The Sequence of *pbp2b* from Penicillin-Resistant *Streptococcus pneumoniae* in Thailand

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Objective: *Penicillin resistance in Streptococcus pneumoniae isolates results from altered penicillin-binding proteins (PBPs), especially PBP2, which has a reduced affinity to penicillin. This study evaluated drug resistance and the gene sequence of the conserved motif pbp2b of penicillin-resistant isolates in Thailand.*
Material and Method: *Penicillin-resistant pneumococci with minimum inhibitory concentrations (MIC) for penicillin ≥ 4 μg/ml and penicillin-susceptible strains were identified from clinical specimens. The pbp2b genes were amplified by polymerase chain reaction (PCR), and the purified PCR product was cloned into E. coli. The recombinant plasmid clones containing pbp2b were sequenced and evaluated for mutations corresponding to penicillin and cefotaxime resistance.*
Results: *Penicillin-susceptible S. pneumoniae isolates were susceptible to 12 other antibiotics tested (range 95-100%) while penicillin-nonsusceptible isolates were resistant to most antibiotics except amoxicillin/clavulanate and levofloxacin. Sequence analysis of pbp2b showed a substitution of A for T_{451} next to the region of the SSN triad in all six resistant isolates tested and mutations clustered around the KTG triad in two isolates. Using the ClustalW alignment program, Thai isolates differed from those of European countries, but were more similar to those from Japan than Korea.*
Conclusion: *Penicillin or cefotaxime resistance in S. pneumoniae in Thailand was due to affinity reduction of PBP2b, similar to changes found in other Asian isolates.*

Keywords*: Streptococcus pneumoniae, Pneumococci, Genotype, pbp2b*

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Streptococcus pneumoniae is a normal inhabitant of the upper respiratory tract in 5-40% of the general population[1]. It primarily causes infection of the middle ear, paranasal sinuses, mastoids and lung parenchyma, and may spread to other sites, including joints, peritoneum, endocardium, biliary tract and, in particular, meninges. It is genetically flexible as demonstrated by frequent recombination events between different strains[2-4].

β-lactam antimicrobial agents act on penicillin binding proteins (PBPs), which are involved in cell wall synthesis. Resistance is most often caused by the presence of β-lactamase, but mutations in PBPs resulting in reduced affinity for β-lactam antibiotics are also commonly observed[5]. Penicillin resistance among pneumococci is due to the presence of altered PBPs, especially PBP2, which has a reduced affinity for penicillin. And β-lactamases have never been detected in pneumococci[6]. Pneumococci encode six PBPs, termed PBP1a, PBP1b, PBP2a, PBP2b, PBP2x, and PBP3. In susceptible strains, these PBPs are highly conserved. The genes for low-affinity PBP1a, PBP2b, and PBP2x found in resistant strains diverge significantly[7,8]. It is assumed that all PBPs retain the same basic tertiary structure in their penicillin binding module[9], with three conserved motifs forming the catalytic center: SXXK, SXN, and KTG (X denotes any amino acid residue). In *S. pneumoniae*, PBP2b is the main target for penicillin, but PBP2x is the target for cefotaxime[10]. The purpose of this study was to evaluate drug resistance and analyze the gene sequences of the conserved motifs of *pbp2b* in penicillin-resistant isolates.

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Material and Method

Clinical isolates

Two hundred *S. pneumoniae* isolates from clinical specimens were obtained from the Bacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital; the Queen Sirikit National Institute of Child Health; Ramathibodi Hospital; and the Department of Medical Sciences, Thailand-Culture Collection (DMST-CC) as part of antimicrobial resistance surveillance. They were isolated from patients during July 2002-August 2003 from different regions of Thailand. Most (138) isolates were from non-sterile sites (*e.g.*, sputum, nasal swabs, adenoids, ear drainage, eyes) and 62 isolates were from sterile sites (*e.g.*, blood, cerebrospinal fluid, and pleural fluid).

Antimicrobial susceptibility tests

In vitro antimicrobial susceptibility to oxacillin, erythromycin, clindamycin, levofloxacin and co-trimoxazole was determined for all isolates by disk-diffusion, according to guidelines of the Clinical and Laboratory Standards Institute, 2005[11]. In addition, minimum inhibitory concentrations (MIC) to penicillin, amoxicillin/clavulanic acid, cefuroxime, cefotaxime, cefdinir, cefprozil, levofloxacin, roxithromycin and azithromycin were determined by Etest (AB BIODISK, Sweden). For quality control, five standard bacteria (*i.e.*, *S. pneumoniae* ATCC 49619, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213) were tested against all appro priate antimicrobial agents.

