Molecular Analysis of *pbp2b* in *Streptococcus pneumonia* Isolated From Clinical and Normal Flora Samples

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Abstract *Streptococcus pneumoniae* is an important bacterial pathogen responsible for respiratory infections, bacteraemia, and meningitis remains an important cause of disease and mortality in infants and younger children around the world, with penicillin being considered the drug of choice for the treatment of infections. However, penicillin-resistant *S. pneumonia* is now becoming endemic worldwide. In this study, a total of 80 pneumococcal isolates were collected from different clinical sources as well as normal flora. These isolates were subjected to antimicrobial susceptibility testing and MIC determination. The penicillin-binding proteins, *pbp*2b, were amplified by PCR, and they were sequenced. The genetic relationship of the penicillin-resistant isolates was performed by BOX PCR. Overall, 36 pneumococcal (45 %) isolates were found to be resistant to penicillin with different MICs. The majority of them (80 %) were intermediately resistant with MIC of 0.12–1 µg/ml, whereas 20 % of isolates were penicillin resistant with MICs of >2 µg/ml. The results identified seven groups which were based on the amino acid substitutions of *pbp2b*. Sequencing analysis revealed that the most prevalent mutation was the substitution of Adenine for Thymine at the position 445 which is next to the second PBP2b-conserved motif (SSN). This study indicates that resistance to penicillin appears to be dependent on specific mutations in *pbp2b*, and the substitution in S620 → T near to the third PBP2b-conserved motif appears to be important in developing highly antibiotic-resistant isolates. Moreover, there was a positive correlation between the mutations in *pbp2b* gene and MIC.

Introduction

Antimicrobial resistance of *Streptococcus. pneumoniae* against antimicrobial drugs, in particular β-lactam, is increasing worldwide [1, 2]. Penicillin as the representative of this class of antibiotics has been the drug of choice for the treatment of pneumococcal infections [2]. The increasing number of reports on penicillin-resistant *pneumococci* (PRSP) throughout the world has made it essential to determine the prevalence of PRSP regionally [2, 3].

The major resistance determinant in the *S. pneumonia* is the alteration of penicillin-binding proteins (PBPs) [4] which results in reduced binding affinity to penicillin [5]. Mutation is the leading resistance mechanism to penicillin which usually is in the transpeptidase (TP)-penicillin-binding domain (PBD) [6]. Six *pbp* genes including *pbp1a, pbp1b, pbp2a, pbp2b, pbp2x* and *pbp3,* have been identified in *S. pneumonia* [6]. For reduced affinity to penicillin, multiple mutations occurred in PBP, and high-level resistance is attained by the acquisition of more than one low-affinity PBP variant [7]. The six genes are divided into three classes: the three with high molecular weight in class

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A are *pbps 1a, 1b* and *2a;* in class B, those with high molecular weight are *pbps 2x* and 2b. The low molecular weight gene is *pbp3* [8]. Although all the six PBPs in *S. pneumoniae* have been implicated in the resistance process [9], changes in the conserved motifs in PBP2B are associated with resistance to penicillin and amoxicillin [5, 7], and changes in PBP2X cause low-level resistance to cephalosporins, and mutations in PBP1a raise MICs of penicillin G and cefotaxime to ≥ 1 μg/ml and $0.5\geq$ μg/ml, respectively [10].

The active site of *pbp2b* contains three conserved motifs including Ser-Val-VAL-Lys (SVVK), Ser-Ser-Asn (SSN) and the Lys-Thr-Gly (KTG) [11]. Mutations within or in flanking regions of these motifs cause reduced affinity to penicillin [12]. Since the MICs of penicillins and carbapenems are firmly influenced by mutations in PBP2B, it is highly important to know about the exact kind of mutations within the *pbp2b* and its relation to the penicillin and carbapenems resistance [4].

Genetic and molecular analyses of *pbp2b* in *S. pneumonia* isolates have been reported in several countries [1]. To the best of the current authors' knowledge, however, this is the first report from this region of the world which has determined the nucleotide sequence of transpeptidase domain of *pbp*2b from Iranian *S. pneumoniae* isolates.

Materials and Methods

Bacterial Isolates

A total of 80 *pneumococcal* isolates were collected between 2010 and 2012. Clinical isolates were obtained from blood (13), pleural aspirate (2), sputum (5), cerebrospinal fluid (7), broncho-alveolar lavage (1), tracheal aspirate (3), ascite (2), eye infection (15) and maxillary Sinus (4), and 28 samples were from normal flora. Normal flora isolates were collected from healthy persons who had not had any antibiotic treatment for at least 6 months and were not hospitalized.

