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Improvement of identification of *Capnocytophaga canimorsus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry using enriched database

Amandine Magnette [a], Te-Din Huang [a,*], Francesco Renzi [b], Pierre Bogaerts [a], Guy R. Cornelis [b], Youri Glupczynski [a]

[a] Laboratory of Clinical Microbiology, CHU Dinant-Godinne UCL Namur, B-5530, Yvoir, Belgium
[b] Research Unit in Biology of Microorganisms, Namur Institute for Life Sciences (NARILIS), University of Namur, B-5000, Namur, Belgium

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ABSTRACT

Capnocytophaga canimorsus and *Capnocytophaga cynodegmi* can be transmitted from dogs or cats and cause serious human infections. We aimed to evaluate the ability of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to identify these two *Capnocytophaga* species. Ninety-four *C. canimorsus* and 10 *C. cynodegmi* isolates identified by 16S rRNA gene sequencing were analyzed. Using the MALDI BioTyper database, correct identification was achieved for only 16 of 94 (17%) *C. canimorsus* and all 10 *C. cynodegmi* strains, according to the manufacturer's log score specifications. Following the establishment of a complementary homemade reference database by addition of 51 *C. canimorsus* and 8 *C. cynodegmi* mass spectra, MALDI-TOF MS provided reliable identification to the species level for 100% of the 45 blind-coded *Capnocytophaga* isolates tested. MALDI-TOF MS can accurately identify *C. canimorsus* and *C. cynodegmi* using an enriched database and thus constitutes a valuable diagnostic tool in the clinical laboratory.

1. Introduction

The genus *Capnocytophaga* is a group of fastidious gram-negative bacteria belonging to the family of *Flavobacteriaceae* (Mally et al., 2009). It includes eight species, namely *C. ochracea*, *C. sputigena*, *C. gingivalis*, *C. granulosa*, *C. leadbetteri*, *C. haemolytica*, *C. canimorsus* and *C. cynodegmi* (Ehrmann et al., 2013). The first six species reside in the human oral cavity (Zangenah et al., 2012; Oehler et al., 2009) and may cause periodontal disease and invasive infections in immunocompromised patients (Zangenah et al., 2012; Jolivet-Gougeon et al., 2007). The last 2 species, *C. canimorsus* and *C. cynodegmi*, reside in the oral cavities of dogs and cats only and may occasionally be transmitted to humans via bites, scratches or even by licks (Mally et al., 2009; Ehrmann et al., 2013; Butler, 2015; Shin et al., 2009).

Formerly called "Dysgonic fermenter 2" (DF-2), *C. canimorsus* can cause wound infections and disseminated infections leading to sepsis, meningitis and endocarditis (Zangenah et al., 2012; Oehler et al., 2009; Jolivet-Gougeon et al., 2007; Shin et al., 2009). On the other hand, *C. cynodegmi* (formerly known as DF-2-like) has been mainly detected in wound infections but systemic infection is very rare (Zangenah et al., 2012; Jolivet-Gougeon et al., 2007; Shin et al., 2009; Brenner et al., 1989). The prognosis of *C. canimorsus* infection is poor especially in chronic alcohol users, in asplenic and in immunocompromised patients with an overall mortality of 30% (Oehler et al., 2009; Jolivet-Gougeon et al., 2007; Butler, 2015; Shin et al., 2009). This justifies the importance of rapid accurate identification to species level and the rapid initiation of treatment with appropriate antimicrobial agents (Zangenah et al., 2012).

The growth of *C. canimorsus* and *C. cynodegmi* requires 2–3 days in an atmosphere of 5% CO_2. Presumptive identification to the genus level is classically performed using colony morphology, gram staining (thin and slender gram-negative fusiform rods) and phenotypical tests (oxidase and catalase positive) (Zangenah et al., 2012; Oehler et al., 2009; Jolivet-Gougeon et al., 2007; Shin et al., 2009). The differentiation between species among the genus *Capnocytophaga* is based on biochemical tests but remains difficult as most current commercial identification kits are unable to identify this organism to genus or species level. Molecular reference methods such as 16S rRNA gene sequencing are time-consuming and are not available in many laboratories (Suzuki et al., 2010). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) therefore appears as a potentially attractive and rapid tool for bacterial identification of these fastidious organisms.

Zangenah et al. (2012) evaluated the performance of the MALDI-TOF MS for identification of *C. canimorsus* and *C. cynodegmi* and showed that it was both faster and more accurate than conventional identification methods but that its use for identification of *C. canimorsus* and *C. cynodegmi* might be significantly improved when more reference spectra were to be added to the database.

* Corresponding author. Tel.: +32-81-42-32-07; fax: +32-81-42-32-04.
E-mail addresses: te-din.huang@uclouvain.be, danhua814@yahoo.fr (T.-D. Huang).

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The aim of this study was to evaluate the ability of MALDI-TOF MS for identification of *C. canimorsus* and of *C. cynodegmi* and the impact of enlarging the number of mass spectra of these two species included in the reference database for improving their identification to species level.

2. Materials and methods

2.1. Bacterial strains and culture growth

A panel of 94 *Capnocytophaga canimorsus* and of 10 *Capnocytophaga cynodegmi* strains was included. The human or animal origin and clinical sources of the different isolates are reported in Table 1.

All strains stored in glycerol stocks at −80°C were thawed and subcultured twice on 5% sheep blood Schaedler agar supplemented with vitamin K1 (Becton-Dickinson, Erembodegen, Belgium) and incubated for 2–3 days at 35°C with 5% CO_2.

