Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for species identification of Acinetobacter strains isolated from blood cultures

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Abstract

The clinical relevance of Acinetobacter species, other than A. baumannii, as human pathogens has not been sufficiently assessed owing to the insufficiency of simple phenotypic clinical diagnostic laboratory tests. Infections caused by these organisms have different impacts on clinical outcome and require different treatment and management approaches. It is therefore important to correctly identify Acinetobacter species. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been introduced to identify a wide range of microorganisms in clinical laboratories, but only a few studies have examined its utility for identifying Acinetobacter species, particularly those of the non-Acinetobacter baumannii complex. We therefore evaluated MALDI-TOF MS for identification of Acinetobacter species by comparing it with sequence analysis of rpoB using 123 isolates of Acinetobacter species from blood. Of the isolates examined, we identified 106/123 (86.2%) to species, and 16/123 (13.0%) could only be identified as acinetobacters. The identity of one isolate could not be established. Of the 106 species identified, 89/106 (84.0%) were confirmed by rpoB sequence analysis, and 17/106 (16.0%) were discordant. These data indicate correct identification of 89/123 (72.4%) isolates. Surprisingly, all blood culture isolates were identified as 13 species of Acinetobacter, and the incidence of Acinetobacter pittii was unexpectedly high (42/123; 34.1%) and exceeded that of A. baumannii (22/123; 17.9%). Although the present identification rate using MALDI-TOF MS is not acceptable for species-level identification of Acinetobacter, further expansion of the database should remedy this situation.

Keywords: Acinetobacter, blood culture isolates, MALDI-TOF MS, non-A. baumannii complex, rpoB

Original Submission: 17 May 2013; Revised Submission: 10 July 2013; Accepted: 21 August 2013

Editor: G. Greub

Article published online: 11 October 2013

Clin Microbiol Infect 2014; 20: 424–430
10.1111/1469-0691.12376

Introduction

The genus Acinetobacter comprises 30 named and nine genomic species [1,2]. Its most clinically important representative, A. baumannii, has emerged as one of the most problematic pathogens for healthcare institutions worldwide because of its resistance to several antibiotics [1,3]. Phenotypic tests only identify members of the Acinetobacter calcoaceticus–A. baumannii complex [4–7], in contrast, several molecular methods are more effective. Among these, amplified 16S rRNA gene restriction analysis, amplified fragment length polymorphism and 16S rRNA and RNA polymerase β-subunit (rpoB) gene sequence analyses are most frequently used [1,8]. The most effective technique may be rpoB gene sequence analysis because of abundant rpoB polymorphisms [9], and it has facilitated species identification [8,9]. Species other than
We report here a comparison of MALDI-TOF MS with the ability of MALDI-TOF MS to identify application of MALDI-TOF MS to fermenting Gram-negative bacilli, including conventional methods [20,21]. Nevertheless, some non-identification using MALDI-TOF MS is more cost-effective than offers equivalent accuracy [19]. Moreover, complete bacterial inexpensive alternative to molecular genetic identification and bacteria, yeasts and even filamentous fungi [18]. It is a rapid and laborious advantage for use in clinical laboratories. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is increasingly used to identify a wide range of microorganisms in clinical laboratories, including Gram-positive and Gram-negative bacteria, yeasts and even filamentous fungi [18]. It is a rapid and inexpensive alternative to molecular genetic identification and offers equivalent accuracy [19]. Moreover, complete bacterial identification using MALDI-TOF MS is more cost-effective than conventional methods [20,21]. Nevertheless, some non-fermenting Gram-negative bacilli, including Acinetobacter species, have been misidentified because of an incomplete database [19]. Further, few published studies are available regarding the application of MALDI-TOF MS to Acinetobacter species.

Therefore, the purpose of the present study was to evaluate the ability of MALDI-TOF MS to identify Acinetobacter species. We report here a comparison of MALDI-TOF MS with rpoB sequence analysis of Acinetobacter strains isolated from blood cultures.

Materials and Methods

Bacterial isolates
This study included 123 consecutive blood culture isolates of Acinetobacter species collected between April 2003 and March 2011 from 123 patients in two University Hospitals in Japan. Isolates were routinely identified as Acinetobacter species using phenotypic methods, the Microscan Walkaway System (Siemens Healthcare Diagnostics Japan, Tokyo, Japan) and the Vitek 2 System (Sysmex-bioMérieux Japan, Kobe, Japan). The 16 type and reference strains of Acinetobacter used in this study were as follows: A. baumannii ATCC17978, A. baylyi KCTC12413T, A. berezinae LMG1003T, A. calcoaceticus KCTC2357T, A. guillouiae LMG988T, A. grimontii KCTC12416T, A. jundi KCTC12406T, A. johnsonii KCTC12405T, A. lwaffii KCTC12407T, A. nosocomialis LMG10619T, A. oleivorans KCTC23045T, A. pittii LMG1035T, A. radioresistens NBRIC102413T, A. soli KCTC22184T, A. ursingii KCTC12410T and Acinetobacter gen. sp. 14BJ LMG10627. All strains were stored at −80°C, precultured for 12–24 h, and cultured aerobically overnight on 5% sheep blood agar at 37°C.

