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Evaluation of Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry for identification of human oral *Capnocytophaga* species

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28 **Abstract**

29 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-
30 TOF MS) was evaluated for rapid identification of *cfxA* PCR positive and negative
31 *Capnocytophaga* strains. Colonies were grown on blood agar, incubated anaerobically at
32 37°C for 48 h, and were then evaluated by MALDI-TOF MS and 16S rRNA gene sequencing.
33 Both methods identified all colonies to the genus level. The MALDI-TOF MS method gave the
34 same result, at the species level, as 16S rRNA gene sequencing for 41/53 *Capnocytophaga*
35 *sp.* strains (77.4%), but the limit of this technique was the absence of some species (*C.*
36 *leadbetteri*, *C. AHN*) in the Biotyper-Bruker® database used in this study. Distinction
37 between the cefotaxime resistant and susceptible strains was unsuccessful using the MALDI-
38 TOF MS method. This technique had low discriminatory power to rapidly detect beta-
39 lactamase-producing *Capnocytophaga* strains in clinical samples. However, the results from
40 a score-oriented dendrogram confirmed MALDI-TOF MS is a rapid, inexpensive, and reliable
41 method for *Capnocytophaga* species identification. Enrichment of the reference database
42 used (Biotyper®) will improve future results.

43

44

45 **Key words:** MALDI-TOF Mass Spectrometry; *Capnocytophaga*; *cfxA*; 16S rRNA; PCR;
46 dendrogram

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48

49 **1. Introduction**

50

51 *Capnocytophaga* are part of the normal bacterial flora of the oral cavity of humans
52 and animals. Generally, they are considered commensal bacteria but, in immunocompetent
53 or immunocompromised patients, they can be responsible for systemic diseases [1]. These
54 bacteria belong to the family *Flavobacteriaceae*, order *Flavobacteriales*, class *Flavobacteria*,
55 *Bacteroidetes* phylum, and *Bacteria* domain. This genus includes 10 different commensal
56 species. There are six isolated from the oral cavity of humans: *Capnocytophaga ochracea*, *C.*
57 *gingivalis*, *C. granulosa*, *C. haemolytica*, *C. sputigena*, *C. leadbetteri*, and four isolated from
58 the oral cavity of animals: *C. canimorsus*, *C. cynodegmi*, *C. stomatis*, and *C. canis*. The
59 classification of many described strains (AHN) remains uncertain.

60 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-
61 TOF MS) is based on the protein composition of the microbial cell, particularly ribosomal
62 protein contents, and can discriminate clinical and environmental species. This technique is
63 effective to identify anaerobic bacteria in the routine clinical microbiology laboratory setting
64 [2-4]. Some clinical case reports used this technique to correctly identify *Capnocytophaga*
65 species at the genus level [5, 6].

66 The MALDI-TOF MS has also been used to detect several beta-lactamases, such as
67 extended-spectrum β -lactamase (ESBL) [7, 8] and carbapenemases [2, 9, 10]. Only a few
68 studies screened beta-lactamases in anaerobes, such as the class B metallo-beta-lactamase
69 encoded by *cfiA* in *Bacteroides fragilis* [2] or carbapenemases [9, 11]. The prevalence of
70 beta-lactamase producing *Capnocytophaga sp.* is increasing in the human oral cavity, as
demonstrated by the high prevalence of *cfxA*-positive PCR strains [12-14], which is the major

71 cause of third generation cephalosporin resistance in *Capnocytophaga* sp. The *cfxA* gene,
72 conferring constant resistance to third generation cephalosporins, especially cefotaxime
73 [15], has been described on plasmids and/or on chromosomes harbouring mobile genetic
74 elements. This ability to gene mobilisation can be responsible for resistance gene
75 dissemination and transfer [12]. When a beta-lactamase-producing *Capnocytophaga* strain is
76 detected in a clinical sample, the empirical antimicrobial treatment administered to the
77 patient should often be rapidly modified. However, the detection of the *cfxA* gene is not
78 sufficient to assert the resistance. In previous work, we showed that the expression of this
79 gene is not always constant: if 1st and 2nd generation cephalosporins are most often
80 affected by the CfxA beta-lactamase, the phenotypic expression of resistance for
81 cephalosporins of 3rd generation is reported variable according to the strains [16].
82 Expression of *cfxA* genes (*cfxA* mRNA levels) was quantified by using quantitative PCR, in
83 *cfxA* PCR-positive isolates differentiated by their beta-lactam resistance profiles. One isolate
84 remained susceptible to beta-lactams (despite a positive *cfxA* PCR), and the *cfxA* gene was
85 not expressed (C_t value identical to distilled water used as negative control). But the
86 normalized C_t values for the third-generation cephalosporins were not directly related to
87 MIC levels. This expression could also be influenced by replacing the sequence in the *cfxA*
88 gene, and enhanced expression related to the proximity of mobile elements such as *mobA*
89 [16]. A simple and rapid method for detecting the presence of the CfxA beta-lactamase
90 would therefore be useful for better interpreting the PCR results. Wybo *et al.* [2] managed to
91 differentiate *cfiA*-positive from *cfiA*-negative isolates in a routine laboratory setting and so
92 pinpoint *B. fragilis* strains potentially resistant to carbapenems. In the same way, we hoped

