Genetic Relatedness of Recently Collected Spanish Respiratory Tract *Streptococcus pneumoniae* Isolates with Reduced Susceptibility to Amoxicillin

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Of *Streptococcus pneumoniae* isolates from 17 hospitals, 9.8% were amoxicillin nonsusceptible (MIC ≥ 4 $\mu\text{g/ml}$). The genetic relatedness of 138 isolates was studied by pulsed-field gel electrophoresis. Although 44 different clones were detected, more than 62% of these isolates were related to four clones (Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, and Spain¹⁴-5).

In Spain, the first penicillin-resistant *Streptococcus pneumoniae* isolates appeared in the late 1970s and early 1980s (1, 2). Since then, the rate of penicillin nonsusceptible isolates has steadily increased, reaching 40 to 44% during 1989 and 1990 (1, 5) and, 10 years later, 50.2% among isolates from community-acquired respiratory infections (12).

Although penicillin-resistant isolates usually show increased resistance to other β -lactam antibiotics, the MICs of amoxicillin are generally equal to or lower than penicillin MICs (10). In addition, in vivo studies have proved oral amoxicillin to be better than oral cephalosporins for the treatment of *S. pneumoniae* infection (11).

In this study we analyzed 165 clinical isolates of *S. pneumoniae* obtained in Spain during a 1-year period; the amoxicillin MICs for these isolates, 138 of which were studied in depth, were ≥ 4 $\mu\text{g/ml}$ (12).

All *S. pneumoniae* isolates were consecutively collected between November 1998 and October 1999 by 17 different hospitals, distributed at different locations throughout Spain, from patients suffering from community-acquired respiratory infections (12). Susceptibility testing was performed by the microdilution method with Mueller-Hinton broth supplemented with 3% lysed horse blood according to previously described NCCLS guidelines (12). Nonviable isolates and isolates showing amoxicillin-clavulanate susceptibility but for which the amoxicillin MICs were determined to be 4 $\mu\text{g/ml}$ in the initial study (12) were excluded from the pulsed-field gel electrophoresis (PFGE) and other genetic relatedness studies.

BOX-PCR and PFGE were performed as previously described (8). Genomic DNA was digested with *Sma*I, and BOX-PCR was performed on all isolates that were closely related by PFGE ($>70\%$ of homology) to the four reference isolates (9) of the Spanish multidrug-resistant clones, Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, and Spain¹⁴-5.

Each different PFGE pattern with a similarity of $<70\%$ to the others was defined as a clone. Isolates with a PFGE ho-

mology of $\geq 85\%$ were assigned to the same clones. Isolates with a PFGE homology between 70 and 84% and the same BOX-PCR patterns were assigned to the same clones, while those with a PFGE homology between 70 and 84% and different BOX-PCR patterns were considered different clones.

Isolates belonging to the four multidrug-resistant Spanish clones were named as described elsewhere (9), while all the other clones found were named arbitrarily.

For 165 (9.8%) of the 1,684 *S. pneumoniae* isolates originally studied, 201 isolated from middle ear samples and 1,483 from patients suffering from lower respiratory tract infections, the amoxicillin MICs were ≥ 4 $\mu\text{g/ml}$ (12). The amoxicillin MICs were 4 $\mu\text{g/ml}$ for 79 isolates and 8 to 16 $\mu\text{g/ml}$ for 86 isolates. Amoxicillin MICs of ≥ 4 $\mu\text{g/ml}$ for the isolates occurred at similar rates in the pediatric (10.2%, 29/285) and adult (9.7%, 136/1399) populations ($P = 0.8$). The percentages of these amoxicillin-nonsusceptible *S. pneumoniae* (ANSP) isolates varied among all the hospitals participating in the study, ranging from 1.9 to 28.3%. For 5 of 17 hospitals, the percentage of ANSP was less than 5%.

Penicillin MICs for these ANSP isolates were two- to three-fold dilutions lower than the amoxicillin MICs (penicillin MICs ranged from 1 to 4 $\mu\text{g/ml}$) (Table 1).

The ANSP isolates belonged mainly to the most typical serogroups of *S. pneumoniae* associated with multidrug resistance. Serogroups 6, 9, 14, and 23 represented 14.5% (24 isolates), 11.5% (19 isolates), 29.1% (48 isolates) and 15.8% (26 isolates) of the ANSP isolates, respectively.

For the ANSP isolates, erythromycin resistance was similar in pediatric (63.6%, 21/33) and adult (62.1%, 82/132) patients, while isolates for which the ciprofloxacin MICs were ≥ 4 $\mu\text{g/ml}$ were isolated exclusively from adult patients (11.4%, 15/132). Of the 138 isolates studied by PFGE, 44 different clones were detected. Considering only the clones with more than two isolates, all these isolates could be grouped among six different clones (Table 1), and they represented 70.3% (97/138) of the total number of the isolates studied. The well-known Spanish multiresistant clones, Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, and Spain¹⁴-5 (9), comprised 62.3% (86/138) of all isolates for which the amoxicillin MICs were ≥ 4 $\mu\text{g/ml}$.

