Emergence of Ciprofloxacin-Nonsusceptible *Streptococcus pyogenes* Isolates from Healthy Children and Pediatric Patients in Portugal[▽]

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We describe 66 ciprofloxacin-nonsusceptible *Streptococcus pyogenes* isolates recovered from colonized and infected children. The ParC S79A substitution was frequent and associated with the *emm6*/sequence type 382 (*emm6*/ST382) lineage. The ParC D83G substitution was detected in two isolates (*emm5*/ST99 and *emm28*/ST52 lineages). One isolate (*emm89*/ST101) had no quinolone resistance-determining region codon substitutions or other resistance mechanisms. Five of 66 isolates were levofloxacin resistant. Although fluoroquinolones are not used in children, they may be putative disseminators of fluoroquinolone-nonsusceptible strains in the community.

Streptococcus pyogenes clinical isolates with reduced susceptibility to fluoroquinolones (1, 3, 8, 14, 16, 18, 27, 29) or with high-level resistance (15, 16, 23–25, 28) have been described previously, and the reduced susceptibility to fluoroquinolones is mediated by point mutations in the quinolone resistance-determining region (QRDR) of the *parC* gene (3, 16, 18) whereas high-level resistance has been associated with mutations in the QRDRs of both *parC* and *gyrA* genes (23, 28). To the best of our knowledge, there are no reports documenting the prevalence and characterization of fluoroquinolone-nonsusceptible *S. pyogenes* associated with asymptomatic colonization. Since 1999 and 2000, we have been collecting *S. pyogenes* isolates from pediatric patients from different clinical origins and from carriers for the surveillance of antimicrobial susceptibility and for the epidemiological characterization of the isolates. This study aimed to describe the prevalence of ciprofloxacin-nonsusceptible *S. pyogenes* isolates from colonized and infected Portuguese children from 1999 to 2006 and to characterize the associated clones and resistance mechanisms.

Strains and antibiotic susceptibility. A total of 1,354 non-duplicated *S. pyogenes* isolates were collected from children in the Lisbon area in Portugal; 901 were associated with asymptomatic colonization (2000 to 2006), 399 were associated with tonsillitis/pharyngitis (2000 to 2006), 48 were associated with skin/soft tissue infections (1999 to 2005), and 6 isolates were associated with invasive diseases (1999 to 2005). Identification was performed by standard methods (19).

Susceptibility testing of ciprofloxacin was done for all isolates by disk diffusion (6) using previously described breakpoints (28). Figure 1 shows the strategy followed for the de-

tection of ciprofloxacin-nonsusceptible isolates. The MIC to ciprofloxacin was tested by the agar dilution method (Fig. 1) (6). MICs to other antimicrobial agents (Table 1) were determined by microdilution (6), except for tigecycline (Etest strips were used).

Sixty-six of the 1,354 isolates (4.9%) were considered putative ciprofloxacin nonsusceptible (MIC ≥ 2 μ g/ml; range, 2 to 8 μ g/ml) (Fig. 1 and Table 1). Ciprofloxacin nonsusceptibility was higher among clinical isolates (6.0%, $n = 27/453$) than among carriage isolates (4.3%; $n = 39/901$) ($P = 0.001$). Similar rates were found among skin/soft tissue infection (6.3%; $n = 3/48$) and tonsillitis/pharyngitis (6.0%; $n = 29/487$) isolates ($P = 0.004$), and no ciprofloxacin-nonsusceptible invasive disease isolates were detected ($n = 0/6$) ($P = 0.13$). To the best of our knowledge, this is the first study describing the rate of ciprofloxacin nonsusceptibility among *S. pyogenes* isolates collected from oropharyngeal colonization during a 7-year period (2000 to 2006). The rate of ciprofloxacin nonsusceptibility among our sample of clinical isolates was comparable to those previously reported among pediatric patients in Brazil, Belgium, and the United States (6% to 9%) (15, 27, 29); however, it was slightly lower than that found in a study from Spain (13.6%) (16) and much lower than the one found in Brussels (22.5%) (27).

Overall, ciprofloxacin-nonsusceptible isolates ($n = 66$) presented high rates of susceptibility ($>90\%$) to other antimicrobial agents (Table 1). Nonsusceptibility to levofloxacin was 7.6% ($n = 5/66$). One isolate was resistant to both erythromycin and clindamycin and presented the constitutive macrolide-lincosamide-streptogramin B (cMLS_B) resistance phenotype and *erm*(B) genotype, as detected by PCR (19). Another isolate was tetracycline resistant, carrying *tet*(M) and *tet*(T) but not *tet*(K), *tet*(L), *tet*(O), *tet*(Q), *tet*(S), or *tet*(W) genes, according to PCR screening assays (19, 22).

