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Role of DNA gyrase and topoisomerase IV mutations in fluoroquinolone resistance of Capnoctophaga spp. clinical isolates and laboratory mutants

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Abstract

Objectives: *Capnocytophaga* species (*C. spp.*) are often reported to cause bacteremia and extra-oral infections and are characterized by their significant contribution to resistance to β-lactam and macrolide-lincosamide-streptogramin in the human oral microbiota. The implication of mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase A and B (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) of fluoroquinolone (FQ) resistant *C. spp.*, hitherto unknown, was explored in this study.

Methods: Two reference strains (*C. gingivalis* ATCC 33624 and *C. sputigena* ATCC 33612) and four *C. spp.* isolated from clinical samples were studied. Nine in-vitro FQ-resistant mutants, derived from two reference strains and one FQ-susceptible clinical isolate, were selected by successive inoculations onto media containing levofloxacin. MICs were determined for ofloxacin, norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin. The presumed QRDRs of GyrA, GyrB, ParC and ParE from *C. spp.* were determined by sequence homology to *Bacteroides fragilis* and *Escherichia coli*. PCR primers were defined to amplify the presumed QRDR genetic region of *C. spp.*, and sequence analyses were performed using the BLAST program at the National Center for Biotechnology Information.

Results and Conclusions: *gyrA* mutations leading to a substitution from amino acid position 80 to 86 were systematically detected in *C. spp.* with ciprofloxacin MIC >1 mg/L, and considered as the primary target of FQ. No mutational alteration in the QRDR of *gyrB* was detected. Other mutations in *parC* and *parE* led to spontaneous amino acid substitutions of DNA topoisomerase IV subunit B with no alteration in FQ-susceptibility.
INTRODUCTION

*Capnocytophaga* species (C. spp.) are fastidious capnophilic/anaerobic (able to grow in an atmosphere containing 5-10% carbon dioxide) Gram-negative gliding bacilli isolated from the human oropharyngeal flora.¹² C. spp. are “difficult-to-cultivate” bacteria whose carriage in adults and children has recently been estimated at 100%.¹³ Human oral species are often reported to cause bacteremia and various extra-oral infections in both immunocompromised and immunocompetent hosts.⁴⁻⁶ The genus *Capnocytophaga* is characterized by its high contribution to β-lactam resistance (from 16% in healthy patients to 82% in periodontitis patients of CfxA2, CfxA3 or CSP-1 β-lactamase-producing isolates) and macrolide-lincosamide-streptogramin resistance (29% of Erm(F) or Erm(C) methylase-producing isolates) in the human oral microbiota.³⁷⁸ Fluoroquinolones (FQs) have been widely used to treat nosocomial Gram-negative bacterial infections over the past two decades. The clonal spread of strains with FQ resistance mutations has become a serious problem. To our knowledge, FQ resistance has only been reported in eleven cases of general infection due to C. spp.⁴⁶⁹¹⁰ FQ resistance could be more frequent, as shown by the study by Martino *et al.* (56% of tested *Capnocytophaga* isolates).⁴ Its presence, especially in respiratory infections, is probably underestimated.⁶ Quinolones target DNA gyrase and topoisomerase IV. DNA gyrase consists of two subunits encoded by *gyrA* and *gyrB*, while topoisomerase IV consists of two subunits encoded by *parC* and *parE*, which are respectively homologous to *gyrA* and *gyrB* of DNA gyrase.¹¹ Alterations through mutations in the quinolone resistance-determining regions (QRDRs) of chromosomal DNA gyrase and topoisomerase IV genes confer FQ resistance.¹²
The aim of this study was 1) to determine the role of DNA gyrase and topoisomerase IV mutations (gyrA/gyrB and parC/parE, respectively) in resistant clinical *Capnocytophaga* isolates and in laboratory mutants selected *in vitro* on levofloxacin-supplemented media and 2) to study the mutation frequencies of the strains.

