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Identification of *Campylobacter* species and related organisms by matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry

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Abstract

The identification of *Campylobacter* species and related organisms at the species level has always been difficult using phenotypic methods because of their low metabolic activity, whereas molecular methods are more reliable but time-consuming. In this study, 1007 different strains were identified using three different methods: conventional methods, molecular biology (real-time PCR and sequencing) and matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Molecular methods were considered the gold standard. The accuracy of MALDI-TOF mass spectrometry reached 100% compared with the gold standard for all of the *Campylobacter* species, except *Campylobacter jejuni* (99.4%). The accuracy of conventional methods compared with the gold standard ranged from 0% to 100% depending on the species. However, MALDI-TOF mass spectrometry was not able to identify a mixture of two different species present in the same sample in four instances. Finally, MALDI-TOF mass spectrometry is highly recommended to identify *Campylobacter* spp. as only 0.4% discrepancy was found, whereas conventional methods led to 4.5% discrepancy.

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Introduction

The incidence of *Campylobacter*-associated food poisoning has gradually increased, and this organism is now considered to be the leading cause of bacterial gastroenteritis worldwide. Infectious complications may occur, especially in patients with immune deficiency, and these bacteria are also implicated in several potentially severe autoimmune diseases, e.g. Guillain–Barré syndrome [1]. *Campylobacter jejuni* and *Campylobacter coli* are the species most frequently isolated from stool specimens in this context and they accounted for 80% and 16%, respectively, of 15 000 isolates received in our laboratory over the last 6 years [2]. However, other *Campylobacter* species [3] or related bacteria from the genera *Arcobacter* [4] and *Helicobacter* are also responsible for diarrhoeal diseases in humans. At the French National Reference Centre for *Campylobacter* and *Helicobacter*, standard identification at species level is performed using phenotypic tests and molecular techniques [5] to obtain the most accurate results, but these tests are time-consuming. Matrix associated laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry has proven interesting in terms of rapidity and cost compared with standard methods [6–8]. Therefore, the aim of this study was to compare its accuracy with the accuracy of the currently used phenotypic and molecular methods to identify *Campylobacter* and related species of Epsilonproteobacteria.

Material and Methods

Bacterial strains

The French National Reference Centre for *Campylobacter* and *Helicobacter* receives *Campylobacter*-like strains isolated in clinical laboratories all over the country, mainly from stool samples, and sent for an analysis of their resistance to antimicrobial agents and epidemiological surveillance.

The strains are sent by courier and are subcultured upon arrival.

Successive *Campylobacter*-like strains received in the laboratory from September to December 2009 were included in this study, comprising a total of 1003 strains. Each strain was identified after being subcultured on a trypticase soy blood agar plate (bioMérieux, Marcy l'Etoile, France) and incubated overnight in a microaerobic atmosphere at 37°C.

The strains were submitted to the standard protocol, which includes performance of phenotypic tests and molecular identification using real-time PCR for the main species and other PCRs and sequencing for the other species.

Conventional methods

Routine identification included observation of motility, Gram staining and oxidase and catalase activities of the isolates. The hippurate test was used to differentiate *C. jejuni* from *C. coli*, and growth at 25°C and resistance to nalidixic acid were used for the identification of *Campylobacter fetus*. For species other than *C. jejuni*, *C. coli* and *C. fetus*, an API-Campy strip (bioMérieux) was used for species identification.

DNA extraction

Genomic DNA was extracted using a lysis buffer composed of 10 mL 10% Triton X-100, 5 mL 10% Tween-20, 1 mL 1 M Tris–HCl (pH 8), 200 μL 0.5 M EDTA and 100 mL water. A few colonies were added to 200 μL of the buffer and incubated for 10 min in a 100°C water bath. After 2 min of centrifugation, the supernatant was diluted 1/10.

