ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2001, p. 2955–2957 Vol. 45, No. 10
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.10.2955–2957.2001

Streptococcus pneumoniae Isolates with Reduced Susceptibility to Ciprofloxacin in Spain: Clonal Diversity and Appearance of Ciprofloxacin-Resistant Epidemic Clones

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Received 12 April 2001/Returned for modification 7 June 2001/Accepted 9 July 2001

Analysis of the pulsed-field gel electrophoretic profiles of 82 pneumococcal isolates with reduced susceptibility to ciprofloxacin (RSC) and of 90 co-occurring susceptible isolates indicates a considerable genetic diversity among isolates with RCS and points to a close relation between the two groups. This finding suggests that pneumococci with RCS emerge through independent mutational events.

The recent emergence and increase in incidence of *Streptococcus pneumoniae* clinical isolates with reduced susceptibility to fluoroquinolone antibiotics (2, 7, 9) has raised questions concerning the mechanisms through which this trait is acquired and spread within populations of *S. pneumoniae.* The issue is whether the resistant isolates represent only a few clonal types or a genetically diverse set of strains that emerged through the antibiotic's selective pressure upon existing bacterial clones. These two alternatives have different implications as to the choice of the most appropriate interventions to curtail further increase in the number of resistant isolates.

Bacterial strains. *S. pneumoniae* isolates were selected from two previous studies performed in Spain (1, 6), where 2.22 defined daily doses of quinolones per 1,000 inhabitants were prescribed in 1997 (19). Strains were collected during 1996 to 1999 in 20 different hospitals from patients suffering from community-acquired respiratory infections. Of the 179 pneumococci identified with reduced susceptibility to ciprofloxacin (MICs $\geq$ 4 μg/ml) (RSC), 82 were selected for molecular typing. All but five of these 82 isolates were from adults; 63 were from the respiratory tract, 14 were from blood, and 5 were from the middle ear. An additional 90 strains were also selected from among isolates susceptible to ciprofloxacin (MIC = 1 μg/ml) collected at the same surveillance sites and during the same surveillance period. Most of these 90 isolates were from the respiratory tract, 22 were from blood, and 12 were from the middle ear. The ciprofloxacin-susceptible isolates were selected to match as closely as possible the characteristics of the isolates with RSC in terms of antimicrobial resistance profile and serogroup. For 63.4% of the 82 isolates with RSC the ciprofloxacin MIC was 4 μg/ml, for 18.2% the MIC was 8 μg/ml, for 15.8% the MIC was 16 μg/ml, and for 3.6% the MIC was >16 μg/ml. Regarding their distribution by serogroup, the most frequent serogroups were 19 (19.2%); 3 (13.2%); 9, 14, and 23 (each 12.0%); 6 (8.4%); and 18 (4.8%). Nontypeable strains accounted for 3.6% of the isolates, and the remaining 14.8% belonged to other serogroups.

PFGE. Total DNA was prepared, and chromosomal DNA fragments generated by *Sma*I digestion were separated by pulsed-field gel electrophoresis (PFGE) as previously described (16). PFGE patterns were assigned by visual inspection of the macro-restriction profiles, using accepted criteria (17). Profiles displayed by at least two isolates were assigned letters arbitrarily, except with previously recognized clones, which were named according to the newly recommended nomenclature (10) (Table 1).

The majority of the clones identified in this study were recovered in more than one hospital, and different clones were identified in all hospitals. Notable exceptions are three clones with RSC—B, E, and K—each of which was restricted to a single hospital.

Serogroup and PFGE type. In Table 1 are identified isolates that, in spite of sharing the same clonal type, as defined by PFGE, expressed different serotypes. Particularly interesting is the acquisition of capsular type 3 by a representative of the France^{9V}-3 clone, since change to this serotype by a representative of the Spain^{23F}-1 clone was shown to have a profound impact on virulence (12). These isolates are believed to result from a capsular switch in vivo through transformation or transduction with foreign DNA (3, 12, 14).

