ORIGINAL ARTICLE 10.1111/j.1469-0691.2005.01334.x

Analysis of mutations in the *pbp* genes of penicillin-non-susceptible pneumococci from Turkey

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ABSTRACT

Sequence analysis of the *pbp* genes from 20 *Streptococcus pneumoniae* isolates from Turkey (eight with high-level penicillin-resistance, nine with low-level penicillin-resistance, and three that were penicillin-susceptible) was performed and phylogenetic trees were constructed. Most isolates clustered together within a single branch that was distinct from sequences deposited previously in GenBank, which suggests that these isolates have probably evolved following new recombination events. The most prominent active-site mutations, which have also been associated previously with resistance, were T371A in PBP1a, E481G followed by T451A in PBP2b, and T338A in PBP2x. All isolates also possessed a ^{570}SVES/TK^{574} block in the PBP2b sequence, instead of the QLQPT sequence of R6, which is fairly uncommon in GenBank sequences. This is the first study to analyse alterations in the *pbp* sequences of pneumococci isolated in Turkey.

Keywords *pbp* mutations, penicillin-non-susceptible pneumococci, penicillin resistance, sequence analysis, *Streptococcus pneumoniae*

Original submission: 17 May 2005; **Revised submission:** 21 June 2005; **Accepted:** 1 August 2005

Clin Microbiol Infect 2006; 12: 150–155

INTRODUCTION

Penicillins and cephalosporins inhibit bacteria by targeting the penicillin-binding proteins (PBPs) that play a role in cell-wall synthesis [1–8]. PBPs are transpeptidases and carboxypeptidases, with a central transpeptidase domain flanked by N-terminal and carboxy-terminal regions. The transpeptidase region includes the nucleophylic serine (Ser37) and is responsible for cross-linking of peptidoglycan. It is also the region at which the active site of the enzyme is located. In all PBPs, the penicillin-binding domain (PBD) in the transpeptidase site includes three restricted motifs, the SXXK sequence, which has a penicillin-binding serine, and SXN and KT(S)G triple motifs [7–9]. Penicillins have been used for the treatment of pneumococcal infections for more than five decades. Unfortunately, this has resulted in the emergence and spread of resistant clones worldwide. Mutations in *pbp1a*, *pbp2b* and *pbp2x* are the most important markers of penicillin resistance in *Streptococcus pneumoniae* [10–12].

In recent studies, the prevalence of low- and high-level penicillin resistance amongst pneumococcal isolates from Turkey has been reported as 29–41.5% and 3–8%, respectively [13–16]. As no data had been published previously concerning alterations in the *pbp* genes of isolates from Turkey, the present study investigated the mutations in the active site of the *pbp1a*, *2b* and *2x* genes in penicillin-non-susceptible *S. pneumoniae* isolates collected in Turkey during the period 1997–2001.

MATERIALS AND METHODS

Bacterial isolates

Twenty isolates of *S. pneumoniae*, eight with high-level penicillin resistance (Pen^R), nine with low-level penicillin resistance (Pen^I), and three that were penicillin-susceptible (Pen^S),

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Table 1. Serotypes and penicillin MICs for the isolates from Turkey

Isolate number	MIC (mg/L)	Serotype	Isolation date
6	2	9 V	17.11.1997
10	0.50	23F	04.05.1999
12	0.125	15	30.11.1999
16	2	9 V	18.10.2000
26	0.50	9 V	25.07.2000
28	1	14	08.04.1999
34	0.19	23	19.01.2000
35	2	15B	26.06.1997
44	1.5	14	15.12.2000
45	3	15B	21.11.2000
51	2	23	10.05.1999
64	1.5	23F	18.12.2000
78	0.50	9 V	15.02.1999
81	1	19	10.07.1999
82	0.38	23F	14.12.1999
83	1.5	23F	25.10.1999
88	0.25	14	12.05.2000
102	0.016	9	04.10.2000
105	0.016	3	21.12.2000
110	0.08	7	06.01.2001

were selected randomly from 110 isolates collected between 1997 and 2001. There was no significant clonal relationship among these isolates on the basis of BOX-PCR results obtained using the BOX A1R primer and the protocol of the Pneumococcal Molecular Epidemiology Network (PMEN) [17]. The serotypes and penicillin MICs for the isolates are shown in Table 1.

