High prevalence of β-lactam and macrolide resistance genes in human oral Capnocytophaga species

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Objectives: To determine macrolide–lincosamide–streptogramin (MLS) resistance determinants in the Capnocytophaga genus and to describe the prevalence of β-lactam resistance genes in human oral Capnocytophaga species.

Methods: Forty-eight Capnocytophaga isolates identified by analysis of 16S rRNA sequences were isolated from subgingival samples from 14 haematology patients (HPs), 11 periodontitis patients (PPs) and 17 healthy volunteers (HVs). MICs of β-lactam and MLS antibiotics were obtained for all isolates. blaCfxA, blaCSP-1 (encoding a new class A β-lactamase) and MLS resistance genes [erm(F), erm(B), erm(Q), erm(D), erm(C) and erm(A)] were evaluated using specific PCR and sequencing.

Results: In HVs, which had the lowest prevalence of β-lactamase-producing isolates in comparison with the other groups (16%; P < 0.001), Capnocytophaga ochracea was the prominent species (68%; P < 0.03). In PPs, which had a high prevalence of β-lactamase-positive isolates (82%; P < 0.001), Capnocytophaga sputigena was more frequently identified (64%; P < 0.03). In HPs, 50% of isolates were β-lactamase-positive. The more rarely identified species (15%) Capnocytophaga gingivalis, Capnocytophaga granulosa and Capnocytophaga leadbetteri were isolated only from PPs and/or HPs. All β-lactam-resistant isolates (44%) were PCR-positive for blaCfxA (31%) or blaCSP-1 (12.5%). Interestingly, blaCSP-1 was identified only in a subgroup of the C. sputigena species. Twenty-nine percent of isolates were MLS-resistant independently of species identification, β-lactamase production or patient group. The MLS-resistant isolates carried the erm(F) or erm(C) gene (93% and 7%, respectively), previously unknown in the Capnocytophaga genus.

Conclusions: Our findings illustrate that Capnocytophaga species are important contributors to the β-lactam and MLS resistance gene reservoir in the oral microbiome.

Keywords: epidemiology, antibiotic resistance, oral microbiota

Introduction

The genus Capnocytophaga encompasses a group of fastidious capnophilic/facultatively anaerobic Gram-negative bacilli.1 Species from the human oral microbiota (Capnocytophaga ochracea, Capnocytophaga sputigena, Capnocytophaga gingivalis, Capnocytophaga granulosa, Capnocytophaga leadbetteri and Capnocytophaga haemolytica) are associated with varying degrees of periodontal health or disease and are responsible for a number of opportunistic infections.1–3 An epidemiological study of carriage and species distribution according to clinical context is necessary.1,3,4

Handal et al.5 reported that blaCfxA and blaCSP-1 were responsible for the extended-spectrum resistance to β-lactam antibiotics in Capnocytophaga spp. Recently, a new β-lactamase, CSP-1, produced by a clinical isolate of C. sputigena, has been characterized,6 but the distribution and prevalence of the gene blaCSP-1 are currently unknown in Capnocytophaga spp. No data concerning resistance to macrolide–lincosamide–streptogramin (MLS) antibiotics are currently available for the Capnocytophaga genus.

The aims of the present study were to identify the determinants of MLS resistance and to analyse the phenotypic expression, distribution and prevalence of β-lactamases (including CSP-1) and MLS resistance genes in Capnocytophaga species according to clinical context.