Molecular typing. The relationship among all the ciprofloxacin-nonsusceptible isolates was assessed by pulsed-field gel

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FIG. 1. Diagram representing the methodologies used for the selection and characterization of ciprofloxacin-nonsusceptible *S. pyogenes* isolates. CIP, ciprofloxacin; NOR, norfloxacin.

electrophoresis (PFGE) (20). Detection of *emm* types was carried out as described at <http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm>, and representative isolates of different PFGE pattern/*emm*-type associations ($n = 14$) were analyzed

by multilocus sequence typing (MLST) (<http://spyogenes.mlst.net>).

The 66 ciprofloxacin-nonsusceptible isolates were included in seven different PFGE patterns, arbitrarily named with capital letter codes (Table 2). PFGE pattern AD was observed in 87.9% of the isolates ($n = 58/66$), which were mainly *emm6* ($n = 52$). Three other PFGE patterns (DX, DY, and AM) also included *emm6*/sequence type 382 (*emm6*/ST382) isolates ($n = 5$). PFGE patterns AD, DX, DY, and AM were grouped into the same lineage, ST382, which included 95.5% of the ciprofloxacin-nonsusceptible isolates ($n = 63/66$). The remaining three PFGE patterns, BT (*emm28*/ST52 lineage), CJ (*emm89*/ST101 lineage), and K (*emm5*/ST99 lineage), included single isolates. The origins of the isolates are shown in Table 2.

Quinolone resistance characterization. Detection of point mutations in the *parC* quinolone resistance-determining region (QRDR) was performed by PCR (4, 25) and by restriction fragment length polymorphism (RFLP) (Fig. 1) (2).

All but one of the ciprofloxacin-nonsusceptible isolates had *parC* QRDR mutations, generating the amino acid substitutions S79A ($n = 63$) and D83G ($n = 2$).

The ParC S79A substitution was found among clinical and colonization ciprofloxacin-nonsusceptible *emm6* ($n = 57$), *emm1* ($n = 5$), and *emm89* ($n = 1$) isolates of the major lineage ST382 (Table 2).

Substitutions in codon S79 of the ParC QRDR have been found to be prevalent in *emm6* fluoroquinolone-resistant *S. pyogenes* isolates (1, 3, 14, 16, 18, 29) and are considered to be intrinsic (18) and not related to selective pressure by antibiotic usage.

While *emm89* was previously found among sporadic ciprofloxacin-nonsusceptible strains (8, 23), *emm1* was associated for the first time in this study with fluoroquinolone nonsusceptibility.

The ParC D83G substitution was also found for the first time in this study, particularly among isolates of lineages *emm28*/ST52 ($n = 1$) and *emm5*/ST99 ($n = 1$), and was previously described for *Streptococcus pneumoniae* (21).

We also detected these two ciprofloxacin-nonsusceptible lineages, together with the *emm22*/ST46 lineage with D83G/Y

TABLE 1. MICs to ciprofloxacin and 12 other antimicrobial agents and susceptibility rates among 66 ciprofloxacin-nonsusceptible *S. pyogenes* isolates collected from different origins in Portugal (1999–2006)^a

Antimicrobial agent(s)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	MIC range (µg/ml)	%S	%IR	%R
Ciprofloxacin	2	4	2–8	— ^b	—	—
Levofloxacin	2	2	2–4	92.4	7.6	0
Moxifloxacin	0.5	0.5	≤0.25–1	—	—	—
Erythromycin	≤0.25	≤0.25	≤0.25–>32	98.5	0	1.5
Clindamycin	≤0.25	≤0.25	≤0.25–>5	98.5	0	1.5
Penicillin	≤0.03	≤0.03	≤0.03	100	0	0
Ampicillin	≤0.25	≤0.25	≤0.25	100	0	0
Cefotaxime	≤0.06	≤0.06	≤0.06	100	0	0
Vancomycin	0.5	0.5	≤0.25–0.5	100	0	0
Teicoplanin	≤0.25	≤0.25	≤0.25	100	0	0
Quinupristin-dalfopristin	≤1	≤1	≤1	100	0	0
Tetracycline	≤2	≤2	≤2–>4	98.5	0	1.5
Tigecycline	0.047	0.064	0.025–0.064	—	—	—

^a Susceptibility breakpoints are according to CLSI interpretative criteria (6). S, susceptible; IR, intermediate resistant; R, resistant.

^b —, no interpretative criteria.

