Original Article

Detection of *pbp2b* and *ermB* genes in clinical isolates of *Streptococcus pneumoniae*

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Abstract

Background: *Streptococcus pneumoniae* is a major human pathogen. The emergence of penicillin resistant strains since the 1970s has been life threatening and the evolution of the bacteria have enabled itself to develop resistance to many other antibiotics such as the macrolides and the fluoroquinolones. This study aims to characterize *S. pneumoniae* isolates for the presence of penicillin and macrolide resistance genes.

Methodology: One hundred and twenty clinical isolates of *S. pneumoniae* were obtained from patients of University Malaya Medical Centre (UMMC). The strains were screened using a multiplex real-time PCR method for the presence of alterations in the genes encoding the penicillin binding proteins: *pbp2b*, macrolide resistance determinant *ermB* and the pneumolysin gene, *ply*. Dual-labelled Taqman probes were used in the real-time detection method comprising three different genes labeled with individual fluorophores at different wavelengths. One hundred and twenty isolates from bacterial cultures and isolates directly from blood cultures samples were analyzed using this assay.

Results: A multiplex PCR comprising the antibiotic resistance genes, *ermB* and *pbp2b* and pneumolysin gene (*ply*), a *S. pneumoniae* species specific gene, was developed to characterize strains of *S. pneumoniae*. Out of the 120 pneumococcal isolates, 58 strains were categorized as Penicillin Sensitive *Streptococcus pneumoniae* (PSSP), 36 as Penicillin Intermediate *Streptococcus pneumoniae* (PISP) and 26 as Penicillin Resistant *Streptococcus pneumoniae* (PRSP). All the 58 PSSP strains harboured the *pbp2b* gene while the 36 PISP and 26 PRSP strains did not harbour this gene, thus suggesting reduced susceptibility to penicillin. Resistance to erythromycin was observed in 47 of the pneumococcal strains while 15 and 58 were intermediate and sensitive to this drug respectively. Susceptibility testing to other β-lactams (CTX and CRO) also showed reduced susceptibility among the strains within the PISP and PRSP groups but most PSSP strains were sensitive to other antibiotics.

Conclusion: The characterization of pneumococcal isolates for penicillin and erythromycin resistance genes could be useful to predict the susceptibility of these isolates to other antibiotics, especially β-lactams drugs. We have developed an assay with a shorter turnaround time to determine the species and resistance profile of *Streptococcus pneumoniae* with respect to penicillin and macrolides using the Real Time PCR format with fluorescent labeled Taqman probes, hence facilitating earlier and more definitive antimicrobial therapy which may lead to better patient management.

Key Words: penicillin, macrolide, *Streptococcus pneumoniae*, Real-Time PCR, antibiotic resistance, pneumolysin.

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Introduction

Streptococcus pneumoniae is a major cause of mortality and has been reported to cause fatality among patients hospitalized for community-acquired pneumonia, bacteremia and meningitis [1]. The increasing rate of penicillin resistance in *S. pneumoniae* since its first description in the early 1970s has resulted in therapeutic challenges in treating patients with pneumococcal infections. Antimicrobials used in the treatment of pneumococcal infections include the β-lactams, macrolides and newer fluroquinolones.

Penicillin resistance in *S. pneumoniae* is due to production of altered penicillin-binding proteins (PBPs), which are essential in cell wall synthesis [2-5]. PBPs are the active-site serine peptidases which catalyze polymerization as well as cross-linking of peptidolglycan presurcors in the assembly of bacterial cell walls [6-7]. *S. pneumoniae* has been reported to possess six pbps: *pbp*1A, *pbp*1b, *pbp*2a, *pbp2b*, *pbp*2x and *pbp*3, of which *pbp*1a, *pbp2b* and *pbp*2x are

required to confer high-level penicillin resistance [8], while altered *pbp*1a and *pbp*2x are required to confer cefotaxime resistance [9]. However, *pbp2b* is identified to be the primary determinant of penicillin resistance in this organism. Resistance to other classes of antibiotics such as the macrolides and fluoroquinolones has posed therapeutic challenge. Unlike penicillin resistance, macrolides resistance is conferred by two different mechanisms. It is either by modification of the ribosomal target site, which reduces the binding of this antibiotic, encoded by the *ermB* gene, or by another mechanism, which is the efflux mechanism of the drug and assigned as *mefA*.