Relation between clonal types and ciprofloxacin MIC. The 82 *S. pneumoniae* clinical isolates with RSC presented 44 different PFGE patterns. Of these, 29 patterns were unique to isolates with RSC and were each represented by a single isolate. Three internationally disseminated clones were identified among both isolates with RSC and susceptible isolates (Table 1). Additionally, other isolates identified in this study sharing the same PFGE pattern and serotype were susceptible and showed RSC (clones A, F, I, and J). Moreover, three clones, with the exception of the internationally disseminated clones,

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TABLE 1. Diversity of microbiological profiles among the 172 isolates analyzed[a]

Susceptibility to ciprofloxacin or no. of isolates in MIC classes[b]	PFGE type	Capsular group or type	Antibiotype(s) (no. resistant)[c]	Total no. of strains
Susceptible	A	3		9
3, 1, 1, 0	A	3	P (1), C (1), F (1)	5
1, 0, 0, 0	*B*	*3*		*1*
1, 0, 0, 0	*B*	*19*	*P*	*1*
1, 0, 2, 0	C	6	P (2), E, C (2)	3
2, 0, 0, 0	D	8		2
2, 0, 0, 0	E	14	P, C, F	2
Susceptible	F	18		1
3, 0, 0, 0	F	18		3
2, 0, 0, 0	G	19	P (1), E (1), C (1)	2
2, 0, 0, 0	H	19	E	2
Susceptible	I	19		1
0, 1, 0, 0	I	19	E	1
Susceptible	J	19	P (1), E, C (1)	2
1, 0, 0, 0	J	19	E	1
1, 0, 1, 0	K	35	E	2
Susceptible	L	ND[d]		1
Susceptible	L	NT[e]		1
1, 0, 0, 0	L	ND		1
Susceptible	M	3		2
Susceptible	N	6	C (1)	2
Susceptible	O	9		2
Susceptible	P	14	P, E (1), C, F (1)	2
Susceptible	R	15	E (1)	2
Susceptible	S	19	E (1)	2
Susceptible	T	19	P, E, C	2
Susceptible	V	11		2
Susceptible	W	24		1
Susceptible	W	NT	E, C (1)	2
Susceptible	*France^{9V} −3*	*9*	*P, A (1), C*	*3*
Susceptible	*France^{9V} −3*	*14*	*P (3), C, F (1)*	*4*
1, 0, 0, 0	*France^{9V} −3*	*3*		*1*
5, 2, 0, 0	*France^{9V} −3*	*9*	*P (4), E (2), C, F (2)*	*7*
1, 2, 0, 0	*France^{9V} −3*	*14*	*P (2), A (1), E (1), C (1), F (1)*	*3*
Susceptible	Spain^{14}−5	14	P, A (1), E, C	1
0, 0, 2, 0	Spain^{14}−5	14	P, E, C, F (1)	2
Susceptible	*Spain^{23F} −1*	*19*	*P (3), E (3), C*	*4*
Susceptible	*Spain^{23F} −1*	*23*	*P (2), E (2), C, F (1)*	*4*
4, 1, 1, 1	*Spain^{23F} −1*	*19*	*P (5), E (4), C, F (3)*	*7*
3, 0, 4, 0	*Spain^{23F} −1*	*23*	*P (4), E (5), C (6), F (2)*	*7*
Susceptible	Unique	6	E	3
Susceptible	Unique	9	P (2), E (1), C (3), F (1)	5
Susceptible	Unique	14	P (1), A (1), E (1), C (1), F (1)	2
Susceptible	Unique	18	C (1)	2
Susceptible	Unique	19	P (2), E (1), C (2), F (1)	5
Susceptible	Unique	23	P (2), E (1), C (2)	4
Susceptible	Unique	28		1
Susceptible	Unique	34		1
Susceptible	Unique	ND		6
Susceptible	Unique	NT	E (2), C (4)	9
3, 1, 0, 0	Unique	3	E (1)	4
1, 0, 0, 0	Unique	4		1
1, 2, 0, 1	Unique	6	P (1), E (3), C (1), F (1)	4
2, 0, 1, 0	Unique	9	E (1)	3
1, 0, 0, 0	Unique	11	E	1
0, 1, 1, 1	Unique	14	P (2), E (2), C (2), F (1)	3
1, 0, 0, 0	Unique	18		1
1, 0, 1, 0	Unique	19	E (1), C (1), F (1)	2
1, 0, 0, 0	Unique	22		1
3, 0, 0, 0	Unique	23	E (1), C	3
1, 0, 0, 0	Unique	31		1
0, 1, 0, 0	Unique	35	P, C	1
1, 0, 0, 0	Unique	ND		1
1, 2, 0, 0	Unique	NT	P (2), E, C, F (1)	3

[a] Isolates having the same PFGE type but expressing different serogroups are highlighted in italics. Isolates with the same PFGE type and serogroup are in roman type. Single isolates with unique PFGE profiles are labeled as such and grouped by serogroup.