Pbp2b gene sequencing

Pbp2 genes were amplified from chromosomal DNA for seven pneumococcal isolates with penicillin MICs $\geq$ 4 μg/ml by polymerase chain reaction (PCR). The 2.5 kb *pbp2b* fragment was amplified using two primers (H.2131 and H.2132), as previously described[12]. The 2.4 kb *pbp2b* fragment was amplified by using two primers (H.2131 and Pn2B down)[13]. The PCR amplification of the PBP-encoding genes was performed in a 25 ml volume containing approximately 50 ng of chromosomal DNA, 1.5 mM $MgCl_2$, 1x Promega PCR buffer, 0.01 U of *Taq* DNA polymerase (Promega, USA), 0.2 mM deoxynucleoside triphosphates and 0.2 mM of each primer. Thermal cycling was performed using the GeneAmp PCR System 9700 (Applied Biosystems, USA) and consisted of pre-denaturation at 94°C for 1 min followed by 30 cycles of 1 min at 94°C, 1 min at 52C and 2 min at 72°C, with a single final extension step at 72°C for 3 min. The amplified products were detected by electrophoresis in a 0.8% agarose gel, stained in ethidium bromide solution and photographed. The PCR products were purified with the QIAquick Gel Extraction kit (Qiagen, UK) and cloned into *E. coli* using a TOPO vector kit (Invitrogen, USA). The *pbp2b* gene was sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

Bioinformatic analysis of pbp2b

The protein alignment of a putative amino acid sequence of PBP2b was used to determine the mutation of the *pbp2b* gene comparing penicillin-resistant strains and penicillin-susceptible strain R6 by a ClustalW alignment program. The latter program and the phylogenetic tree program (http://www.ebi.ac.uk/cgibin/clustalw) were used to compare the penicillin-resistant isolates with the pneumococcal strains from other countries, focusing only on the transpeptidase domain. The BLAST program (http://www. ncbi. nlm.nih. gov/BLAST/Blast.cgi) was used to compare the resistant isolates with pneumococcal strains from other countries.

Results

Antimicrobial susceptibility

Of 200 *S. pneumoniae* isolates tested, 120 (60%) were penicillin nonsusceptible (PNSP). None of the PNSP produced β-lactamase enzymes based on testing by the nitrocefin method. The cross-resistance of pneumococci to penicillin and other antimicrobial agents as determined by disk-diffusion method and MIC analysis is shown in Table 1. Based on the disk-diffusion results, penicillin-susceptible isolates were also susceptible to levofloxacin (100%), erythromycin (96.2%), clindamycin (95%) and co-trimoxazole (70%). PNSP isolates had high rates of resistance to co-trimoxazole (86.7%), erythromycin (80%) and clindamycin (36.7%) but a low rate of resistance to levofloxacin (1.7%). Based on Etests, most penicillin-susceptible isolates were also susceptible to amoxicillin/clavulanate (100%), cefuroxime (100%), cefotaxime (100%), cefdinir (100%), cefprozil (100%), levofloxacin (100%), azithromycin (96.2%) and roxithromycin (95%). PNSP isolates had high rates of resistance to roxithromycin (81.7%), cefdinir (80.8%), azithromycin (74.2%), cefuroxime (64.2%) and cefprozil (32.5%) but low rates of resistance to cefotaxime (5%), levofloxacin (1%), and none was resistant to amoxicillin/clavulanate. Minimum inhibitory concentrations (MICs) of

Table 1. Cross-resistance of pneumococcal isolates to penicillin and other antimicrobial agents

Antimicrobial agents	Percentage of isolates with indicated resistant profile[a]					
	Penicillin-susceptible (n = 80)			Penicillin-nonsusceptible (n = 120)		
	S	I	R	S	I	R
Disk diffusion						
Oxacillin	92.5	-	7.5	-	-	100
Erythromycin	96.2	-	3.8	17.5	2.5	80
Clindamycin	95	2.5	2.5	63.3	-	36.7
Levofloxacin	100	-	-	98.3	-	1.7
Co-trimoxazole	70	13.8	16.2	6.7	6.7	86.7
Etest						
Penicillin	100	-	-	-	48.3	51.7
Amoxicillin/clavulanate	100	-	-	100	-	-
Cefuroxime	100	-	-	20.8	15	64.2
Cefotaxime	100	-	-	59.2	35.8	5
Cefdinir	100	-	-	15	4.2	80.8
Cefprozil	100	-	-	30.8	36.7	32.5
Levofloxacin	100	-	-	98.3	0.7	1
Azithromycin	96.2	-	3.8	20	5.8	74.2
Roxithromycin	95	1.2	3.8	18.3	-	81.7

[a] S; susceptibility, I; intermediate, R; resistant. Breakpoints used to define percentages of susceptible, intermediate and resistant categories are those recommended by CLSI[11]

Table 2. Minimum inhibitory concentrations (MICs) (μg/ml) of pneumococcal strains that were selected for sequencing of the *pbp2b* gene

Strain	Source of specimen	MICs (μg/ml)[a]								
		PEN	CTX	CXM	CDR	CPZ	AUG	AZI	ROX	LEV
S171	Gastric wash	0.008	0.032	0.016	0.125	0.125	0.032	2	0.25	1
S146	NP wash	4	0.5	4	8	16	1	256	256	2
S197	Sputum	4	16	16	16	8	2	16	16	0.5
S250	Bronchial wash	8	4	8	16	16	2	32	16	1
S275	Sputum	4	1	4	8	8	1	8	8	1
GP136/46	CSF	8	4	16	16	8	2	256	256	0.5
124/jun 03	Tracheal wash	4	2	8	8	8	2	16	16	2

[a] PEN, penicillin; CTX, cefotaxime; CXM, cefuroxime; CDR, cefdinir; CPZ, cefprozil; AUG, amoxicillin/clavulanate; AZI, azithromycin; ROX, roxithromycin; LEV, levofloxacin

pneumococcal strains that were selected for *pbp2b* gene sequencing are shown in Table 2.