Methods

From two prospective cohort studies examining the subgingival flora in the Centre Hospitalier Universitaire de Nice, France, 18 clinical isolates were
sampled from 14 haematology patients (HPs) undergoing a course of chemotherapy for cancer, and 11 isolates were taken from 11 periododontitis patients (PPs) treated in the dental outpatient department. In addition, a study was conducted to evaluate the oral presence of Capnocytophaga spp. from healthy volunteers (HV) without medical or oral pathology and with or without prior or concomitant exposure to β-lactam or MLS antibiotics. Nineteen isolates were sampled from 17 HVs. The composition of the groups (age and sex) and the exposure of all patients and volunteers to antimicrobial agents prior to (6 months) or during the conduct of the study are indicated in Table S1 (available as Supplementary data at JAC Online). All participants provided written informed consent in accordance with local ethics committee requirements. Subgingival plaque was collected as previously described. The samples were serially diluted and inoculated into blood agar medium (Bio-Rad Laboratories, Marnes-la-Coquette, France), bacitracin chocolate blood agar medium (Bio-Rad Laboratories), vancomycin–kanamycin medium (VK; Beckton Dickinson, Le Pont de Claix, France) and vancomycin–colistin–ampicillin B–trimethoprim medium (VCAT; Oxoid, Dardilly, France). All plates were kept for 3 days under 5% (v/v) CO2 at 37°C except blood agar and VK plates, which were incubated in an anaerobic atmosphere.

Bacterial species identification was performed by analysis of the 16S rRNA gene sequence. Briefly, a 16S rRNA gene fragment (1468 bp) was amplified by PCR and sequenced according to a previous study. The Bioinformatics Bacterial Identification software (http://pubmlst.org/bibli/) was used for phylogenetic analysis and bacterial identification. For all isolates, MICs were determined by Etest as previously described, for the following antibiotics: amoxicillin, amoxicillin/clavulanic acid, cefotaxime, cefazidime, erythromycin and clindamycin. The presence of β-lactam (blaCef, and blacsp-1) and MLS [erm(A), erm(B), erm(C), erm(Q), erm(D) and erm(A)] resistance genes was assessed by PCR with specific primers (Table 1), followed by subsequent sequencing. DNA was amplified by PCR 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. We selected the MLS resistance genes to be included in this study by reviewing the literature on MLS resistance in anaerobes and oral flora and by analysing the phylogenetic relationships between methylases. The positive control strains used were as follows: Escherichia coli (pCAP01) for blacsp-1; C. sputigena strain 507 for blacsp-1 (GenBank GQ919044.1); E. coli (pYK028) for erm(F); E. coli (pJIR432) for erm(Q); Staphylococcus aureus RN1389 for erm(A); E. coli (pAT273) for erm(D); Enterococcus faecalis (pJH1) for erm(B); and S. aureus (pE194) for erm(C). A negative control without template DNA was included in each experiment.

R software (R-2.15.0 for Microsoft Windows, http://www.R-project.org/) was used for statistical analysis of species and resistance marker distributions (Fisher’s exact test). A P value of <0.05 was considered to indicate statistical significance.

### Results and discussion

The diversity of media used allowed isolation of Capnocytophaga spp. from every patient. Therefore, our present study, which focused on the Capnocytophaga genus, allowed us to estimate the carriage of these fastidious bacteria in adults and children, for the first time as 100%. In the literature, data regarding the isolation frequency of Capnocytophaga spp. from oral clinical samples are conflicting due to the different microbial procedures employed, and due to the fastidious growth of this bacterial genus in the rich oral microbiota. With the exception of C. haemolytica, all Capnocytophaga spp. species were detected, including the new species C. leadbetteri. The species distributions of the subject groups were significantly different (P<0.03; Table 2). C. ochracea was the main species (68%) detected in the HV group, consistent with its role in periodontal health. In the PP group, C. putitigena was the main species isolated (64%; C. ochracea, 18%); C. granulosa and C. leadbetteri were also isolated. Samples from HPs included a more diversified species distribution (50% C. putitigena and 22% C. ochracea), with three additional species: C. gingivalis, C. granulosa and C. leadbetteri. In the HP and PP groups, the proportion of β-lactamase-producing isolates was higher (50% and 82%, respectively) versus the HV group (16%; P<0.001; Table 2). Sixty-four percent of C. putitigena isolates were β-lactamase-positive versus 11% of C. ochracea isolates (P<0.001). Periodontitis is one of the most common chronic inflammatory diseases and may promote colonization by antibiotic-resistant resident bacteria.

