Breakthrough in penicillin resistance? *Streptococcus pneumoniae* isolates with penicillin/cefotaxime MICs of 16 mg/L and their genotypic and geographical relatedness

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**Objectives:** To phenotypically and genotypically characterize 11 strains (isolated in four different centres) exhibiting penicillin MIC of 8–32 mg/L among isolates of the SPICE project. Nine isolates were from Romania (9/162; 5.56%) and two from Poland (2/305; 0.66%).

**Methods:** *In vitro* susceptibility was determined in triplicate by microdilution (CLSI guidelines), and additionally, MICs of penicillin, cefotaxime and amoxicillin were confirmed in triplicate by agar dilution. Multilocus sequence typing (MLST), PFGE and gene amplification and sequencing were performed.

**Results:** For the nine Romanian isolates, MICs were $\geq 16$ mg/L for penicillin, cefotaxime and amoxicillin, $\geq 32$ mg/L for cefuroxime and cefpodoxime, 4–8 mg/L for cefditoren and $\geq 128$ mg/L for erythromycin and gentamicin. All isolates were non-susceptible to imipenem (MIC $\geq 0.5$ mg/L) and susceptible to levofloxacin (MIC $= 0.5–1$ mg/L) and vancomycin (MIC $= 0.25–0.5$ mg/L). These Romanian strains presented a new cluster in the 595–600 region of PBP2X (YSGIQL→LS TPWF) conferring 98% homology with *Streptococcus mitis* PBP2X, with a new MurM allele (seven strains) with eight amino acid changes versus R6. PBP nucleotide sequences were highly conserved suggesting a common origin. Allelic profiles of two strains gave sequence type 321, three strains exhibited a single- and four a double-locus variance. MLST-predicted serotype was 23F in all but one strain (19F), but three strains were 19A by Quellung.

**Conclusions:** The multidrug high resistance (precluding adequate oral therapy in children), its origin, the prevalence found in Romania and the presence of non-vaccine (7-valent) serotypes should worry the medical community because of a possible clonal diffusion that would limit therapeutic alternatives.

Keywords: MurM, multidrug resistance, serotype 23F, serotype 19A

**Introduction**

Bacterial evolution towards resistance occurs in two steps: emergence and dissemination.1 Although both concepts are closely related in *Streptococcus pneumoniae*, their specific weight on resistance varies. Point mutations in the host are responsible for resistance to quinolones,2 whereas acquisition of resistance genes by transformation from either viridans streptococci3,4 or penicillin non-susceptible *S. pneumoniae*5 is mostly responsible for the resistance to β-lactams and macrolides, with *de novo* resistance being rare.2 Resistance to penicillin/β-lactams is associated with modifications in genes encoding penicillin-binding proteins (PBP). Development of high β-lactam resistance is a complex process in which the involvement of the...
**S. pneumoniae** with penicillin/cefotaxime MICs ≥ 16 mg/L

*MurMN* operon appears to be dependent on specific mutations in PBPs2X, 2B and/or 1A.5,7 Particular PBP–MurM combinations tend to be preserved and may have an independent evolutionary history in particular clones with respect to high-level penicillin resistance.5 As shown by molecular studies, penicillin/β-lactam resistance is a combination of the spread of resistant clones, acquisition of resistance genes within those clones and the spread of resistance genes to new lineages.9

While some developed countries have experienced shifts towards higher penicillin MICs,10,11 in many developing countries intermediate penicillin resistance continues to predominate.12 In countries of Southern Europe such as Spain, rates of penicillin intermediate and full resistance were similar (≈26% compared with 20%) in the period 1998–2002,13,14 but among penicillin-resistant strains, rates of non-susceptibility to amoxicillin (≈40%), oral cephalosporins (cefaclor and cefuroxime-axetil) (close to 100%) and macrolides (≥60%) reached high values,13,14 with MIC90 of 4 mg/L (penicillin), 8 mg/L (amoxicillin) and 2 mg/L (cefotaxime).14 However, in these studies, none of the penicillin-resistant pneumococci exhibited penicillin MIC of ≥8 mg/L.13,14 In a population-based surveillance carried out in the USA (1995–2001), 1% strains exhibited high resistance (HR) to penicillin (MIC = 8 mg/L) with none of the isolates exhibiting higher penicillin MICs.11

The clinical impact of resistance has been clearly demonstrated for pneumococcal meningitis,15 otitis media,16 and bacteremic pneumonia (with macrolides).17 In the case of pneumococcal pneumonia, the relationship between penicillin/cefotaxime resistance and treatment failures is less clear, penicillin and cefotaxime being appropriate treatments with the current levels of penicillin resistance (MIC up to 4 mg/L).18 However, the emergence of multidrug-resistant pneumococci with ‘very’ high level penicillin/cefotaxime resistance (MIC ≥ 16 mg/L) may clearly imply a challenge for empirical treatment of pneumococcal respiratory tract infections.