DNA extraction, PCR and DNA sequencing

DNA extraction and PCR for the *pbp* genes were performed as described previously [18]. The primers used for PCR and sequence analysis are shown in Table 2. Amplification products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Nucleotide sequence analysis was performed on single strands using a Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were cleaned using Centrisep spin columns (Princeton Separations, Adelphia, NJ, USA) and analysed on an ABI Prism 377 DNA sequencer (Applied Biosystems).

Table 2. Primers used for PCR and nucleotide sequence analysis

Primer	Sequence
PBP1a 1(P)	CGGCATTC GATTTGATTCGCTTCT
PBP1a 2(P)	CTGAGAAGATGTCTTCTCAGG
PBP1a F1(S)	ATGAACAAACCAACGATTCTG
PBP1a F2(S)	GCTTGGTTAGCGATTCAGTTAG
PBP1a F3(S)	AATGTAGACCAAGAAGCTC
PBP1a F4(S)	GGTCTTGGTATCGACTATC
PBP1a F5(S)	AATCGTTTAACTCCTATCG
PBP2b 1(P)	ATCCT CTAAATGATTCTCAGG TGG
PBP2b 2(P)	CAATTAGCTTAGCAATAGGTGTTGG
PBP2b F1(S)	GATCCTCTAAATGATTCTCAGGTGG
PBP2b F2(S)	AAGGTGTCTATGCAGTCGC
PBP2b F3(S)	CATTGACCTTCCAGATGAGTC
PBP2b F4(S)	GCTAACAATACTAATGCTG
PBP2x F1(P,S)	CGTGGGACTATTTATGACCGAAATGG
PBP2x F2(S)	CTTGCAGGGACAGACGGC
PBP2x F3(S)	AATGAAGGATTGACTGGTG
PBP2x F4(S)	ATGTATAACCACAGCACAG
PBP2x R1(P,S)	AATTCCAGCACTGATGGAAATAAACATATTA

(P), PCR primer; (S), sequencing primer.

Phylogenetic analysis

The *pbp1a*, *pbp2b* and *pbp2x* active site sequences from the isolates studied (nine *pbp1a*, 14 *pbp2b*, ten *pbp2x*) were aligned, together with 20 *pbp1a*, 43 *pbp2b* and 38 *pbp2x* sequences from GenBank, with Clustal X v.1.81. Phylogenetic trees of aligned sequences were constructed with Mega v.2.1, using the neighbour-joining method and Kimura-2 parameter correction [19]. CIs of the tree were calculated by bootstrap analysis. Synonymous (K_S) and non-synonymous (K_A) mutation rates and K_A/K_S values, as well as transition/transversion ratios for all the isolates, were determined with Mega v.2.1.

Complete DNA sequences of *pbp1a*, *pbp2b* and *pbp2x* were determined and phylogenetic trees were constructed for five, 14 and seven study isolates, respectively. The GenBank sequences used for the phylogenetic trees and the corresponding references are available in Table S1 (see Supplementary Material).

RESULTS AND DISCUSSION

Active site mutations in the *pbp1a*, *pbp2b* and *pbp2x* genes

T371A in the active site STMK motif, which has been described previously as being important for resistance, was the most prominent mutation within the conserved sequence motifs of *pbp1a*. This alteration, and a P432T mutation, was detected in all $Pen^{I/R}$ isolates; identical mutations have been reported previously [20,21]. As the T371A mutation is adjacent to the serine amino-acid in the SXXN motif of the penicillin-binding region, this change modifies the active site and lowers the binding affinity to penicillin. However, as the P432T mutation is close to the $^{428}SRN^{430}$ motif, it also affects binding to penicillin. A $^{574}NTGY^{577}$ block, instead of the TSQF sequence of R6, was also detected in five isolates (Fig. S1; see Supplementary Material). This substitution has also been reported by other investigators [20,22], and Smith and Klugman [23] have demonstrated the importance of mutations at positions 574–577.

