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Comparison of two matrix-assisted laser desorption ionisation-time of flight mass spectrometry methods for the identification of clinically relevant anaerobic bacteria

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Abstract

Two commercially available MALDI-TOF MS systems, Bruker MS and Shimadzu MS, were compared for the identification of clinically relevant anaerobic bacteria. A selection of 79 clinical isolates, representing 19 different genera, were tested and compared with identification obtained by 16S rRNA gene sequencing. Correct genus identification was achieved for 71% of isolates by Shimadzu MS and for 61% by Bruker MS. Correct identification at the species level occurred in 61% and 51%, respectively. Shimadzu showed markedly better results for identification of Gram-positive anaerobic cocci. In contrast, the Bruker system performed better than Shimadzu for the *Bacteroides fragilis* group. When strains not present in the database were excluded from the analyses for each database, both systems performed equally well, with 76.7% and 75.0% correct genus identification for Shimadzu and Bruker, respectively. Similarly, when the most recently updated Bruker database was applied, no difference was observed. We conclude that the composition and quality of the database is crucial for a correct identification. The databases currently available for both systems need to be optimized before MS can be implemented for routine identification of anaerobic bacteria.

Keywords: Anaerobic, bacteria, identification, MALDI-TOF, mass spectrometry

Original Submission: 6 October 2010; **Revised Submission:** 11 December 2010; **Accepted:** 28 December 2010

Editor: G. Greub

Article published online: 17 January 2011

Clin Microbiol Infect 2011; **17:** 1501–1506

10.1111/j.1469-0691.2011.03467.x

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Introduction

The identification of anaerobic bacteria in routine diagnostics is difficult and time consuming [1]. To obtain more accurate and rapid identification, molecular methods have been developed, such as fluorescent *in situ* hybridization [2] and PCR-based diagnostics [3]. Recently, matrix-assisted laser desorption and ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced as a rapid and reliable identification method for routine application in diagnostic laboratories. Several studies have found MALDI-TOF MS to be a promising tool for the identification of anaerobic bacteria [4–6]. However, the majority of studies that compared MALDI-TOF identification with a reference standard, contained low numbers of anaerobic species and/or used conventional phenotypic identification as a reference [5,7–9].

Currently, two MALDI-TOF MS systems are commercially available in Europe: Bruker MS (Microflex; Bruker Daltonik, Bremen, Germany) and Shimadzu MS (AXIMA; Shimadzu Corporation, Kyoto, Japan). The methodology of the two systems is similar, but differences are present in the composition of the databases and application of software packages for data analyses. The aim of this study was to compare the performance of both systems, with 16S rRNA gene sequencing as the reference standard, for identification of clinically relevant anaerobic bacteria.

Materials and Methods

Settings

The measurements performed with the Bruker system were performed at the Leiden University Medical Centre (LUMC) and the measurements using the Shimadzu system were

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performed at the University Medical Centre Groningen (UMCG).

Bacterial strains

Anaerobic strains were derived from clinical specimens collected by the UMCG. A selection of 79 isolates from 19 different genera was made to include all clinically relevant anaerobes (Table 1). Strains were stored at −80°C and were subcultured on Brucella Agar with 5% sheep blood, hemin and vitamin K (BBA) at the UMCG. Strains were sent to the LUMC in Stuart medium and were subcultured on Trypticase Soy Agar with 5% sheep blood (TSA). At both centres the strains were incubated at 35°C in an anaerobic atmosphere for 48 h prior to the measurements.

16S rRNA gene sequencing

DNA of the strains was isolated as described previously by Boom *et al.* [10] and the 16S rRNA genes were amplified and sequenced using universal 16S rRNA-specific primers [11]. The sequences obtained were compared with sequences present in GenBank using the Blastn.

Measurement with MALDI-TOF MS Bruker

Measurements were performed with a Microflex mass spectrometer (Bruker Daltonik) using FlexControl software (version 3.0). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1, voltage at 20 kV; ion source 2, voltage at 18.4 kV; lens voltage, 9.1 kV; mass range, 2000–20 000 Da). Spectra were internally calibrated daily by using *Escherichia coli* ribosomal proteins. The spectra were imported into the integrated Biotyper software (version 2.0) and were analysed by standard pattern matching with default settings.

