Alterations of the penicillin-binding proteins and murM alleles of clinical Streptococcus pneumoniae isolates with high-level resistance to amoxicillin in Spain

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Aims: The aim of this study was to analyse the nucleotide sequences of regions encoding the penicillin-binding domains of pbp1A, pbp2B and pbp2X genes and murM alleles from 14 selected amoxicillin-resistant Streptococcus pneumoniae isolates (MICs 8–16 mg/L) obtained in Spain.

Methods: PFGE and dideoxynucleotide chain termination sequencing were used.

Results: Analysis of PFGE profiles showed that the amoxicillin-resistant S. pneumoniae strains belonged to six different PFGE patterns including the Spain23F-1, Spain6B-2, Spain9V-3 and Spain14-5 international clones; however, 8 of the 14 strains belonged to the Spain9V-3 clone. These strains showed the typical changes in penicillin-binding proteins (PBPs) 1A and 2X and had 10 unique changes in the 590–641 region of PBP2B as described previously. Transformation experiments tried to incorporate the transpeptidase domain of PBP2B including the 590–641 region associated with amoxicillin-resistant pneumococci. Sequencing of the pbp2B genes revealed that part of the 3' region of the pbp2B sequence encoding a region of the domain (around amino acid 514–538 to the C terminus of PBP2B) did not recombine with the R6 pbp2B gene. The murM sequence analysis showed that 6, 6 and 2 amoxicillin-resistant S. pneumoniae strains had murMA, murMB5 and murMB6 alleles, respectively. However, strains with murMB5 or murMB6 alleles showed a single mutation (N537D) in the 537–581 region of PBP2B, while strains with the murMA allele had 12 unique changes.

Conclusions: Ten unique changes in the 590–641 region of PBP2B and no specific murM alleles were found in S. pneumoniae strains isolated in Spain with an amoxicillin MIC ‡8 mg/L (MICs from 6 to 12 mg/L by 1 mg/L step dilution). In addition, the presence of specific mutations in PBP2B seems to play a key role in the presence of different murM alleles in these amoxicillin-resistant pneumococcal strains.

Keywords: MurM, PBPs, S.pneumoniae, high-level amoxicillin resistance

Introduction

The higher capability of spread of penicillin-resistant pneumococci has led to a high penicillin resistance prevalence in Spain although a significant decrease in penicillin resistance has been recently observed in children and in adults.1,2 This decrease coincided with the introduction of a heptavalent conjugate pneumococcal vaccine (June 2001) and with a global reduction in antibiotic consumption levels. Thus, the total antibiotic use decreased from 21.66 to 19.71 defined daily doses/1000 inhabitants/day between 1998 and 2002. While consumption of broad-spectrum penicillins, cephalosporins and erythromycin has decreased in Spain, use of amoxicillin/clavulanate and quinolones has increased by 17.5% and 27%, respectively.1

However, the prevalence of amoxicillin-resistant Streptococcus pneumoniae remains as low as in previous years (5.1% in 1998–99).3 Thus, in a recent report carried out in 25 hospitals throughout Spain from 2001 to 2002, the prevalence of amoxicillin-resistant...
pneumococci was 4.4%. In addition, among amoxicillin-resistant pneumococci with an MIC of 8 mg/L, the predominant serotype was 14 (45.9%), followed by serotypes 6, 9, 23 and 15. Moreover, the Spanish multiresistant clones Spain23F-1, Spain6B-2, Spain9V-3 and Spain14-5 composed 62.3% (86 of 138 strains) of all isolates for which the amoxicillin MICs were 4 mg/L.

A recent report has shown that pneumococcal strains with amoxicillin MICs (8–16 mg/L) higher than penicillin MICs (2–8 mg/L) had 10 unique changes in PBP2B, including an A618G substitution, in addition to typical changes in penicillin-binding proteins (PBPs) 1A and 2X. These PBP2B changes appear to be the key alteration associated with amoxicillin resistance.

