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Elodie EHRMANN[1,2], Anne JOLIVET-GOUGEON[3,4*], Martine BONNAURE-MALLET[3,4#], Thierry FOSSE[5,6#]

[1]Pôle odontologie, CHU de Nice, Nice, France ; [2]Faculté d'Odontologie, Université de Nice-Sophia-Antipolis, Nice, France ; [3]Equipe de Microbiologie EA 1254/ Inserm U 1241 NUMECAN, Université de Rennes 1, France ; [4]CHU de Rennes, 35043 Rennes, France ; [5]Service d'hygiène et vaccinations, CHU de Nice, 06003 Nice, France; [6]Faculté de Médecine, Université de Nice Sophia-Antipolis, Nice, France; [#]author equivalent

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***Corresponding author**. Mailing address: Anne Jolivet-Gougeon, INSERM U1241 NUMECAN, Université de Rennes 1, 2, avenue du Professeur Léon Bernard, 35043 Rennes, France. Phone: (33) 2 23 23 43 05 – Fax: (33) 2 23 23 49 13. E-mail: anne.gougeon@univ-rennes1.fr

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.[1,2] *C.* spp. are "difficult-to-cultivate" bacteria whose carriage in adults and children has recently been estimated at 100%.[1,3] 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.[3,7,8] 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.[4,6,9,10] 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 moxifloxacin[3] 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.[13,14] 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.[13,15,16] According to Moon *et al*.[12], 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.*[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*[18] and *Aeromonas* spp.[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.

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List of Tables

Primer names[a]	Primer sequences (5' - 3')	Amplicon size (bp)	Target genes	Amino acid positions (aa)[b]	Annealing temperatures (°C)
CgyrA	GAAGGAGAAAAATTGATTCCT ACTGT[CT]TC[CT]TTGTCGATATC	419	*gyrA*	2 - 141	53
CgyrB	GAAGAAAA[CT]CCT[AG]A[AT]GATGCCAA GAACCATC[CG]ACATCGGCATC	440	*gyrB*	377 - 523	54
ParC3	CGTAATCCTCGAACGCGC CATCGTCGCCWGTGAGGAT	248	*parC*	33 - 114	56
ParC6FC4R[c]	GAGGACGGTCGCTATAACA GAGGATATTCCCCCAG	147		65 - 114	48
ParE1	ACCGAAATGGGAGAAGGAATGCC GGGAAGAAYTGCAAAAAGAAAGT	554	*parE*	325 - 509	52
ParE2[b]	GGTTCCACTGAAATGGGAG CACGGAATAGGGGTGTTTG	613		323 - 527	56

TABLE 1. Primer sequences used to amplify the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* of *Capnocytophaga* spp.

[a]Pairs of specific PCR primers of the table were designed in this study , except CgyrA.[6]

[b]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).

[c]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.

	MIC (µg/ml)[a]					Mutation in QRDR of[b]:			
	OFX	CIP	LVX	MXF	NOR	GyrA	GyrB	ParC	ParE
FQ-susceptible wild type (reference strains n = 2; clinical isolates n = 2)									
C. sputigena									
S5 (ATCC 33612)	0,38	0,125	0,19	0,064	0,064	-	-	-	-
C. gingivalis									
S1 (ATCC 33624)	0,38	0,125	0,19	0,047	0,064	-	-	-	-
Ag44	0,094	0,032	0,047	0,064	0,5	-	-	-	-
Aa6	0,064	0,032	0,032	0,012	0,064	-	-	-	I502X T503X F504J
FQ-resistant wild type (clinical isolates n=2)									
C. gingivalis									
A0	>32	>32	>32	0,38	>256	G80N	-	-	E515D
C. sputigena									
Ah47	>32	>32	>32	1,5	32	T82I	-	M55I	-
FQ-resistant mutants (n=9)									
C. sputigena									
S5M1[c]	2	1,5	0,5	0,125	16	D86Y	-	-	-
S5M2[c]	>32	>32	2	4	24	D86Y	-	-	-
S5MD[e]	>32	>32	>32	3	32	G80D	-	-	-
C. gingivalis									
S1M1[d]	2	2	0,5	0,125	32	S82F	-	E101Q	G377D K410Q F508X
S1M2[d]	2	2	0,5	0,125	32	S82F	-	E101Q	G377D K410Q
S1M3[d]	2	4	0,5	0,125	32	S82F	-	E101Q	G377D K410Q F508X
S1M4[d]	8	>32	2	0,19	>256	S82F	-	E101Q	G377D K410Q
S1MD[e]	>32	>32	>32	1,5	>256	D81G	-	E101Q	G377D K410Q
Ag44MD[e]	>32	>32	>32	2-3	>256	S82Y	-	-	F509X E511X

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.

[b]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)[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)[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)[12]; X : stop codon.

[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).

[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.

[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).