Analysis of amino acid motif of penicillin-binding proteins 1a, 2b, and 2x in invasive Streptococcus pneumoniae nonsusceptible to penicillin isolated from pediatric patients in Casablanca, Morocco

Idrissa Diawara1,2,3*, Kaotar Nayme4, Khalid Katfy1,2, Abouddihaj Barguigua5, Mohamed Kettani-Halabi3, Houria Belabbes1,2, Mohammed Timinouni4, Khalid Zerouali1,2 and Naima Elmdaghri1,2

Abstract

Objectives: This study aimed to investigate the nature of the amino acid motifs found in PBPs of Streptococcus pneumoniae isolates in invasive diseases from pediatric patients at Casablanca, Morocco. Five penicillin-susceptible (PSSP), ten penicillin-intermediate (PISP), and fifteen penicillin-resistant S. pneumoniae (PRSP) were studied by PCR–RFLP and DNA sequencing of the pbp1a, −2b, and −2x genes.

Results: There were no changes in the conserved motifs of PBP1a, PBP2b and PBP2x for PSSP strains. Substitution close to PBP1a conserved motifs were found in all PRSP isolates and six/five PISP. Analysis of PBP2b showed that all but one of the 10 PISP strains and all PRSP had substitutions. Substitution close to PBP2x motifs showed that all but three of the 10 PISP strains and all PRSP had substitutions in two conserved motifs. A total of 6, 11 and 10 genotypes were found after analysis of pbp1a, pbp2b, and pbp2x, respectively. The penicillin-nonsusceptible S. pneumoniae isolated in Casablanca share most amino acid substitutions of those reported worldwide, but they occurred among pneumococci with low level resistance to β-lactams.

Keywords: Streptococcus pneumoniae, Penicillin-binding proteins, β-lactams, Serotypes, Penicillin resistance, pbp gene

Introduction

Streptococcus pneumoniae is worldwide a common cause of invasive diseases such as meningitis, bacteraemia [1]. The treatment of pneumococcal infection has been compromised due to the acquisition of several antibiotic resistance, especially to β-lactam drugs [2, 3]. Resistance to β-lactams has been associated in pneumococcus, to alterations of the penicillin-binding proteins (PBP) which reduce their affinity [4]. β-Lactam antibiotics exert their biological effects by interacting with the PBPs. Resistance in clinical pneumococci to this antibiotic family is associated mainly to the alteration of PBP1a, PBP2b and PBP2x [5]. The active site of these PBPs is formed by three conserved amino acid motifs, SXXX, SXN, and KT(S)G. These motifs are found at amino acid positions 370–373, 428–430, and 557–559 in PBP1a, at positions 337–340, 395–397, and 547–549 in PBP2x, and at positions 385–388, 442–444, and 614–616 in PBP2b [4]. Changes in these motifs, or in the positions flanking, are associated with low-affinity variants of the PBPs. It has been previously described that penicillin resistance in S. pneumoniae is mediated by stepwise alterations of PBPs [6, 7]. Several studies have described the genetic profile

*Correspondence: idiawara@um6ss.ma
1 Laboratoire de Microbiologie, Faculté de Médecine et de Pharmacie, Hassan II University of Casablanca, B.P 5696, Casablanca, Morocco

© The Author(s) 2018. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
of the three major plp genes: pbp1a, pbp2b and pbp2x genes in S. pneumoniae around the world. But currently, there are no data on penicillin resistant S. pneumoniae in Morocco. Furthermore, given that the proportion of penicillin resistance in S. pneumoniae has considerably increased in Morocco since 1998 [8], and Casablanca is part of this emerging trend, we described the nature of penicillin resistance in S. pneumoniae in Morocco. Furthermore, given that the proportion of penicillin-resistant S. pneumoniae (PRSP): MIC \( \geq 2 \) mg/L, and 15 penicillin-intermediate (PISP): > 0.06 mg/L, and 15 penicillin-resistant S. pneumoniae (PRSP): MIC > 2 mg/L, were randomly chosen (from 2007 to 2016) to represent the different ranges of MICs of S. pneumoniae found IPD in Casablanca. Antibiotic susceptibility testing was done following Clinical Laboratory Standard Institute guidelines 2016, with the same antibiotics and the same methods used in our previous study [3].

