ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2005, p. 418–420 Vol. 49, No. 1
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.1.418–420.2005

Streptococcus pyogenes Pharyngeal Isolates with Reduced Susceptibility to Ciprofloxacin in Spain: Mechanisms of Resistance and Clonal Diversity

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Received 13 April 2004/Returned for modification 5 August 2004/Accepted 23 September 2004

A survey of *emm* gene sequences and an analysis of the pulsed-field electrophoretic profiles of 30 *Streptococcus pyogenes* isolates with reduced susceptibilities to ciprofloxacin detected the prevalence of isolates with *emm* type 6 and considerable genetic diversity among isolates. The mechanism of ciprofloxacin resistance in these isolates was based on point mutations in topoisomerase IV subunit C encoded by *parC*, mainly replacement of serine-79 by alanine.

Streptococcus pyogenes is the etiologic agent of a wide range of human infections, including streptococcal sore throat, skin and soft tissue infections, and the postinfectious syndromes of glomerulonephritis and acute rheumatic fever. Penicillin remains the drug of choice for the treatment of these infections because *S. pyogenes* remains susceptible to this antibiotic despite its intensive use. By contrast, an increasing frequency of *S. pyogenes* isolates that are resistant to macrolides, probably due to the increasing use of these antibiotics for the treatment of other bacterial respiratory pathogens, i.e., *Streptococcus pneumoniae* (5), has been reported in different countries (2, 7, 13). Similarly, the increasing use of fluoroquinolones, due to their excellent activities against some bacterial pathogens, has led to the emergence of *S. pyogenes* isolates with reduced susceptibilities to these antibiotics (4, 15). Increased resistance of *S. pyogenes* to ciprofloxacin has been reported in Spain at the highest rates ever published (10). Using *emm* gene typing and pulsed-field electrophoresis, we studied whether those resistant isolates represent only a few clonal types or a genetically unrelated set of strains that have emerged through the antibiotic's selective pressure.

Bacterial strains. *S. pyogenes* isolates included in this study belong to the SAUCE II surveillance collection and were collected between November 1998 and October 1999 from patients with acute pharyngitis in 17 different hospitals selected on the bases of population and geographical location in Spain (10). Among 70 *S. pyogenes* isolates with reduced susceptibilities to ciprofloxacin (for MICs of ≥4 μg/ml, no NCCLS susceptibility breakpoint was available) collected during this period, a random sample of 30 isolates was selected for *emm*-typing studies. All but 6 of these 30 isolates were from pediatric samples. An additional 32 pharyngeal isolates were also selected from isolates susceptible to ciprofloxacin (MIC ≤ 1 μg/ml) so as to match the resistant strains in terms of location, age group, and temporal proximity during the same surveillance period.

***emm* gene typing.** The *emm* gene types of the *S. pyogenes* isolates were determined by amplification and sequencing of the *emm* genes as described by Beall et al. (3). Lysates of the *S. pyogenes* isolates were prepared with mutanolysin as described previously (1). Primers GASM1 and GASM2 were used in PCRs carried out according to a method described previously (3). PCR products were sequenced with primer GASM1 with a dye terminator mix (Perkin Elmer, Applied Biosystems, Madrid, Spain) and were subjected to automated sequence analysis on a model 377 DNA sequencer (Perkin Elmer, Applied Biosystems). DNA sequences were subjected to homology searches against the bacterial DNA database with BLASTN. Sequences were given GenBank *emm* designations based on previously described criteria (3). We found a strong association between *emm* type 6 and resistance to ciprofloxacin. Thus, 19 of the ciprofloxacin-resistant isolates (63.3%) exhibited *emm* type 6, while only 1 ciprofloxacin-susceptible isolate (3.1%) exhibited this *emm* type. The remaining resistant isolates exhibited other *emm* types (Table 1). *emm* type distribution among susceptible isolates was highly heterogeneous. Up to 12 different *emm* types accounted for 100% of the susceptible isolates (data not shown).