The identification of the organism would take at least 48 hours using the conventional culture-based method, and a further 18- to 24-hour incubation is required to obtain the antibiotic susceptibility testing profile. Tests with shorter turnaround time can potentially impact on therapeutic choices and outcome. In order to better characterize *S. pneumoniae* isolates from UMMC a real time PCR assay was developed to be able to identify strains as well as to characterize the antibiotic profile with respect to penicillin and erythromycin simultaneously.

Materials & Methods

Bacterial Strains

One hundred and twenty pneumococcal isolates were obtained from clinical samples processed at the Microbiology Laboratory of the University Malaya Medical Centre, Malaysia, from March 1999 to December 2003. The isolates were obtained from invasive and non-invasive infection of both paediatric and adult patients. The source of the isolates included blood, nasopharyngeal secretions, tracheal secretions, sputum and bronchoalveolar lavage. Direct blood cultures which were smear positive for *S. pneumoniae* were also obtained to evaluate the assay.

Strain Identification

The strains were identified by standard bacteriological methods, i.e. colonial morphology, Gram Stain, a negative catalase reaction, susceptibility to ethylhydrocupreine (optochin) and bile solubility.

Susceptibility testing

The antibiotic susceptibility of the strains was tested on Mueller Hinton Agar plates containing 5% (v/v) sheep blood incubated at 37^0 C with 5% CO_2 using the agar dilution method described [10]. The antimicrobial agents used were penicillin, cefotaxime, ceftriaxone and erythromycin, and were obtained in powder from Sigma Aldrich. *S. pneumoniae* ATCC 49619 (Penicillin intermediate *S. pneumoniae*) was used as the control. Strains were also tested against ciprofloxacin, and moxifloxacin, both of which were obtained from Bayer, gatifloxacin (Bristol-Myers Squibb) and levofloxacin (Daiichi).

DNA Extraction

Genomic DNA was extracted from pure bacterial cultures using the method adapted from Unal *et al.* [11]. Bacterial colonies suspended in 15μl of dH_2O containing 50 μgml^{-1} lysostaphin (Sigma Aldrich) were incubated at 37^0 C for 10 minutes. This was followed by the addition of 10 μgml^{-1} Proteinase K and 0.1mM Tris HCL (pH 7.5) prior to incubation at 37^0 C for another 10 minutes. Subsequently, the suspension was boiled for 5 minutes and finally centrifuged at 13,000 rpm for 2 minutes. The supernatant obtained was used as the template in the PCR reaction. The extraction of DNA from blood samples was carried out using the standard phenol-chloroform extraction protocol [12].

Probe and primer design

The probes used were the Taqman dual-labeled fluorescence system. Probes and primers were designed to target the *pbp2b* gene, *ermB* and pneumolysin gene respectively. The *pbp2b* gene was targeted as it is the primary resistance marker for penicillin. The *ermB* gene was selected as the macrolide resistance marker while the species specific pneumolysin gene was included for species identification. The probes were short oligonucleotides targeting specific regions of the gene and were designed to flank regions of the gene that are between the forward and reverse primer pair. The probes were labeled with different fluorescent dyes with excitation and emission energy at different wavelengths. The primers used in this study were adapted from [13] and were synthesized by Research Biolabs, Singapore. The sequences of the probe and primers used are listed in Table 1.

Primer and Probe concentration determination

The probe concentration was optimized from a starting material of 100nM, gradually reducing it to a minimum concentration of 40nM. The primer concentration ranged from 10-20 pmol. The primer and probe were optimized separately, initially in a single assay and eventually in the multiplex assay.

Table 1. List of primer and probe sequences used in this study.

Genes	Primer Sequences (5' …3')	
	Forward	Reverse
Pbp2b	CCTATATGGTCCAAACAGCCT	GGTCAATTCCTGTCGCAGTA
*Pbp*1A	AAACAAGGTCGGACTCAACC	ATATACATTGGTTTATAGTAAGTT
ErmB	CGTACCTTGGATATTCACCG	GTAAACAGTTGACGATATTCTCG
ply	GTGATATTTCTGTAACAGCTACC	GAGAATTCCCTGTCTTTTCAAAG-
Genes	**Probe Sequences 5'…3'**	
Pbp2b	(Fam)ATCAACCCAATATGTTTGTCGGCACCAG (BHQ1)	
*Pbp*1A	(TexasRed) AGATGGCTGCTGCTTACGCTG (BHQ2	
ErmB	(HEX) TGCACACTCAAGTCTCGATTCAGC (BHQ1)	
ply	(Cy5) CGCCTCTATCCTGGAGCACTTCACG (BHQ2)	