Pneumococci were obtained from the bacteria bank of the laboratory of Microbiology of the University hospital centre of Casablanca, Morocco. The procedure employed for bacterial growth and capsular typing was previously described [9].

For DNA extraction, we use QIAamp® DNA mini kit (QIAGEN, Valencia, CA) according to manufacturer’s recommendations.

**Main text**

**Materials and methods**

**Bacterial strains, growth conditions and DNA extraction**

Thirty invasive pneumococcal isolates: 5 penicillin-susceptible S. pneumoniae (PSSP): MIC \( \leq 0.06 \) mg/L, 10 penicillin-intermediate S. pneumoniae (PISP): > 0.06 and < 2 mg/L, and 15 penicillin-resistant S. pneumoniae (PRSP): MIC \( \geq 2 \) mg/L, were randomly chosen (from 2007 to 2016) to represent the different ranges of MICs of S. pneumoniae found IPD in Casablanca. Antibiotic susceptibility testing was done following Clinical Laboratory Standard Institute guidelines 2016, with the same antibiotics and the same methods used in our previous study [3].

**PCR–RFLP and DNA sequencing of pbp1a, −2b, and −2x genes**

Genetic polymorphism of the penicillin resistance genes pbp1a, pbp2b, and pbp2x was investigated by restriction fragment length polymorphism (RFLP) analysis as described previously [3, 10]. As previously studied [3], the different pbp genotypes received a three numbers code (e.g., x/y/z) referring to the RFLP patterns of the genes pbp1a (x), pbp2b (y), and pbp2x (z), respectively. The 5 penicillin-susceptible S. pneumoniae were used as positive control. Both strands of the purified amplicons (ExoSAP-IT™ PCR, ThermoFisher Scientific, Carlsbad, CA, USA) were sequenced with a Genetic Analyzer 3130x1 sequencer (Applied Biosystems, Foster City, CA, USA), with the same primers used for PCR amplification as recommended by the Centers for Disease Control and Prevention (CDC, USA) [11]. The results of DNA sequencing were aligned using ChromasPro software version 1.7 (Technelysium Pty, Australia) and Basic Local Alignment Search Tool available on Internet at the National Center for Biotechnology Information website. The nucleotide and derived amino acid sequence data for strains are compared to the corresponding sequence data for the β-lactam susceptible laboratory isolate R6 (sequence available at GenBank Accession Numbers: pbp1aM90527; pbp2bX16022; pbp2xX16367).

**Results**

Clinical isolates from various patients were chosen for this study. The age of the patients from whom the isolates were recovered ranged from 0.1 to 5 years. The geometric mean values of MICs (and MIC ranges) in milligrams per liter for penicillin, amoxicillin and ceftriaxone for the three groups with their serotypes were presented in the Table 1. Co-resistance rates among the isolates showed that, 60% of PISP were nonsusceptible to tetracycline, 50% to erythromycin, and 40% to cotrimoxazole. As for PRSP, 86.6% were nonsusceptible to cotrimoxazole, 20% to tetracycline and erythromycin, and 13% to chloramphenicol (Table 1). There was no co-resistance for PSSP (wild profile).

The amino acid sequences of the three conserved PBP motifs of the three PBP studied and their genotypic profiles are shown in Table 2. There were no changes in the conserved motifs of pbp1a, pbp2b and pbp2x for PSSP. As for the amino acid alterations of the three conserved motifs of all PNSP, no mutation is reported close to the KTG (pbp1a) and SVVK (pbp2b) conserved motifs.

Substitution close to PBPla conserved motifs showed Thr \( ^{371} \) → Ala substitution in the conserved STMK motif in all PRSP and six PISP, Pro \( ^{432} \) → Thr (SRNVP motif) in all PRSP and five PISP.

Analysis of PB2p2b showed that 96% of PNSP had Thr \( ^{445} \) → Ala substitutions in the SNNT motif. Substitution close to PB2p2x motifs showed that all but three of the 10 strains of PISP and all PRSP had Thr \( ^{338} \) → Ala substitutions in the STMK motif and had Leu \( ^{546} \) → Val substitutions close to the LKSG motif.