Identification of *S. pneumoniae*

To determine *S. pneumoniae*, standard microbiological techniques including hemolysis, Gram staining, bile solubility and susceptibility to optochin (1 μg) disc were performed for species identification. *S. pneumonia* ATCC 49619 was used as a quality control strain. The final identification was confirmed by *lytA* and *ply* genes using polymerase chain reaction (PCR) by species-specific primers as shown in Table 1 [13, 14].

Table 1 Primers used for identification of pneumococcal isolates and amplification of *pbp2b* gene

Gene[a]	Primer sequence (5′ → 3′)	Product length (bp)
pbp2b-F	GATCCTCTAAATGATTCTCAGGTGG	1,500
pbp2b-R	CAATTAGCTTAGCAATAGGTGTTGG	
lytA-F	CGGACTACCGCCTTTATATCG	250
lytA-R	GTTTCAATCGTCAAGCCGTT	
ply-F	ATTTCTGTAACAGCTACCAACGA	329
ply-R	GAATTCCCTGTCTTTTCAAAGTC	

[a] *F* forward (*upstream*) primer; *R* reverse (*downstream*) primer

Antimicrobial Susceptibility Testing

The susceptibility to penicillin was determined by the disc diffusion method (oxacillin 1 μg), 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 plates were incubated in 5 % CO_2 at 37°C for 24 h. MICs results were inferred according to Clinical and Laboratory Standards Institute guidelines [15]. The oxacillin disc was purchased from Mast Diagnostics Ltd. (Bootle, Merseyside, UK).

PCR and Nucleotide Sequencing

Extraction of DNA from pneumococcal isolates was done using peq GOLD Bacterial DNA Kit (peQlab, Germany). Amplification of *pbp2b* gene was performed by specific primer (Table 1) [16]. PCR assay was made in a total 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, UK) and forward and reverse primer 40 pmol. PCR was done under the following conditions: an initial cycle at 95 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, 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 [17]. Sequencing of the products was carried out using the ABI capillary system (Macrogen Research, Seoul, Korea) after purification.

DNA sequences were aligned using multiple sequence alignment in T-COFFEE, Version_10.00.r1613 (http://tcoffee.crg.cat/). We compared the sequences of resistant isolates with each other, and with susceptible strain R6 (GenBank Accession No. X16022).

Table 2 Properties of pneumococcal isolates. Penicillin MIC values were obtained by Etest methods

Isolate no	Date	Specimen source	Age	MIC (mg/L)
2	2010	PLA	74	1.50
3	2010	T/ASP	25	.75
6	2010	CSF	62	4.00
8	2010	SP	72	1.50
9	2010	PLA	46	1.50
15	2010	CSF	24	.01
18	2010	SF	.3	3.00
21	2010	BAL	57	1.50
40	2010	Eye	66	.38
56	2010	Eye	34	.19
58	2010	Eye	38	.12
61	2010	Eye	68	1.50
67	2010	Eye	81	1.50
69	2010	BL	9	.50
78	2010	Eye	.1	2.00
79	2011	Eye	45	.12
80	2011	Eye	64	.12
81	2011	BL	3	.38
83	2011	BL	1	3.00
88	2011	BL	85	1.50
92	2011	T/ASP	75	1.00
98	2011	MS	28	2.00
100	2011	BL	89	3.00
105	2011	SP	64	.01
107	2011	BL	79	.75
111	2011	MS	59	1.00
113	2011	T/ASP	62	1.50
127	2011	SP	59	2.00
128	2011	SP	60	1.00
131	2012	SP	80	1.50
134	2012	CSF	.1	.75
411	2012	Eye	52	.06
2F	2012	Nasopharynx	13	1.50
28F	2012	Nasopharynx	4	3.00
309	2012	Nasopharynx	3	1.50
315	2012	Nasopharynx	7	.75
316	2012	Nasopharynx	7	1.00
320	2012	Nasopharynx	14	.02
321	2012	Nasopharynx	7	1.00
362	2012	Nasopharynx	9	.75

SP sputum; *SF* synovial fluid; *BL* blood; *MS* Maxillar Sinus; *CSF* cerebrospinal fluid; *BAL* broncho-alveolar lavage; *T/ASP* tracheal aspirate; *PLA* Pleural aspirate