TABLE 2. Genotypes, phenotypes, and origins of the 66 ciprofloxacin-nonsusceptible *S. pyogenes* isolates collected in Portugal (1999–2006)

PFGE pattern (no. of isolates)	<i>emm</i> type (no. of isolates)	Sequence type ^a	MIC range (μg/ml) ^b			No. of isolates with CIP and LVX coresistance	Amino acid change in ParC ^c		Origin ^e (no. of isolates)
			CIP	LVX	MXF		S79	D83	
AD (58)	<i>emm6</i> (52)	ST382	2–8	2–4	≤0.25–0.5	2	A	— ^d	OC (30), T/P (20), S/STI (2)
	<i>emm1</i> (5)	ST382	2–4	2–4	0.5	1	A	—	OC (2), T/P (3)
	<i>emm89</i> (1)	ST382	4	4	1	1	A	—	OC (1)
DX (2)	<i>emm6</i> (2)	ST382	2–4	2	0.5	0	A	—	OC (2)
DY (2)	<i>emm6</i> (2)	ST382	4	2–4	0.5–1	1	A	—	OC (2)
AM (1)	<i>emm6</i> (1)	ST382	2	2	0.5	0	A	—	OC (1)
BT (1)	<i>emm28</i> (1)	ST52	2	2	≤0.25	0	—	G	S/STI (1)
CJ (1)	<i>emm89</i> (1)	ST101	2	2	0.5	0	—	—	T/P (1)
K (1)	<i>emm5</i> (1)	ST99	2	2	0.5	0	—	G	OC (1)

^a Only one isolate of each PFGE/*emm* association was analyzed by MLST.

^b CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin.

^c The *parC* gene was sequenced in selected isolates. The remaining isolates had mutation detected by PCR-RFLP. The *emm89*/ST101 isolate presented the triple substitution D91N/S107L/S140P and did not present any *gyrA*, *gyrB*, or *parE* mutation (see the text).

^d —, no change.

^e OC, oropharyngeal colonization; T/P, tonsillitis/pharyngitis; S/STI, skin/soft tissue infection.

substitutions, among infection products from adults (data not shown). This D83Y codon replacement was previously associated with nonsusceptibility to fluoroquinolones among other streptococci (4, 7).

No ParC substitutions at codon S79 or D83 were detected in the *emm89*/ST101 isolate, but instead, replacements were observed at codons D91, S107, and S140 (Table 2). This isolate was also investigated for the presence of mutations in the QRDRs of *gyrA*, *gyrB*, and *parE*, as described previously (4, 25, 29), and no mutations were found. Moreover, specific genes *qnrA*, *qnrB*, *qnrC*, and *aac(6')-Ib-cr*, which confer resistance to fluoroquinolones in *Enterobacteriaceae*, were not detected after PCR screening assays (5, 13). Also, the MICs of ciprofloxacin, levofloxacin, and moxifloxacin did not decrease after the addition of 10 μg/ml of reserpine, which is an efflux pump inhibitor (11). Substitutions at positions D91 and S140 are most likely not involved in fluoroquinolone resistance because they can be found in both susceptible and resistant isolates (9). Similarly, the substitution at position S107, which was not previously reported for *S. pyogenes*, may also not be involved in fluoroquinolone resistance since it was detected in one ciprofloxacin-susceptible isolate of our study (data not shown).

Fluoroquinolones have been widely used in Portugal for many years (10), and continuous use has been implicated in selection for resistance (14). Particularly, older fluoroquinolones, like ciprofloxacin, were previously suggested to promote dissemination of fluoroquinolone nonsusceptibility among different *S. pyogenes* clones (1, 14). On the other hand, horizontal genetic exchange by interspecies recombination has also been reported to lead to quinolone resistance in *S. pyogenes* (9).

The finding of a few ciprofloxacin-nonsusceptible strains, other than *emm6* strains, may suggest the occurrence of horizontal gene transfer involving the *parC* QRDR. The recognition of ciprofloxacin nonsusceptibility among isolates colonizing healthy children attending day care centers and schools highlights their possible role as disseminators of ciprofloxacin-nonsusceptible strains, as has been recognized for other bacteria (26).

In conclusion, our study demonstrated that in Portugal fluoroquinolone resistance in *S. pyogenes* isolates from pediatric

patients is mediated by both clonal dissemination and unrelated events of development of resistance. Whether or not high and sustained fluoroquinolone consumption in Portugal has been implicated in selection for resistance in *S. pyogenes*, the recognition of colonization and clinical isolates nonsusceptible to ciprofloxacin, particularly in adult populations, should be a cause for concern and may compromise the therapeutic importance of these antimicrobials. A continuous surveillance of fluoroquinolone resistance is important to monitor the evolution of nonsusceptible *S. pyogenes* isolates.

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