The aim of this study was to phenotypically and genotypically characterize 11 multidrug-resistant *S. pneumoniae* isolates with very high penicillin/cefotaxime MIC values (one dilution higher than the value previously used to define ‘very high level’ resistance to penicillin11) among isolates of a surveillance study.

### Materials and methods

**Strains**

Of the 789 *S. pneumoniae* clinical isolates received in the central laboratory (Department of Medical Microbiology and Antimicrobial Chemotherapy, Fundación Jiménez Díaz, Madrid, Spain) from eight Eastern European countries (Czech Republic, Slovakia, Republic of Estonia, Hungary, Poland, Romania, Lithuania and Latvia) participating in the SPICE project (1 November 2005 to 31 December 2006), the 11 strains (received from four different centres) exhibiting penicillin MIC of 8–32 mg/L were further phenotypically and genotypically characterized.

**Susceptibility testing**

MICs of penicillin, cefotaxime and amoxicillin were determined in triplicate by microdilution following CLSI guidelines19 at the central laboratory and confirmed in triplicate by agar dilution19 in the Spanish National Reference Pneumococcal Laboratory, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain, to discard susceptibility incongruences. In addition, at the central laboratory, the MICs of the following antibiotics were determined by micro-dilution: cefditoren, cefuroxime, cefpodoxime, erythromycin, levofloxacin, vancomycin, imipenem and gentamicin.

Breakpoints considered were those defined by CLSI20 except for gentamicin for which a susceptibility breakpoint of 1 mg/L was used. Serotyping was performed by Quellung reaction and/or dot blot assay.21

**Multilocus sequence typing (MLST)**

MLST analysis was performed as described previously.22 Briefly, internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *spa* and *ddl* genes were amplified by PCR from chromosomal DNA using the primer pairs described by Enright and Spratt.23 The internet-accessible database23 was used for assignment alleles numbers and, on the basis of the resulting allelic profiles, the sequence type (ST).

**PFGE**

Total DNA was prepared, and chromosomal DNA fragments generated by *Smal* digestion were separated by PFGE as described previously.24 PFGE patterns were assigned by visual inspection of the macrorestriction profiles using accepted chromosomal DNA restriction patterns criteria.25

**Gene amplification and sequencing**

Chromosomal DNA was obtained using the QIAamp DNA kit (Qiagen, Hilden, Germany). PB2X-R, F and PBP2X-R26 and PBP2B-F and PBP2B-R primers27 were used for *pbp2X* and *pbp2B* amplifications, respectively. The *pbp1A* gene was amplified using primers described by Coffey *et al*.28 For sequencing, we used previously described additional primers for *pbp1A*, *pbp2X* and *pbp2B*.7 PCR products were purified with a Qiaquick PCR purification kit (Qiagen) and then sequenced with a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For amplification of *murM* alleles, the primers used were those described previously.7 Sequences were compared using the BLAST program.30 The ClustalW2 program31 was used to construct PBPIA, 2B, 2X and MurM phylogenetic trees.

### Results

The 11 strains studied represented 1.4% of all pneumococci received in the SPICE project: two isolates (strains 424 and 434) from the 305 isolates (0.66%) received from Poland, and the remaining nine strains from the 162 isolates (5.56%) received from Romania. All 11 strains had been isolated between December 2005 and April 2006 from children: one from blood, five from upper respiratory tract and five from lower respiratory tract specimens.

MIC determination by broth microdilution and agar dilution showed the same MIC values for penicillin, cefotaxime and amoxicillin (Table 1). MICs of penicillin and cefotaxime were 8 and 4–8 mg/L, respectively, for strains 424 and 434 (Polish isolates), showing an HR pattern, while penicillin, cefotaxime and amoxicillin MICs were ≥16 mg/L for the nine Romanian isolates, showing a very-HR (VHR) pattern. Cefuroxime and cefpodoxime MICs were ≥32 mg/L in all cases, while cefditoren MIC was 2 mg/L for the Polish isolates (HR) and 4–8 mg/L for the Romanian isolates (VHR).
Romanian ones (VHR), being the most active cephalosporin tested. All strains were resistant to erythromycin (MIC ≥ 128 mg/L), non-susceptible to imipenem (MIC = 0.5–1 mg/L) and susceptible to levofloxacin (MIC = 0.5–1 mg/L) and vancomycin (MIC = 0.25–0.5 mg/L). High MIC values of gentamicin were also determined: 8 mg/L for strains 424 and 434 (HR) and ≥256 mg/L for VHR strains.

Serotype determined by Quellung was 23F (eight strains) and 19F (three strains). However, these latter 19A strains matched serotype 23F (two strains) and 19F (one strain) in the MLST database (Table 1).