The strains were tested with the Bruker system both directly and after pretreatment, both in duplicate (four measurements per isolate). Pretreatment consisted of suspending several colonies in 150 μL of distilled (RNAse-free) water. Ethanol absolute (450 μL) was added and the sample was centrifuged (14 000 **g** for 2 min). The supernatant was discarded, the procedure of centrifuging was repeated, and the remaining pellet was air-dried. The pellet was then suspended in formic acid (70%; 25 μL) and acetonitrile (25 μL) and after mixing the sample was centrifuged again (14 000 **g** for 2 min). The supernatant (1 μL) was deposited on the MALDI-plate.

For measurement without pretreatment, a colony was directly spotted on the MALDI-plate. Pretreated and untreated samples were overlaid with 1 μL of matrix solution (α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried. Measurements were performed as described previously [9]. The Biotyper database contained 3476 spectra and was updated until 17 February 2010. The spectrum of each isolate was compared with those in the database and identification was provided with an accompanying score (log score 0–3) of reliability. This score is based on (i) matching of the spectrum in general, (ii) matching of the locus of the peaks and (iii) matching of the height of the peaks. Scores <1.7 represent no reliable identification. A score ≥1.7 and <2.0 is considered identification at the genus level, scores ≥2.0 identification at the species level. Of the four measurements executed per isolate, the highest reliable identification was used as the definitive result. Direct results were also analysed separately. If duplicate measurements were equally reliable but contradictory, this was classified as 'no uniform result'.

Measurement with MALDI-TOF MS Shimadzu

Colonies were directly spotted on the MALDI-plate and covered with 1 μL matrix solution (α-cyano-4-hydroxy-cinnamic acid) and air-dried. The measurements were performed with an AXIMA Confidence MALDI-TOR mass spectrometer (Shimadzu). Mass spectra were acquired in a linear positive ion extraction mode using an acceleration of 20 kV and a low mass gate of 1500 Da. The system was calibrated externally with a mass spectrum obtained from fresh cells of an *E. coli* K12 strain (CCUG). Spectra were accumulated from 1000 laser pulse cycles, automatically processed with the Shimadzu Biotech Launchpad software, and exported to and analysed with the SARAMIS software package (AnagnosTec, Golm, Germany). The database, which was updated until 4 February 2010, contained 2875 SuperSpectra and 37 804 reference spectra of 701 and 1439 taxonomic units, respectively. A SuperSpectrum is derived from several reference spectra of different strains of the same species and represents the reference peaks suitable for identification. They were used for the fully automated identification of bacterial strains. If no identification with a SuperSpectrum could be obtained, measurements were compared with the original reference spectra. Species were separated by setting a threshold of 50% matching peaks. All measurements were performed in duplicate and the measurement with the highest percentage of matching peaks was considered to be correct. When the percentage of matching peaks was <50%, strains were manually identified using the software. If a conclusive identification was obtained, this was accepted. If the results of two duplicate measurements were different, while the percentage of matching peaks was similar, they were considered to be 'not uniform'.

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TABLE 1. Identification results obtained with MALDI-TOF MS using two different systems, compared with 16S rRNA gene sequencing