Sequencing of the pbp2b gene

Within the penicillin-binding domain (PBD) of *pbp2b*, isolates with intermediate and full penicillin resistance carried a variety of non-synonymous substitutions (Fig. 1). The first group occurred within the region of the S_{448}-S-N (SXN) triad, and these mutations were observed in all seven isolates. Mutations occurring in this region, in particular the substitution of A for T_{451}, have been shown to mediate the interaction of PBPs with μ-lactam and may contribute to the development of penicillin resistance.

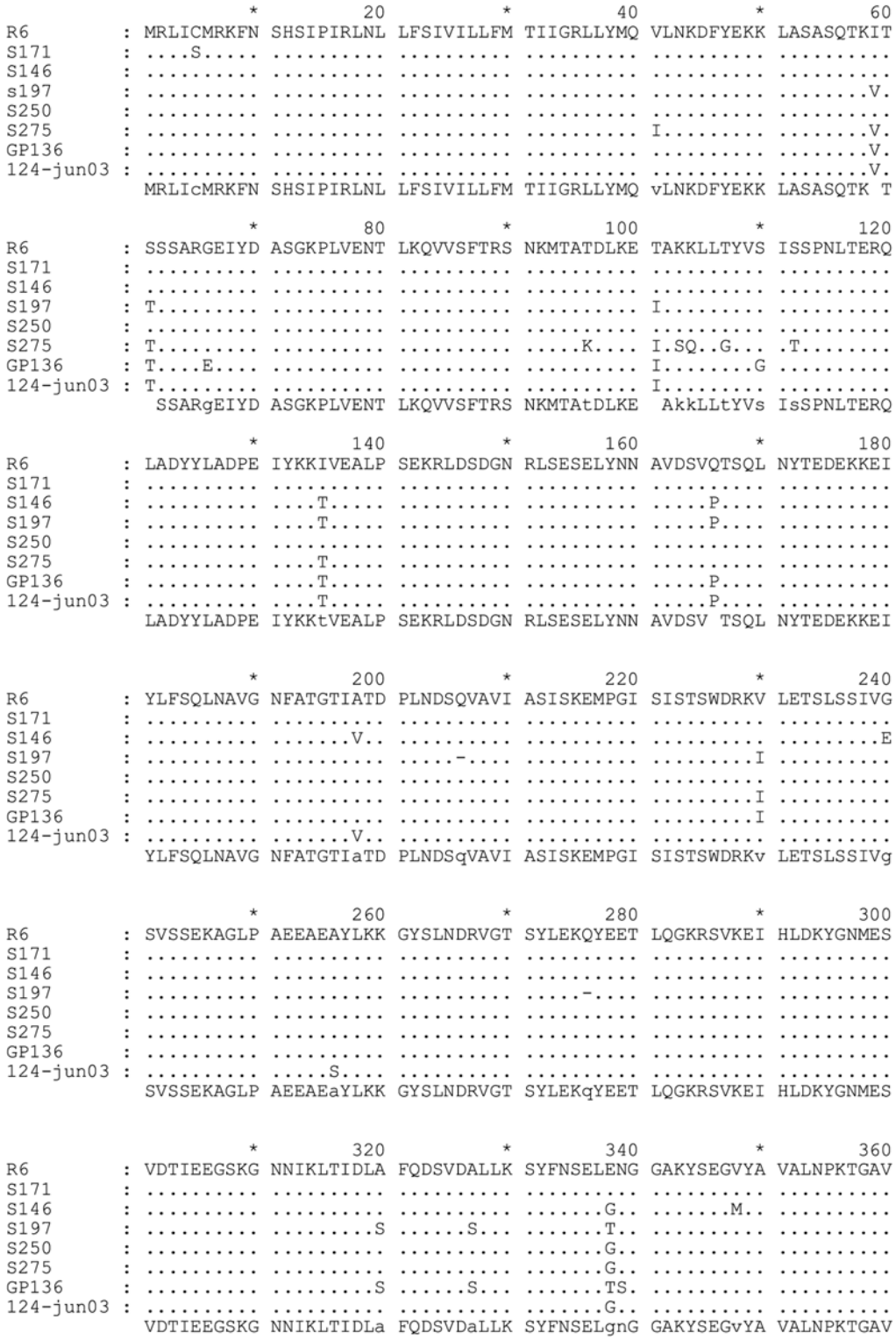

Fig. 1 The ClustalW protein alignment of a putative amino acid sequence of PBP2b. The dots indicate identical amino acids, shaded areas indicate same amino acids, black line group indicate active site, thick gray lines indicate classification based on amino acid alteration, and dashes indicate non-sense codon in the sequence.