The allelic profile of strains 424 and 434 (HR isolates) differed only in locus ddl (α-alanine-α-alanine ligase) from the Taiwan23F-15 clone (157 in the studied strains and 14 in the reference clone) (Table 1). Two of the VHR strains (2541 and 2552) exhibited an allelic profile that matched with ST321, six strains exhibited an allelic profile for which the nearest match was ST321, with three of them showing a single-locus variance in locus gki (glucose kinase) (strain 2098) or xpt (xanthine phosphoribosyltransferase) (strains 2550 and 2553) and three strains exhibiting a double-locus variance in locus gki (strains 2099, 2162 and 2539) and/or locus xpt (strains 2099). The remaining strain (strain 2554) exhibited an allelic profile that matched with ST2615 differing only in a double locus variance from ST321.

By PFGE, three types were identified: one for HR strains and two for the VHR strains (with two and three subtypes) (Table 1).

Table 2 shows amino acid alterations in the transpeptidase domain of PBP1A, 2X and 2B and in MurM. PBP2B showed modifications in PBP2X, 2B and 1A have been classically associated to β-lactam resistance, mainly penicillin/cefotaxime resistance in relation to PBP2X and amoxicillin resistance in relation to PBP2B.32 In the present study, two types of PBP mutation/configuration profiles were found: one associated with HR (MIC = 8 mg/L) and the other associated with VHR (MIC ≥16 mg/L) to penicillin/cefotaxime/amoxicillin. In the first case, the two Polish strains, the already described classic mutations in PBPs were present,7,33 but with a new murM allele with six amino acid changes with respect to R6. The second case (VHR strains), the mutation/configuration profile has not been described previously and showed an absence of mutations in the conserved domain of PBP1A together with the presence of a new cluster in PBP2X (not previously seen in S. pneumoniae) and a new murM allele, this time with eight amino acid changes. The new cluster in the 595–600 amino acid region (where five changes were clustered: YSGIQL→LSTPWF). In MurM, only two strains presented the R6 allele (MurMA); the other strains presented alleles not described previously (six amino acid changes in HR strains and eight changes in VHR strains) (Figure 1).

Figure 2 shows phylogenetic trees of PBP2X, 2B and 1A. Very high homology was found between VHR strains in PBP1A and PBP2B sequences, with two strains diverging in PBP2X sequence. In the case of HR strains, high homology was found in the three sequences.

**Discussion**

Modifications in PBP2X, 2B and 1A have been classically associated to β-lactam resistance, mainly penicillin/cefotaxime resistance in relation to PBP2X and amoxicillin resistance in relation to PBP2B.32 In the present study, two types of PBP mutation/configuration profiles were found: one associated with HR (MIC = 8 mg/L) and the other associated with VHR (MIC ≥16 mg/L) to penicillin/cefotaxime/amoxicillin. In the first case, the two Polish strains, the already described classic mutations in PBPs were present,7,33 but with a new murM allele with six amino acid changes with respect to R6. The second case (VHR strains), the mutation/configuration profile has not been described previously and showed an absence of mutations in the conserved domain of PBP1A together with the presence of a new cluster in PBP2X (not previously seen in S. pneumoniae) and a new murM allele, this time with eight amino acid changes. The new cluster in the 595–600 region of PBP2X had been previously seen in *Streptococcus mitis* and the homology in the 440 amino acids sequenced of PBP2X between these

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**Table 1.** Serotype (Quellung/MLST), allelic profile, ST, PFGE type and MICs of penicillin (PEN), cefotaxime (CTX) and amoxicillin (AMX) for the 11 strains studied with HR (MIC = 8 mg/L) and VHR (MIC ≥16 mg/L) patterns

<table>
<thead>
<tr>
<th>Strain/resistance type</th>
<th>Country</th>
<th>Serotype Quellung/MLST</th>
<th>Allelic profilea</th>
<th>ST</th>
<th>PFGEb</th>
<th>PEN</th>
<th>CTX</th>
<th>AMX</th>
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<tbody>
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<tr>
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<td>23F/23F</td>
<td>15-29-4-21-30-1-157</td>
<td>2338</td>
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<tr>
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<td>15-29-4-21-30-1-157</td>
<td>2338</td>
<td>1</td>
<td>8</td>
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<tr>
<td>VHR</td>
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<tr>
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<td>2-5-15-16-6-3-62</td>
<td>321</td>
<td>2a</td>
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<td>16</td>
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<tr>
<td>2552 Romania</td>
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<td>2b</td>
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</tr>
<tr>
<td>2098 Romania</td>
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<td>321</td>
<td>2a</td>
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<tr>
<td>2162 Romania</td>
<td>19A/23F</td>
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<tr>
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<td>19A/19F</td>
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<td>2615</td>
<td>3c</td>
<td>16</td>
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</tbody>
</table>