Strains (*n*)[a]	Bruker (pretreatment)			Shimadzu (direct)		
	Correct species ID (*n*)	Correct genus ID (*n*)	Spectra in database (*n*)	Correct species ID (*n*)	Correct genus ID (*n*)	Spectra in database (*n*)
Actinomyces						
israelii (3)	0	0	0	0	0	13
meyeri (1)	0	0	1	1	1	6
naeslundii (1)	0	0	2	0	0	4
odontolyticus (2)	1	1	2	1	1	21
Anaerococcus						
lactolyticus (1)	1	1	1	1	1	10
murdochii (1)	1	1	1	0	1	6
prevotii (1)	0	1	2	0	0	4
tetradius (1)	1	1	1	1	1	8
vaginalis (2)	0	2[b]	1	2	2	34
Atopobium						
parvulum (1)	0	0	1	1	1	8
Bacteroides						
dorei (2)	0	2[b]	0	0	2	35
fragilis (4)	4	4	9	4	4	90
ovatus (2)	2	2	4	0	2	26
thetaiotaomicron (3)	3	3	8	3	3	27
uniformis (2)	2	2	3	0	2[d]	48
ureolyticus (2)	0	0	0	0	0	5
vulgatus (2)	2	2	4	1	1	78
Bilophila						
wadsworthia (3)	0	2	0[c]	3	3	15
Campylobacter						
rectus (3)	0	0	0	0	0	0
Clostridium						
butyricum (1)	0	0	1	1	1	4
clostridioforme (1)	0	1[b]	2	0	0	0
difficile (2)	2	2	10	2	2	839
hathewayi (1)	0	1	0	0	0	0
perfringens (2)	2	2	9	2	2	104
ramosum (1)	1	1	4	1	1	8
septicum (1)	1	1	2	0	0	4
sporogenes (1)	1	1	3	0	1	7
Eggerthella						
lenta (2)	0	0	0	0	0	6
Finegoldia						
magna (3)	3	3	6	3	3	202
Fusobacterium						
necrophorum (1)	1	1	2	1	1	21
nucleatum (1)	0	1	4	0	0	25
Parabacteroides						
distasonis (2)	2	2	5	2	2	32
Parvimonas						
micra (3)	0	1	1	3	3	153
Peptococcus						
niger (1)	0	0	0	1	1	6
Peptoniphilus						
gorbachii (2)	0	0	0	1	2	22
harei (3)	1	1	2	3	3	113
ivorii (1)	0	1	1	1	1	8
lacrimalis (1)	0	0	0	1	1	16
Peptostreptococcus						
anaerobius (2)	2	2	2	2	2	31
stomatis (1)	0	0	0	0	1[b]	0
Porphyromonas						
species (1)	0	0	0	0	0	0
gingivalis (1)	1	1	2	1	1	12
Prevotella						
buccae (1)	1	1	1	1	1	44
nigrescens (1)	1	1	1	1	1	42
Propionibacterium						
acnes (3)	3	3	5	3	3	85
propionicum (1)	0	1	1	0	0	0
Veillonella						
parvula (1)	1	1	2	0	1	0[c]

[a]Number of strains for each species.
[b]Minor error: correct genus, incorrect species.
[c]Genus present in database.
[d]No uniform results.

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MALDI-TOF MS identification vs. 16S rRNA gene sequencing

Outcomes of MALDI-TOF identification as compared with the reference standard were classified as: 'no identification, species present or not present in the database', 'major error', 'minor error', 'correct genus identification', 'correct species identification' and 'no uniform result'. 'Major error' represents misidentification at the genus level; 'minor error' represents correct genus identification but incorrect species.

Statistics

PASW Statistics 17.0 (SPSS Inc., Chicago, IL) was used. Chi-square tests were used for the statistical analyses.

Results

Table 1 depicts the 79 isolates belonging to 19 genera and 47 species that were tested in both the Bruker MS and the Shimadzu MS systems. No effect of culture medium was found when a subset of 58 strains was grown on both TSA and BBA medium and subsequently tested with Bruker MS (data not shown). In general, Shimadzu MS performed better than Bruker MS (p 0.024 and p 0.139, see Table 2). When only direct measurements were analysed, correct species identification occurred in 60.8% using Shimadzu and 35.4% using Bruker MS, whereas correct genus identification was achieved in 70.9% and 51.9%, respectively. When the results with pretreatment were taken into account, the outcomes of Bruker MS improved to correct species identification of 50.6% and correct genus identification of 60.8% of the isolates.

The number of strains that could not be identified because of absence in the database was significantly larger for the Bruker system as compared with the Shimadzu system (19.0% vs. 7.6%, p 0.035).

TABLE 2. Results of identification by MALDI-TOF MS Bruker and Shimadzu

	Bruker Direct, *n* = 79 (%)	Bruker Including pre-treatment, *n* = 79 (%)	Shimadzu Direct, *n* = 79 (%)
Major error[a]	2 (2.5)	2 (2.5)	2 (2.5)
Minor error[b]	3 (3.8)	5 (6.3)	1 (1.3)
No id, present in database	18 (22.8)	9 (11.4)	12 (15.2)
No id, not in database	15 (19)	15 (19)	6 (7.6)
Correct genus	41 (51.9)	48 (60.8)	56 (70.9)
Correct species	28 (35.4)	40 (50.6)	48 (60.8)
No uniform results	0 (0)	0 (0)	2 (2.5)
p-value[c]	0.024	0.139	

[a]Incorrect genus identification.
[b]Correct genus, incorrect species identification.
[c]Bruker vs. Shimadzu.

Results for the various species

With the Bruker system duplicate measurements yielded uniform results. Results of Shimadzu MS were not uniform for two isolates (2.5%), both concerning *Bacteroides uniformis*. The first was manually identified as *B. uniformis* and *B. fragilis*, the second as *B. uniformis*, *B. fragilis* and *Bacteroides* sp.