The Ala \( ^{618} \) → Gly substitution close to the third PB2p2b conserved motif (KTG) was identified in only one PISP and one PRSP strain (Table 2). The only change close to the motifs SSN in the PB2p2x was a His \( ^{394} \) → Leu substitution. This change was found in only one strain with MIC to PG at 0.125 mg/L.

A total of 6, 11 and 10 restriction profiles were found after analysis of pbp gene by PCR–RFLP specific for pbp1a, pbp2b, and pbp2x, respectively (Fig. 1). Twenty four different composite pattern profiles for the three resistance genes were found among the 30 isolates. There
were 3, 9 and 12 different composite pattern profiles for PSSP, PISP and PRSP strains, respectively.

We found only one profile for \(pbp1a\) and \(pbp2x\) while \(pbp2b\) presented three different profiles for PSSP strains (Table 2). Concerning the PISP and PRSP strains, there were several genotypes for the three genes. However, among the PRSP strains, we found only two types of profile for \(pbp1a\) gene and the major composite profile was 2/4/6 present in three strains with serotype 14 and 1/4/6 in two strains (one serotype 14 and one serotype 23F).

**Discussion**

PBPs are the major resistance determinants in the pneumococcus. The low-affinity variants of PBPs are the results of recombination of the genes coding for these proteins with genes of other species, such as viridans streptococci. Previous studies have suggested that \(pbp1a\), \(-2x\), and \(-2b\) are generally recognized as the major PBPs associated with the activities of penicillins and some cephalosporins [4, 12]. In our study, changes found in \(pbp1A\), \(pbp2b\) and \(pbp2X\) are globally similar to those previously reported [6, 13, 14].
### Table 2 PBP1a, PBP2b and PBP2x genetic profiles of the 30 pneumococcal isolates in Casablanca, Morocco

| Strain no. | Penicillin-binding protein motifs | Strain no. | Penicillin-binding protein motifs | Strain no. | Penicillin-binding protein motifs |
|------------|----------------------------------|------------|----------------------------------|------------|----------------------------------|
|            | PBP1a                            |            | PBP2b                            |            | PBP2x                            |
|            | STMK (370–373)                   |            | SVVK (385–388)                   |            | STMK (337–340)                   |
|            | SRNVP (428–432)                 |            | SNNT (442–445)                   |            | HSSN (395–397)                   |
|            | KTG (557–559)                   |            | KTGTA (614–618)                  |            | LKSG (546–549)                   |
| Penicillin-susceptible | | | | | |
| 1          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 2          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 3          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 4          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 5          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| Penicillin intermediate | | | | | |
| 6          | −−−−−                            |            | −−−−−                            |            | −A−−−                            |
| 7          | −−−−−                            |            | −−−−−                            |            | −A−−−                            |
| 8          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 9          | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 10         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 11         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 12         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 13         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 14         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 15         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| Penicillin resistant | | | | | |
| 16         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 17         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 18         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 19         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 20         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 21         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 22         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 23         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 24         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 25         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 26         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 27         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 28         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |
| 29         | −−−−−                            |            | −−−−−                            |            | −−−−−                            |

**PBP1a motifs:**
- STMK (370–373)
- SRNVP (428–432)
- KTG (557–559)

**PBP2b motifs:**
- SVVK (385–388)
- SNNT (442–445)
- KTGTA (614–618)

**PBP2x motifs:**
- STMK (337–340)
- HSSN (395–397)
- LKSG (546–549)

**Penicillin-susceptible:**
- Strains 1, 2, 3, 4, 5

**Penicillin intermediate:**
- Strains 6, 7, 8, 9, 10, 11, 12, 13

**Penicillin resistant:**
- Strains 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29
Table 2 (continued)

| Strain no. | Penicillin-binding protein motifs | PBP profile |
|------------|----------------------------------|-------------|
|            | PBP1a | PBP2b | PBP2x | plp1a | plp2b | plp2x |
|            | STMK (370–373) | SRNVP (428–432) | KTG (557–559) | SVVK (385–388) | SNNT (442–445) | KTGTA (614–618) | STMK (337–340) | HSSN (395–397) | LKSG (546–549) |
| 30         | A−−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |

S: serine, T: threonine, M: methionine, K: lysine, R: arginine, N: asparagine, V: valine, P: proline, G: glycine, A: alanine, H: histidine, L: leucine, PBP: penicillin-binding protein
The maximal level of resistance of PNSP responsible of invasive pneumococcal disease reported in Casablanca, is relatively low with the maximal MICs = 2 mg/L compared to MICs of PNSP in many countries where MICs ≥ 8 mg/L were reported [14, 15]. Our investigation of the PBP1a, −2x, and −2b amino acid sequences of 30 clinical pneumococci demonstrates that the degree of diversity within these amino acid sequences correlates with increasing resistance to β-lactam antibiotics. Analysis of PBP1a, −2x, and −2b penicillin-binding motifs revealed the absence of substitution in or close to the active site of all motif analyzed in PSSP strains in this study. These findings differed from previously studies. Indeed Nagai et al. are found Thr445 → Ala substitutions close to SNNT motif in PBP2b gene and Leu546 → Val substitutions close to the LKSG motif in PBP2x gene in PSSP strains [6]. Granger et al. are also found Thr445 → Ala substitutions in the SNNT motif in PBP2b in one PSSP strain in Canada [16]. It is not clear how some PSSP isolates can harbor these two mutations without becoming non-susceptible to penicillin. PSSP analyzed in this study are probably associated with a limited number of clones according to the RFLP profiles of the three pbp genes.

Analysis of PBP1a and PBP2b motifs revealed the absence of substitution in or close to the active site of conserved KTG and SVVK motifs. These findings are in agreement with results from other studies, suggesting that these motifs are not involved in the development of penicillin resistance [17, 18].

Interestingly, PISP isolates with amoxicillin MICs ≥ 0.125 mg/L and MICs ≥ 0.25 mg/L harbored amino acid substitutions close to PBP1a conserved motifs STMK (Thr371 → Ala) and SRNVP (Pro432 → Thr), respectively. This result suggests that alteration in conserved motif of PBP1a may be occurred among S. pneumoniae with low level resistance to penicillin and amoxicillin. The diversity of the pattern of amino acid motifs in the PNSP as well as pbp2b and pbp2x genes suggests these isolates have emerged independently as previously described [19].

For PRSP strains, they shared a similar pattern of amino acid motifs but had different genotypes of the three pbp genes. All of these isolates harbored the same amino acid substitutions close to PBP1a, PBP2b and PBP2x conserved motifs. Similar results were published by Zhou et al. in China [14]. In addition, one strain had Ala618 → Gly substitutions close to KTGTA motif in the PLP2B. These changes are identical to those previously reported [6, 13]. However, we reported in this study, amino acid alteration among PNSP with low-levels of MICs (2 mg/L). In several study, amino acid alteration, especially for PBP1a, is reported for high-level penicillin resistance MICs > 4 mg/L [16, 18, 19]. Our explanation for this difference is the origin of our isolates. Indeed, non-invasive pneumococcal isolates frequently have a higher prevalence and high-levels of antimicrobial non-susceptibility, compared to invasive isolates, but they can share the same amino acid alterations.

Moreover, we found that all PNSP strains had generally some co-resistances associated with other antibiotics families especially to cotrimoxazole, tetracycline and erythromycin as reported elsewhere [20].

PCR–RFLP analysis of the pbp1a, pbp2b and pbp2x genes yielded six, eleven and ten distinct fingerprint patterns, respectively. Genotype 5/2/7 was found most frequently among PSSP isolates and there was a single genotype for plp1a and pbp2x. In contrast, genotype of PNSP strains, showed several composite pattern profiles for the three resistance genes. Variations in the RFLP patterns demonstrate the highly variable nature of the pbp genes.
genes, suggesting a high frequency recombination or point mutations that they undergoes over the time [21].

Conclusions
This study constitutes the first investigation of \( pbp \) gene alterations in invasive \( S.\ pneumoniae \) isolates in Morocco. Our study reveals that penicillin-nonsusceptible \( S.\ pneumoniae \) isolated among children in Casablanca share most \( \text{PBP1a} \), \( \text{PBP2b} \) and \( \text{PBP2x} \) amino acid substitutions with those reported worldwide. Alteration of \( \text{PBP} \) reported here occured among pneumococci with low level resistance to \( \beta \)-lactams. Surveillance of antibiotic-resistant pneumococci in Casablanca should be continued, with due attention to the mechanisms of resistance.