Molecular identification

The method used was a real-time PCR developed in the laboratory targeting a 444-base-pair fragment of the *gyrA* gene [5]. Briefly, the amplification was performed in glass capillary tubes in a LightCycler® thermocycler (Roche Diagnostics, Meylan, France). Each tube contained 7 μL of reaction mixture including 0.7 μL of FastStart DNA Master Hybridization probe mixture (Roche Diagnostics), 3 mM $MgCl_2$, 0.72 μM each of forward and reverse primers, 0.2 μM of each probe and 1–10 ng of template DNA. Following initial denaturation at 95°C for 10 min, 50 amplification cycles (95°C for 6 s, 54°C for 12 s and 72°C for 25 s) were performed, all with a temperature transition rate of 20°C/s. Fluorescence was measured at 640 nm after each cycle. Amplification was followed by a melting programme of 95°C for 60 s and 38°C for 50 s at a temperature transition rate of 20°C/s, and 80°C for 0 s (hold time) at a rate of 0.1°C/s, with continuous monitoring of the fluorescence. The final step consisted of cooling at 20°C/s to 40°C with a 30-s hold. For species other than *C. jejuni* and *C. coli*, another real-time PCR under the same conditions as previously described but with different primers was used to identify *C. fetus*, as well as a PCR designed to identify *Arcobacter butzleri* [9]. In the case of a negative result for these last two PCRs, 16S rRNA gene sequencing was performed [10].

Mass spectrometry identification

Sample preparation. A part of a colony of each isolate, taken directly from the agar plate after 18–24 h of incubation to obtain fresh bacteria, was deposited on a microtitre 384 target plate ground steel T F, (Bruker Daltonics, Bremen, Germany) in a single spot and allowed to dry at room temperature. One microlitre of matrix solution (saturated solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile) was added to the sample and was then crystallized by air-drying at room temperature for 5 min.

MALDI-TOF mass spectrometry measurements. Measurements were performed with an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 200-Hz smartbeam I laser. The parameter settings were as follows: delay, 80 ns; ion source, 1 volt, 25 kV; ion source, 2 volts, 23.4 kV; lens voltage, 6 kV; and mass range, 0–20 137 kDa. Each run included an *Escherichia coli* control sample provided by Bruker Daltonics where the presence of eight specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analysed by MALDI Biotyper 2.0 software (Bruker Daltonics) using the default settings (all of the settings are potentially adjustable). The whole process from MALDI-TOF mass spectrometry measurement to identification was performed automatically without any user intervention. Briefly, the software generated a list of peaks up to 100. The threshold for peak acceptance was a signal-to-noise ratio of 3. After alignment, peaks with a mass-to-charge ratio difference of <250 ppm were considered to be identical. The peak list generated was used for matches against the reference library, by directly using the integrated pattern-matching algorithms of the software. All parameters were the same regardless of the presumptive bacterial species analysed. Concerning only *Campylobacter* and related species, the Biotyper 2.0 database was composed of four *A. butzleri*, two *Arcobacter cibarius*, two *Arcobacter cryaerophilus*, one *Arcobacter halophilus*, one *Arcobacter nitrofigilis*, two *Arcobacter skirrowii*, three *C. coli*, five *C. fetus*, four *Campylobacter helveticus*, two *Campylobacter hyointestinalis*, six *C. jejuni*, four *Campylobacter lari*, one *Campylobacter sputorum* and four *Campylobacter upsaliensis*. Spectra were obtained in the positive linear mode after 1000 shots (size, 61 794 points; delay, 232 points). A

score was attributed to each identification. When this score was >2.00, the identification was considered correct at the species level; between 1.7 and 1.999, the identification was considered correct at the genus level; and <1.7, the identification was not similar enough to a spectrum to draw a conclusion.

Results

One thousand and three isolates were included in this study. Molecular identifications were considered as the gold standard and allowed us to identify 999 single species and four mixtures of two species (Table 1). For the single-species samples, *C. jejuni* represented the main species found (78%), followed by *C. coli* (14%). As usual, *C. fetus* (4%) and *A. butzleri* occupied the third and fourth positions, the other species being seldom encountered. When compared with these results, the MALDI-TOF mass spectrometry identification gave very similar results. The only discrepancy concerned four strains identified as *C. jejuni* by the reference methods whereas the MALDI-TOF mass spectrometry led to the identification of three *C. coli* and one *C. fetus* with a score >2. The corresponding strains were then submitted to 16S rRNA gene sequencing, which confirmed the accuracy of the gold standard. Discrepancies were more frequent with conventional methods (45) than with MALDI-TOF mass spectrometry (four). These methods have led to the misidentification or non-identification of 24 *C. jejuni*, three *C. coli*, 14 *A. butzleri* and four *C. lari* compared with MALDI-TOF mass spectrometry, and in one case the culture identified a *C. jejuni* that the MALDI-TOF mass spectrometry misidentified as a *C. fetus*. The percentage of accuracy for each species is given on Table 2. The standard methods as well as the gold standard were also able to identify a mixture of two strains in four samples. In two cases there was a mixture of *C. coli* and *C. jejuni* and MALDI-TOF mass spectrometry only identified *C. coli*. In one case, a mixture of *C. fetus* and *C. jejuni* was present and MALDI-TOF mass spectrometry identified only *C. fetus*, and finally in the fourth case MALDI-TOF mass spectrometry identified *A. butzleri* (Table 1). The second strain (*C. jejuni*) was only detected because a second run of this sample was performed.