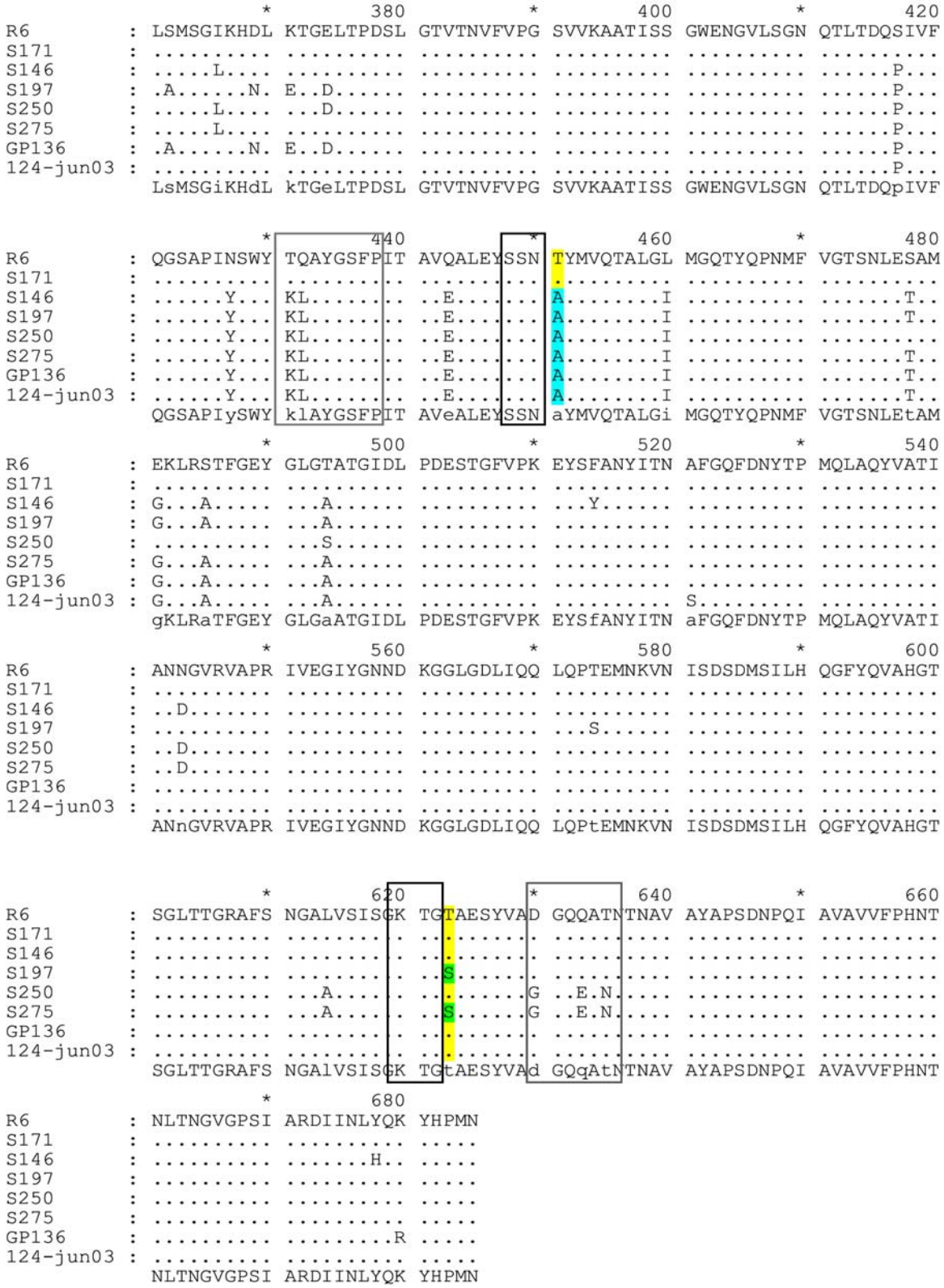
R6 : LSMSGIKHDL KTGELTPDSL GTVTNVFVPG SVVKAATISS GWENGVLSGN QTLTDQSIVF
S171 : .....
S146 : .....L.... .....P...
S197 : .A......N. E..D..... .....P...
S250 : .....L.... ...D..... .....P...
S275 : .....L.... .....P...
GP136 : .A......N. E..D..... .....P...
124-jun03 : .....P...
LsMSGiKHdL kTGeLTPDSL GTVTNVFVPG SVVKAATISS GWENGVLSGN QTLTDQpIVF
R6 : QGSAPINSWY TQAYGSFPIT AVQALEYSSN TYMVQTALGL MGQTYQPNMF VGTSNLESAM
S171 : .....
S146 : ......Y... KL..... ..E..... A.....I ....T..
S197 : ......Y... KL..... ..E..... A.....I ....T..
S250 : ......Y... KL..... ..E..... A.....I .....
S275 : ......Y... KL..... ..E..... A.....I ....T..
GP136 : ......Y... KL..... ..E..... A.....I ....T..
124-jun03 : ......Y... KL..... ..E..... A.....I ....T..
QGSAPIySWY klAYGSFPIT AVeALEYSSN aYMVQTALGi MGQTYQPNMF VGTSNLEtAM
R6 : EKLRSTFGEY GLGTATGIDL PDESTGFVPK EYSFANYITN AFGQFDNYTP MQLAQYVATI
S171 : .....
S146 : G...A..... ...A..... ...Y..... .....
S197 : G...A..... ...A..... .....
S250 : ....S..... .....
S275 : G...A..... ...A..... .....
GP136 : G...A..... ...A..... .....
124-jun03 : G...A..... ...A..... S.....
gKLRaTFGEY GLGaATGIDL PDESTGFVPK EYSfANYITN aFGQFDNYTP MQLAQYVATI
R6 : ANNGVRVAPR IVEGIYGNND KGGLGDLIQQ LQPTEMNKVN ISDSDMSILH QGFYQVAHGT
S171 : .....
S146 : ..D..... .....
S197 : ....S..... .....
S250 : ..D..... .....
S275 : ..D..... .....
GP136 : .....
124-jun03 : .....
ANnGVRVAPR IVEGIYGNND KGGLGDLIQQ LQPtEMNKVN ISDSDMSILH QGFYQVAHGT
R6 : SGLTTGRAFS NGALVSISGK TGTAESYVAD GQQATNTNAV AYAPSDNPQI AVAVVFPHNT
S171 : .....
S146 : .....
S197 : ....S..... .....
S250 : ....A..... .....G ..E.N..... .....
S275 : ....A..... ..S.....G ..E.N..... .....
GP136 : .....
124-jun03 : .....
SGLTTGRAFS NGAlVSISGK TGtAESYVAd GQqAtNTNAV AYAPSDNPQI AVAVVFPHNT
R6 : NLTNGVGPSI ARDIINLYQK YHPMN
S171 : ....
S146 : ....H.. .....
S197 : ....
S250 : ....
S275 : ....
GP136 : ....R .....
124-jun03 : ....
NLTNGVGPSI ARDIINLYQK YHPMN