In addition, the inactivation of the murMN operon was shown to result in a complete loss of penicillin resistance in several of the studied resistant strains. The diversity of the murM sequences has revealed that the great majority of the strains (761 of 814), including both penicillin-susceptible and penicillin-resistant isolates, reacted exclusively with the murMA probe. A smaller group of penicillin-resistant strains (48 of 814 isolates) reacted only with the murMB probe.

Thus, the development of high-level β-lactam resistance is a complex process and the involvement of MurMN in penicillin resistance appears to be dependent on specific mutations in PBPs 2X, 2B and/or 1A. Furthermore, an additional non-PBP-mediated resistance determinant appears to be required for full resistance development in some pneumococci.

The aim of this study was to analyse the nucleotide sequences of regions encoding the penicillin-binding domains of pbp1A, pbp2B and pbp2X genes and murM alleles from 14 selected amoxicillin-resistant S. pneumoniae isolates (MICs 8–16 mg/L) obtained in Spain.

Materials and methods

Bacterial strains

A total of 14 selected amoxicillin-resistant, 1 amoxicillin-intermediate and 3 amoxicillin-susceptible S. pneumoniae strains were studied. All strains were clinical isolates from community-acquired respiratory tract infections recovered from 2000–2002 in Spain. MICs were performed by the microdilution method according to the guidelines of the CLSI10 using double dilutions and also for amoxicillin in 1 mg/L steps formed by the microdilution method according to the guidelines of the CLSI10.

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PFGE

Total DNA was prepared, and chromosomal DNA fragments generated by Smal digestion were separated by PFGE as described previously. PFGE patterns were assigned by visual inspection of the macrorestriction profiles, using accepted criteria.

Gene amplification and sequencing

Chromosomal DNA was obtained using the Bactools Kit (Biotools, B&M Labs, Spain). PBP2X-F and PBP2X-R and PBP2B-F and PBP2B-R primers were used for pbp2X and pbp2B amplifications, respectively. The pbp1A gene was amplified using primers described by Coffey et al. For sequencing the following additional primers: for pbp2X, 5'-GGGCAACAGAGAACTTTCCCAAC-3', 5'-GATGACGATGGCAAGGATGGG-3' and 5'-TTTACGCTATGTGATGATGG-3'; for pbp2B, 5'-TTTGCTGAAAGTATCTTCCATTTTAACCA-3' and 5'-ATTGCTTTCCAGGAAGCTCG-3'; and for pbp1A, 5'-AAGCTCAAAAACATCTGGG-3', 5'-TACTCCACTCTACACTTGCCG-3' and 5'-CCACAAAAACATTTTCATCGGACC-3'. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and then sequenced with an 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For the amplification of murM alleles the primers used were 5'-GCTGGAATCCCATGAGAAGGTTGTTTATTA-3' and 5'-GCTGAAATCCTGATGAGGCTTGT-3' as described by Filipe et al.

Transformation

Cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with CaCl2 at an OD490 of 0.058 and diluted 10-fold in the same medium. After the addition of competence-stimulating peptide 1 (100 ng/mL) and the PCR product (10 µg), cultures were incubated for 30 min at 30°C and for 120 min at 37°C. Of these cultures, 0.1 mL was plated on Mueller–Hinton agar supplemented with 5% blood containing antibiotic in appropriate concentrations for mutant selection. The gene fragments of the PCR products of pbp1A, pbp2B and pbp2X and the R6 strain as the recipient were used in the transformation studies. Transformations were confirmed by PCR and sequenced.

Results

PFGE profiles

Serotypes and pulotypes of the studied strains are shown in Table 1. Analysis of PFGE profiles showed that the amoxicillin-resistant S. pneumoniae strains belonged to six different PFGE patterns including the Spain23F-1, Spain6B-2, Spain9V-3 and Spain14-5 international clones. It is noteworthy that 8 of the 14 strains belonged to the Spain9V-3 clone.