TABLE 1. Identification of *Campylobacter* species and related organisms according to the methods used

Reference[a]	Conventional methods	MALDI-TOF	Total
Single species			
C. jejuni	*C. jejuni*	*C. jejuni*	760
C. jejuni	*C. jejuni*	*C. fetus*	1
C. jejuni	*C. coli*	*C. coli*	3
C. jejuni	*C. coli*	*C. jejuni*	21
			Total = 785
C. coli	*C. coli*	*C. coli*	146
C. coli	*C. jejuni*	*C. coli*	2
C. coli	No identification	*C. coli*	1
			Total = 149
C. fetus	*C. fetus*	*C. fetus*	40
			Total = 40
C. lari	*C. lari*	*C. lari*	4
C. lari	*A. cryaerophilus*	*C. lari*	1
C. lari	*C. coli*	*C. lari*	1
C. lari	*C. jejuni*	*C. lari*	1
C. lari	No identification	*C. lari*	1
			Total = 8
A. butzleri	*A. cryaerophilus*	*A. butzleri*	11
A. butzleri	No identification	*A. butzleri*	2
A. butzleri	*C. coli*	*A. butzleri*	1
			Total = 14
C. upsaliensis	*C. upsaliensis*	*C. upsaliensis*	2
			Total = 2
C. sputorum	*C. sputorum*	*C. sputorum*	1
			Total = 1
Total single species = 999			
Mixed species			
C. coli + *C. jejuni* (x2)	*C. coli* + *C. jejuni* (x2)	*C. coli* + *C.coli* (x2)	
A. butzleri + *C. jejuni*	*A. cryaerophilus* + *C. jejuni*	*A. butzleri* + *C. jejuni*	
C. fetus + *C. jejuni*	*C. fetus* + *C. jejuni*	*C. fetus*	
Total = 1007	Total = 1007	Total = 1004	

[a]Gold standard includes real-time-PCR and sequencing.
A., *Arcobacter*; MALDI-TOF, matrix associated laser desorption ionization–time of flight.

Discussion

The identification of *Campylobacter* species and related organisms based on phenotypic characteristics has always been difficult because these bacteria do not use carbohydrates and have little metabolic activity. In addition, these phenotypic traits are subject to environmental pressure, for example the occurrence of quinolone resistance has decreased the value of the nalidixic acid susceptibility test. Miniaturized strips such as ApiCampy using specific biochemical tests have been

TABLE 2. Accuracy of conventional methods or matrix associated laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry compared with the gold standard, according to the species and excluding mixed samples

Species	Accuracy of conventional methods compared with gold standard[a] (%)	Accuracy of MALDI-TOF mass spectrometry compared with gold standard[a] (%)
Campylobacter jejuni	96.7	99.4
Campylobacter coli	97.7	100
Campylobacter fetus	100	100
Campylobacter lari	50	100
Campylobacter sputorum	100	100
Campylobacter upsaliensis	100	100
Arcobacter butzleri	0	100

[a]Gold standard includes real-time PCR and sequencing.

used [11,12], however, the absence of growth of certain species (*C. lari*, *C. upsaliensis*) in a minimal medium and the lack of update of the database, despite the description of new species, limits the efficiency of this technique. Molecular methods were therefore developed with success allowing a quicker and more accurate diagnosis. Among them the real-time PCR developed and used in our laboratory has been used on over 20 000 isolates since 2002. However, molecular techniques are time-consuming and real-time PCR requires in some instances additional 16S rRNA gene sequencing. The classical PCR requires at least 3–4 h of manipulation from extraction to amplification, and at the other extreme 16S rRNA gene sequencing requires 48–72 h in our local setting. Furthermore, these techniques are not implemented in all laboratories; they are only used in specialized laboratories because they are expensive and adapted to the identification of a large number of isolates.