Fig. 1 Cont.

The second group of mutations was clustered around the K_{619}-T-G (KXG) triad, and both isolates with this mutation had penicillin MICs of 4 μg/ml (isolates S197 and S275). In resistant *S. pneumoniae* strains we found the T_{451} to A and T_{623} to S changes that were described previously in penicillin-resistant pneumococci; these changes were reportedly responsible for low-level penicillin resistance, lower lysis rate, and piperacillin resistance[14]. However, alignments showed additional substitutions, which were present only in resistant *S. pneumoniae* strains. The penicillin-binding domain and transpeptidase domain of PBP2b isolated from six penicillin-resistant strains contained 16 to 30 amino acid changes over the sequence of PBP2b from the R6 β-lactam-sensitive strain (Table 3).

Analysis of *pbp2b* revealed similar patterns of nucleotide and amino acid sequence variations in all resistant isolates (MICs ≥ 2 μg/ml) and penicillin-susceptible isolates (MIC = 0.008 μg/ml). However, DNA sequence analysis of *pbp2b* revealed extensive sequence divergence in all penicillin-resistant isolates compared to the sequences of the genes of penicillin-susceptible strain R6. BLAST analysis of the S146, S197, S250, S275, GP136/46, and 124/jun03 strains revealed PBP2 sequences nearly identical to the PBP2b sequences of the *S. pneumoniae* strains SP00083 (98%), SP00086 (99%), SP00077 (95%), SP00081 (96%), SP00086 (98%) and SP00079 (97%), respectively, as published by Yumiko Sanbongi, Meiji Seika Kaisha Ltd., Pharmaceutical Research Center; Clinical Isolates Collected in Japan (GenBank code; AB119903, AB119906, AB119897, AB119901, AB119906 and AB119899, respectively). From the ClustalW alignment (Fig. 1) and phylogenetic tree program (Fig. 2) (http://www.ebi.ac.uk/cgi-bin/clustalw), the Thai isolates were different only in the part of the transpeptidase domain when compared to those from other countries, especially Kenya, the Netherlands and Vietnam.

Isolates S250, S275 and 124/jun03 were closely related to French isolates. Isolates S146, S197, and GP136/46 were more closely related to Japanese isolates than Korean isolates. The transpeptidase domain of Thai isolate 124/jun03 was closely related to the *S. sanguis* protein accession number D35965 from the United Kingdom [identities = 352/352 (100%)][15], but the PBP dimer domain had low simi- larity [identities = 123/134 (91%)].

Interestingly, there were two non-sense mutations found in strain S197 in the gene coding region for the transpeptidase domain of the PBP2b. The DNA sequences have been confirmed in two different clones of *E. coli* transformants to have the same nonsense codons.

