

# Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) systems for the identification of *Candida* species

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## Abstract

*Candida* spp. are responsible for severe infections in immunocompromised patients and those undergoing invasive procedures. The accurate identification of *Candida* species is important because emerging species can be associated with various antifungal susceptibility spectra. Conventional methods have been developed to identify the most common pathogens, but have often failed to identify uncommon species. Several studies have reported the efficiency of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for the identification of clinically relevant *Candida* species. In this study, we evaluated two commercially available MALDI-TOF systems, Andromas™ and Bruker Biotyper™, for *Candida* identification in routine diagnosis. For this purpose, we investigated 1383 *Candida* isolates prospectively collected in eight hospital laboratories during routine practice. MALDI-TOF MS results were compared with those obtained using conventional phenotypic methods. Analysis of rDNA gene sequences with internal transcribed regions or D1-D2 regions is considered the reference standard for identification. Both MALDI-TOF MS systems could accurately identify 98.3% of the isolates at the species level (1359/1383 for Andromas™; 1360/1383 for Bruker Biotyper™) vs. 96.5% for conventional techniques. Furthermore, whereas conventional methods failed to identify rare or emerging species, these were correctly identified by MALDI-TOF MS. Both MALDI-TOF MS systems are accurate and cost-effective alternatives to conventional methods for mycological identification of clinically relevant *Candida* species and should improve the diagnosis of fungal infections as well as patient management.

**Keywords:** Andromas system, Bruker Biotyper system, *Candida* spp. identification, conventional methods, MALDI-TOF mass spectrometry, molecular identification

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## Introduction

*Candida* yeasts are responsible for frequent and severe infectious complications in immunocompromised patients and those undergoing invasive procedures [1]. While six

species of *Candida* (i.e. *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida kefyr* and *Candida krusei*) are currently considered the leading aetiological agents of candidiasis, several other *Candida* species have been reported as significant pathogens [2]. At present, up to 40 distinct *Candida* species have been shown to infect humans [3]. Identification of these new clinically relevant species has been made possible due to the development of molecular identification [4–6]. An accurate identification of *Candida* species is of major importance, as several of these emerging species have distinct antifungal susceptibility profiles and/or could be

associated with specific clinical settings [7]. Conventional methods routinely used in a mycology laboratory for identification of yeasts traditionally included a combination of phenotypic methods, including morphological and biochemical features. However, these methods have been developed to identify only the most common pathogens and often failed to identify emerging/cryptic species.

Indeed, molecular taxonomic studies have revealed several new *Candida* species among phenotypically distinct species [8–10]. For instance, recent studies have revealed that some clinical isolates previously identified as *C. glabrata* using conventional methods actually belong to the closely related species *Candida nivariensis* and *Candida bracarenis* [5]. Similarly, some isolates formerly misidentified as *C. parapsilosis* actually represent isolates of *Candida metapsilosis*, *Candida orthopsilosis* or *Lodderomyces elongisporus* [9,10]. Molecular identification, by sequencing genomic regions of the rDNA genes, is considered the 'reference standard' for accurate identification of *Candida* at the species level [4–6]. However, sequencing of clinical isolates is still time-consuming and not standardized; it cannot therefore be used as a routine method for yeast identification.

In recent years, several studies have reported the performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for the identification of clinically relevant *Candida* species [11–14]. This method could represent a valid and rapid alternative to both conventional and molecular methods for yeast identification in routine practice. Currently, there are four commercial systems available: the MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the AXIMA@SARAMIS database (AnagnosTec, Potsdam, Germany and Shimadzu, Duisburg, Germany), and lately the Andromas (Andromas, Paris, France) and VITEK MS systems (bioMérieux, Marcy l'Etoile, France)[15]. However, this method has not yet been comparatively evaluated in a large number of clinical isolates concomitantly with accepted standards.

In this study, we compared the identification efficiency of two MALDI-TOF MS systems, Andromas™ and Bruker Maldi Biotyper™, with that of conventional methods on a large panel of 1383 clinical isolates of *Candida* obtained from eight French university hospital laboratories. Our results demonstrate that both techniques are reliable, the Andromas™ strategy being easier to perform in clinical microbiology laboratories as it does not require protein extraction.

## Materials and Methods

### *Candida* isolates

The *Candida* isolates included in this study have been prospectively isolated over a 2-month period from patients

hospitalized in haematology, intensive care and renal transplant units of eight French university hospitals. All isolates, from both superficial and deep sites, were collected by the laboratories in sequential order without any selection criteria. Isolates were cultured on chromogenic media (CHROMagar *Candida*™ medium (Becton-Dickinson, Le Pont-de-Claix, France)) for 48 h at 35°C. Growing colonies were checked for mixed cultures and transferred to a new plate. The isolates growing in pure culture were identified routinely in each of the eight laboratories by conventional methods using the same protocol of identification described below. The isolates were cryopreserved at –80°C until use. In total, 1383 *Candida* isolates have been included.