2.2. Sequencing of 16S rRNA gene

All isolates included in the study were analyzed by comparing 1.07 kb of the 16S rRNA (from nucleotide 27 to 1100, *E. coli* 16S rRNA numbering) to all 16S rRNA genes in the ribosomal database according to the method described previously which proved to be an accurate tool for the species identification and differentiation of *C. canimorsus* and *C. cynodegmi* (Mally et al., 2009).

2.3. MALDI-TOF MS

MALDI-TOF MS measurements were performed on a Microflex LT (Bruker Daltonik, Leipzig, Germany) provided with a 20-Hz nitrogen laser. Spectra were recorded in the positive linear mode with an accelerating voltage of 20 kV. For species level identification, the mass range used is between 2 and 20 kDa. The Bruker bacterial test standard (BTS 8255343; Bruker Daltonik) was used as a calibrator for each sample run (Verroken et al., 2010).

2.3.1. Establishment of a C. canimorsus and C. cynodegmi database

Of the total 104 collection strains, 51 randomly selected *C. canimorsus* isolates (32 from animals, 19 from humans) and 8 *C. cynodegmi* isolates were extracted and their mass spectra were analyzed by Microflex LT as per manufacturers' recommendations to build a specific database.

The extraction procedure was performed according to previously published methods (Verroken et al., 2010). After 2 or 3 days of incubation, freshly grown colonies were suspended in distilled water and ethanol solution, centrifuged and suspended in formic acid solution and acetonitrile. The resulting supernatant was subjected to MALDI-TOF MS analysis.

One microliter of the final extraction product of the strain was spotted ten times on the steel surface of a target plate (Bruker Daltonics). Each spot was overlaid with 1 μl of matrix (saturated α-cyano 4-hydroxy cinnamic acid in 50 % acetonitrile with 2.5% tri-fluoro-acetic acid) and was measured three times by the MBT_FC.par flexControl method and the MBT-autoX.axe autoExecute method. Every measurement resulted from six series of 40 laser shots at different positions on the spotted product. The 30 spectra obtained were closely analyzed in the flexAnalysis program with major attention to intrusive peaks. Finally, a minimum of 20 accurate spectra out of 30 were downloaded to the MALDI BioTyper software in order to generate a single mean spectrum accounting for the extracted *Capnocytophaga* strain with the BioTyper MSP creation standard method.

As a means of estimating the similarity between the organisms and visualizing the clustering, a dendrogram was calculated with the MALDI BioTyper software (Verroken et al., 2010).

2.3.2. MALDI-TOF MS identification of C. canimorsus and C. cynodegmi challenge strains

In a first step, all isolates were initially submitted for species identification by MALDI-TOF MS using Microflex LT based on the MALDI BioTyper database (version 3.0 updated on January 2014) bearing the spectra of 5627 cellular organisms which contains 27 strains belonging to seven *Capnocytophaga* species yet only including one isolate each of *C. canimorsus* and of *C. cynodegmi*. Bacterial colonies were applied directly onto a steel target. Samples were overlaid with 1 μL of matrix, dried at room temperature and analyzed by the MBT_FC.par flexControl method. A first score was obtained after submitting the downloaded raw spectra to the original MALDI BioTyper database.

In a second step, 51 *C. canimorsus* strains (32 animal and 19 clinical) and 8 strains of *C. cynodegmi* were extracted in order to build a complementary reference database.

Then, 43 randomly selected *C. canimorsus* strains and 2 *C. cynodegmi* strains not included in the new database were subsequently submitted for species identification using the generated database expanded by the inclusion of these 59 isolates.

According to the specifications of the manufacturer, a high log score of ≥2 is required for identification to the species level and an intermediate log score lying between <2 and ≥1.7 is required for identification to the genus level. A low score of <1.7 is considered unreliable for identification (Verroken et al., 2010).

Table 1
Capnocytophaga canimorsus and *Capnocytophaga cynodegmi* isolates included in the study.

Species	Human/ animals	Clinical sites	Country	No. of isolates
Capnocytophaga canimorsus	Dogs	Dog mouth (n = 75)	Belgium	13
			Italy	1
			Switzerland	61
	Human	Blood cultures (n = 19)	Belgium	10
			Germany	1
			Netherlands	1
			Switzerland	3
			Sweden	1
			USA	3
Capnocytophaga cynodegmi	Dogs	Dog mouth (n = 7)	Belgium	1
			Switzerland	5
			USA	1
	Human	Hand wound (n = 2)	USA	2
	Unknown origin	Unknown (n = 1)		1

3. Results

With the mass spectra of the 51 extracted *C. canimorsus* strains and the 8 *C. cynodegmi* strains, we generated a dendrogram with species-specific cluster patterns (Fig. 1). The dendrogram delineated all the 8 *C. cynodegmi* strains (including reference type strains ATCC49044, ATCC49045, LMG11538 and LMG11539) in a single cluster while two distinct clusters (clusters I and II) were observed among the isolates of *C. canimorsus*. Cluster I included 9 isolates, all originating from dogs (none from human specimens) while cluster II regrouped 23 and 19 isolates from dogs and humans (including reference type strains ATCC35979, ATCC35978, LMG11551, LMG11510 and LMG11511), respectively.

When using the original MALDI BioTyper database, correct identification was achieved for 16 out of 94 (17%) *C. canimorsus* strains. Fifteen strains (16%) were identified to the genus level (log score between <2 and ≥1.7) and one single strain only was correctly identified to the species level (log score ≥2). Unreliable identification results were obtained for the 78 (82.9%) remaining strains (log score <1.7). On the other hand, correct identification to the species level was achieved for all the 10 strains of *C. cynodegmi*.

Following addition into the original database of the mass spectra of the 59 extracted *C. canimorsus* and *C. cynodegmi* isolates, MALDI-TOF