BOX PCR

The clonal relatedness of forty isolates was established by BOX-PCR, and the same was done using the *boxA* primer

5′- CTA CGG CAA GGC GAC GCT GAC G -3′. The PCR program was designed as follows: predenatured at 95° C for 7 min, 30 cycles each of denaturation at 90 °C for 30 s, primer annealing at 48 °C for 1 min, chain extension at 65 °C for 8 min, and a single cycle extension at 65 °C for 16 min [18]. The amplified products were separated by size on a 1 % agarose gel run in 0.5X Tris–borate-EDTA at 100 V for 3 h. The gel was stained with ethidium bromide (1 mg/ml) and viewed with UV light [19]. The details were evaluated by visual examination of the banding patterns that differed from each other by more than three bands.

Statistical Analysis

Statistical analyses were done in IBM SPSS Statistics, Version 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp). The relationship between the number of mutations and penicillin resistance was examined using Pearson product-moment correlation coefficient, and the relationship between BOX PCR and penicillin MIC was determined using Kruskal–Wallis Test. A cutoff P value of ≤0.05 (two tailed) was considered statistically significant.

Result

Susceptibility of Pneumococcal Strains

The MICs results for penicillin revealed that 44 (55 %) isolates were penicillin-susceptible pneumococci (PSSP) (MIC ≤ 0.06 μg/ml). On the other hand, 36 (45 %) isolates were resistant to penicillin including 29 (80 %) isolates with MICs ranging from 0.12 to 1 μg/ml as intermediately resistant (PISP) and 7 (20 %) isolates were resistant to penicillin (PRSP) with MICs of ≥2 μg/ml. All of resistant isolates were from blood (6), pleural aspirate (2), sputum (4), cerebrospinal fluid (2), broncho-alveolar lavage (1), tracheal aspirate (3), synovial fluid (1), eye infection (8), maxillary Sinus (2) and the seven normal flora isolates (Table 2).

Amino Acid Alterations in PBP2b

Of the 36 resistant pneumococcal isolates, seven groups based on the amino acid substitutions of *pbp2b* gene were recognized (Table 3) including: group 1 with 21 mutations and MICs between 0.1 and 1 μg/ml; group 2 with 37 mutations (MIC 0.1–4 μg/ml); group 3 with 30 mutations (MIC 0.7–3 μg/ml); group 4 with 23 mutations (MIC of 1 μg/ml); group 5 with 26 mutations (MIC 0.38–2 μg/ml); group 6 with 29 mutations (MIC 1.5–3 μg/ml); and group 7 with 35 mutations (0.75–3 μg/ml). In total, we detected 76

Table 3 Distribution of amino acid substitution in the PBD of PBP 2B from *S. pneumonia* isolates

Pbp2b						
Group (no of isolates)	SVVK[a] 385–388	SSNT 442–445	KTGTAES 614–620	No of change in 590–641	Total mutation (%)	BOX PCR
I (8)	- - - -	- - - -A	- - - - - - -	3	21 of 459 (4.6)	1-1-1-2-1-4-1-8
II (2)	- - - -	- - - -A	- - - - - - -	8	37 of 455 (8.1)	1,1
III (4)	- - - -	- - - -A	- - - - - - T	6	30 of 459 (6.5)	8-8-7-7
IV (1)	- - - -	- - - -A	- - - - - - -	3	23 of 435 (5.2)	8
V (9)	- - - -	- - - -A	- - - - - - T	6	26 of 463 (5.6)	8-5-7-1-8-7-6-4-8
VI (6)	- - - -	- - - -A	- - - - - - T	6	29 of 453 (6.4)	1-8-1-7-1-3
VII (6)	- - - -	- - - -A	- - - - - - -	5	35 of 454 (7.7)	1-9-4-7-1-1
Susceptible group (4)	- - - -	- - - - -	- - - - - - -	0	0	1-8-7-7