Identification of Acinetobacter species
Species identification of isolates was performed by partial sequence analysis of rpoB, using the primers Ac696F and Ac1093R [8]. All isolates were considered correctly identified when the rpoB sequence yielded ≥98% identity with the closest species sequence match in the GenBank database. Identification of A. baumannii was confirmed by PCR amplification of blaoxa-51-blk [12]. A neighbour-joining (NJ) tree was constructed using the MEGA software, version 5 [22].

MALDI–TOF MS
Strains were extracted as described [18]. A sample of each colony was suspended in 300 μL distilled water and adjusted to McFarland standard 2, and 900 μL absolute ethanol was added. The suspension was vortexed vigorously and centrifuged at 20 000 g for 5 min. The supernatant was discarded, and the pellet was dried at 55°C for at least 30 min. Ten microlitres of 70% formic acid (Wako Pure Chemical Industries, Osaka, Japan) was then added and thoroughly mixed by pipetting. Next, 10 μL of acetonitrile (Wako) was added, and the sample was centrifuged again at 20 000 g for 5 min, and then 1 μL of supernatant was placed onto a stainless steel target plate (Bruker Daltonik GmbH, Lepizig, Germany) and dried for 10 min at room temperature. Finally, 1.5 μL of matrix solution, comprising a saturated solution of α-cyano-4-hydrocinnamic acid (Bruker Daltonik) in 50% acetonitrile and 2.5% trifluoroacetic acid (Wako), was applied to the samples and co-crystallized at room temperature for 10 min.

The samples prepared using the standard extraction method described above were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik). Each measurement was performed once for each culture. Escherichia coli DH5α was used as a quality control for each experiment, as recommended by the manufacturer.

Data analysis
The log score identification criteria recommended by the manufacturer (Bruker Daltonik) were used as follows: ≥2.300,
reliable species: 2.00–2.299, probable species; 1.700–1.999, genus; and <1.700, unreliable. Duplicate experiments were performed. For identifying genus and species, the least stringent identification criterion was used. If the genus of one strain was identified in the first experiment and the species in the second, the genus was used for analysis. If the results of two experiments were different, such as genus or unreliable identification, the unreliable identification result was used. A dendrogram was constructed using the correlation distance measure and the average linkage algorithm settings of the BIOTYPER 3.0 software (Bruker Daltonik).

Results

Identification of Acinetobacter species

Thirteen species were identified according to rpoB sequence analysis (Table 1). The identities of the 123 isolates were as follows: A. pittii (n = 42); A. baumannii (n = 22); A. nosocomialis (n = 19); A. ursingii (n = 15); A. grimontii (n = 8); A. oleivorans (n = 4); A. bereziniae (n = 2); A. soli (n = 2); A. johnsonii (n = 1); A. junii (n = 1); A. baylyi (n = 1); A. radiotolerans (n = 1); and Acinetobacter gen. sp. 14BJ (n = 1). The sequences of four strains were >98% identical to the rpoB sequence of any species in the GenBank database and could not be reliably identified; of these four strains, three strains were 96% identical and one strain was 97% identical to the rpoB sequence of A. baumannii.