93 to discriminate negative and positive *cfxA* strains: culture of bacteria with cefotaxime was
94 also tested to confirm the experiment.

95 The aim of this study was to use MALDI-TOF MS for rapid species identification of
96 *Capnocytophaga*. The study also tested the discriminatory power of this technique to
97 differentiate the cefotaxime resistant from cefotaxime susceptible *Capnocytophaga* strains.

98

99 **2. Materials And Methods**

100 *2.1 Strains used*

101 Reference strains (n=5) were used for this study: *C. sputigena* ATCC 33595, *C. sputigena*
102 ATCC 33612, *C. gingivalis* ATCC 33624, *C. ochracea* ATCC 27872, and *C. granulosa* ATCC
103 51502. Clinical strains of *Capnocytophaga* sp. (n=48) were isolated from routine cultures, at
104 the Teaching Hospital of Rennes (France). Clinical strains were isolated from oral swabs
105 (n=30) and sputum samples (n=8) on TBBP (Trypticase-blood-bacitracin-polymyxin B) agar
106 [17], and from surgical bone samples (n=4), abscesses (n=4), and blood samples (n=2) on
107 horse blood agar (Oxoid). All samples were conserved in frozen stock cultures (Cryobanks,
108 MAST diagnostics), and cultured on horse blood agar in an anaerobic chamber for 24 to 48 h
109 at 37°C for further experiments.

110

111 *2.2 Susceptibility testing*

112 The MICs were determined by Etest methodology (Etest[®], BioMérieux, France), and
113 interpreted according to CLSI recommendations for anaerobes [18], since no breakpoint for
114 third generation cephalosporins (as cefotaxime) is currently available for anaerobes,
115 according to EUCAST recommendations [19]. The CLSI breakpoints of cefotaxime for

116 susceptible, intermediate, and resistant strains were ≤ 16 mg/L, 32 mg/L, and ≥ 64 mg/L,
117 respectively.

118

119 2.3 PCR amplification

120 Amplification of the *cfxA* gene by PCR was performed as previously described [20]. Briefly,
121 the primers used were: *cfxA1* (5'-CTTTGTCGGCAAATAAAGAT-3') and *cfxA4* (5'-
122 TGAACGAGGAATGAGTGTGG-3'), generating a 580bp fragment and *cfxA5* (5'-
123 TGGTTAATGTCGCTCAAACA-3') and *cfxA6* (5'-TCAAAGCAAGTGCAGTTTAAGA-3'),
124 generating a 507bp fragment. A 966-bp consensus sequence of *cfxA3* was then determined.
125 The amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35
126 cycles).

127 Sequencing of the 16S rRNA gene was used as a reference method for *Capnocytophaga*
128 species identification, based on previous work [21]. Real-time PCR was performed with Sybr
129 green to target the 5' part of the 16S rRNA gene (forward primer 27F, 5'-AGA GTT TGA TCM
130 TGG CTC AG-3'; reverse primer 685R3, 5'-TCT RCG CAT TYC ACC GCT AC-3'; 658-bp
131 amplification product; GenBank accession number NR_024570). The corresponding
132 amplicons were sequenced in both strands and assembled, and the consensus sequences
133 were compared with those in the Bioinformatics Bacteria Identification (BIBI) and BLAST
134 databases. The rates of concordance between 16S rRNA gene PCR and bacteriological results
135 were based on results at the genus ($\geq 96\%$ similarity) and species ($\geq 98\%$ similarity) levels.
136 Positive and negative controls were added in each series [21]. To control DNA extraction and
137 confirm the absence of PCR inhibitors, a fragment of the human beta-globin gene was
138 amplified for each negative sample.