PFGE. Our *emm*-typing results suggest that ciprofloxacin-resistant *S. pyogenes* isolates could be genetically related, since a high percentage of them exhibited *emm* type 6. To test this hypothesis, we analyzed by pulsed-field gel electrophoresis (PFGE) the genomic DNA of all the ciprofloxacin-resistant *S.*

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TABLE 1. Characteristics of pharyngeal ciprofloxacin-resistant *S. pyogenes* isolates collected in Spain (1998 to 1999)[a]

No. of isolates	*emm* type	PFGE pattern	QRDR mutation(s)
9	6	A	Ser79Ala
3	6	D	Ser79Ala
2	6	B	Ser79Ala
2	6	H	Ser79Ala
1	6	G	Ser79Ala
1	6	E	Ser79Ala
1	6	N	Ser79Ala
2	73	M	Ser79Ala
1	28	R	Ser79Phe, Asp91Asn
1	28	C	Asp91Asn
1	75	J	Ser79Phe, Asp91Asn
1	75	L	Ser79Ala
1	22	F	Asp91Asn
1	78	I	Asp91Asn
1	3	K	Ser79Ala
1	st1815	B	Ser79Ala
1	12	B	Ser79Ala

[a] A MIC of ≥4 μg/ml was used as the ciprofloxacin resistance breakpoint. No NCCLS breakpoint was available.

pyogenes isolates. Total DNA was prepared, digested with SfiI, and resolved by PFGE as previously described (2). Differences in banding patterns were documented by visual examination and indexed by capital lettering. Interpretation of restriction fragments was performed in accordance with recent consensus publications (14).

Results of PFGE analysis are shown in Fig. 1. Nine *emm* type 6 isolates presented PFGE pattern A, confirming our hypothesis that these isolates were genetically related. However, the remaining 10 *emm* type 6 isolates exhibited six different PFGE patterns. Moreover, the remaining ciprofloxacin-resistant isolates, which exhibited different *emm* types, also presented different PFGE patterns (Table 1). These findings suggest that ciprofloxacin-resistant *S. pyogenes* strains are the products of independent mutational events selected by the drugs from among a diverse population of *S. pyogenes* strains,

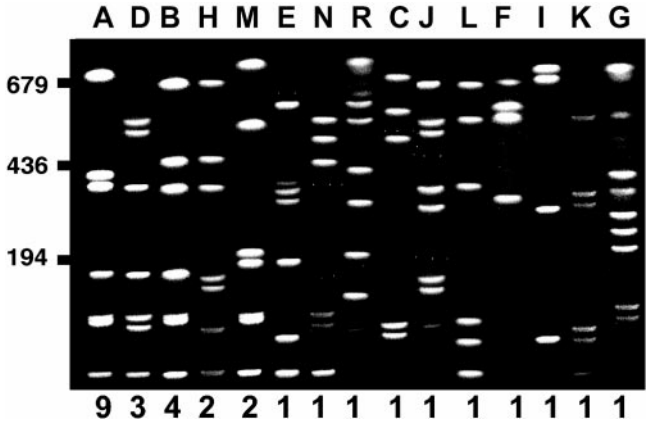

FIG. 1. Representative PFGE types of ciprofloxacin-resistant *S. pyogenes* isolated between 1998 and 1999 in Spain. *S. pyogenes* genomic DNA was isolated, restricted with SfiI, and analyzed by PFGE. Letters above the lanes refer to PFGE types, and the numbers below the lanes refer to the number of isolates of each type. Molecular size markers (in kilobases) are indicated on the left.

presumably during treatment of other bacterial pathogens with these antibiotics. However, certain clones (of *emm* type 6 and PFGE pattern A) are preferentially selected and disseminated. Interestingly, eight of the nine isolates of this prevalent clone were also resistant to erythromycin (data not shown). The mechanism of resistance to erythromycin of these eight isolates is based on the presence of an efflux pump encoded by *mefA* as previously described (2). Moreover, all these isolates were collected from different patients in the same city. Thus, it is more probable that this specific clone was preferentially selected due to the use of macrolides than due to the use of quinolones, since all isolates were from children, in whom quinolone consumption, if any, is extremely low.