PCR amplification

The optimal PCR condition for a 50µl reaction included 1X PCR buffer, 7mM $MgCl_2$, 0.2 mM dNTP mix, 2U Taq Polymerase, 10 to 20 pmol of each PCR primers, and probe concentration ranging from 50nM -150nM. The PCR cycling parameters were as follows: an initial denaturation step of 95^0 C for 3 minutes followed the denaturation at 95^0 C for 30 seconds and completed with an annealing step at 60^0 C for 30 seconds. The cycling parameter was carried for 30 amplification cycles. The amplification reaction was performed in an iCycler thermalcycler (Biorad). The PCR amplification was monitored continuously on the computer, which was connected to the Real-Time PCR thermalcycler, integrated with the analysis software. Analysis of the results was performed using the available software. Single probe amplification was conducted prior to developing the Real-Time multiplex PCR.

Determination of specificity and sensitivity of the assay

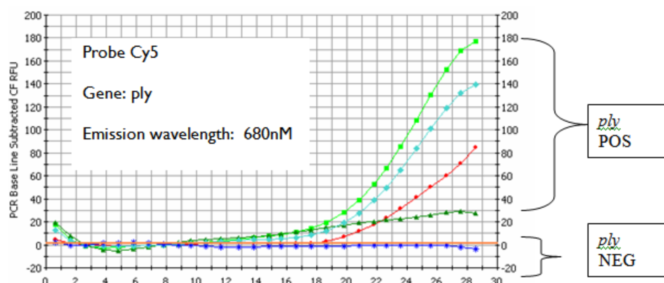
The sensitivity of the assay was determined using pure bacterial cultures and spiked blood samples. Colonies from pure bacterial cultures incubated overnight on sheep blood agar were suspended in 0.85% NaCl and the turbidity adjusted to an OD equivalent to 0.5 McFarland standards, which is approximately 10^8 cfu/ml. Further 10-fold dilutions of the suspension were made up to 10 cfu/ml. DNA extraction was conducted on each dilution which was later subjected to real-time PCR amplification. The sensitivity of the assay in the clinical setting was carried out by simulating known concentration of bacterial cultures and human blood samples into blood culture bottles. This was done by inoculating 5ml of healthy volunteer blood into an aerobic blood culture media (BD Biosciences, Becton Dickinson,USA), and the bottle was spiked with 100µl of diluted culture suspension which ranged from 10^8 to 10^3 cfu. The final concentration of the bacteria in the blood culture bottles ranged from 10^6 to 10 cfu allowing for the volume of the culture media in the blood culture bottles. The blood culture bottle was incubated overnight at 37^0 C prior to DNA extraction and PCR amplification. Viable bacterial count in the blood culture media was determined before and after the incubation period. The DNA from blood cultures was extracted using the method previously described by Dagan *et al*. (1998) [12]. The DNA extracted from each blood culture bottle with known concentration of bacterial culture was subjected to PCR. The specificity of the assay was also evaluated using DNA extracted from other gram positive bacteria such as *Streptococcus pyogenes* ATCC 4543, *Streptococcus sanguis* ATCC 10556 and *Staphylococcus aureus* ATCC 43300, as well as gram negative bacteria such as *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 15308.

Results and Discussion

Current methods to detect and then determine resistance profiles take at least two to three days. In this study a multiplex real time PCR assay was developed to detect *S. pneumonia* specifically as well as to amplify genes responsible for penicillin and erythromycin resistance. Figure 1 shows a representative illustration of a real-time PCR amplification. The figure shows positive amplification for the pneumolysin gene in all the

representative strains used in the study. These strains were biochemically confirmed as *S. pneumoniae*. The assay was then evaluated using *S. pneumoniae* strains from 120 pure bacterial cultures and 20 direct blood cultures. The detection of genes using the Taqman approach has a high level of specificity as the probes were designed to target a specific region of the genes and will only bind to complementary sequences of that region. Using a range of gradually diluted bacterial suspension the lower limit of detection of S. *pneumoniae* was 10 cfu (Figure 2). The blood sample that was intentionally inoculated was to test for the sensitivity of the assay. One sample (pure bacterial culture) was used for this purpose but repeated thrice to observe reproducibility. Blood cultures which were identified to be positive and identified (smear positive) to harbour *S. pneumoniae* were also used to evaluate the assay.