139

140 *2.4 Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF*
141 *MS)*

142 MALDI-TOF MS (Biotyper, Bruker®) analyses were performed in positive linear mode in the
143 range of 2000–20,000 masses-to-charge ratio (m/z), and used to identify the isolates to the
144 level of species. Colonies were selected after culturing on horse blood agar in an anaerobic
145 chamber for 24 to 48 h, in the absence of antibiotic or around the cefotaxime disk
146 (BioRad, 30 μ g). Colony spotting was performed in triplicate and spectra were obtained and
147 analyzed with MALDI Biotyper 2.0 software and references (Bruker® Daltonik, GmbH,
148 Bremen, Germany), according to the manufacturer's instructions. For the direct colony
149 method, bacteria were applied as a thin film onto a 96-spot steel plate (Bruker® Daltonics)
150 and allowed to dry at room temperature. Subsequently, 1.2 μ l of MALDI matrix (a saturated
151 solution of α -cyano-4-hydroxycinnamic acid [HCCA; Bruker® Daltonics] in 50% acetonitrile
152 and 2.5% trifluoroacetic acid) was applied to the colony and allowed to dry before testing.
153 For the extraction method, 1 to 2 colonies were extracted as already described [22]. Each
154 series of measurements was preceded by calibration with a bacterial test standard (BTS
155 255343; Bruker Daltonik), to calibrate the instrument and validate the run. Negative controls
156 were a matrix deposit alone and Brucella broth (supplemented with 5% blood, vitamin K1
157 and cefotaxime (0.5mg/L)). For identification, scores and reproducibility were considered
158 according to the manufacturer's instructions (Bruker®). Results of the pattern matching
159 process were expressed as log (score) values ranging from 0 to 3. Values from 0-1.6999
160 indicated not suitable identification, >1.7 indicated relationships on the genus level, and >2.0
161 indicated relationships on the species level (score: 2.3 to 3: high probability of identification

162 to the species level, 2 to 2.2999: high probability of identification to the genus level and
163 probable identification to the species level, 1.7-1.999: probable identification to the genus
164 level). The interpretation considered two independent parameters: the value of the
165 homology score and the reproducibility of the identification obtained (from 10
166 measurements carried out after laser impacts where the same bacterial species must be
167 found at least three times with the highest scores).

168 To test if MALDI-TOF MS was able to differentiate between *cfxA*-negative and *cfxA*-positive
169 isolates, bacterial pellets were also extracted after incubation of each isolate in the presence
170 of cefotaxime (0.5 mg/L) in Brucella broth supplemented with 5% blood and vitamin K1 (1
171 mg/L) under anaerobic conditions for 6 h. Identification was performed, as indicated above,
172 from a deposit of a drop of pellet left to dry and score-oriented dendrogram of matrix-
173 assisted laser desorption ionization time-of-flight mass spectrometry profiles were
174 compared.

175 The MALDI-TOF MS spectral data resulting from the analysis of 53 isolates were processed
176 using the Bruker database software to identify species-specific masses that were then used
177 to generate dendrograms. A class dendrogram of all the study isolates was constructed (with
178 the correlation distance measured by the average linkage algorithm; Euclidean distances) of
179 the Biotyper 2.0 software (Bruker Daltonics) [23]. The MALDI-TOF spectra similarity
180 dendrogram between *cfxA* positive and negative strains, based on the mass spectral
181 patterns, was used to study dispersion of spectra [24].

182

183 **3. Results**

184 *3.1 Identifications of strains by MALDI-TOF MS and 16S rRNA gene sequencing*

185 The MALDI-TOF MS and 16S *rRNA* gene sequencing of the clinical and reference
186 isolates clearly identified all strains as *Capnocytophaga* (100% agreement) at the genus level,
187 and 41/53 (77.4%) were identified as the same at the species level (Table 1), with a score
188 >1.8 and a reproducibility >4 (minimum of 3/10 replicates had MALDI confidence scores
189 >1.8). Both methods identified the *C. sputigena* (n=31) and *C. ochracea* (n=3) strains. The
190 four *Capnocytophaga* AHN (AHN9576/AHN9798/AHN8471/ChDc) strains identified by 16S
191 *rRNA* sequencing were characterized as *C. sputigena* by MALDI-TOF MS. The six *C.*
192 *leadbetteri* strains recognized by 16S *rRNA* sequencing were identified as *Capnocytophaga.*
193 *sp.* by MALDI-TOF MS. One strain classified as *Capnocytophaga* *geno sp.*, *C. granulosa* and
194 another as *C. gingivalis* by 16S *rRNA* sequencing were identified as *C. sputigena*, *C. gingivalis*
195 and *C. granulosa*, respectively, by MALDI-TOF MS. The MALDI-TOF MS technique had limited
196 ability to discriminate between *C. gingivalis* and *C. granulosa* (two misidentified strains), but
197 these species were similar, as shown on the dendrogram of all *Capnocytophaga* species
198 identified using MALDI-TOF MS (Figure 1).

