Differentiation of Penicillin Susceptible and Nonsusceptible *Streptococcus pneumoniae*

Ali Ahmadi [1], Farzaneh khodaei [2], Maryam Esghaei [3], Gholamreza Irajian [4],

Malihe Talebi [4] ∗

[1]*Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.*

[2]*Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.*

[3]*Department of Virology, Iran University of Medical Sciences, Tehran, Iran.*

[4]*Department of Microbiology, School of Medicine, Iran University of Medical Sciences , Tehran, Iran.*

ARTICLE INFO	ABSTRACT
Article type: Original Article	**Introduction**: *Streptococcus pneumoniae* cause morbidity and mortality in infants and younger children. Because of high prevalence of penicillin resistance, rapid and reliable diagnostic techniques for penicillin non-susceptible *S. pneumoniae* (PNSSP) are important for prevention and treatment. We investigated the association of the restriction length polymorphism (RFLP) patterns for *pbp2b* to distinguish between penicillin susceptible and resistant *S. pneumoniae* isolates.
	Methods: In this study, a total of 70 pneumococcal isolates were collected from different clinical sources. MIC of these isolates was determined and *pbp*2b gene was amplified by PCR and they were digested by HaeIII enzyme.
	Results: Of the 70 isolates, 86% (60) and 14% (10) pneumococcal isolates were found to be PNSSP (penicillin intermediate *S. pneumoniae* (PISP) and penicillin resistant *S. pneumoniae* (PRSP)) and penicillin susceptible *S. pneumoniae* (PSSP). In addition, 10 RFLP patterns (A-J) which were based on the HaeIII digestion of *pbp2b* gene were observed. All PSSP isolates showed that they belonged to pattern D, whereas all PNSSP showed 10 different patterns. **Conclusion**: In general, the present study suggests that RFLP can be a powerful tool in differentiation between the penicillin resistant and susceptible strains.

* *Corresponding Authors:* Malihe Talebi, Department of Microbiology, School of Medicine, Iran University of Medical Sciences, IR Iran. *Tel*: +982182943193, E-*mail*: talebi_25@yahoo.com
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Introduction

Streptococcus pneumoniae remains a leading cause of morbidity and mortality between children less than five years of age with 11% of all deaths and other groups including the immunocompromised and the people above 65 years of age (1, 2). Globally, up to 5 million deaths are reported annually including children with pneumonia and meningitis (2, 3). Bacterial meningitis is the most serious infectious diseases in central nervous system (CNS). *Neisseria meningitidis* along with *Streptococcus pneumoniae* cause approximately 80% of cases in acute bacterial meningitis in unvaccinated *Haemophilus influenzae* type b countries (16). Ever since the identification of PRSP in late 70's, it has increased worldwide (4, 22). Mechanisms involved include the acquisition of penicillin agents useful for treatment of PRSP infections consist of other extended-spectrum antibiotics such as cephalosporins, rifampin, linezolid, glycopeptides, and fluoroquinolones, although the economic cost has been enormously (5). Presently, the culture-based susceptibility testing is time consuming and tedious requiring classical bacterial identification techniques (7). The use of molecular tests in identification of the resistant mechanism such as altered *pbp2b* and *pbp1a* genes (high level of resistance) could benefit rapid identification of the resistant strains. The use of the restriction length polymorphism (RFLP) for *pbp2b* could be used to distinguish between penicillin susceptible and resistant *S. pneumoniae.* The aim of this study was to determine the prevalence of penicillin resistance in clinical isolates of *S. pneumoniae* and distinguish between penicillin susceptible and resistant *S. pneumonia* by PCR-RFLP.

Materials & Methods

Bacterial strains

Seventy pneumococcal isolates were obtained from clinical samples collected between 2010 and 2012. The isolates were obtained from invasive and non-invasive infection of both paediatric and adult patients hospitalized ten major hospitals in Tehran, Iran. The strains of *S. pneumoniae* were isolated from the following clinical materials: blood (16), eye infection (18), sputum (11), cerebrospinal fluid (8), maxillary Sinus (5), tracheas aspirate (5), ear swab (2), ascite (1), broncho-alveolar lavage (BAL) (1), abscess (1), pleural aspirate (1), and urine (1).