Limitation of the study
The development of resistance to \( \beta \)-lactams is a complex mechanism and can be influenced by mutations in other \( \text{pbp} \) and non-\( \text{pbp} \) genes. In our study, the main limitation was that the substitutions outside the specific areas of \( \text{pbp} \) genes were not examined. These substitutions might also contribute to resistance, in addition to other mechanisms, in the activities of \( \beta \)-lactams in pneumococci [6, 7].

Abbreviations
\( \text{PBP}: \) penicillin binding protein; \( \text{PSSP}: \) penicillin-susceptible \( \text{Streptococcus pneumoniae} \); \( \text{PISP}: \) penicillin-intermediate \( \text{Streptococcus pneumoniae} \); \( \text{PRSP}: \) penicillin-resistant \( \text{Streptococcus pneumoniae} \); \( \text{S}: \) serine; \( \text{T}: \) threonine; \( \text{A}: \) alanine; \( \text{H}: \) histidine; \( \text{L}: \) leucine.

Authors’ contributions
ID, MT and NE conceived and designed the study. ID, KZ, KK and HB conducted the case surveillance and collected clinical data. ID and KN conducted the laboratory assays. ID, KZ, AB, MKH and NE analyzed the data. ID, AB, MKH and KZ drafted the manuscript. All authors read and approved the final manuscript.

Author details
1 Laboratoire de Microbiologie, Faculté de Médecine et de Pharmacie, Hassan II University of Casablanca, B.P 5696, Casablanca, Morocco. 2 Service de Microbiologie, CHU Ibn Rochd, B.P 2698, Casablanca, Morocco. 3 Mohammed VI University of Health Sciences (UM6SS), Casablanca, Morocco. 4 Molecular Bacteriology Laboratory, Institut Pasteur du Maroc, Casablanca, Morocco. 5 Laboratory of Biotechnology and Sustainable Development of Natural Resources, Polydisciplinary Faculty, Université Sultan Moulay Slimane, Beni Mellal, Morocco.

Acknowledgements
We thank all members of Laboratory of Microbiology, University Hospital Centre Ibn Rochd of Casablanca for their collaboration. We would like to acknowledge Pr. Mohamed Benbachir for his generous scientific advisories.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The data supporting the conclusions are included within the manuscript.

Consent for publication
Not applicable.

Ethics approval and consent to participate
This study was approved by the Ethical committee for biomedical research of the University Mohammed V—Soussi, Faculty of Medicine, Pharmacy and Dental Medicine of Rabat, Morocco (N°408, 10 May 2013). The patients/legal guardians were informed about the study (for post-vaccination period); they signed a consent form, and the study was carried out in an anonymous way.

Funding
No funding.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 4 June 2018 Accepted: 20 August 2018
Published online: 31 August 2018