All of the 24 isolates belonging to the Spain^{9V}-3 clone

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TABLE 1. MICs of antibiotics for 138 *S. pneumoniae* clinical isolates and their clonal distributions^a

Parameter	Value for:										
	Spain ^{23F} -1	Spain ^{6B} -2	Spain ^{9V} -3	Spain ¹⁴ -5	15-I	15-II	19-I	19-II	35	Other	Total
No. of isolates	19	17	24	26	7	2	2	2	4	35 ^b	138
No. of hospitals	11	8	9	8	4	2	1	1	2	12	16
No. of pediatric isolates	3	7	6	3	1	0	0	0	0	9	29
No. of adult isolates	16	10	18	23	6	2	2	2	4	26	109
No. of isolates with PFGE homology of >85%	8	8	24	26	7	2	2	2	4		
Origin of isolates											
Sputum or bronchial aspirate	14	12	14	21	7	1	1	1	4	25	100
Otic exudate	3	5	3	1						6	18
Blood	2		7	4		1	1	1		4	20
Drug ^c											
Penicillin											
MIC ₅₀	4	2	4	4	4				4	2	4
MIC ₉₀	4	4	4	4	4				4	4	4
MIC range	1-4	2-4	1-4	1-4	2-4	2-4	2-4	2-4	2-4	1-4	1-4
Amoxicillin											
MIC ₅₀	4	8	8	8	4				4	8	8
MIC ₉₀	8	16	8	16	8				8	8	8
MIC range	4-8	4-16	4-16	4-16	4-8	8	4	4	4-8	4-16	4-16
Cefotaxime											
MIC ₅₀	2	1	1	1	2				1	1	1
MIC ₉₀	2	2	2	4	2				2	2	2
MIC range	0.5-2	0.5-2	0.5-2	0.5-4	0.5-2	0.5-2	1-2	2-4	1-2	0.5-2	0.5-4
Erythromycin											
MIC ₅₀	0.12	>32	0.12	>32	>32				0.12	>32	>32
MIC ₉₀	>32	>32	>32	>32	>32				>32	>32	>32
MIC range	0.12->32	>32	0.03->32	>32	0.12->32	>32	>32	>32	0.12->32	0.03->32	0.03->32
Ciprofloxacin											
MIC ₅₀	1	0.5	1	1	2				1	1	1
MIC ₉₀	4	2	2	>16	2				1	2	2
MIC range	0.25->16	0.25-4	0.25-2	0.5->16	0.5-2	1-4	1	2	1	0.25-4	0.25->16

^a The amoxicillin MICs for the isolates were ≥ 4 $\mu\text{g/ml}$. All isolates were collected in Spain from 1998 to 1999. MIC₅₀, the MIC at which 50% of the strains are inhibited; MIC₉₀, the MIC at which 90% of the strains are inhibited.

^b Serogroups (number of isolates): 4 (1), 6B (5), 9V (1), 10 (2), 14 (6), 15 (1), 18 (1), 19F (4), 22 (2), 23F (4), 28 (1), 31 (1), nontypeable (6).

^c MICs are given in micrograms per milliliter.

showed a PFGE genetic relationship of $\geq 90\%$ with the corresponding reference strain. This clone grouped 15 serotype 9V and 8 serotype 14 isolates and 1 serotype 19F isolate.

The 26 isolates of the Spain ¹⁴-5 clone belonged to serotype 14 and showed the greatest genetic homology of all the Spanish clones (the overall PFGE genetic relationship of all the isolates was $\geq 95\%$ with the reference strain). All of the isolates were resistant to erythromycin, and for six of them the ciprofloxacin MICs were >16 $\mu\text{g/ml}$.

Nearly all serotypes can acquire resistance to penicillin (1), but it is generally accepted that penicillin resistance is associ-

ated with certain serogroups or serotypes (7). According to genomic DNA macrorestriction patterns, the majority of the isolates for which the amoxicillin MICs were ≥ 4 $\mu\text{g/ml}$ (62.3%) belonged to the previously characterized Spanish multiresistant clones. It is noteworthy that the clone with the highest number of isolates was the Spain ¹⁴-5 clone, one of the most, if not the most, multiresistant of the international clones described (13). The mosaic *pbp* genes encoding the low-affinity PBP 1a, 2x, and 2b of penicillin-resistant isolates probably arose by homologous recombination following transformation with DNA from other pneumococci or from closely related

species (4). Although some isolates of this study had no clonal relationship and could have acquired amoxicillin resistance through a natural process of transformation and genetic recombination, the resistance observed for the majority of the isolates could have been the result of a nationwide dissemination of the previously multiresistant Spanish clones. In a study performed in France on *S. pneumoniae* isolates for which the amoxicillin MICs were ≥ 4 $\mu\text{g/ml}$, the majority of the resistant isolates belonged to three of the four clones found in the present study (3).

The appearance and diffusion of multidrug-resistant isolates with amoxicillin nonsusceptibility, in particular, the four Spain ^{23F}-1, Spain ^{6B}-2, Spain ^{9V}-3, and Spain ¹⁴-5 clones originating in Spain, make it more difficult to find possible therapies for community-acquired *S. pneumoniae* respiratory infections. New pharmacokinetically enhanced amoxicillin formulations able to cope with these amoxicillin nonsusceptible strains could represent a solution to this problem (6).

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