Figure 1. Representative results obtained in a Real-Time Multiplex PCR assay. Five colors represent 5 different strains. The samples are *S. pneumoniae* ATCC 49619 (Light green amplicon curve), 3 clinical isolates (light blue, red and dark green amplicon curve), and no template control (dark blue line).

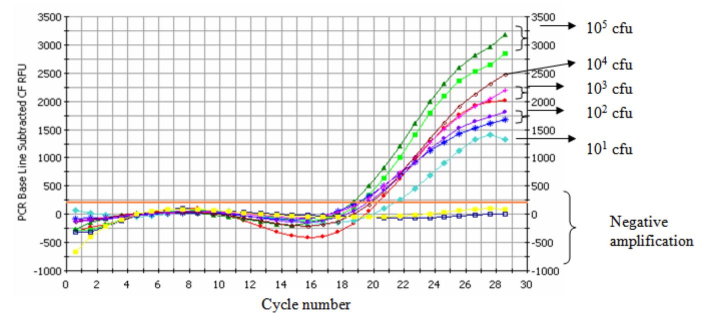


The resistance of *S. pneumoniae* to penicillin and macrolides has increased in recent years. The fundamental factor that leads to antimicrobial resistance is the widespread use of antimicrobials, resulting in selective pressure to promote evolution and transmission of resistant organisms. Penicillin binds to one or more of the six known penicillin binding proteins (PBPs), located in the *S. pneumoniae* cell membrane (1A, 1B, 2A, 2B, 2X and 3). The normal function of these PBPs is to enzymatically promote cross-linking of bacterial cell wall precursors in the cell development process. This action is inhibited when PBPs are bound to penicillin.

The binding of penicillin to individual PBPs occurs with variable affinity. Resistance occurs when the target penicillin binding proteins become physically altered so that the binding sites have reduced affinity for the drug. Therefore, amplification of the gene indicates susceptibility or no alterations within the sequence, and absence of the gene infers resistance. Jalal *et al.* (1997) [14] and Ubukata *et al.* (1996) [15] have also used this principle to differentiate penicillin susceptible strains from non-susceptible strains using PCR. In their studies, the target gene, *pbp2b*, was similar but the real-time PCR assay had a higher rate of specificity when compared to the conventional PCR assay because of the probe design which is specific to the complementary target region. Kearns *et al.* (2002) [16] used the real time PCR technique to identify penicillin susceptibility in cases of pneumococcal meningitis, but used SybrGreenI as an intercalating dye.

It has been shown that the SybrGreenI assay is less specific when compared to the Taqman probe approach [17] probably due to non-specific binding as the SybrGreenI dye would generally bind to any double-stranded structured DNA.

Figure 2. Taqman PCR amplification of the ply gene by real-time PCR. Ten-fold serial dilution of a representative strain (10^5-to 10 cfu) was used. The assay could detect up to 10cfu.



Molecular based assays have been developed in recent years to increase the sensitivity for the detection of *S. pneumoniae*. Messmer *et al.* (1997) [18] described a PCR assay to detect *S. pneumoniae* in clinical samples via the detection of the autolysin gene. In another study by Toikka *et al.* (1999) [19], blood-based pneumolysin PCR was described to be the most sensitive assay when compared to other conventional diagnostic assays. In this study, we have described a method for detecting *S. pneumoniae* from clinical isolates and

also to detect antibiotic resistance genes within this organism.

The three genes that could be incorporated in a single tube were the pneumolysin gene, *pbp2b* and the *ermB* gene. The assay could detect two antibiotic resistance genes and also identify the organism. The ability of the assay to detect as low as 10 cfu in a sample makes the assay highly sensitive and this would be of great advantage, especially in clinical samples with low bacterial load. This specific and sensitive assay could also be used for detection of *S. pneumoniae* directly from other body sites such as nasopharyngeal swabs, tracheal aspirates, and sputum. This multiplex real time PCR could thus reduce the laboratory turnaround time of a patient sample and aid in better patient management. Although the assay shortens the turnaround time of diagnosis, the use of expensive equipment limits the establishment of this assay in all laboratories.