Identification of S. pneumonia

The isolates were identified to the species level using the following biochemical tests; colonial morphology, hemolysis, Gram staining, bile solubility and susceptibility to optochin (1µg) disc. *S. pneumonia* ATCC 49619 was used as a quality control strain (6).

Antimicrobial susceptibility testing

The susceptibility to penicillin was determined according to the standard guideline of the CLSI by using the disc diffusion method (oxacillin 1µg) from Mast Diagnostics Ltd. (Bootle, Merseyside, UK) and MICs were determined by the Etest (Liofilchem, Via Scozia, Roseto d. A. Italy) on Mueller-Hinton agar with 5% defibrinated sheep blood according to the manufacturer's instructions. All isolates were incubated in 5% CO_2 at 37ºC for 24 h. MICs results were inferred according to CLSI guidelines.

PCR and RFLP

Extraction of DNA from pneumococcal isolates was done with peq GOLD Bacterial DNA Kit (peQlab, Germany). The *pbp2b* gene was amplified using the primers pbp2b-for (5'GATCCTCTAAATGATTCTCAGGTGG3' and pbp2b-rev (5'CAATTAGCTTAGCAATA GGTG TTGG3') described as previously (11). PCR assay was made in a volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.2 mM each dNTPs, 0.5 U of Taq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) and forward and reverse primer 40 pmol. The PCR cycle was as follows;: an initial cycle at 95°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1min, annealing at 57°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min (12). The PCR products of *pbp2b* gene were digested with HaeIII (Fermentas, Canada, Ontario) (11). The digestion mixture consisted of 2 microl of HaeIII enzyme (10 U/micro liter) in 10 mM Tris-HCl (pH 8.5), and 18 micro liter of distied water. A total of 10 ml of PCR product was added to the digestion mixture. Digestion proceeded for 4h at 37°C.

Gel electrophoresis and pattern analysis

The digested products were run on a 2% agarose gel in 0.5X TBE buffer. The details were evaluated by visual examination of the banding patterns; the RFLP patterns of *pbp2b* received an alphabetical code (A-J).

Statistical analysis

Statistical analysis was done in IBM SPSS Statistics, Version 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) using the Chi-square test (X^2) applying Yates correction, with a level of significance of 95% ($p < 0.05$). The sensitivity, specificity, and positive and negative predictive values of the PCR-RFLP assays in detecting penicillin resistance and susceptible among all *S. pneumoniae* isolates were calculated by using standard dentitions (7).

Results

Susceptibility of pneumococcal strains

Table 1 shows the MIC results with the source of isolates. The results showed that majority of isolates obtained from eye infection and blood site was resistant to penicillin. Thirty percent of isolates were PRSP (MIC ≥ 2micro g/ml) and fifty six percent were PISP (MIC 0.1-1micro g/ml) (86% of PNSSP) while fourteen percent of isolates were penicillin susceptible *S. pneumoniae* (MIC ≤ 0.06 micro g/ml).
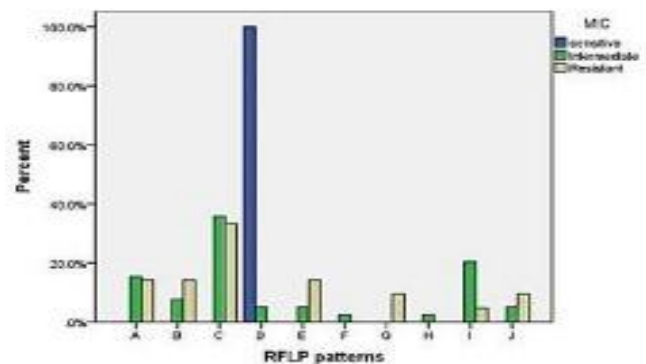

Figure 1. RFLP patterns and MIC of *S. pneumoniae* isolates.