<table>
<thead>
<tr>
<th>rpoB (zone 1) sequencing</th>
<th>MALDI-TOF identification</th>
<th>Score value</th>
<th>Number of isolates</th>
<th>blaoxa-51</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. pittii (n = 42)</td>
<td>A. pittii</td>
<td>≥2.0</td>
<td>37 (21)</td>
<td>–</td>
</tr>
<tr>
<td>A. baumannii (n = 22)</td>
<td>A. baumannii</td>
<td>≥1.700–1.999</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>A. nosocomialis (n = 19)</td>
<td>A. nosocomialis</td>
<td>≥2.0</td>
<td>21 (10)</td>
<td>+</td>
</tr>
<tr>
<td>A. ursingii (n = 15)</td>
<td>A. ursingii</td>
<td>≥2.0</td>
<td>14 (9)</td>
<td>–</td>
</tr>
<tr>
<td>A. grimontii (n = 8)</td>
<td>A. junii</td>
<td>≥2.0</td>
<td>8 (6)</td>
<td></td>
</tr>
<tr>
<td>A. oleivorans (n = 4)</td>
<td>A. calcoaceticus</td>
<td>≥1.700–1.999</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A. pittii</td>
<td>A. pittii</td>
<td>≥1.700–1.999</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A. soli (n = 2)</td>
<td>A. baylyi</td>
<td>≥2.0</td>
<td>1 (0)</td>
<td></td>
</tr>
<tr>
<td>A. johnsonii (n = 1)</td>
<td>A. johnsonii</td>
<td>≥2.0</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>A. junii (n = 1)</td>
<td>A. junii</td>
<td>≥2.0</td>
<td>1 (0)</td>
<td></td>
</tr>
<tr>
<td>A. baylyi (n = 1)</td>
<td>A. baylyi</td>
<td>≥2.0</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>A. radiotolerans (n = 4)</td>
<td>A. radiotolerans</td>
<td>≥2.0</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter gen. sp. 14BJ (n = 1)</td>
<td>Not reliable identification</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Not reliable identification (n = 4)</td>
<td>A. baumannii</td>
<td>≥2.0</td>
<td>3 (0)</td>
<td>–</td>
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<td></td>
<td>A. baumannii</td>
<td>≥1.700–1.999</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

The number of isolates with score ≥2.3.

The dendrogram indicated that these four strains comprise a cluster, which is closely related to a cluster represented by A. nosocomialis: LMG10619 (Fig. 1). Further analysis of these four isolates is planned. PCR analysis detected the blaoxa-51 gene in 22 of the A. baumannii strains; PCR analysis of the other 101 strains failed to detect a product (Table 1).

MALDI-TOF MS

MALDI-TOF MS analyses yielded scores of ≥2.3, 53/123 (43.1%); 2.00 ≤ ≥ 2.3, 53/123 (43.1%); 1.7 ≤ ≥ 2.0, 16/123 (13.0%); and ≤ ≥ 1.7, 1/123 (0.8%) for reliable species, probable species, genus and unreliable identifications, respectively. Based on their rpoB sequences, 106 isolates with scores ≥2.0, 89/106 (84.0%) were confirmed at the species level and 17/106 (16.0%) were not (Table 1). Hence, species identification of 89/123 (72.4%) was achieved. Interestingly, seven of the 16 unconfirmed results scoring 1.7 ≤ ≥ 2 were confirmed by their rpoB sequences. Therefore, the overall level of concordance between MALDI-TOF MS (valid and invalid results) and rpoB sequence analysis at the species level was 78.0% (96/123).

Among the 17 discordant results for species, the three isolates identified as A. baumannii by MALDI-TOF MS, in which blaoxa-51 sequences were undetectable, were identified as A. nosocomialis according to rpoB sequence analysis. The eight isolates identified as A. junii by MALDI-TOF MS were identified instead as A. grimontii isolates according to their rpoB sequences. This discrepancy may be attributed to the synonymy of A. grimontii and A. junii [23]. Sequence analysis of rpoB revealed that the isolates of A. guillouiae and A. baylyi (one each) were actually A. bereziniae and A. soli, respectively. This discrepancy was caused by their absence from the database.

A dendrogram was created using the 123 isolates and 16 reference strains (Fig. 2). Three A. nosocomialis isolates erroneously identified as A. baumannii with scores of ≥2.0 by MALDI-TOF MS cluster with A. nosocomialis LMG10619. This cluster includes two A. nosocomialis isolates erroneously identified as A. baumannii (MALDI-TOF MS scores, 1.7 ≤ ≥ 2). These erroneous identifications were caused by inaccurate taxonomic assignment of the given spectra within the Bruker database. One A. baylyi isolate erroneously identified as A. guillouiae clusters with reference strains A. guillouiae LMG988 and A. bereziniae LMG1003. Acinetobacter baylyi is known to be transformable by DNA from other Acinetobacter species [24]. In some cases, it has picked up the rpoB gene from A. guillouiae [24]. These intragenic recombination events may have caused this erroneous identification. Four isolates, which were not reliably identified by rpoB sequence analysis, represent a cluster with no reference strain. Two were identified as A. baumannii (MALDI-TOF MS, ≥2.0) and the others as A. baumannii (1.7 ≤ ≥ 2).
FIG. 1. Dendrogram generated from partial *rpoB* sequences for all isolates and reference strains. The scale bar indicates a genetic distance of 0.02, and the numbers shown next to each node represent bootstrap values (1000 replicates). The strain numbers next to a species name indicate that sequence types of the isolates with those strain numbers are identical to that of the species.
FIG. 2. Dendrogram generated using the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) -specific protein signatures for all isolates and reference strains from the MALDI Biotyper 3.0 database. The species names in parentheses indicate identifications that are discordant with the rpoB sequence. Distance values are relative and normalized to a maximum value of 1000. ▲ indicates a score of $1.7 \leq \chi < 2$ (genus identification) and ● indicates a score of $\chi \leq 1.7$.
Discussion