199 3.2 Susceptibility testing

200 From the total clinical *Capnocytophaga* *sp.* (n=48), 35 strains were cefotaxime-susceptible,
201 three were intermediate, and 10 were resistant, according to CLSI criteria [17]. The MICs of
202 cefotaxime were: MIC₅₀ = 1.5 and MIC₉₀ ≥256 mg/L (range: 0.016->256 mg/L). Among these
203 *Capnocytophaga* *sp.* isolates, the *cfxA* gene was amplified by PCR in 42 (87.5%) all resistant
204 to cefalotin (data not shown): 13 strains were resistant/intermediate and 29 remained
205 phenotypically susceptible to cefotaxime despite a positive *cfxA* PCR. Five of the latter (four
206 from saliva and one from osteo-articular fluid) *cfxA*⁺, susceptible to cefotaxime with MIC
207 range: 0.016-0.5 mg/L were found to be gene misexpressed and/or beta-lactamase

208 inactivated, as previously suggested. The five reference strains were *cfxA* PCR negative and
209 susceptible to beta-lactams.

210 3.3 MALDI-TOF spectra similarity dendrogram

211 The MALDI-TOF spectra similarity dendrogram, in which the strains were dispersed
212 irrespective of their mass spectral patterns, was unsuccessful in differentiating susceptible
213 and resistant strains (data not shown).

214

215 4. Discussion

216 In a previous study, Conrads *et al.* [25] used oligonucleotide probes and
217 demonstrated that the dendrogram of 16S *rRNA* sequences of the *Capnocytophaga*
218 reference strains showed a very close relationship, especially *C. granulosa* and *C. gingivalis*
219 (97.7% similarity) and *C. ochracea* and *C. sputigena* (95.0% similarity). Previously, MALDI-TOF
220 MS was used with success to identify strict anaerobes, such as *Bacteroides* [26] or *Prevotella*
221 [27], with clinical isolates of *Prevotella* sp. identified to the species (62.7%) and genus
222 (73.5%) levels. Recently, Magnette *et al.* [28] showed that MALDI-TOF MS provided a reliable
223 identification to the species level for 100% of 45 blind-coded *Capnocytophaga canimorsus*
224 and *C. cynodegmi*, but only following the establishment of a complementary homemade
225 reference database. Using MALDI-TOF MS in this study, we found improved *Capnocytophaga*
226 genus level identification (100%) and a good human oral *Capnocytophaga* species level
227 identification (77.4%). The six *C. leadbetteri* and four *C. AHN* strains (recognized by 16S *rRNA*
228 sequencing) were identified by MALDI-TOF MS as *C. spp.* and *C. sputigena*, respectively. This
229 result was not surprising because *C. leadbetteri* and *C. AHN* do not exist in the Biotyper-
230 Bruker® database used, as was previously observed [4]. In addition, the MALDI-TOF MS

231 method could not completely discriminate between *C. gingivalis* and *C. granulosa*, a finding
232 that is consistent with previous reports using multilocus enzyme electrophoretic analysis and
233 serotyping of immunoglobulin A1 proteases [29]. Thus, our results suggest that MALDI-TOF
234 MS can be used for rapid and reliable identification of most clinical *Capnocytophaga* strains.

235 We also investigated if MALDI-TOF MS could be used to detect the presence of the
236 beta-lactamase CfxA (mainly responsible for cefotaxime resistance) or one of its metabolites
237 in *Capnocytophaga* strains. The MALDI-TOF MS method failed to detect *cfxA* positive
238 *Capnocytophaga* strains (compared to *cfxA* negative strains), cultured in the presence or
239 absence of cefotaxime. Most previous studies used inhibitors of beta-lactamases (ESBL or
240 carbapenemases) to reveal by MALDI-TOF MS the intermediates of beta-lactamases [30] or
241 enzyme inhibition [31]. We did not do this because the intrinsic activity of all beta-lactamase
242 inhibitors was demonstrated in *Capnocytophaga* species [32]. Wybo *et al.* [2] demonstrated
243 that MALDI-TOF MS could be used to differentiate between *cfiA*-positive and *cfiA*-negative
244 *Bacteroides* strains, cultured on fastidious anaerobic agar at 35°C in an anaerobic chamber
245 for 24 to 48 h, without use of antibiotic or beta-lactamase inhibitors. This result was
246 confirmed by Fenyvesi *et al.* [33], who characterized 5/60 *cfiA* positive strains using this
247 method. In our study, we could not discriminate between cefotaxime susceptible (*cfxA*-
248 positive) and resistant (*cfxA*-negative) strains using the MALDI-TOF MS method and MALDI
249 Biotyper 2.0 software, as recommended by Wybo *et al.* [2].