The overall results are representative of the results for the various genera, except for the *Bacteroides fragilis* group and Gram-positive anaerobic cocci (GPAC). The Bruker system showed a significantly higher percentage of correct species identification of members of the *Bacteroides fragilis* group than the Shimadzu system; 87% (13 out of 15, no difference between pretreated and direct measurement) and 53% (8 out of 15, p 0.046), respectively. On the other hand, the Shimadzu system performed significantly better for the identification of GPAC ($p < 0.05$). Of the 24 isolates in this group, including *Anaerococcus* spp., *Atopobium parvulum*, *Finegoldia magna*, *Parvimonas micra*, *Peptococcus niger*, *Peptoniphilus* spp. and *Peptostreptococcus* spp., only three (12.5%) were correctly identified at the species level and nine (37.5%) at the genus level by direct measurement with Bruker MS. With pretreatment these percentages increased to 37.5% (n = 9) and 50% (n = 12), respectively. Direct measurements with Shimadzu MS reached much higher levels of correct species (n = 20, 83%) and genus identification (n = 22, 92%).

Two major errors occurred with Bruker MS: *Actinomyces israelii* (not in the database) was misidentified as *Lactobacillus catenaformis* and *Actinomyces naeslundii* was misidentified as *Neisseria gonorrhoea*. Shimadzu MS erroneously determined *Campylobacter rectus* (not in the database) as being *Staphylococcus aureus* (n = 1) and *Fusobacterium nucleatum* as *Clostridium* sp. (n = 1). Only *Actinomyces israelii* was repeatedly misidentified.

Minor errors by the Bruker system were: *Anaerococcus hydrogenalis* instead of *A. vaginalis* (n = 2, only with pretreatment), *Bacteroides vulgatus* instead of *B. dorei* (n = 2, not in the database) and *Clostridium hathewayi* instead of *C. clostridioforme* (n = 1). Log scores of these five strains were not significantly different from the log scores of 10 strains that were correctly identified to these particular species (data not shown). The one minor error by Shimadzu was: *Peptostreptococcus anaerobius* instead of *P. stomatis* (n = 1, not in the database).

Discussion

MALDI-TOF MS is a promising tool for the identification of bacteria that can only be identified using elaborate

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phenotypic determination methods, such as anaerobes. In this study, two commercially available MALDI-TOF MS systems were compared using 16S rRNA gene sequencing as the reference standard. Identification of anaerobes by Shimadzu was better, due to the need for pretreatment of the samples in Bruker's methodology and differences in the composition of the databases.

A limitation of this study is the relatively small number of isolates per species. Therefore, we focused on the overall results and on two selections of isolates: GPAC and the *Bacteroides fragilis* group. Strengths of this study are the wide range of clinically relevant anaerobes tested and the use of 16S rRNA gene sequencing as the reference standard. In addition, this study is the first analysis of two different MALDI-TOF MS systems for performance on anaerobic bacteria. Cherkaoui *et al.* [7] compared Bruker and Shimadzu MS for a variety of mainly aerobic bacteria and found high-confidence identification in 94.4% and 88.8%, respectively, >99% of which was correct. However, only a small number of anaerobic bacteria were included and identification was only reported at genus level. The majority of studies that included anaerobes in comparing MALDI-TOF MS with standard identification [5,7–9], used phenotypic identification as the first line reference. When discrepancies were found, 16S rRNA gene sequencing was performed. A disadvantage of this approach is that if the results of MS and biochemical tests are incorrect but similar, this error will not be noted.

The performance of MALDI-TOF MS in bacterial identification is known to depend highly on the quality of the database present in the software that accompanies the system [8,9,12]. At the moment of this study, the Shimadzu system contained more reference spectra than the Bruker system, which explains the better performance. This was supported by two findings. First, when strains not present in the database were excluded from the analyses for each database, the Shimadzu and Bruker systems performed equally well, with respectively 76.7% and 75.0% correct genus identification. Secondly, in May 2010 Bruker provided the LUMC with an update of the database (upgrade from 3476 to 3740 spectra). Comparing the measured peaks of all isolates with the spectra in the updated database led to a remarkable improvement of the results: 63.3% correct species identification and 72.2% correct genus identification (measurements after pretreatment). The percentage of isolates that could not be identified due to current absence of reference spectra decreased from 19% to 7.6%. Both the Bruker and Shimadzu system would benefit from further expansion of their databases, to cover this final 7.6% of strains. However, a significant number of species remains unidentified even though the database contains their reference spectra (Bruker, 10–11%; Shimadzu, 15%). For the Bruker system this may be explained by variation within species and the small number of spectra per species that are currently available. For the Shimadzu system, strains that could not be identified in spite of available reference spectra are generally represented by <10 spectra (see Table 1). Nevertheless, it is unclear why the Shimadzu system cannot recognize *F. nucleatum* and *A. israelii*, with 25 and 13 reference spectra, respectively. Perhaps pretreatment would improve the results, in line with our findings for the Bruker system.

