcal serotypes found in India". *Journal of Microbiological Methods* 130 (2016): 133-135.

21. Antonio M., *et al*. "Evaluation of sequential multiplex PCR for direct detection of multiple serotypes of Streptococcus pneumoniae from nasopharyngeal secretions". *Journal of Medical Microbiology* 58.3 (2009): 296-302.

22. Bogaert D., *et al*. "Streptococcus pneumoniae colonisation: the key to pneumococcal disease". *Lancet Infectious Diseases* 4.3 (2004): 144-154.

23. Gray BM., *et al*. "Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life". *Journal of Infectious Diseases* 142.6 (1980): 923-933.

24. Thomas CM and Nielsen KM. "Mechanisms of and barriers to, horizontal gene transfer between bacteria". *Nature Reviews Microbiology* 3.9 (2005): 711-721.

25. Hanage WP., *et al*. "Hyper-recombination, diversity, and antibiotic resistance in pneumococcus". *Science* 324.5933 (2009): 1454-1457.

26. Chewapreecha C., *et al*. "Dense genomic sampling identifies highways of pneumococcal recombination". *Nature Genetics* 46.3 (2014): 305-309.

27. Everett DB., *et al*. "Genetic characterization of Malawian pneumococci prior to the roll-out of the PCV13 vaccine using a high-throughput whole genome sequencing approach". *PLoS ONE* 7 (2012): e44250.

28. Kamng'ona AW., *et al*. "High multiple carriage and emergence of Streptococcus pneumoniae vaccine serotype variants in Malawian children". *BMC Infectious Diseases* 15 (2015): 234.

29. Bronsdon MA., *et al*. "Immunoblot Method To Detect Streptococcus pneumoniae and Identify Multiple Serotypes from Nasopharyngeal Secretions". *Journal of Clinical Microbiology* 42.4 (2004): 1596-1600.

30. Huebner RE., *et al*. "Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes". *Pediatric Infectious Disease Journal* 19.10 (2000): 1017-1020.

31. O'Brien KL and Nohynek H. "Report from a WHO Working Group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae". *Pediatric Infectious Disease Journal* 22.2 (2003): e1-e11.

32. Kandasamy R., *et al*. "Multi-Serotype Pneumococcal Nasopharyngeal Carriage Prevalence in Vaccine Naïve Nepalese Children, Assessed Using Molecular Serotyping". *PLoS ONE* 10.2 (2015): e0114286.

33. Selva L., *et al*. "Rapid and Easy Identification of Capsular Serotypes of Streptococcus pneumoniae by Use of Fragment Analysis by Automated Fluorescence-Based Capillary Electrophoresis". *Journal of Clinical Microbiology* 50.11 (2012): 3451-3457.

34. Facklam RR and Washington II JA. "Streptococcus and related catalase negative gram- positive cocci". In: Balows A, Hausler WJ, Herman JKL, *et al*. eds. Manual of clinical microbiology. 9th edition. American Society for Microbiology. Washington, D.C.: ASM press (1991): 238-257.

35. Saha SK., *et al*. "Identification of Serotype in Culture Negative Pneumococcal Meningitis Using Sequential Multiplex PCR: Implication for Surveillance and Vaccine Design". *PLoS ONE* 3.10 (2008): e3576.

36. Yu J., *et al*. "Development of an Automated and Multiplexed Serotyping Assay for Streptococcus pneumoniae". *Clinical and Vaccine Immunology* 18.11 (2011): 1900-1907.

37. Cardozo DM., *et al*. "Prevalence and risk factors for nasopharyngeal carriage of Streptococcus pneumoniae among adolescents". *Journal of Medical Microbiology* 57.2 (2008): 185-189.

38. Yun KW., *et al*. "Streptococcus pneumoniae Type Determination by Multiplex Polymerase Chain Reaction". *Journal of Korean Medical Science* 26.8 (2011): 971-978.

39. Hausdorff WP., *et al*. "Geographical differences in invasive pneumococcal disease rates and serotype frequency in young children". *Lancet* 357.9260 (2001): 950-952.

40. Finland M and MW Barnes. "Changes in occurrence of capsular serotypes of Streptococcus pneumoniae at Boston City Hospital during selected years between 1935 and 1974". *Journal of Clinical Microbiology* 5.2 (1977): 154-166.

41. Bentley SD., *et al*. "Genetic Analysis of the Capsular Biosynthetic Locus from All Pneumococcal Serotypes". *PLoS Genetics* 2.3 (2006): e31.

42. Carvalho M da G., *et al*. "Revisiting Pneumococcal Carriage by Use of Broth Enrichment and PCR Techniques for Enhanced Detection of Carriage and Serotypes". *Journal of Clinical Microbiology* 48.5 (2010): 1611-1618.

43. Dias CA., *et al*. "Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children". *Journal of Medical Microbiology* 56.9 (2007): 1185-1188.

44. Iraurgui P., *et al*. "Modified sequential multiplex PCR for determining capsular serotypes of invasive pneumococci recovered from Seville". *Clinical Microbiology and Infection* 16.9 (2010): 1504-1507.

45. Coskun-Ari FF., *et al*. "One-Step Multiplex PCR Assay for Detecting Streptococcus pneumoniae Serogroups/Types Covered by 13-Valent Pneumococcal Conjugate Vaccine (PCV13)". *PLoS ONE* 7.12 (2012): e50406.

46. Lin YW., *et al*. "Laser induced fluorescence technique for DNA and proteins separated by capillary electrophoresis". *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* 793.1 (2003): 37-48.

47. del Amo E., *et al*. "Estimation of the invasive disease potential of Streptococcus pneumoniae in children by the use of direct capsular typing in clinical specimens". *European Journal of Clinical Microbiology and Infectious Diseases* 34.4 (2015): 705-711.

48. del Amo E., *et al*. "Serotypes and Clonal Diversity of Streptococcus pneumoniae Causing Invasive Disease in the Era of PCV13 in Catalonia, Spain". *PLoS ONE* 11.3 (2016): e0151125.

49. del Amo E., *et al*. "High invasiveness of pneumococcal serotypes included in the new generation of conjugate vaccines". *Clinical Microbiology and Infection* 20.7 (2014): 684-689.

50. Selva L., *et al*. "Serotype 3 is a common serotype causing invasive pneumococcal disease in children less than 5 years old, as identified by real-time PCR". *European Journal of Clinical Microbiology and Infectious Diseases* 31.7 (2012): 1487-1495.

51. Selva L., *et al*. "Direct Identification of Streptococcus pneumoniae Capsular Types in Pleural Fluids by Using Multiplex PCR Combined with Automated Fluorescence-Based Capillary Electrophoresis". *Journal of Clinical Microbiology* 52.7 (2014): 2736-2737.

52. Saha Sk., *et al*. "Identification of Serotype in Culture Negative Pneumococcal Meningitis Using Sequential Multiplex PCR: Implication for Surveillance and Vaccine Design". *Plos One* 3.10 (2008): e3576.

53. Carvalho M da G., *et al*. "Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract". *PeerJ* 1 (2013): e97.

54. Marimon JM., *et al*. "Single-step Multiplex PCR assay for determining 92 pneumococcal serotypes". *Journal of Clinical Microbiology* 54.8 (2016): 2197-2200.

55. Lawrence ER., *et al*. "Evaluation of semiautomated multiplex PCR assay for determination of Streptococcus pneumoniae serotypes and serogroups". *Journal of Clinical Microbiology* 41.2 (2003): 601-607.

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