### Table 1. Primers and cycling conditions used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′ – 3′)</th>
<th>Positions of amplification products (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>blacsp-1</td>
<td>TCAATTTCAACATGCAATTG 227–908 52 this study</td>
<td>227–908</td>
<td>52</td>
<td>this study</td>
</tr>
<tr>
<td>erm(A)</td>
<td>TCTTGATATGATGAGATATATATG 44–686 53</td>
<td>44–686</td>
<td>53</td>
<td>this study</td>
</tr>
<tr>
<td>erm(B)</td>
<td>GAAARGTACTCAACCAAAAAAATA 42–681 54</td>
<td>42–681</td>
<td>54</td>
<td>this study</td>
</tr>
<tr>
<td>erm(C)</td>
<td>CTGACTATATGACTCT 100–683 53</td>
<td>100–683</td>
<td>53</td>
<td>this study</td>
</tr>
<tr>
<td>erm(D)</td>
<td>GATGTTGCCTATATATGTC 85–712 56</td>
<td>85–712</td>
<td>56</td>
<td>this study</td>
</tr>
<tr>
<td>erm(F)</td>
<td>ATGACAAAAAAAGATAATTGGCC 1–799 53</td>
<td>1–799</td>
<td>53</td>
<td>this study</td>
</tr>
<tr>
<td>erm(Q)</td>
<td>CTCCATCATTGCCTGTGAAT 135–541 54</td>
<td>135–541</td>
<td>54</td>
<td>this study</td>
</tr>
<tr>
<td>ermA</td>
<td>GATTGTTYAAMAGCATTTTCATG 44–686 53</td>
<td>44–686</td>
<td>53</td>
<td>this study</td>
</tr>
<tr>
<td>ermB</td>
<td>GCACTATTTAGGTYGAG 459–712 56</td>
<td>459–712</td>
<td>56</td>
<td>this study</td>
</tr>
<tr>
<td>ermC</td>
<td>GATGGTTATAAGGTYGAG 459–712 56</td>
<td>459–712</td>
<td>56</td>
<td>this study</td>
</tr>
<tr>
<td>ermD</td>
<td>GATGGTTATAAGGTYGAG 459–712 56</td>
<td>459–712</td>
<td>56</td>
<td>this study</td>
</tr>
</tbody>
</table>

8R, Y and M correspond, respectively, to A or G, C or T, and A or C nucleotides.
Lactamase-positive isolates were also found among more rarely identified species (C. leadbetteri, C. gingivalis and C. granulosa). All β-lactamase-producing isolates (44%) were PCR-positive for \( \text{bla}_{\text{CfxA}} \) (31%) or \( \text{bla}_{\text{CSP-1}} \) (12.5%). With Prevotella, the Capnocytophaga genus constitutes the main source of β-lactam resistance genes in the oral microbiota. \( \text{bla}_{\text{CfxA}} \) was more frequently detected than \( \text{bla}_{\text{CSP-1}} \), probably due to its location on a plasmid (pCAP01 or other unrelated plasmid) or a transposon, such as in Bacteroides and Prevotella.\(^5,8,15\) \( \text{bla}_{\text{CSP-1}} \) was identified only in a subgroup of C. sputigena species (\( P < 0.05 \)), which was PCR-negative for \( \text{bla}_{\text{CfxA}} \) (Table 2). CfxA2, CfxA3 and CSP-1 β-lactamase-producing isolates presented high MIC\(_{50}\) and MIC\(_{90}\) values of amoxicillin, cefotaxime and ceftazidime (8–64 and 64–256 mg/L, respectively) and thus could easily be differentiated from β-lactamase-negative isolates (Table 3). C. sputigena \( \text{bla}_{\text{CfxA}} \) or \( \text{bla}_{\text{CSP-1}} \) isolates could not be differentiated by their phenotypic resistance patterns or 16S rRNA sequences. β-Lactamase-producing isolates remained susceptible to the combination of clavulanic acid and amoxicillin.\(^7\) All \( \text{bla}_{\text{CSP-1}} \) PCR-positive isolates clearly expressed β-lactam resistance (amoxicillin MIC >16 mg/L), in contrast with the observations of Guillon et al.,\(^6\) who found \( \text{bla}_{\text{CSP-1}} \) in two β-lactam-susceptible C. sputigena bacterial reference strains (CIP 104301T and CRBIP 1739) by PCR.