Discussion

By the mid 1980s, ceftriaxone or cefotaxime were typically recommended for empirical treatment of children with suspected bacterial meningitis[16]. *S. pneumoniae* isolates were uniformly susceptible to these extended-spectrum cephalosporins. However, by the early 1990s, as PNSP became more common throughout the world, treatment failures associated with cefotaxime or ceftriaxone administration for pneumococcal meningitis were reported[16]. In this

Table 3. Distribution of nucleotide and amino acid substitutions in the penicillin binding domain of PBP2b from clinical isolates of *S. pneumoniae*

Strains	Penicillin MIC (μg/ml)	Cefotaxime MIC (μg/ml)	Serotype	% of I dentity	No.(%) of[a]:		Amino acid motif[b]		
					Nucleotides altered	Amino acids altered	SVVK	SSNT	KTGTA
S.171	0.008	0.032	23F	99	4 (0.2)	1 (0.1)	——	——	——
S.146	4	0.5	19F	94	96 (4.7)	21 (3.1)	——	—A	——
124/jun 03	4	2	23F	94	99 (4.8)	20 (2.9)	——	—A	——
S.250	8	4	19F	93	116 (5.6)	16 (2.3)	——	—A	——
GP.136/46	8	4	23F	92	148 (7.2)	28 (4.1)	——	—A	——
S.197	4	16	23F	92	147 (7.1)	28 (4.1)	——	—A	—S-
S.275	4	1	19F	91	164 (8)	30 (4.4)	——	—A	—S-

[a] Published sequence of penicillin-susceptible *S. pneumoniae* R6 was used for comparison

[b] Only amino acid residues different from the PBP2b conserved motif sequences of *S. pneumoniae* R6 are shown. Conserved amino acid motifs are underlined

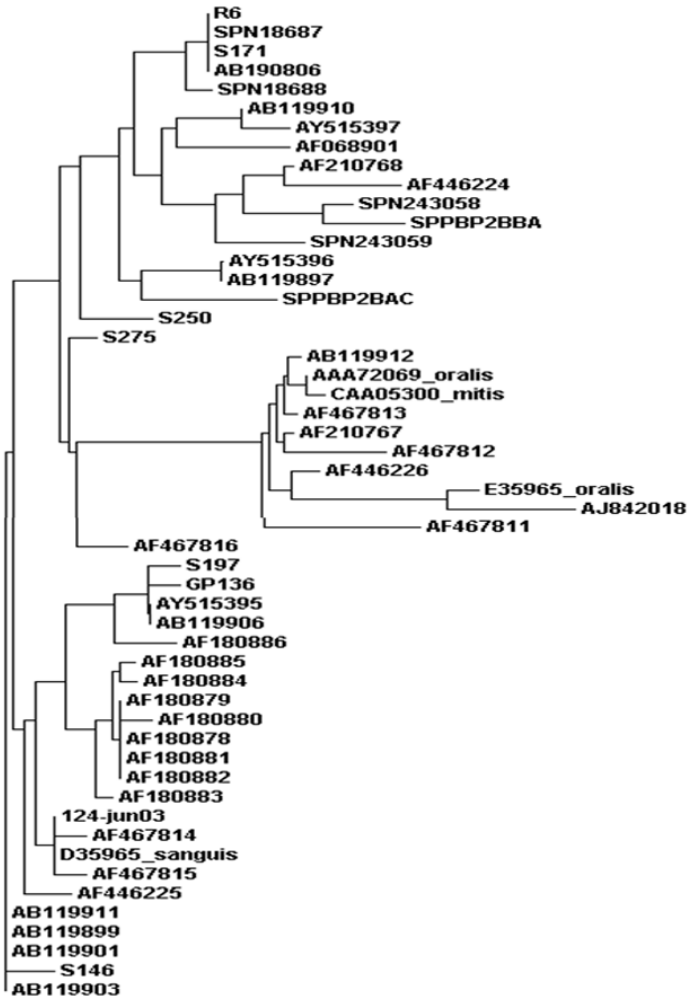

Fig. 2 Phylogram constructed from transpeptidase domain data showing the genetic relatedness of the 58 pneumococcal strains and 3 viridans streptococcal strains from other countries. EMBL/GenBank/DDBJ
Nucleotide sequence accession numbers: AB119897, AB119899, AB119901, AB119903, AB119906, AB119910, AB119911 and AB11912 were isolated in Japan. AF467811 to AF467816, AF210767, AF210768, SPN18687 and SPN18688 were isolated in France. AY609304 to AY609306, AY515395 to AY515397 and AF180878 to AF180886 were isolated in Korea, AF446224 to AF446226 were isolated in Spain, SPN243058, SPN243059, AAA72069 (*S. oralis*) and D35965 (*S. sanguis*) were isolated in UK. AF068901 was isolated in Italy. D35965, E35965 and AAA72069 (*S. oralis*) were isolated in USA. CAA05300 (*S. mitis*) was isolated in Germany

study, up to 41% of isolates were cefotaxime-nonsusceptible (Table 1).

The National Committee for Clinical Laboratory Standards guidelines[17] state that a pneumococcal isolate that is susceptible to penicillin can be considered susceptible to other β-lactams. Consequently, it has been suggested that penicillin MICs can be used to predict the MICs of other β-lactam antimicrobials for *S. pneumoniae*[18]. It is generally accepted that the MICs for amoxicillin and extended-spectrum cephalosporins are usually equal to or two to four times less than the MIC of benzylpenicillin[19]. In our study all PNSP isolates were sensitive to amoxicillin/clavulanate. The reason for this result is unclear given that pneumococci do not produce β-lactamase. However, amoxicillin and clavulanate have been reported to bind selectively to different target penicillin-binding proteins[20], which may lead to synergy of bactericidal effects and subsequent susceptibility to amoxicillin/clavulanate.