**MATERIALS AND METHODS**

Two reference strains S1/*C. gingivalis* ATCC 33624 and S5/*C. sputigena* ATCC 33612 and four *C. spp.* isolated from clinical samples were studied. Clinical *C. spp.* were isolated from the subgingival flora of patients with hematological cancer (*C. gingivalis* Aa6, *C. gingivalis* Ag44 and *C. sputigena* Ah47)³ and from a lung infection (*C. gingivalis* A0).⁶ Species-level identification was performed by analysis of 16S rDNA sequences.¹

MICs were determined by the Etest method (Biomérieux, Craponne, France) for ofloxacin, norfloxacin, ciprofloxacin, levofloxacin, and moxifloxacin³ without standard interpretation available for the *Capnocytophaga* genus.

To evaluate mutation frequency, the reference strains S1/*C. gingivalis* ATCC 33624 and S5/*C. sputigena* ATCC 33612 were used as parental strains to select *in vitro* quinolone-resistant mutants (S1M1-4; S5M1-2) in two successive steps, by plating an inoculum of $10^{10}$ bacteria onto blood agar medium (Columbia + 5% sterile sheep blood, Bio-Rad Laboratories, Marnes-la-Coquette, France) and containing levofloxacin at either two or four times the MIC. After anaerobic incubation at 37°C for 48h, colonies were counted and mutation frequencies were determined, relative to the total viable count of bacteria plated.

Laboratory mutants (S1MD, S5MD and Ag44MD; MD for mutant disc) derived from reference strains and from a clinical isolate (S1/*C. gingivalis* ATCC 33624, S5/*C. sputigena* ATCC 33612
and Ag44/ *C. gingivalis* were also obtained by successive inoculations, onto blood agar, of colonies taken from the vicinity of a disc containing 5 µg levofloxacin (Bio-Rad Laboratories). Pairs of PCR primers were designed to amplify the presumed QRDR of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) of *C. spp.* (Table 1). Presumed QRDRs of *C. spp.* were positioned by sequence homology with *B. fragilis* or *E. coli* (accession numbers from the NCBI (National Center for Biotechnology Information) database; Table 2). DNA was amplified with an initial denaturation step at 94°C for 5 min, followed by 30 s at the annealing temperature (Table 1), then a final extension step at 72°C for 7 min. Reference strains were used as positive controls and sterile water as negative control. To amplify *parC*, an extra pair of primers was necessary because of the low ParC sequence homology among the different species of *Capnocytophaga*, especially around the QRDR (up to only 73%). Amplifications were followed by subsequent sequencing.³ Sequence analyses were performed using the NCBI BLAST program.

**RESULTS AND DISCUSSION**

The two reference strains and two of the four clinical isolates (Aa6 and Ag44) presented low FQ MIC values (ofloxacin, norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin 0.012-0.5 mg/L; Table 2). The two other clinical isolates (Ah47 and A0) presented high MIC values for all FQs (ofloxacin, norfloxacin, ciprofloxacin and levofloxacin 32-256 mg/L), except for moxifloxacin (0.38 - 1.5 mg/L). The reference strain S1/*C. gingivalis* ATCC 33624 was plated onto agar containing levofloxacin at 0.5 and 1 mg/L (Table 2). First-step mutants S1M1 and S1M2 were selected at a frequency of $10^{-5}$ and $10^{-8}$, respectively. Second-step mutants S1M3 and S1M4, derived from S1M2 (plated onto agar containing levofloxacin at 1 and 2 mg/L) were selected at a mean frequency of $10^{-3}$ and $10^{-9}$, respectively. Two *in vitro* mutants, *C. gingivalis*
S1MD and Ag44MD, respectively derived from the reference strain S1/C. gingivalis ATCC 33624 and clinical isolate Ag44/C.gingivalis, were obtained respectively after 4 and 5 successive inoculations. A first-step mutant S5M1 derived from reference strain S5/C. sputigena ATCC 33612 was selected only on the plate at twice the MIC, at a frequency of $3.10^{-9}$. Bacterial growth was inhibited on the plate containing 1 mg/L levofloxacin. A second-step mutant S5M2 derived from S5M1, was obtained at a mean frequency of $5.10^{-9}$. A mutant S5MD derived from S5 was obtained after 4 successive inoculations. The duration and frequency of exposure to levofloxacin had an adverse impact on C. spp. resistance rates. Exposure to low antibiotic concentrations led to greater numbers of selected mutants. Both FQ-resistant isolates and in vitro mutants displayed higher MICs to 2nd generation fluoroquinolones (ofloxacin, ciprofloxacin, norfloxacin) compared to 3rd generation fluoroquinolones (levofloxacin and moxifloxacin) (Table 2). These data therefore suggest that continued exposure to levofloxacin may lead to increased levels of FQ-resistance in the genus Capnocytophaga.