The development of MALDI-TOF mass spectrometry appears to have improved the bacterial identification process in terms of delay, cost and also accuracy according to recent studies [13–15]. We applied this method to this large number of campylobacters and related organisms, which are representative of the species isolated from humans in France because they are collected by clinical laboratories all over the country (http://www.cnrch.u-bordeaux2.fr/Campylobacter_Bilan_surveillance_reseau_2008.pdf). An excellent agreement was obtained between molecular methods and MALDI-TOF mass spectrometry. Misidentifications obtained with MALDI-TOF mass spectrometry occurred in only 0.4% of the samples. Indeed *C. jejuni* and *C. coli* are genetically similar species. It may be possible to avoid this problem by enriching the BIOTYPER 2.0 database. When the study was performed there were only seven different *Campylobacter* species and six *Arcobacter* species and there were only six different isolates of *C. jejuni*, three *C. coli* and five *C. fetus* included. This is probably not enough to obtain a 100% correct identification. It is interesting to compare the performance of MALDI-TOF mass spectrometry with that of the conventional methods to evaluate the utility of MALDI-TOF mass spectrometry in routine laboratories that do not employ molecular techniques. Misidentifications made with conventional methods occurred in 4.5% versus 0.4% with MALDI-TOF mass spectrometry. The errors were mainly the result of hippurate-negative *C. jejuni* strains, which were identified as *C. coli* by conventional methods because the species identification is mainly based on this test, whereas the MALDI-TOF mass spectrometry found the correct identification, concordant with molecular methods. Moreover, conventional methods are not accurate to identify species other than *C. coli*, *C. jejuni* and *C. fetus* even if an APICampy strip is used [11,12]. Finally, MALDI-TOF mass spectrometry is undoubtedly more efficient than conventional methods in identifying *Campylobacter* species and saves time and costs.

In terms of delay, a MALDI-TOF identification requires 2 min after the colony has been deposited on the target and placed in the automat, whereas a conventional identification requires several hours, approximately 4 h if only a hippurate test is needed and up to 24–48 h when an ApiCampy strip is used. In terms of cost, the MALDI-TOF mass spectrometry does not require any consumable except the matrix (€ 0.049 for one run), the main investment being the price of the mass spectrometer and the database, which can reach almost € 150 000. In comparison, an ApiCampy strip costs € 7.77. Another advantage obtained with MALDI-TOF mass spectrometry concerns the amount of biological material required for the identification. Only a part of a colony is necessary to identify the isolates with MALDI-TOF mass spectrometry whereas a suspension equivalent to a McFarland 3 opacity standard is needed to seed an APICampy strip. This represents an interesting point for suspensions typical of *Campylobacter* species, which are quite laborious to grow.

A previous study performed by Martiny *et al.* [16] showed that MALDI-TOF mass spectrometry was able to identify *Campylobacter* species and related organisms with a sensitivity of 98.3% whereas conventional methods (APICampy + additional tests) gave 88.9% correct identification. Their result for conventional methods was inferior to ours. We do not have a good explanation for this discrepancy. However, both studies concluded that *Campylobacter* species and related organisms can be reliably identified with MALDI-TOF mass spectrometry.

One limit of MALDI-TOF mass spectrometry concerns samples in which several species are present, because the technique could only identify one of the two species. However, since the beginning of our study, Bruker Daltonics has developed an algorithm that allows the identification of several species contained in the same sample. This tool will be help to solve this problem [17].

While waiting for the availability of this future algorithm, it is important to keep in mind that mixtures of *Campylobacter* species are not identified by MALDI-TOF mass spectrometry and consequently if some colonies have a different macroscopic aspect these different colonies should be tested. This is not a problem because a run is not expensive and a quick result can be obtained.

In conclusion, MALDI-TOF mass spectrometry offers an excellent identification of campylobacters and related organisms, and is completely adapted to our National Reference Centre for *Campylobacter* and *Helicobacter* because this new

technology allows the identification of a large number of isolates in a very short time compared with standard methods. It is also adapted to routine identification of Epsilonproteobacteria in a laboratory which has access to this technology. Currently, molecular biology is no longer performed in our laboratory for identification of this latter group of bacteria. Moreover, MALDI-TOF mass spectrometry has the potential for further characterization of the strains and could be used to develop a typing schema, for example to identify strains which have the same phylogenetic origin.

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Transparency Declaration

The authors declare no conflicts of interest.

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