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*a In the order aroE-gdh-gki-recP-spi-xpt-ddl.*

*b Type (number) and subtype (letter) using accepted criteria.24*

*c Nearest matched ST.23*
strains and *S. mitis* is 98%. This probably indicates that VHR was acquired by transformation from *S. mitis* as has been previously described for PBP2B (conferring lower cefotaxime resistance, 4 mg/L). Alterations in the 595–600 region might affect β-lactam binding since in the three-dimensional structure of the protein this region reaches a position close to the catalytic site of the transpeptidase domain. These changes, in the absence of mutations in PBP1A, are probably responsible for the very high MICs determined, while changes in MurM protein are probably more related to the maintenance of these PBP changes (probably by lowering its biological cost) as previously described for PBP2B, although further experiments are needed to fully clarify the molecular nature of VHR. It should be highlighted that this VHR pattern (nucleotide sequences of PBPs, including the new cluster) was highly conserved, as seen in the phylogenetic trees, suggesting a possible common origin.

The clonality of these VHR strains, when studying the allelic profile, showed that all but one strains presented ST321 (two strains) or this was the nearest matched type, with only a single- (three strains) or double-locus variance (three strains). The VHR strain presenting ST2615 also differed in a double-locus variance from ST321. The assigned serotype of these strains by MLST was 23F except in one case; however, two of these 23F strains were serotype 19A by Quellung, suggesting capsular switching. The clonality of HR strains showed only a single locus variance with respect to the clone Taiwan 23F-15, but with significantly higher penicillin/cefotaxime MICs than those of reference (1–2 mg/L).

All VHR strains had been isolated in Romania, while HR strains were from Poland. Globally, when considering the eight countries of the surveillance, the prevalence of penicillin resistance with MICs ≥8 mg/L was 1.4% (11 of 789 isolates). On a

### Table 2. Amino acid alterations of PBP1A, 2X and 2B in *S. pneumoniae* R6 and the 11 studied strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Changes in amino acids in conserved motifs forming or surrounding active PBP binding sitea</th>
<th>PBP1A</th>
<th>PBP2X</th>
<th>PBP2B</th>
<th>No. of changes in the 590–641 region</th>
<th>MurM</th>
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<td>-A--</td>
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<td>V---</td>
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</table>

aChanges in amino acid motifs of test strains from those of *S. pneumoniae* R6.

bR6 sequence.

Changes not described previously, conforming two new alleles.

### Figure 1. Changes in MurM protein in *S. pneumoniae* R6 and the 11 strains studied.

Figure 1. Changes in MurM protein in *S. pneumoniae* R6 and the 11 strains studied.
country basis, the prevalence of HR in Poland found in this study was negligible (0.66%; 2 of 305 strains), but on the contrary, VHR among strains from Romania was worrisome (5.56%; 9 of 162 strains). In addition, this very-high penicillin/cefotaxime/amoxicillin resistance (MICs ≥ 16 mg/L), with MICs of penicillin as high as 32 mg/L and of cefotaxime and amoxicillin as 64 mg/L, was associated to multidrug resistance, all strains being resistant to β-lactams and macrolides with very-high MIC values (≥ 32 mg/L). Even more, none of the strains was susceptible to vancomycin and levofloxacin; however, the presence of this resistance pattern among clinical isolates is highly troubling because therapeutic alternatives are seriously limited.

This study reports a breakthrough increase in the magnitude of β-lactam resistance when compared with MIC values of penicillin, cefotaxime and amoxicillin reported up to now, because although scarce number of strains exhibiting HR (up to 16 mg/L) to penicillin or cefotaxime had been reported, to our knowledge, this is the first report of VHR to penicillin (MICs up to 32 mg/L) concomitant to VHR to cefotaxime and amoxicillin (MICs up to 64 mg/L), and with a biological cost not incompatible with the possible diffusion of the strains. Human population movements may contribute to the diffusion of these strains, and there are two facts that makes them specially troublesome: (i) all strains had been isolated in children and its resistance profile precludes available oral therapy for children (quinolones are not licensed for use in children); and (ii) three strains exhibited a 19A capsular serotype by Quellung (two of them showing capsular switching) and this serotype is not included in the 7-valent pneumococcal conjugate vaccine.

In a previous study where two serotype 23 isolates from the UK with penicillin, amoxicillin and cefotaxime MICs of 8 mg/L were reported, pharmacodynamic considerations concluded that this marked reduction of susceptibility to β-lactams will be associated with therapeutic failures for standard antibiotic doses. The new VHR described, its possible common origin, the non-negligible prevalence found in Romania, the association to multidrug HR (precluding adequate oral therapy in children) and the presence of serotypes not included in the 7-valent vaccine should worry the medical community because of a possible clonal diffusion of this resistance that would clearly limit the therapeutic alternatives.

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Transparency declarations

None to declare.

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