The pneumococcal strains were assigned into 3 groups based on the susceptibility levels to penicillin, i.e. Penicillin Sensitive *S. pneumoniae* (PSSP) for strains with penicillin MIC of <0.125µg ml^{-1}; Penicillin Intermediate *S. pneumoniae* (PISP) for strains with penicillin MIC of ≥0.125 - <1.0 µg ml^{-1}; and Penicillin Resistant *S. pneumoniae* (PRSP) for strains with penicillin MIC of >1.0 µg ml^{-1}. The antibiotic profiles of the 120 strains against penicillin, cefotaxime, cefriaxone and erythromycin are shown in Table 2. As seen in the table, there were 58 PSSP strains, 36 PISP strains and 26 PRSP strains. With regard to erythromycin susceptibility, 58 strains were sensitive to erythromycin, 15 intermediate, and 47 resistant to erythromycin. The 58 erythromycin sensitive strains were from the PSSP (53/58) and PISP strains (5/36), while the erythromycin resistant strains were from the PISP (25/36) and PRSP (22/26) strains. The erythromycin intermediate strains were from the PSSP (5/58) and PISP (6/58) group of strains. Susceptibility testing to CRO and CTX showed that 75/120 strains were sensitive to CRO while 78/120 strains were sensitive to CTX. Thirty-four and 21 of the 120 strains were intermediate to CRO and CTX respectively, while 11/120 and 21/120 strains were resistant to these drugs. These results are shown in Table 3.

The *pbp2b* gene was detected in all the 58 PSSP strains but not in the 36 PISP and 26 PRSP strains. Due to the polymorphism in the penicillin binding proteins sequences, the conserved region of the *pbp2b* gene was targeted in this assay. Therefore, amplification of the gene indicates susceptibility or no alterations within the sequence, and absence of the gene infers resistance. This suggests no alteration within the penicillin binding protein *pbp2b* among the sensitive strains but the absence of the gene among the PISP and PRSP strains suggests alteration within the gene. It was also observed that most of the strains categorized as PSSP were also sensitive to the other antibiotics, CRO, CTX and ERY, but the number of strains decreased as it progressed into the PISP and PRSP categories (Table 3). This suggests that decrease in the susceptibility to penicillin could be used to predict the level of susceptibility to other drugs to a certain extent.

Table 2. MICs and gene profile of 120 strains screened using the developed multiplex real-time PCR.

NO	STRAIN ID	MIC PG	MIC CRO	MIC CTX	MIC ERY	*PBP2B*	*ERMB*	mefE
(i) Penicillin sensitive *S. pneumoniae* (PSSP) strains.								
	ATCC 49619	0.5	0.064	0.125	0.125	-	-	NT
1	1	0.032	<0.032	0.064	0.064	+	-	-
2	3	0.032	<0.032	0.064	0.125	+	-	-
3	4	<0.016	<0.032	<0.032	0.064	+	-	-
4	7	0.032	<0.032	0.064	0.125	+	-	-
5	9	0.032	<0.032	<0.032	0.064	+	-	-
6	10	0.032	<0.032	0.064	0.064	+	-	-
7	12	0.032	<0.032	0.064	0.125	+	-	-
8	13	<0.016	0.5	4	0.064	+	-	-
9	14	0.032	<0.032	0.064	0.125	+	-	-
10	15	0.032	<0.032	0.064	0.125	+	-	-
11	19	<0.016	<0.032	<0.032	0.125	+	-	-
12	22	<0.016	<0.032	0.064	0.125	+	-	-
13	23	<0.016	0.5	0.064	0.125	+	-	-
14	25	0.064	0.064	0.064	0.25	+	-	-
15	26	<0.016	0.064	0.064	0.5	+	-	-
16	27	<0.016	<0.032	<0.032	0.064	+	-	-
17	28	<0.016	0.125	2	0.5	+	-	-
18	29	<0.016	0.064	0.064	0.125	+	-	-
19	30	<0.016	0.25	0.064	0.064	+	-	-
20	31	<0.016	0.064	0.064	1	+	-	-
21	34	<0.016	0.064	0.064	0.064	+	-	-
22	35	0.032	<0.032	0.064	0.064	+	-	-
23	36	<0.016	2	0.064	0.064	+	-	-
24	37	0.064	0.064	0.064	1	+	-	-
25	40	<0.016	0.064	0.064	0.064	+	-	-
26	46	0.032	<0.032	0.125	0.064	+	-	-
27	47	0.064	0.125	0.064	0.064	+	-	-
28	52	<0.016	<0.032	0.064	0.064	+	-	-
29	53	<0.016	0.125	0.064	0.125	+	-	-
30	54	0.032	<0.032	0.064	0.064	+	-	-
31	55	0.032	<0.032	1	0.064	+	-	-
32	56	0.032	<0.032	0.064	0.125	+	-	-
33	61	0.032	<0.032	<0.032	0.064	+	-	-
34	64	0.032	<0.032	0.064	0.064	+	-	-
35	65	0.032	<0.032	0.25	0.064	+	-	-
36	66	<0.016	<0.032	<0.032	0.064	+	-	-
37	70	<0.016	<0.032	0.064	0.064	+	-	-
38	72	<0.016	<0.032	<0.032	0.064	+	-	-
39	74	<0.016	<0.032	0.064	0.064	+	-	-
40	83	<0.016	<0.032	<0.032	0.064	+	-	-
41	88	0.064	0.5	0.125	>1	+	-	-
42	89	0.032	<0.032	0.125	0.064	+	-	-
43	90	<0.016	<0.032	<0.032	0.064	+	-	-
44	96	<0.016	<0.032	0.064	0.064	+	-	-
45	98	0.032	<0.032	1	0.064	+	-	-
46	99	0.032	0.25	<0.032	0.064	+	-	-
47	5A	0.032	<0.032	0.064	<0.032	+	-	-
48	6A	<0.016	<0.032	<0.032	<0.032	+	-	-