[a] Only amino acids that were changed from the reference sequence of R6 strains are shown. Dashes indicate residues identical with those of R6

amino acid modifications compared with the R6-PBP2b control sequences (Table 3). No mutation was detected close to the SVVK-conserved motifs in all groups. The T445 → A replacement next to the second PBP2b-conserved motif (SSN) was detected in 36 resistant isolates, and S620 → T replacement near to the third PBP2b-conserved motif (KTG) was recognized in 3, 5 and 6 groups with 11 isolates. All PSSP isolates had amino acid sequences highly homologous to the R6-PBP2b control sequence. The amino acid substitutions of non-susceptible *S. pneumonia* (NSSP: PISP and PRSP) isolates differed from 21 (4.6 %) to 37 (8.1 %) residues compared with the R6-PBP2b control sequence (Table 3). An initial analysis was performed to confirm no deviation of the assumptions of normality, linearity and homoscedasticity. There was a strong, positive, partial correlation between the two variables [$r = .55$, $n = 40$, $P < 0.05$], with high levels of mutation being associated with high levels of resistance to penicillin. On the other hand, the mutation number (≥ 26) in isolates with MIC 1.5 μg/ml were significantly higher than ($P < 0.05$) the mutation rate in isolates with MIC <1.5 μg/ml.

BOX-PCR

Nine different BOX PCR types were detected (Table 3). The more common BOX-PCR types were types 1 with 15 and 8 with 9 isolates. The more prevalent type was type 1 in five NSSP and PSSP groups, and no statistical difference between types of BOX PCR and penicillin MIC was found ($P = 0.53$).

Discussion

After the first report of β-lactam clinical resistance, pneumococcal penicillin resistance has been gradually growing, with its dissemination greatly threatening the clinical

efficacy of these compounds [20]. In the present investigation, our results showed a high prevalence of resistance to penicillin (45 %) in pneumococci isolates, among which 20 % of isolates with MIC being more than 2 μg/ml. This is higher than the reports by other investigators from China and Greece [21, 22] who reported low prevalence of penicillin resistance among their isolates. However, the reports from Vietnam, South Korea and Spain have shown high rates of resistance [2, 23].

PBP1a, 2x and 2b are generally recognized as the major PBPs associated with the activities of penicillins and some cephalosporins [10]. It has been suggested that the mutation in PBP2b results in increased penicillin MIC compared to that in strain R6 [4]. According to Granger et al. [24], the PBP2b amino acid sequence of NSSP strains can be categorized based on sequence relatedness of their mosaic blocks, and grouped into two classes: class A and B. Class A strains contained a characteristic altered sequence that resulted in the substitution of six contiguous residues; AFSVPM or AFSRPM (amino acids 426–431), and class B sequence included different strains that have several identical block mutations. According to this classification, 8 (22 %) isolates were identified as class A mutations (Group I in Table 3), and 28 (78 %) isolates did not belong to either class A or B mutation blocks. In contrast with the previous studies by other investigators where substitution Thr252 → Ala was a common and specific mutation in NSSP [25, 26], no such mutations were found in our study. In the present study, there was no change at or close to the serine-active site of conserved SVVK. Our results were in agreement with the results from other studies [10, 13]. On the other hand, Thr445 → Ala replacement next to the second conserved (SSN) motif was found in all PISP and PRSP isolates, which supported the importance of such substitution in β-lactam resistance [1, 27]. The amino acid substitution Ser620 → Thr next to the third conserved (KTG) motif was found in groups 3, 5 and 6 with high level of MIC. Based on sequencing, some isolates with different MIC ranges

showed identical mutation(s) and were clustered in same group. For example, two isolates in group 2 with different MIC ranges of 0.75 and 4 μg/ml showed identical mutations. This suggests that additional PBP and non-PBP genes may participate in the antibiotic resistance [1].

The results obtained in this study suggested that there is a direct association of number of mutations in *pbp2b* gene with MIC. Other factors have also been suggested to affect penicillin MIC such as mutation in *pbp1a*, *murM* and *murN* genes, histidine protein kinase CiaH and the glycosyl-transferase CpoA [13]. These aspects were not explored in the present study.

Using BOX PCR typing method, consistent with Pinar et al., we showed the penicillin-resistant isolates to be polyclonal [28]. This is in contrast to the findings of other studies [3, 29] that reported their isolates that are clonally related.

Collectively, our results revealed that PRSP and PISP strains isolated in this study concurred with most results of PBP2b amino acid substitutions reported elsewhere [1, 4].

Moreover, we found cases of high prevalence of penicillin resistance in this study. The presence of Ser620 → Thr, adjacent to the third conserved (KTG) motif, may have important role in penicillin resistance. Furthermore, we observed that many of the mutation characteristics of NSSP in our isolates were similar to those of isolates reported from other countries.

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