T451A (T252A) and E481G (E282G) mutations were found in the *pbp2b* sequences from 92% of the $Pen^{I/R}$ isolates, and these mutations have also been reported previously [10,21,24]. Dowson *et al.* [24] reported six or seven amino-acid substitutions between T431/Q432-F437 of the *pbp2b* sequence from mutants of $Pen^{I/R}$ *S. pneumoniae* isolates. An additional T451A mutation, adjacent to the $^{448}SSN^{450}$ motif, has been shown to decrease the binding affinity of PBP2b to penicillin. Other active site mutations detected in $Pen^{I/R}$ isolates

were T494A and L460I. All isolates also possessed a SVES/TK block between amino-acids 570 and 574, instead of the QLQPT sequence of R6 (Fig. S2; see Supplementary Material). The same mosaic genes have also been reported in some strains from Iceland [25]. As one of the Pen^S isolates also had the same changes, this alteration does not seem to cause penicillin resistance. Nevertheless, the block is located in the active site and may affect the decrease in penicillin susceptibility associated with other mutations located in the active site, or it may simply be a molecular evolution marker forming part of a mosaic sequence acquired via homologous recombination by the Turkish isolates in the present study.

Analysis of the *pbp*2x genes revealed a T338A alteration in the ^{337}STMK^{340} motif in three of seven $Pen^{I/R}$ isolates. Another two isolates had an alteration of T338P, indicating positive Darwinian selection under antibiotic pressure. An A369V mutation was found exclusively in five $Pen^{I/R}$ isolates. Four isolates, including a Pen^S strain, also possessed a Q552E change located near the ^{547}KSG^{549} box (Fig. S3; see Supplementary Material). T338A/P and Q552E mutations have also been reported in previous studies [11,21,26]. As these mutations take place near the active site, or just on its periphery, they may change the configuration of the active site and cause drug resistance by the same mechanism as the other mutations mentioned above.

In PBP2x of *S. pneumoniae*, one of the basic amino-acids that plays a role in penicillin resistance is T338 in the STMK motif, located in the primary part of the α_2 helix. This amino-acid is situated next to the serine to which the antibiotic binds, and is located in a cavity separated from the active site by the main chain (α_3) of the protein. This cavity also includes a water molecule that is essential for acylation and deacylation of β-lactams. The water molecule forms hydrogen bonds with OH^- groups of the T338, S571 and Y536 amino-acids [11]. As a result of T338A alteration, the rate of acylation is reduced. Mouz et al. [26] showed that a T338A/G/P/V substitution in the SXXK restricted motif changed the location of the water molecule in the active site (because of the absence of the OH^- group), leading to difficulty in binding of PBP2x to β-lactams. The T338A mutation causes a decrease in the β-lactam-binding affinity of PBP2x greater than that caused by T338G and T338P mutations. Besides these mutations, S571A/P and Y536F mutations also lead to a decrease in affinity because of the absence of the OH^- group in mutant amino-acids.

One of the major amino-acids in PBP2x that plays a role in penicillin resistance is Q552, which is often different in penicillin-resistant strains. This amino-acid is situated close to the ^{547}KSG^{549} restricted motif. Gln552 is a positively-charged molecule, whereas E is negatively-charged. This substitution changes the acylation activity of PBP2x by two- or three-fold [26]. In strains carrying T338A and Q552E mutations together, the affinity of PBP2x for penicillin and cefotaxime is decreased by 90% and 80%, respectively [11,26]. Furthermore, the β-lactam affinities of strains carrying triple mutations of T338A, Q552E and S571P were significantly lower [26]. However, in the present study, no increase was observed in the β-lactam MICs for the two isolates (numbers 12 and 82) that carried double mutations in comparison with isolates carrying a single mutation. In addition, two isolates (numbers 45, penicillin MIC of 3 mg/L, and 88, penicillin MIC of 0.25 mg/L) were penicillin-resistant although they lacked these mutations. The combined effects of other genes (i.e., *pbp* genes other than *pbp1a*, *pbp2b*, *pbp2x* and the *murMN* operon) have also been shown to be important for the expression of Pen^I or Pen^R [27–31], and this may be the case for the isolates in the present study.

Overall, when the active site mutations were considered in association with penicillin MICs, the results from the present study were compatible with the suggestion that *pbp1a* mutations are of greatest importance for Pen^R.