Table 2. (continued)

NO	STRAIN ID	MIC PG	MIC CRO	MIC CTX	MIC ERY	*PBP2B*	*ERMB*	mefE
49	21A	<0.016	<0.032	2	<0.032	+	-	-
50	22A	<0.016	<0.032	<0.032	<0.032	+	-	-
51	41A	<0.016	<0.032	<0.032	0.016	+	-	-
52	54A	<0.016	0.125	<0.032	<0.032	+	-	-
53	60A	<0.016	<0.032	<0.032	<0.032	+	-	-
54	64A	<0.016	<0.032	<0.032	<0.032	+	-	-
55	89A	<0.016	<0.032	<0.032	0.016	+	-	-
56	93A	0.032	<0.032	<0.032	0.016	+	-	-
57	95A	0.032	<0.032	<0.032	<0.032	+	-	-
58	97A	0.032	<0.032	<0.032	<0.032	+	-	-
(ii) Penicillin intermediate *S. pneumoniae* (PISP) strains								
1	2	0.5	0.25	0.25	>1.0	-	+	-
2	5	1	0.5	1	>64	-	+	-
3	6	0.125	0.064	0.125	>64	-	+	-
4	11	1	0.5	4	>64	-	+	-
5	17	1	0.5	4	2	-	+	
6	18	1	0.5	1	2	-	+	-
7	20	0.125	<0.032	0.25	>64.0	-	+	-
8	21	0.5	0.5	0.25	>1.0	-	+	-
9	32	1	2	4	>64.0	-	+	-
10	33	1	2	4	4	-	-	+
11	51	1	1	<0.032	>1.0	-	-	+
12	60	1	1	0.5	>1.0	-	+	-
13	67	1	1	0.25	>1.0	-	+	-
14	68	1	0.5	2	0.064	-	-	-
15	77	0.25	0.25	0.125	0.064	-	-	-
16	78	1	0.5	0.25	>1.0	-	-	+
17	79	1	1	2	>1.0	-	-	+
18	80	0.25	<0.032	<0.032	0.064	-	-	-
19	81	1	1	2	>1.0	-	-	+
20	82	1	1	0.5	>1.0	-	-	+
21	85	0.125	<0.032	<0.032	0.064	-	-	-
22	86	0.125	<0.032	<0.032	0.5	-	+	-
23	94	0.25	1	0.5	>1.0	-	+	-
24	20A	0.5	1	0.5	>1.0	-	+	-
25	24A	1	1	0.5	0.5	-	-	+
26	28A	1	1	2	>1.0	-	-	+
27	29A	1	1	4	>1.0	-	-	+
28	31A	1	1	0.125	0.5	-	-	+
29	48A	1	0.5	4	0.5	-	+	-
30	53A	0.25	0.25	0.064	<0.032	-	-	-
31	71A	1	1	0.5	0.5	-	+	-
32	72A	1	1	0.5	>1.0	-	-	+
33	76A	1	1	1	>1.0	-	+	+
34	77A	1	1	0.5	>1.0	-	+	+
35	81A	1	1	1	>1.0	-	-	+
36	85A	0.5	0.5	0.25	0.5	-	+	-
(iii) Penicillin resistant *S. pneumoniae* (PRSP) strains								
1	8	2	1	4	128	-	+	-
2	16	2	1	4	0.125	-	-	-
3	41	>2	2	1.0	128	-	+	-
4	44	2.0	0.125	1.0	128	-	+	-
5	48	>2	1	1.0	2	-	+	-
6	49	>2	1	1.0	4	-	+	-
7	50	>2	2	1.0	128	-	+	-
8	57	2	2	0.25	8	-	-	+
9	58	2	>2	1.0	4	-	+	-
10	59	>2	2	1.0	1	-	-	+
11	62	2	2	1.0	128	-	+	-
12	63	>2	>2	1.0	0.064	-	-	-
13	75	2	1	1.0	128	-	+	-
14	87	2.0	1.0	1.0	128	-	+	-
15	91	2.0	1.0	1.0	2.0	-	+	-
16	95	2.0	1.0	1.0	2.0	-	+	-
17	97	2.0	1.0	1.0	2.0	-	-	+
18	100	2.0	1.0	1.0	2.0	-	-	+
19	15A	2.0	1.0	2.0	2.0	-	+	-
20	17A	2.0	1.0	4.0	1.0	-	+	-
21	18A	2.0	1.0	2.0	128	-	+	+
22	18A	2.0	1.0	2.0	128	-	+	-
23	55A	2.0	2.0	2.0	1.0	-	-	+
24	88A	2.0	1.0	<0.032	2.0	-	+	-
25	98A	4.0	1.0	2.0	2.0	-	+	+
26	94A	2.0	2.0	0.25	1.0	-	-	+