MALDI-TOF MS is a promising clinical microbiological laboratory technique that can rapidly identify a wide range of bacterial and fungal species [18,19]. MALDI-TOF MS correctly identifies the species of 85% of routine clinical isolates [19]. In the present study, however, valid species identification was achieved for only 89/123 (72.4%) of the isolates. Discordant results at the species level were obtained for 17/123 (13.8%) of the isolates and were mainly caused by recent changes in the taxonomy of a given species, the absence of the species name, or inaccurate taxonomic assignment of a given spectrum in the MALDI-TOF MS database. These taxonomic discordances were corrected by updating the database.

We selected 16 type and reference strains for the present study to create a set of reference spectra to complement the MALDI-TOF MS database, and spectra from 123 isolates were reanalysed using the local database. Hence, the original sensitivity was improved from 74.8% to 82.4% (Table 2). This update improved identification of genus (16/123, 13.0%) as well. Specifically, the discordances and errors were largely caused by an incomplete database. An extensive database is critical for accurate identification of Acinetobacter species by MALDI-TOF MS.

In the present study, rpoB-sequence analysis assigned the 123 isolates to 13 species and could not reliably identify them all (Fig. 1). In contrast, MALDI-TOF MS analysis assigned the 123 isolates to nine species and did not reliably identify them all (Fig. 2). These results indicate that the discriminatory power of MALDI-TOF MS is less than that of sequence analysis of rpoB; however, the latter method is laborious and not suitable for clinical laboratories. The main advantages of MALDI-TOF MS are that species can be routinely identified faster than sequencing rpoB, and the costs of consumables are lower. Further, MALDI-TOF MS spectra may permit an educated guess regarding the identity of an unknown.

The clinical relevance of Acinetobacter species as human pathogens, other than A. baumannii, has not been sufficiently assessed because of the scarcity of simple phenotypic tests used in diagnostic laboratories. In the present study, 78.8% (97/123) blood culture isolates were identified as 12 species of Acinetobacter other than A. baumannii. Six of the 12 species, A. ursingii, A. grimontii, A. johnsonii, A. juni, A. baylyi and A. radioreistantes, were identified as A. lwaffi according to phenotypic tests, and the other six species, A. pittii, A. nosocomialis, A. oleivorans, A. bereziniiae, A. soli and Acinetobacter gen. sp. 14B, and A. baumannii were identified as A. baumannii by phenotypic identification. Surprisingly, the incidence of A. pittii was high, 42/123 (34.1%), and exceeded that of A. baumannii, 22/123 (17.9%). Acinetobacter pittii is implicated in endocarditis and can cause life-threatening infections, as do A. baumannii, A. nosocomialis, A. johnsonii, A. lwaffi and A. beijerinckii [4,25,26]. The relatively high incidence of A. ursingii, which accounted for 15/123 (12.2%) of isolates, was unexpected but agrees with observations from hospitals in the UK, the Netherlands and Northern Ireland [4,27,28]. This organism infects the bloodstream of hospitalized patients [7,11,29], and it has been associated with a nosocomial outbreak of fatal bloodstream infections in a neonatal intensive care unit [10]. Infections caused by these organisms have different impacts on clinical outcome and require different treatment and management approaches [30]. It is therefore extremely important to correctly identify Acinetobacter species, including non-A. baumannii isolates.

In conclusion, MALDI-TOF MS is currently not adequate for species-level identification of Acinetobacter. However, our present studies show that further expansion of the database to include Acinetobacter species other than A. baumannii will make MALDI-TOF MS an efficient method for identification of nosocomial Acinetobacter species. Some Acinetobacter species other than A. baumannii, particularly A. pittii, A. nosocomialis and A. ursingii, have been associated with outbreaks [10,28], suggesting that they may become an increasingly important healthcare problem. We expect that MALDI-TOF MS will provide a useful method for clinical laboratories to identify Acinetobacter.

Acknowledgements

We thank the technical staff of the diagnostic laboratories of the University of Tokyo Hospital and the Dokkyo University...
Hospital for permission to use their bacterial strains. This study was supported in part by a research grant from the Kurozumi Medical Foundation and a Grant-in-Aid (S0991013) from the Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT) for the Foundation of Strategic Research Projects in Private Universities. 

**Transparency Declaration**

The authors have no conflicts of interest.

**References**


*The Acknowledgements section was updated on 17/12/2013 after initial online publication 11/10/2013. Funding information is now correct.*