Two differences in identification results between the Bruker and Shimadzu systems were clearly observed: Bruker MS performed better for *Bacteroides* spp, while Shimadzu MS performed better for the identification of GPAC. Nagy *et al.* [5] previously showed that Bruker MS was superior to phenotypic identification for members of the *Bacteroides fragilis* group. *B. dorei*, which in our study was erroneously identified by Bruker MS as *B. vulgatus*, was not included in the study by Nagy *et al.* As *B. dorei* and *B. vulgatus* share the same biochemical features, it might be possible that the same 'minor error' has occurred in the study reported by Nagy *et al.*, but remained unnoticed. It is unclear why Shimadzu MS performs less well on *Bacteroides* spp, especially considering the large number of spectra for this genus in the reference database (Shimadzu, 304; Bruker, 28). The superior results of Shimadzu regarding GPAC may be explained by the fact that the Shimadzu database is more extensive.

Bruker MS measurements were performed with and without pretreatment, as this mimics the diagnostic routine at the LUMC (for clinical specimens, if no direct result is obtained the measurement is repeated with pretreatment). Measurements with and without pretreatment resulted in identical results if a log score of ≥1.7 was assigned. In general, pretreatment raised the log scores. In two cases this led to a minor error instead of correct genus identification, but overall pretreating the samples increased the percentage of correctly identified, mainly Gram-positive strains. This may be explained by the thicker peptidoglycan layer of Gram-positive bacteria, which may interfere with the laser ionisation. Cherkaoui *et al.* [7], comparing the performance of the Shimadzu and Bruker systems for aerobes and anaerobes, noted that isolates not identified by MALDI-TOF MS were mostly Gram-positive bacteria. In contrast, Van Veen *et al.* [9] noted that pretreatment for Gram-positive bacteria was not necessary using the Bruker system. Grosse-Herrenthey *et al.* [4] used pretreatment for clostridial identification with the Bruker system, but did not test it against direct measurement.

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In contrast to the Bruker methodology, pretreatment of samples does not seem necessary with the Shimadzu system. There might be several reasons for this. First, in the Bruker database only reference spectra of pretreated strains are present, while the Shimadzu database contains spectra obtained without pretreatment. Secondly, the 'time of flight' for the Shimadzu system is longer than for the Bruker system, which may result in a more distinct peak pattern. A major disadvantage of pretreatment is the prolongation of the turnaround time. The turnaround time for one sample is approximately 5 min for both MS systems, which is prolonged to 35 min with pretreatment.

Bacterial identification by Shimadzu MS is based on comparison of the measured spectrum with SuperSpectra and reference spectra. If the percentage of matching peaks is <50%, manual identification can be performed. The results of manual identification are less favourable than those achieved by automated identification (data not shown). However, it contributes to a higher rate of identification, while this opportunity is not used by the Bruker system, which may explain another small part of the difference in performance between the systems.

Based on our study, we can not yet recommend implementing MALDI-TOF MS for routine identification of anaerobic bacteria in clinical microbiology. However, the study was not designed to evaluate the potential advantages of MALDI-TOF identification over currently used methods. If MALDI-TOF MS is applied, simple microbiological tests such as Gram-staining are still required to recognize major errors made by MS. MALDI-TOF systems need optimization, (i) by adding reference spectra of bacteria that are not yet represented, (ii) by expanding the number of available spectra per species, and (iii) by gaining insight into the reasons why identification sometimes fails, even with sufficient reference spectra present in the database. We are confident that this will ultimately lead to a method of rapid identification of most clinically relevant anaerobic bacteria.

Acknowledgements

We thank Petra Dofferhoff for her contribution.

Transparency Declaration

The authors declare that they have no conflicts of interest.

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