PEN(S : ≤ 0.06, I : 0.12 – 1.0, R: ≥2.0), CRO (S: ≤ 0.5, I :1.0, R: ≥2.0), CTX= (S: ≤ 0.5, I : 1.0, R: ≥2.0), ERY (S: ≤ 0.25, I: 0.5, R: ≥2.0). All strains were positive for the pneumolysin gene.

In order to determine other mechanisms of macrolide resistance, the strains were also screened for the *erm* gene using an in-house conventional triplex PCR assay (primer sequence of Nagai *et al*. 2001 [13]. Thirty-seven of the 120 strains harboured the *ermB* gene. Of these, 32 of the strains were resistant to erythromycin while 5 were intermediate to erythromycin. The *ermB* gene was not detected in the sensitive strains. There were, however, strains that were either intermediate or resistant to erythromycin but did not harbour the *ermB*, but instead harboured the *mefE* gene, which was detected in 22 strains.

Table 3. Susceptibility of the strains to other antibiotics in comparison to penicillin.

Category of strains	Susceptibility of other antibiotics (number of strains, n)								
	CRO			CTX			ERY		
	S	I	R	S	I	R	S	I	R
PSSP (n=58)	57	1	0	53	2	3	53	5	0
PISP (n=36)	17	17	2	22	4	10	5	6	25
PRSP (n=26)	1	16	9	3	15	8	0	4	22

S: Sensitive, I: Intermediate, R: Resistant

The presence of the *mefE* gene in the 22 of the erythromycin intermediate and resistant strains, which had negative amplification for the *ermB* gene, shows that there are 2 different mechanisms involved in conferring macrolide resistance in *S. pneumoniae*. The *ermB* was incorporated into the multiplex because of its high prevalence. The *ermB* determinant is borne by conjugative transposons related to Tn1545, Tn1545-like elements, or a Tn 917-like element, that is part of a larger composite transposon, Tn 3872. This accounts for horizontal transfer of the element and results in a higher prevalence of the *ermB* determinant.

Conclusions

In conclusion, the characterization of *S. pneumoniae* for the presence of penicillin and macrolide resistance genes could be conducted using this in house real time PCR assay in a shorter time. Characterization has revealed that the loss of *pbp2b* gene confers both intermediate and total resistance to penicillins while also

conferring reduced susceptibility to other β-lactams.

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