PBPs

The amino acid alterations of the three conserved motifs of PBPs 1A, 2X and 2B in 14 amoxicillin-resistant, 1 amoxicillin-intermediate and 3 amoxicillin-susceptible S. pneumoniae strains are shown in Table 1. PBP analyses showed that all but two of the 18 strains had T371A substitutions in the STMK373 motif and had P374T substitutions close to the SRN430 motif of PBPA1. DNA analysis of pbp2x revealed a new substitution (T338G) located in the STMK340 motif, which was found in two of the three strains with amoxicillin MICs of 16 mg/L. The rest of the isolates, including all strains with amoxicillin MICs of 8 mg/L, showed the same mutation (T338A), but two isolates with MICs of 4 and 0.25 mg/L showed the substitutions TM339AF and T338S, respectively. All but one strain (MIC 0.25 mg/L) of the 18 strains had an L546V substitution close to the KSG549 motif of PBP2X. As for pbp2B the gene, all strains showed the same substitution T345V in the STMK345 motif. However, a pattern of 10 substitutions (A591S, G596P, N605D, L608T, A618G, S639T and D640E) was found to be unique to the 14 amoxicillin-resistant isolates (MICs 8–16 mg/L), with 6 substitutions being specific for this group: G596P, N605D, L608T, A618G, S639T and D640E. One of these unique alterations, A618G, is close to the active site stimulating peptide 1 (100 ng/mL) and the PCR product (10 µg).

Transformation

Two strains were the donors of the pbp2X gene, strain 17 carried the T338G substitution in the STMK motif and strain 4 carried the
### Table 1. Serotypes, susceptibility, PFGE profiles and amino acid alterations of the three conserved motifs of PBP 1A, 2X and 2B in *S. pneumoniae* R6 and 18 studied strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serotype/PFGE type</th>
<th>MIC (mg/L)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PBP1A</th>
<th>PBP2X</th>
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<td>----</td>
</tr>
<tr>
<td>4</td>
<td>14/Spain&lt;sup&gt;14-5&lt;/sup&gt;</td>
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</tr>
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<td>14/unique</td>
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</tbody>
</table>

<sup>a</sup> Amoxicillin MIC determined in 1 mg/L steps appears in parentheses.

<sup>b</sup> Changes in amino acid motifs of test strains from those of *S. pneumoniae* R6.

Bold font indicates conserved amino acid motifs.
PBPs/MurM in highly amoxicillin-resistant *S. pneumoniae*

Table 2. Alterations of penicillin-binding protein 2B associated to murM alleles of clinical *Streptococcus pneumoniae* isolates with a high level of resistance to amoxicillin


*a*Changes in the amino acids of test strains from those of *S. pneumoniae* R6.

TM339AF substitutions in the same motif. Two transformants reached the same MICs of penicillin and amoxicillin (0.12 mg/L and 0.03 mg/L, respectively). In order to determine the impact of the substitutions observed in the 590–641 region we transformed the strains R6⁴/²ˣ and R6¹⁷/²ˣ with the *pbp2B* gene of three strains with different profiles. Strains 11 and 14 carried 10 substitutions in the 590–641 region. Strain 4 presented no change in this region. All six double transformants (R6⁴/²ˣ-⁴/²ᵇ, R6⁴/²ˣ-¹¹/²ᵇ, R6⁴/²ˣ-¹⁴/²ᵇ, R6⁷/²ˣ-¹²/²ᵇ, R⁶⁷/²ˣ-¹¹/²ᵇ and R⁶⁷/²ˣ-¹⁴/²ᵇ) produced the same MICs of the two antimicrobial compounds (0.25 mg/L for penicillin and 0.25 mg/L for amoxicillin). Sequencing of the *pbp2B* genes isolated from the transformant strains showed partial integration in the *pbp2B* gene of the R6 strain. Incomplete integration was observed from amino acid 538; thus, all transformants showed the same profile of the *pbp2B* gene, with no substitutions in the 3′ region of the gene.