Phylogenetic analysis of *pbp* genes

Phylogenetic trees based on the *pbp* sequences indicated that most Turkish isolates clustered together, with high bootstrap values of >80 for *pbp1a* (Fig. 1) and *pbp2x* (Fig. S4; see Supplementary Material), and of 69–100 for *pbp2b* (Fig. S5; see Supplementary Material) in a branch separate from the published GenBank sequences. This was especially the case for *pbp1a*. This finding suggests that different recombination events have occurred in these isolates. Two other sequences reported for isolates from France and Iceland were also on the same branch as the *pbp1a* sequences for the

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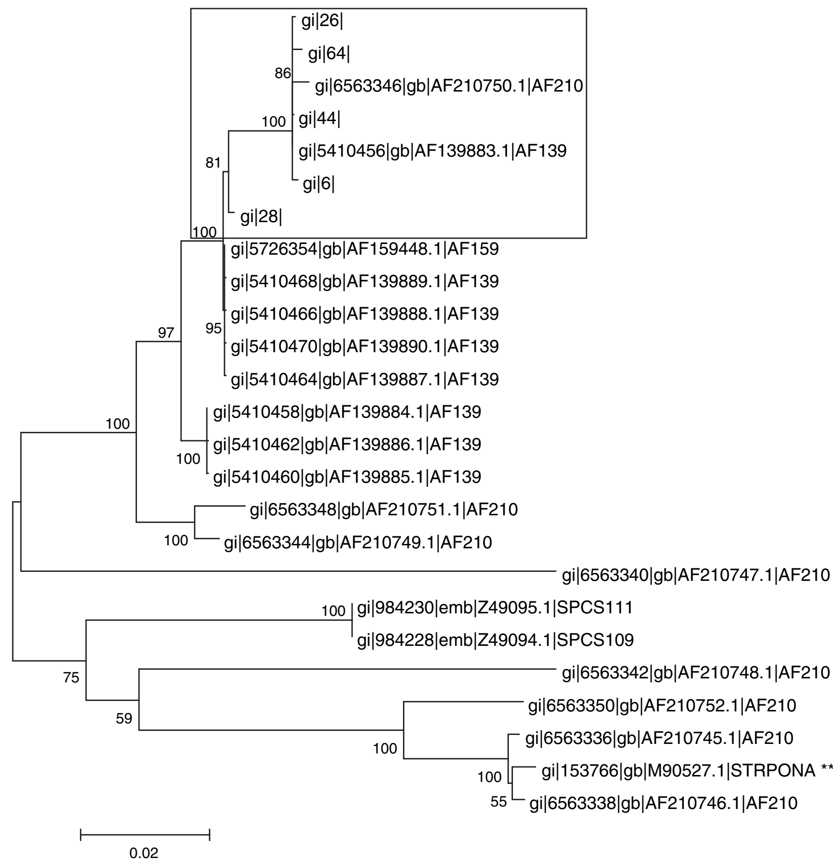

Fig. 1. Phylogenetic tree, obtained by the neighbour-joining method and Kimura-2 parameter correction, for the *Streptococcus pneumoniae* 1995-bp *pbpla* sequences (the frame marks the isolates from Turkey). Bootstrap values are shown on the tree. ***pbpla* sequence of R6.

isolates from Turkey. In particular, sequences from isolates 6, 26, 44 and 64 were very similar to those from the isolates from France and Iceland. However, similarity in only one gene may not be sufficient to prove an evolutionary relationship among these isolates. In the phylogenetic tree drawn using the *pbp2b* sequences, the isolates from Turkey also clustered within a branch that was separate from the GenBank sequences, with the exception of isolate 82, which was located on the same branch as the isolates from France and Iceland. No epidemiological connection was found between these countries and the patient from whom isolate 82 was cultivated.

In terms of branch length, the tree topologies suggest that the PBPs of the Turkish isolates have evolved in a local geographical niche, with *pbp1a* showing evidence of more recent recombination processes than the sequences obtained from GenBank.

Variation analysis of nucleotide and amino-acid sequences

Variation analysis showed that the *pbp* sequences of the isolates in the present study were similar at both the nucleotide and amino-acid level (mean divergence of nucleotide bases was 0.6%, 6.6% and 7.8% for *pbp1a*, *pbp2b* and *pbp2x*, respectively). This is probably the result of the same environmental factors acting on these isolates, and recovery of the isolates from the same geographical area with similar commensal streptococci.