In the genus Capnocytophaga, FQ exposure led to an increase in FQ MIC values. Then, in accordance with what was observed in B. fragilis, the MIC values evolved differently depending on the FQ agent. Bacterial tolerance to norfloxacin was the highest, followed by ciprofloxacin, ofloxacin, levofloxacin and finally moxifloxacin. Therefore, all FQs could be affected, attesting to cross-resistance to FQs in C. spp. For the genus Capnocytophaga, because no data exists, FQ MIC data could be interpreted according to the interpretation standards for anaerobes issued by CA SFM (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM2013vjuin.pdf), EUCAST (http://www.eucast.org/clinical_breakpoints/) or by the Clinical and Laboratory Standards Institute (http://clsi.org/). According to CA SFM 2013, MIC breakpoints were only available for
ofloxacin ≤ 1 and > 4 mg/L, and for moxifloxacin ≤ 1 and > 2 mg/L (or ≤ 2 and ≥ 8 mg/L, in cases of bone or brain infection). In C. spp., from MIC value of moxifloxacin ≥ 0.38 mg/L (and even ≥ 0.125 mg/L for the mutants), ofloxacin ≥ 2 mg/L, ciprofloxacin ≥ 1.5 mg/L, or ≥ 0.5 mg/L levofloxacin, a resistance mechanism to FQs was observed (Table 2).

The gyrA, gyrB, parC and parE QRDR nucleotide sequences of the clinical isolates and in vitro mutants were analyzed and compared with reference strains S1/C. gingivalis ATCC 33624 and S5/C. sputigena ATCC 33612 (Table 2). The locations of presumed QRDRs of C. spp. were positioned by sequence homology with B. fragilis or E. coli and were described in the Table 2. All resistant isolates and in vitro mutants of C. gingivalis carried a mutation in the QRDR of gyrA, leading to a substitution between amino acids 80 and 82 (G80N, D81G, S82F or S82Y) compared with wild-type reference strain S1/C. gingivalis ATCC 33624 and susceptible clinical isolates (Table 2). Concerning the C. sputigena species, all resistant isolates and in vitro mutants carried a mutation in the QRDR of gyrA leading to a substitution between amino acids 80 and 86 compared with wild-type reference strain S5/C. sputigena ATCC 33612 and susceptible clinical isolates (D86Y for S5M1 and S5M2; G80D for S5MD). The systematic presence of gyrA mutations, in both FQ-resistant clinical isolates and in-vitro mutants, was observed without mutational alteration of gyrB.

These gyrA-mutants had different resistance phenotypes and these data suggested that, similar to what was observed in E. coli, but contrary to B. fragilis, the DNA gyrase of C. gingivalis could be a primary target for FQs, whereas topoisomerase IV would be the secondary target and would confer a high level of resistance. According to Moon et al., FQ-resistant E. coli isolates carried the same substitution in GyrA (S83L and D87N), but different types of substitutions in ParC. These various configurations were compared for their
FQ-MIC values: double substitutions in ParC at S80I and E84G were linked to significantly higher MICs than those of the isolates carrying other types of double mutations. The systematic substitution of the position 80 of ParC was not found in C. spp.. Maeda et al.\textsuperscript{17} highlighted numerous spontaneous amino acid substitutions in the QRDRs of DNA topoisomerase IV with no alteration in ciprofloxacin susceptibility within viridans group Streptococci. We showed that, in C. spp., the mutations observed in parC as in parE (Table 2) implied spontaneous amino acid substitutions of DNA topoisomerase IV subunit A and B with no alteration in FQ-susceptibility.