The prevalence of MLS-resistant isolates was 21%–36%, independent of patient group, species or β-lactamase production (Table 2). MLS-resistant isolates presented equally high MIC\(_{50}\) and MIC\(_{90}\) values (256 mg/L) of erythromycin and clindamycin, suggesting constitutive MLS resistance (Table 3). Resistance determinants unknown in Capnocytophaga spp. were sought by specific PCR. The majority of MLS-resistant isolates (13/14, 93%) were \( \text{erm}(\text{F}) \) PCR-positive with DNA sequences 100% identical to each other, harbouring the same Ile130Val substitution that is absent in the Bacteroides fragilis M14730 \( \text{erm}(\text{F}) \) sequence.\(^11\) Horizontal gene transfer may occur in Capnocytophaga species and probably in the Bacteroides–Cytophaga–Flavobacterium branch of the eubacterial tree.\(^16,17\) Indeed, \( \text{erm}(\text{F}) \) genes are known to have a
broad host range that includes Treponema denticola. The \textit{erm}(F) gene was detected in all species (except \textit{C. granulosa}), including the two \textit{C. leadbetteri} isolates. One \textit{C. ochracea} isolate that was highly resistant to erythromycin and clindamycin (256 mg/L) was \textit{erm}(F) PCR-negative, but \textit{erm}(C) PCR-positive with 100% nucleotide identities with \textit{erm}(C) of \textit{S. aureus} and 96% with \textit{Lactobacillus reuteri} (GenBank FJ489650). All other \textit{erm} methylase genes sought by PCR were not detected in \textit{Capnocytophaga} spp. All \textit{Capnocytophaga} isolates that were methylase PCR-negative were susceptible to erythromycin and clindamycin (0.38–1 and 0.016–1 mg/L, respectively). With a minimum prevalence of 21% methylase-producing isolates, and in comparison with published studies, we showed that \textit{Capnocytophaga} spp. constitute the main reservoir of MLS genes in the oral cavity. This work did not allow demonstration of a link between the prevalence of resistant \textit{Capnocytophaga} isolates and antibiotherapy during the last 6 months, or hospitalization (HP group of inpatients versus HV and/or PP groups of outpatients).

In conclusion, our findings show a high prevalence of \textit{β}-lactam (44%) and MLS (29%) resistance genes in human oral \textit{Capnocytophaga} species. We also describe for the first time the distribution of the new class A \textit{β}-lactamase gene, \textit{bla}_{CSP-1}, only in a subgroup of \textit{C. sputigena} species and characterize the \textit{erm}(F) and \textit{erm}(C) genes incriminated in MLS resistance in \textit{Capnocytophaga} spp.

**Nucleotide sequence accession numbers**

The complete \textit{erm}(F) and partial \textit{erm}(C) nucleotide sequences from \textit{C. sputigena} Be58 and \textit{C. ochracea} Bi75 isolates, respectively, have been deposited in GenBank under accession numbers JQ707297 and JQ886176.

**Acknowledgements**

We dedicate this work to the memory of Chantal Giraud-Morin. We thank Bernard Beill and Julian Rood for providing \textit{S. pyogenes} isolate 9116-03 (pRW35) and \textit{E. coli} (pJR+32), respectively. We thank Sandrine Courtal for technical support.

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**Transparency declarations**

None to declare.

**Supplementary data**

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**