Table 1. Properties of pneumococcal isolates

RFLP pattern	MIC			Total
	S	I	R	
A	0	6	3	9
B	0	3	3	6
C	0	14	7	21
D	10	2	0	12
E	0	2	3	5
F	0	1	0	1
G	0	0	2	2
H	0	1	0	1
I	0	8	1	9
J	0	2	2	4
Total	10	39	21	70

PCR-RFLP for *pbp2b*

Out of 70 pneumococcus isolates, a total of ten different RFLP patterns (A-j) were identified in the *pbp2b* gene digested by HaeIII restriction enzyme. (Table 2). Only one pattern (D) was observed for all 10 susceptible and the 2 intermediate resistance isolates. The 39 intermediately resistant strains exhibited nine different RFLP patterns; pattern C with 14 (36%) isolates were the most observed patterns and this pattern was observed only in PNSSP (PISP and PRSP) isolates. Among the 21 resistant strains, seven different RFLP patterns were observed. Pattern G was only detected in resistance isolates. Pattern C and D with 21 and 12 isolates were the common patterns in seventy isolates.

The MICs and RFLP patterns of the isolates are illustrated in Figure 1. By using conventional dentitions of 'not resistant' and non-susceptible *S. pneumonia* isolates, the sensitivities were 100% and 100%, the specificities were 93.7% and 96.7%, the positive predictive values (PPV) were 83% and 96.7%, and the negative predictive values (PNV) were 100% and 100%, respectively.

Discussion

Several different methods have been approved for the molecular diagnosis of penicillin resistance bacterial isolates (3). This PCR-based method can offer information on penicillin susceptibility within hours, follow-on in perfect suggesting of antimicrobial agents and prevention of the unnecessary consumption of broad-spectrum antimicrobial agents. This assay may also have a part in clinical situations somewhere cultures are negative as a consequence of prior antibiotic treatment (11). The sensitivity of the PCR assay for the finding of penicillin-susceptible genotypes fluctuates with different geographical regions (3). In our research, ten different patterns were found which in susceptible isolates with only one pattern (D). The result was in accordance with a study done by O'Neill *et al* (3) who found the same RFLP pattern for all their susceptible isolates with sensitivity, specificity, PPV and NPV of 97.5%, 100%, 100%, 93.1%, respectively, and 94.4%, 98.9%, 94.4%, 98.8%, for penicillin-resistant isolates, respectively. The present study indicated that there is a sufficient variation between susceptible and resistant pneumococcal isolates to be clearly distinguish them. Whist, failed to separate between intermediate and resistant isolates. Two out of our 39 intermediate resistant strains were identified as susceptible isolates. This inconsistency has also been reported by other investigators (7), although the sensitivity, specificity, PPV and NPV of our study was almost similar to other study (13, 14, 9).

Conclusion

PCR-RFLP can be used for diagnosing of susceptible and resistant isolates and also for the epidemiological analysis as of *S. pneumonia* strains. Rapid diagnosis of penicillin-resistant pneumococcal directly from clinical specimens can be investigated in the future.

Conflict of interest

None declared conflicts of interest.

Table 2: RFLP patterns and MIC of *S. pneumoniae* isolates.

Source of isolates	MIC			Total
	s	I	R	
Abscess	0	1	0	1
Ascite	0	0	1	1
BAL	0	1	0	1
Blood	1	8	7	16
CSF	1	4	3	8
Ear	0	0	2	2
Eye infection	4	9	5	18
Pleural	0	1	0	1
Sinus	0	4	1	5
Sputum	3	7	1	11
Trachea aspiration	0	4	1	5
Urine	1	0	0	1
Total	10	39	21	70

The method presented here was useful in differentiation of susceptible and resistance isolates but not satisfactory because of inadequate sensitivity and specificity to determine between resistance and susceptible isolates. In a publication by du Plessis *et al* (15), they used a a semi nested technique with species- specific conserved primers for pbp2b gene, they identify properly all of the resistant isolates, but needs high- quality polymerase and harsh reaction settings.

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