The study highlighted the role of DNA gyrase A as the primary target of FQs in the genus Capnocytophaga. However, despite the difference between the FQ MICs of S5M1 and S5M2 (derived from S5/C. sputigena ATCC33612), they carried only the same substitution D86Y in GyrA. This raises the possibility of other resistance mechanisms such as membrane impermeability, drug efflux, target protection and drug enzymatic modification. A plasmid-mediated quinolone resistance determinant (such as the qnr gene), previously described in E. coli\textsuperscript{18} and Aeromonas spp.\textsuperscript{19} could also be involved, despite the fact that it has never been detected in anaerobes.

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TRANSPARENCY DECLARATIONS

None to declare.

REFERENCES


**List of Tables**
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<th>Target genes</th>
<th>Amino acid positions (aa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Annealing temperatures (°C)</th>
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**TABLE 1.** Primer sequences used to amplify the QRDRs of gyrA, gyrB, parC, and parE of *Capnocytophaga* spp.

<sup>a</sup>Pairs of specific PCR primers of the table were designed in this study, except CgyrA.<sup>6</sup>

<sup>b</sup>Corresponding to amino acid positions of: GyrA of *C.gingivalis* (accession number EEK 14408.1); GyrB of *C. gingivalis* (accession number EEK13479.1); ParC of *C. ochracea* (accession number WP_002673707.1) and *C. gingivalis* (accession number WP_002669241.1); ParE of *C. sputigena* (WP_040361975.1) and *C. gingivalis* (WP_002668274.1).

<sup>c</sup>The primer sets ParC6FC4R and ParE2 were used for amplification of the parC and parE gene in *C. gingivalis*. Otherwise, all primer sets were universally used to amplify gyrA and gyrB gene loci in each *Capnocytophaga* species analyzed.
<table>
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<th>MIC (µg/ml)</th>
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<td>&gt;32</td>
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</table>

TABLE 2. Phenotype and amino acid substitutions in DNA gyrase and topoisomerase IV of *Capnocytophaga* spp.

*a*OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; NOR, norfloxacin.
The symbol “-” indicates the absence of mutation; presumed QRDR of GyrA (C. gingivalis EEK14408.1 and C. sputigena EEB66463.1; position 81-82) positioned by sequence homology with B. fragilis (CAH08193.1; position 81-82)\textsuperscript{13}, and E. coli (EGT66353.1; position 83-87); presumed QRDR of GyrB (C. gingivalis EEK13479.1; position 421 and 483) positioned by sequence homology with B. fragilis (position 415 and 478; AB017713.1)\textsuperscript{13} and E. coli (position 296 and 359; WP_032218993.1); presumed QRDR of ParC (C. ochracea WP_002673707.1; amino acid position 80) positioned by sequence homology with GyrA from C. ochracea (ZP_10368497.1); presumed ParE QRDR of C. gingivalis (WP_002668274.1; position 438-451) and C. sputigena (WP_040361975.1; position 431-444) positioned by sequence homology with E. coli (U00096.3 ; position 445-458)\textsuperscript{12}; X : stop codon.

\textsuperscript{c}S5M1 mutants were selected from the parental strain S5 on levofloxacin at twice the MIC (0.5 mg/L). S5M2 mutants were selected from S5M1 mutants on levofloxacin at four times the MIC (2 mg/L).

\textsuperscript{d}S1M1 and S1M2 mutants were selected from the parental strain S1, on levofloxacin at two and four times the MIC (0.5 and 1mg/L) respectively. S1M3 and S1M4 mutants were obtained from S1M2 mutants on levofloxacin at two times and four times the MIC (1 and 2 mg/L) respectively.

\textsuperscript{e}S1MD, Ag44MD, and S5MD were mutants respectively selected from S1, Ag44 and S5 on blood agar with a 5 µg levofloxacin disc and able to grow in contact with the levofloxacin disc after successive inoculations (respectively 4, 5 and 4).