When compared with the sequences of R6, the nucleotide and peptide sequences of $\text{Pen}^{I/R}$ isolates showed up to 14.9% and 8.5% divergence, respectively. Moreover, a 406-bp region (1726–2131) of the *pbp2b* sequence, which is 32.7% divergent from the R6 homologue, showed 97% similarity to the *pbp2b* sequence of *Streptococcus mitis*. This supports the hypothesis that mosaic

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genes acquired from commensal streptococci by homologous recombination are responsible for penicillin resistance in pneumococci.

Analysis of locus changes among the *pbp1a*, *pbp2b* and *pbp2x* sequences

When K_S and K_A mutations were investigated for the Turkish isolates and the GenBank sequences, the proportion of K_S mutations was, as expected, higher (Table 3). This finding indicates that mutations in *pbp* genes are K_S mutations that do not alter the amino-acid sequence, indicating that the gene is functional and probably vital for the microorganism [32,33]. Thus, the present findings support the theory that the mutations result only in the development of penicillin resistance, as opposed to enzymic changes in the PBP proteins, which are essential for peptidoglycan synthesis.

K_A/K_S values of the pneumococcal sequences from GenBank were similar for *pbp2b* and *pbp2x*. A higher K_A/K_S ratio for *pbp1a* shows that *pbp1a* has been differentiating within itself [34]. Moreover, a higher K_A/K_S ratio for *pbp1a* than for *pbp2x* and *pbp2b* suggests that *pbp1a* may be less vital functionally in comparison with *pbp2x* and *pbp2b*. Previous studies have proved that PBP2X and PBP2B are essential for cellular function, but that PBP1A is non-essential [22,35]. However, the small number of isolates examined in the present study, and the choice of *pbp* genes for evolutionary analysis, may have affected the results. In previous studies on genes related to resistance and virulence, analysis of clinical isolates (which are more virulent and resistant) has resulted in a bias; therefore, commensal isolates are preferred for phylogenetic analysis [32].

GenBank accession numbers

GenBank accession numbers of the sequences determined in this study for *pbp1a*, *pbp2b* and *pbp2x* are as follows: *pbp*1a: 6, AY937241; 26, AY943313; 28, AY943314; 44, AY943315; 64, AY943316; 78, DQ060831; 82, DQ060832; 83, DQ060833; 110, DQ060834; *pbp2b*: 6, AY970297; 10, AY970298; 12, AY970299; 26, AY970300; 28, AY970301; 34, AY970302; 35, AY970303; 44, AY970304; 51, AY970305; 64: AY970306; 78, AY970307; 81, AY970308; 82, AY970309; 110, AY970310; *pbp2x*: 6, DQ020579; 10, DQ020580; 12, DQ020581; 34, DQ020582; 45, DQ020583; 82, DQ020584; 88, DQ020585; 102, DQ020586; 105, DQ020587; 110, DQ020588.

Table 3. Synonymous (K_S) and non-synonymous (K_A) values for the sequences of the isolates from Turkey included in the study and for GenBank sequences

	GenBank sequences				Turkish sequences			
	n	K_A	K_S	K_A/K_S	*n*	K_A	K_S	K_A/K_S
PBP1a	20	0.02	0.27	0.07	5	0.004	0.010	0.4
PBP2b	43	0.03	0.38	0.07	14	0.015	0.22	0.06
PBP2x	38	0.05	0.58	0.08	7	0.027	0.28	0.09

ACKNOWLEDGEMENTS

This study was presented in part at the 14th European Congress of Clinical Microbiology and Infectious Diseases (Praque, Czech Republic, 2004). This work was supported by Dokuz Eylul University Research Funds (project grant no. 92.301.01.07).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available online from http://www.blackwell-synergy.com

Table S1 Sequences used for the construction of the phylogenetic trees.

Fig. S1 Alignment of active site sequences for *pbp1a*.

Fig. S2 Alignment of active site sequences for *pbp2b*.

Fig. S3 Alignment of active site sequence for *pbp2x*.

Fig. S4 Phylogenetic tree for *pbp2a* sequences.

Fig. S5 Phylogenetic treee for *pbp2x* sequences.