Mechanisms of ciprofloxacin resistance in *S. pyogenes*. The MICs of the antibiotics were determined by the microdilution method according to NCCLS guidelines. To eliminate the possibility that ciprofloxacin resistance was due to the presence of an active efflux pump, we determined the ciprofloxacin MICs in the presence of the efflux pump inhibitor reserpine (10 μg/ml). The ciprofloxacin MICs remained unchanged in the presence of reserpine, indicating that ciprofloxacin resistance in *S. pyogenes* was not due to the expulsion of the antimicrobial drug from the intracellular compartment by an efflux pump. Reduced susceptibility of *S. pyogenes* to ciprofloxacin has been associated with point mutations in either *gyrA* or *parC* (15). Those point mutations are located along the quinolone resistance-determining regions (QRDRs) of both genes (15). We investigated whether the ciprofloxacin-resistant isolates included in this study presented point mutations in the QRDRs of *gyrA* and *parC*. For this purpose, genomic DNA purified as described above was used as the template for PCR amplification of a 614-bp fragment of *gyrA* and a 520-bp fragment of *parC* containing the QRDRs of both genes with the pairs of primers described by Yan et al. (15). PCR products were sequenced on both strands, and sequences were compared with *S. pyogenes gyrA* and *parC* sequences from ciprofloxacin-resistant and -susceptible isolates previously described (15).

All ciprofloxacin-susceptible isolates demonstrated nucleotide sequences for the QRDRs of both *gyrA* and *parC* that were identical to those of the previously described susceptible strain ATCC 700294 (GenBank accession number AF220946) (data not shown). In contrast, mutations were identified only in *parC*, not in *gyrA*, in all the ciprofloxacin-resistant isolates. Specifically, two point mutations within the QRDR were identified in *parC*, with codon TCC (Ser-79; *S. pyogenes* coordinates) being replaced by TTC (Phe) or GCC (Ala) and with GAT (Asp-91) being replaced by AAT (Asn). Almost all ciprofloxacin-resistant isolates (25) showed only the replacement of Ser-79 by Ala, since the most prevalent ciprofloxacin-resistant clone (of *emm* type 6 and PFGE type A) contained this point mutation (Table 1). We found three isolates with a mutation in which Asp-91 was replaced by Asn and two isolates with this mutation together with a replacement of Ser-79 by Phe (Table 1).

Our results are consistent with previous reports which demonstrated that resistance to fluoroquinolones usually results from alterations in the QRDRs of either *gyrA* or *parC* or of both genes. This observation has been reported for *S. pneumoniae* (8, 9) and *S. pyogenes*; Yan et al. demonstrated that one isolate of *S. pyogenes* that was resistant to high levels of quinolones showed mutations in both *gyrA* and *parC* (15). Two

recent studies conducted in the United States (12) and Germany (11) have confirmed the results reported by Yan et al. and identified *S. pyogenes* isolates with high levels of resistance to fluoroquinolones with mutations in both genes *gyrA* and *parC*. In these isolates, some mutations observed in *parC* were identical to those identified in our study, such as the replacement of Ser-79 by Phe (11, 12) and the replacement of Asp-91 by Asn (11). Recently, Alonso et al. showed that reduced susceptibility to ciprofloxacin is mainly associated with single mutations preferentially in *parC*, in which Ser-79 is replaced by Ala (R. Alonso, M. Galimand, and P. Courvalin, Letter, Antimicrob. Agents Chemother. **46:**3686-3687, 2002). In our study, nearly all the resistant isolates exhibited levels of resistance to ciprofloxacin (MIC = 4 μg/ml) similar to those reported by Alonso et al., and consistently, almost all the isolates showed the same mutation, Ser79Ala in *parC*.

The fact that quinolone resistance in *S. pneumoniae* arises through mutations of *parC* before changes in *gyrA* (6), together with the experimental observation that high levels of resistance to quinolones in *S. pyogenes* are associated with mutations in both *gyrA* and *parC* (15), suggests that the ciprofloxacin-resistant isolates identified in Spain are good candidates to become highly resistant to fluoroquinolones.

In summary, since the year 1988, in which ciprofloxacin was introduced into therapeutic practice in Spain, several clones of *S. pyogenes* have developed mutational alterations of key topoisomerases and exhibit reduced susceptibilities to ciprofloxacin. Certain clones may be selected and further disseminated, particularly if they combine resistance to macrolides and fluoroquinolones.

We thank C. Vidal for DNA sequencing and A. Oliver for technical assistance with PFGE.

This study was supported by a grant from GlaxoSmithKline, Madrid, Spain.

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