[b] Isolates were classified as susceptible if the MIC for the isolate was 1 μg/ml. Otherwise, numbers of isolates in the MIC classes, 4, 8, 16, and >16 μg/ml, are indicated.

[c] A, amoxicillin; C, cefuroxime; E, erythromycin; F, cefotaxime; P, penicillin. Breakpoints were according to the NCCLS (12). If not all of the isolates were resistant, the numbers in parentheses indicate how many were.

[d] ND, not determined.

[e] NT, nontypeable.

presented different resistance levels (clones A, C, and K) (Table 1).

Surprisingly, an increase in ciprofloxacin MIC did not cause a reduction in the diversity of PFGE profiles observed. We identified 32 different PFGE patterns among the 52 isolates for which the ciprofloxacin MIC was 4 μg/ml, 12 patterns among the 15 isolates for which the MIC was 8 μg/ml, and 7 patterns among the 13 isolates for which the MIC was 16 μg/ml. None of the three isolates for which the MIC was >16 μg/ml had identical PFGE profiles (Table 1). This situation is in contrast to what is observed with penicillin resistance, with which we find a smaller number of clonal types among highly resistant isolates (18).

Ciprofloxacin was introduced into therapeutic practice in Spain in 1988. Notwithstanding, genetic diversity among ciprofloxacin-resistant *S. pneumoniae* is still widespread among clinical isolates collected from 1996 through 1999, and the same PFGE profiles can be found among isolates with RSC and susceptible isolates. The findings suggest that ciprofloxacin-resistant pneumococci are the products of independent mutational events selected by the drugs from among a diverse population of pneumococci, presumably during treatment with these antibiotics. Most likely the mechanism of resistance involves alteration of the quinolone targets through either mutation (8, 11, 13) or transformation with genes derived from other organisms (5, 20), explaining the observed clonal diversity. The fact that approximately 93% of the strains for which MICs are ≥4 μg/ml from the original collections were isolated from adults correlates well with the exclusive use of fluoroquinolones among adult and not pediatric patients (2, 6, 9). The five pediatric isolates with RSC studied in detail do not show an overrepresentation of the international clones frequently isolated from children; one isolate belonged to the France^{9V}-3 clone and two belonged to the Spain^{23F}-1 clone, but two others presented unique PFGE types.

In addition to the genetic diversity of the pneumococcal isolates with RSC, equally impressive was the fact that a large proportion—25 of the 82 isolates or 30%—of the pneumococci with RSC belonged to two internationally spread multidrug-resistant epidemic clones: France^{9V}-3 and Spain^{23F}-1. In spite of their high numbers, there is no overrepresentation of these clones among isolates with RSC in relation to the number of sensitive isolates selected to be as similar as possible in terms of antibiotype and serotype in our sample (Table 1). This finding suggests that the high prevalence of these clones among isolates with RSC reflects their prevalence in the population, i.e., these clones are not more represented than would be expected if we assume that isolates with RSC are being selected from existing clones. The epidemicity of these clones (4, 15) suggests that dissemination of ciprofloxacin resistance through these isolates is a plausible scenario.

We thank Alexander Tomasz for critical reading of and suggestions concerning the manuscript and Lorenzo Aguilar (Medical Department, GlaxoSmithKline) for technical support.

Partial support for this work was provided by contract PRAXIS/P/SAU14051/1998 from Junta Nacional de Investigação Científica e Tecnológica. L.A. was supported by a grant from the Universidad Complutense de Madrid, Madrid, Spain. M.R. was the recipient of a fellowship from Fundação para a Ciência e Tecnologia (BPD/20185/99).

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