Association between murM alleles and PBP2B

The distribution of *murM* alleles of the strains is shown in Table 2. Six of the strains that carried the *murMA* allele were amoxicillin resistant (MICs ≥8 mg/L; MICs from 6 to 12 mg/L by 1 mg/L step dilution). These strains showed a characteristic cluster of mutations in the 537–581 region of PBP2B (V₅₄₁L, R₅₄₄H, G₅₅₃D, D₅₆₀E, Q₅₇₃P₅₇₄AIDTK, M₅₇₃L, D₅₇₇E and S₅₈₁A). Moreover, the strains that carried the *murMB₅* or *murMB₆* alleles did not show these mutations but they showed a different substitution (N₅₅₇D) in this region.

Discussion

PBPs are the major resistance determinants in the pneumococcus, but there are also other mechanisms that can affect β-lactam activity. Previous studies have suggested that substitutions in the PBP2B transpeptidase domain may play a significant role in contributing to the development of amoxicillin resistance. A recent study, shows a cluster of mutations located in the 590–641 region of PBP2B in strains with higher MICs of amoxicillin.

In our study, changes found in PBP2X and PBP1A are similar to those previously reported. In addition, we have found the same changes in the region 590–641 of PBP2B in all the strains with amoxicillin MICs ≥8 mg/L (MICs from 6 to 12 mg/L by 1 mg/L step dilution) as described previously. The importance of this C-terminal region of PBP2B highlights a special resistance phenotype in strain 4. This strain shows an unusual MIC of penicillin, which is 2-fold greater than the amoxicillin MIC (16 mg/L for 4 mg/L, respectively). One explanation for the differences in penicillin and amoxicillin MICs in this strain is the absence of substitutions in the C-terminal region of PBP2B. Moreover, PBP2X and PBP1A of this strain present the typical substitution of the low-affinity form, and the *murM* allele is identical to those in other strains with a very high level of amoxicillin resistance.

With the aim of understanding the implication of the final region of PBP2B in amoxicillin resistance, transformation assays were performed. In these experiments, three PBP2B from strains with the altered C-terminal region (strains 11, 14 and 4) were introduced in a PBP2X-R6 transformant. Double transformants were selected on amoxicillin plates, but no differences were observed in the MICs (0.25 mg/L). Analyses of the sequences showed an incomplete integration of the *pbp2B* gene in the double transformants, and the C-terminal region in all cases remained as in the R6 strain. This phenomenon was observed in previous studies, and were only able to introduce the complete PBP2B when the recipient strain had been previously transformed with low-affinity forms of PBP1A, -2X, -2A and -1B.
change, N 537D, in this region. These two possibilities could be strains carrying murMB5 in strains with the same MIC carrying murMA alleles; or two murMB6 alleles independently of the amoxicillin MIC (8–16 mg/L). The presence of one of these alleles does not seem to be a determinant of the amoxicillin resistance phenotype. Thus, the murMA allele is present in both susceptible and resistant strains.

Moreover, strains with MICs ≥8 mg/L carrying the murMA allele showed specific mutations clustered in the 537–581 region of the php2B gene (Table 2). These substitutions could not be found in strains with the same MIC carrying murMB5 or murMB6 alleles; strains carrying murMB5 or MurMB6 alleles presented a unique change, N373D, in this region. These two possibilities could be two different pathways to reach high resistance to amoxicillin. In order to understand the PBP2B-mediated amoxicillin resistance and the relation with the mur MA gene it is necessary to transfer the complete gene into the transformant recipient. We can conclude that 10 unique changes in the 590–641 region of PBP2B and no specific mur MA alleles were found in S. pneumoniae strains isolated in Spain with an amoxicillin MIC ≥8 mg/L (MICs from 6 to 12 mg/L by 1 mg/L step dilution). In addition, the presence of specific mutations in PBP2B seems to play a key role in the presence of different mur MA alleles in these amoxicillin-resistant pneumococcal strains.

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Transparency declarations

None to declare.

References


PBPs/MurM in highly amoxicillin-resistant *S. pneumoniae*