A unique mutation was unique in isolates S250 and GP136/46 may contribute to the development of high-level penicillin resistance. The majority of these nucleotides and associated amino acid alterations occurred within a ± 250-bp area between A_{410} and T_{494} and were located within the vicinity of the SVVK tetrad (which houses the active-site serine) and the SSN motif. The exchange of A for T_{451}, which has been identified in all resistant isolates analyzed to date, occurs adjacent to the conserved SSN motif. Because the N residue of this motif has been proposed to form a hydrogen bond with the carbonyl group of the penicillin R1 side chain, the substitution of A for T_{451} presumably disrupts this hydrogen bond and leads to a reduction in the affinity of the protein[21]. Analysis of *pbp2b* revealed very similar patterns of nucleotide and amino acid sequence variation among all penicillin-resistant isolates (MIC ≥ 4 μg/ml), as well as cefotaxime-resistant isolate S197 (MIC = 16 μg/ml). Further analysis of these mutations is required to confirm their role in the development of resistance and their contribution to this unusual phenotype. We hypothesize that precise amino acid changes would be present along with a mosaic structure, at least in primarily affected β-lactam targets (PBP2b and PBP2x), to yield highly resistant isolates. There also may be point mutations among these strains in addition to recombination events that result from mosaics. More information about the three-dimensional structure of these proteins is needed to validate these observations. Further, for the amino acid changes, we found that after protein alignment, the changes occurred at positions common to PBP2b. This is especially true for the cefotaxime-resistant strain S197, which had amino acid changes around the K_{619}-T-Gly triad. From the BLAST program, all penicillin-resistant isolates had nearly identical *pbp2b* gene sequences to clinical *S. pneumoniae* clinical isolates collected in Japan, which were published by Yumiko Sanbongi, Meiji Seika Kaisha Ltd., Pharmaceutical Research Center. The non-sense mutations found in *pbp2b* in strain S197 were confirmed in two different *E. coli* transformants. It is very likely that the mutations found were artifacts from the cloning experiment, as the *pbp2b* gene has been shown experimentally to be essential for growth in *S. pneumoniae*[22]. However, these mutations in the transpeptidase domain in clinical isolates have never been described, and it is possible that the mutations may confer cefotaxime resistance in isolates that express no penicillin binding protein. Further investigation of these mutations is needed to confirm our observation.

The sequence of *pbp2b* from the susceptible isolate (S171) had 99% identity to the sequence of the *S. pneumoniae* PBP2b strain R6, a descendant of the original type 2 capsule S (smooth or encapsulated) clinical isolate used by Avery and coworkers to demonstrate the genetic function of DNA[23], and used worldwide as a standard laboratory strain. The lack of a polysaccharide capsule in R6 renders it avirulent. A few additional substitutions are seen in all penicillin-resistant strains. Based on our analyses, Thai isolates were different from those in other countries, especially those from European countries. Thai isolates were more similar to Japanese than Korean isolates, and had not been reported before; therefore our study could point to interesting results underlying a reduced affinity of the PBP2b in Thai isolates. Most of these amino acid changes are probably responsible for increasing MICs. In PBP2b Japanese isolates, the T_{451}AFS and E_{481}G mutations close to the SSN motif were observed in 24 isolates from Japan[24], some of which showed full penicillin resistance and some had intermediate resistance similar to Thai and Korean isolates. Comparison of the *pbp2b* gene in our isolates with those of Korean isolates revealed that our isolates had no $N_{276(475)}$ to K, $R_{285(484)}$ to C, and $S_{305(504)}$ to F substitution mutations [25]. However, the $T_{252(451)}$ to A and $E_{282(481)}$ to G were similarly found in the translated sequences of our isolates as in all Korean strains and other penicillin-resistant strains from other parts of the world[25,26]. The mutations found near the $S_{442(448)}$-S-N triad, which was A for $T_{445(451)}$ were similar to those described in a report from France[27]. The mutations around the K_{620}-T-G triad (amino acids$_{620-636}$) were also similar to those French isolates, except for the mutations in S197 and S275 that had T_{624} S. Serine is in the same amino acid group as threonine, so the importance of this mutation is not obvious. The MIC data among various β-lactams were somewhat inconclusive, and we had no amoxicillin resistance in our isolates. Further mutagenesis studies of the amino acid in this 624 position would clarify the possible effect of this change.

The higher the penicillin MIC, the more likely it is that the strain will be multidrug resistant. Multidrug-resistant (including resistance to fluoroquinolones) pneumococci have been reported in Hong Kong[28], Canada[26], and Spain[29], and the clonal spread of these strains from country to country and continent to continent is of concern. It may be inferred that resistant strains of Thai isolates were related to Japanese and French isolates.