250 The limits of this technique for beta-lactamase detection have already been
251 highlighted. Trevino *et al.* [11] used MALDI-TOF MS for rapid characterization of
252 carbapenemase-producing *Enterobacteriaceae* and genetic diversity among carbapenemase-
253 producing strains. Trevino *et al.* [11] also showed that fingerprinting analyses by automated

254 repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (DiversiLab system)
255 showed higher discriminatory power than MALDI-TOF MS for clonal strain typing. With this
256 last method, distinct mass spectra were obtained only within a mass range of 2,000 to
257 20,000 Da using whole cell MALDI-TOF MS [34], and the mass range of the CfxA beta-
258 lactamase was estimated to 35,275 Da and CfiA to be 27,260 Da. By MALDI-TOF MS, only
259 degradation products of the beta-lactam cefotaxime might have been detected, but no
260 difference in profiles was observed when *Capnocytophaga* strains were cultured with and
261 without antibiotic. Moreover, the expression of CfxA enzyme has been described as variable
262 with different MICs reported. Wybo *et al.* [2] suggested that the *cfiA* gene was not always
263 activated, and Soki *et al.* [35] hypothesized that the *cfiA* gene was activated by its own
264 promoter. The differentiation of *cfiA*-negative and *cfiA*-positive *Bacteroides fragilis* [2] was
265 probably due to a selection bias in identifying strains, because the *cfiA* gene, encoding the
266 unique carbapenemase found in *Bacteroides*, was restricted to division II *Bacteroides fragilis*
267 strains [36].

268

269 5. Conclusion

270 In conclusion, MALDI-TOF MS can be used for rapid and low cost oral human
271 *Capnocytophaga* genus identification. But the limits of this technique was the absence of
272 some species (*C. leadbetteri*, *C. AHN*) in the Biotyper-Bruker® database used in this study,
273 and its inability to completely discriminate between *C. gingivalis* and *C. granulosa*. This
274 method was not also suitable for quickly detecting beta-lactamase producing
275 *Capnocytophaga* strains.

276

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283

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395

396 **FIGURE LEGEND**

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398 **Figure 1** Score-oriented dendrogram of matrix-assisted laser desorption ionization time-of-
399 flight mass spectrometry profiles generated by the default setting in MALDI Biotyper
400 software version 2.0. for 53 *Capnocytophaga* strains cultured on blood agar for 48 h under
401 anaerobic conditions. Reference strains were *C. sputigena* ATCC 33595 (strain 40), *C.*
402 *sputigena* ATCC 33612 (strain 41), *C. ochracea* ATCC 27872 (strain 42), *C. gingivalis* ATCC
403 33624 (strain 43), and *C. granulosa* ATCC 51502 (strain 44). The misidentified (compared
404 with the 16S rRNA gene sequencing identification technique) species were: *C. granulosa/C.*
405 *gingivalis* (n=1), *C. spp./C. leadbetteri* (n=6), *C. sputigena/C. AHN* (n=4), and *C. sputigena/C*
406 *sp.* (n=1). Two distinct clades appeared on dendrogram using MALDI-TOF corresponding to:
407 clade at the top: clade for *C. gingivalis/granulosa*, and clade down: clade for other
408 *Capnocytophaga* spp.

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411

412 **Table 1** The number of isolates identified at the indicated level by matrix-assisted laser
 413 desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) compared with 16S
 414 rRNA gene sequencing results for 53 *Capnocytophaga* strains.

415

	Identification by 16S rRNA gene sequencing	No. of isolates identified at the indicated level by MALDI-TOF MS using the Bruker® Reference Library 3.2.0 database			
		Total No. of isolates	Species	Genus	Absence of identification
<i>C. sputigena</i>		31	31	0	0
<i>C. ochracea</i>		3	3	0	0
<i>C. gingivalis</i>		3	2	1	0
<i>C. granulosa</i>		5	4	1	0
<i>C. leadbetteri</i>		6	0	6	0
AHN9576/AHN9798/AHN8471/ChDc		4	0	4	0
<i>Capnocytophaga</i> sp.		1	0	1	0
Total		53	40	13	0

416

417

Maldi-Tof MS
identification

16SrRNA sequencing
identification
(strain number)