References
1. Organization WH. Pneumococcal conjugate vaccine for childhood immunization—WHO position paper. Wkly Epidemiol Rec. 2007;82(12):93–104.
2. Appelbaum PC. Resistance among \( \text{Streptococcus pneumoniae} \): implications for drug selection. Clin Infect Dis. 2002;34(12):1637–20.
3. Diawara I, Barguiquia A, Katfy K, Nayme K, Belabbes H, Timiouoni M, Zerouali K, Elmdaghri N. Molecular characterization of penicillin non-susceptible \( \text{Streptococcus pneumoniae} \) isolated before and after pneumococcal conjugate vaccine implementation in Casablanca, Morocco. Ann Clin Microbiol Antimicrob. 2017;16:23.
4. Hakenbeck R, Kaminski K, Konig A, van der Linden M, Paik J, Reichmann P, Zahner D. Penicillin-binding proteins in beta-lactam-resistant \( \text{Streptococcus pneumoniae} \). Microb Drug Resist. 1999;52:91–9.
5. Ghysen JM. Serine beta-lactamases and penicillin-binding proteins. Annu Rev Microbiol. 1991;45:37–67.
6. Nagai K, Davies TA, Jacobs MR, Appelbaum PC. Effects of amino acid alterations in penicillin-binding proteins (PBPs) 1a, 2b, and 2x on PBPs affinities of penicillin, ampicillin, amoxicillin, cefotaxime, cefuroxime, cefazolin, and cefadroxil in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant pneumococci. Antimicrob Agents Chemother. 2002;46:1273–80.
7. Cañi F, del Campo R, Alou L, Sellavino D, Morosini ML, Baquero F, Prieto J. Alterations of the penicillin-binding proteins and mumM alleles of clinical \( \text{Streptococcus pneumoniae} \) isolates with high-level resistance to amoxicillin in Spain. J Antimicrob Chemother. 2006;57:224–9.
8. Benbachir M, Elmdaghri N, Belabbes H, Haddioui G, Benzaid H, Zaki B. Eleven-year surveillance of antibiotic resistance in \( \text{Streptococcus pneumoniae} \) in Casablanca (Morocco). Microb Drug Resiat. 2012;18:157–60.
9. Elmdaghri N, Benbachir M, Belabbes H, Zaki B, Benzaid H. Changing epidemiology of pediatric \( \text{Streptococcus pneumoniae} \) isolates before vaccine introduction in Casablanca (Morocco). Vaccine. 2012;30(Suppl 6):G46–50.
10. Ghirardi G, Whitney CC, FaxKlarm RR, Beall B. Major related sets of antibiotic-resistant Pneumococci in the United States as determined by pulsed-field gel electrophoresis and pbp1a-pbp2b-pbp2x-dhf restriction profiles. J Infect Dis. 2000;181:216–29.
11. Centers for Disease Control and Prevention. Characterization of Neisseria meningitidis, Haemophilus influenzae, and \( \text{Streptococcus pneumoniae} \) by Molecular Typing Methods. 2014. http://www.cdc.gov/meningitis/lab-manual/chpt12-molecular-typing-methods.html. Accessed 1 June 2014.
12. Smith AM, Klugman KP. Alterations in penicillin-binding proteins 1A, 2X, and 28 in \( \text{Streptococcus pneumoniae} \) isolates for which amoxicillin MICs are higher than penicillin MICs. Microb Drug Resiat. 2004;48(1):4020–2.
13. Zhou X, Liu J, Zhang Z, Liu Y, Wang Y, Liu Y. Molecular characteristics of penicillin-binding protein 2b, 2x and 1a sequences in \( \text{Streptococcus pneumoniae} \).
pneumoniae isolates causing invasive diseases among children in Northeast China. Eur J Clin Microbiol Infect Dis. 2016;35:4:633–45.

15. Liu EY, Chang JC, Lin JC, Chang FY, Fung CP. Important mutations contributing to high-level penicillin resistance in Taiwan 15F-14, Taiwan 15F-15, and Spain 13F-1 of Streptococcus pneumoniae Isolated from Taiwan. Microb Drug Resist. 2016;22:8:646–54.

16. Granger D, Boily-Larouche G, Turgeon P, Weiss K, Roger M. Molecular characteristics of pbp1a and pbp2b in clinical Streptococcus pneumoniae isolates in Quebec, Canada. J Antimicrob Chemother. 2006;57:1:61–70.

17. Baek JY, Ko KS, Oh WS, Jung SI, Kim YS, Chang HH, Lee H, Kim SW, Peck KR, Lee NY, et al. Unique variations of pbp2b sequences in penicillin-nonsusceptible Streptococcus pneumoniae isolates from Korea. J Clin Microbiol. 2004;42:4:1746–50.

18. Nichol KA, Zhanel GG, Hoban DJ. Penicillin-binding protein 1A, 2B, and 2X alterations in Canadian isolates of penicillin-resistant Streptococcus pneumoniae. Antimicrob Agents Chemother. 2002;46:10:3261–4.

19. Granger D, Boily-Larouche G, Turgeon P, Weiss K, Roger M. Genetic analysis of pbp2x in clinical Streptococcus pneumoniae isolates in Quebec, Canada. J Antimicrob Chemother. 2005;55:5:832–9.

20. Bean DC, Ikram RB, Klena JD. Molecular characterization of penicillin non-susceptible Streptococcus pneumoniae in Christchurch, New Zealand. J Antimicrob Chemother. 2004;54:1:122–9.

21. Bennett D, Lennon B, Humphreys H, Cafferkey M. Penicillin susceptibility and epidemiological typing of invasive pneumococcal isolates in the Republic of Ireland. J Clin Microbiol. 2003;41:8:3641–8.