For β-lactam antimicrobial agents, high-level

resistance requires a sequential transformation event which may then be followed by point mutations, whereas intermediate resistance can be conferred by a single transformation event[30]. Penicillin resistance in *S. pneumoniae* is the result of the acquisition of series of stepwise mutations in PBP genes[24]. Mutations that reduce the amount of drug binding are selected by β-lactam antibiotics. In the laboratory, very low-level resistance to penicillin can result from a point mutation leading to amino acid substitution within the transpeptidase domain of the PBP. High-level penicillin resistance can be established by alteration in only three of these PBPs, *i.e.* PBPs 2x, 2b and 1a, while only altered PBPs 1a and 2x are required for high-level cefotaxime resistance[31].

In conclusion, the analysis of *pbp2b* revealed highly similar patterns of nucleotide and amino acid sequence variation among all penicillin-resistant isolates (MICs, $\geq$ 4 μg/ml), as well as cefotaxime-resistant isolate S197 (MIC, 16 μg/ml). The importance of the exchange of A for T_{451}, which has similarly been identified in all resistant isolates analyzed to date, occurs adjacent to the conserved SSN motif in penicillin-resistant pneumococci and is reportedly responsible for low-level penicillin resistance. Modifications that are almost always observed upstream and downstream of the SXN motif were found to confer significant resistance. Interestingly, all resistant isolates containing the *pbp2b* gene were closely related to those belonging to pneumococcal clinical isolates in Japan. In addition, from the ClustalW alignment and the phylogenetic tree program, Thai isolates differed from isolates from other countries, especially European countries (except in France), and were more similar to Japanese isolates than Korean isolates. The phylogenetic tree of the transpeptidase domain of Thai isolate number 124/jun03 was closely related with the *S. sanguis* protein accession number D35965, which was isolated in the United Kingdom.

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การศึกษาลำดับเบสของจีน pbp2b ในเชื้อ Streptococcus pneumoniae สายพันธุ์ที่ดื้อต่อยา penicillin ซึ่งแยกได้ในประเทศไทย

ชาญวิทย์ ตรีพุทธรัตน์, ปิติมน พลวิชัย, ปรีชา แซ่มปรีดา, สมพร ศรีเฟื่องฟุ้ง

วัตถุประสงค์: *เชื้อ Streptococcus pneumoniae ดื้อยา penicillin เพราะมีการเปลี่ยนแปลงของ penicillin-binding protein (PBP) โดยเฉพาะอย่างยิ่ง pbp2 การเปลี่ยนแปลงเป็นผลทำให้ penicillin สามารถจับกับเอนไซม์ penicillin-binding protein ซึ่งเป็นเป้าหมายของยาได้ลดลงการศึกษานี้ทำเพื่อประเมินสถานการณ์การดื้อยาและวิเคราะห์หาลำดับเบสของจีน pbp2b ในเชื้อ S. pneumoniae สายพันธุ์ที่ดื้อต่อยา penicillin ซึ่งแยกได้ในประเทศไทย*

วัสดุและวิธีการ: *คัดเลือกเชื้อ S. pneumoniae ที่ดื้อยา penicillin มาโดยดูจากการมีค่าความเข้มข้นน้อยที่สุดของยาที่สามารถยับยั้งการเจริญของแบคทีเรียได้ ≥ 4 ไมโครกรัม/มิลลิลิตร และที่ไวต่อยา penicillin แล้วนำมาเพิ่มปริมาณสารพันธุกรรมของจีน pbp2b ด้วยเทคนิคพอลิเมอเรสเชนรีแอกชัน หรือ พีซีอาร์, ทำให้บริสุทธิ์และโคลนจีนนี้เข้าในเชื้อ E. coli จากนั้นสกัดพลาสมิดให้บริสุทธิ์เพื่อนำไปหาลำดับเบสและวิเคราะห์ความเหมือนของลำดับเบสด้วยโปรแกรมทาง bioinformatic*

ผลการศึกษา: *เชื้อ S. pneumoniae ที่มีความไวต่อยา penicillin จะไวต่อยาทั้งหมดอีก 12 ชนิดที่ทำการทดสอบ (95-100%) ส่วน S. pneumoniae ที่ดื้อยา penicillin ก็จะดื้อต่อยาอื่นๆ ที่ทำการทดสอบด้วยยกเว้นยังคงมีความไวต่อยา amoxicillin/clavulanate และ levofloxacin จากข้อมูลลำดับเบสของจีน pbp2b พบว่ามีการแทนที่ของ Ala ที่ตำแหน่ง Thr_{451} ซึ่งอยู่ติดกับส่วน SSN ทั้ง 6 สายพันธุ์ และพบการเปลี่ยนแปลงบริเวณกลุ่มรอบๆ KTG ใน 2 สายพันธุ์ เมื่อใช้โปรแกรม ClustalW เพื่อเปรียบเทียบความเหมือนจะพบว่าสายพันธุ์ที่แยกได้ในประเทศไทยมีความแตกต่างจากที่พบในแถบยุโรป และพบว่ามีความใกล้เคียงกับเชื้อจากประเทศญี่ปุ่นมากกว่าประเทศเกาหลี*

สรุป: *เชื้อ S. pneumoniae สายพันธุ์ที่ดื้อต่อยา penicillin หรือ cefotaxime ซึ่งแยกได้ในประเทศไทยพบว่ามีการเปลี่ยนแปลงของจีน pbp2b เช่นเดียวกับสายพันธุ์ในคนเอเชีย*