### Conventional methods

Isolates identified as *C. albicans* on CHROMagar *Candida*™ media were screened with the Bichro-Dubli™ test (Sofibel, Levallois-Perret, France) to identify *C. dubliniensis* [10–12]. Isolates suspected to be *C. glabrata* or *C. krusei* were tested using the RTT *Glabrata*™ (Sofibel) and *Krusei-color*™ (Sofibel) methods, respectively [10,13]. All other isolates were identified using ID32C AUX™ identification strips (bioMérieux), according to the manufacturer's instructions. We defined an identification score of  $\geq 98\%$  by the ID32C AUX™ method as the threshold for accurate identification at the species level.

### MALDI-TOF MS

The 1383 isolates were identified blindly by the two MALDI-TOF systems in two independent laboratories.

### Analysis by the MALDI-TOF Andromas™ system

Part of a pure colony of each pure culture was transferred to an Andromas™ target well using a disposable loop, overlaid with 1  $\mu$ L of formic acid 70% (v/v) and dried. One microlitre of a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid, 50% acetonitrile and 2.5% trifluoroacetic acid (HCCA; matrix solution) was then added and allowed to co-crystallize with the sample. Samples were processed in a MALDI-TOF spectrometer (Andromas™, Paris, France) with the control software [12,16–18]. Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. The spectra were analysed and compared in an *m/z* range of 3000–20 000 Da with Andromas software (Andromas™). The Andromas software identified the number of common peaks between the spectra of the tested isolate and the species-specific spectral fingerprints of the reference strains in the database. Briefly, for each isolate, all peaks with an intensity  $>0.1$  were retained and were compared with the peaks for the species-specific spectral fingerprints of each reference strain, taking into account possible variations in the *m/z* value of  $\pm 10$ . Subsequently, the

percentage of common peaks was obtained using the formula:  $100 \times (\text{number of common peaks between the tested isolate and the species-specific spectral fingerprint} / \text{total number of peaks specific to the species-specific spectral fingerprint})$ . An acceptable identification was given if the percentage of common peak was  $\geq 70\%$  of those of a species-specific profile in the database. A 10% difference between the first two species diagnostics having the best match in the database is also required to give species identification.

#### Analysis by the MALDI-TOF Biotyper™ system

Samples were prepared according to the manufacturer's instructions. Briefly, two to three yeast colonies were transferred using a 10- $\mu\text{L}$  inoculating loop into 300  $\mu\text{L}$  of distilled water and 900  $\mu\text{L}$  of ethanol (Sigma-Aldrich, St Louis, MO, USA). The suspension was pelleted after centrifugation at 6000 g for 3 min, dried, and reconstituted in 50  $\mu\text{L}$  of 70% formic acid (Sigma-Aldrich). After incubation for 30 min, 50  $\mu\text{L}$  of acetonitrile (Sigma-Aldrich) was added. The suspension was then centrifuged at 6000 g for 3 min. A volume of 1.5  $\mu\text{L}$  of the supernatant was applied to a 96-spot Anchorchip™ target (Bruker Daltonics, Inc., Bremen, Germany) plate and dried. A saturated solution of 1.8  $\mu\text{L}$  of MALDI matrix (HCCA; Bruker Daltonics, Inc., Bremen, Germany) was applied to the fungal smear and dried. Measurements were performed with a Microflex™ mass spectrometer (Bruker Daltonics, Wissembourg, France) using the FlexControl™ software (version 3.3.108.0). Mass spectra ranging from 2000 to 20 000 Da were acquired in a linear, extraction mode with positive polarity. The spectrum was imported into the Biotyper software (version 3.0; Bruker, Karlsruhe, Germany). The generated spectrum of biomarkers for each sample was then compared with reference spectra in the Bruker library. Identifications from MALDI-TOF were classified using modified score values proposed by the manufacturer: a score  $\geq 2$  indicated identification to the species level; a score between 1.7 and 1.99 indicated identification to the genus level; and a score of  $< 1.7$  indicated no identification.

#### Molecular identification

Molecular identification of isolates was performed by amplification and sequencing of the ITS rDNA regions without a prior DNA extraction step (colony-PCR), as previously described [19]. Amplification of the ITS rDNA was achieved using the universal primers ITS1 (TCCGTAGGTGAACC TGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [20]. Nucleotide sequences were assembled using Seqscape software (Applied Biosystems, Foster City, CA, USA.). Molecular identification was achieved by comparing sequence data with

the GenBank database using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) or with the MycoBank database (<http://www.cbs.knaw.nl/collections/BioLoMICSSequences.aspx>). For some isolates, identification was also confirmed by amplification of the D1-D2 region of the 28S rDNA using NLI (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) primers [20]. A per cent similarity of  $\geq 98\%$  between the unknown sequence and the closest matching sequence from the reference database was used as the criterion to classify an isolate to the species level.

#### Criteria for final species identification of isolates

All isolates were subjected to conventional identification methods, and MALDI-TOF Andromas™ and Biotyper™ systems.

The final species identification of isolates was performed as follows: an accurate identification of the species *C. albicans*, *C. dubliniensis*, *C. krusei* and *C. tropicalis* was confirmed if the three methods (conventional methods, and Andromas™ and Biotyper™ MALDI-TOF systems) yielded the same species identification. When discrepant results were observed for the three methods or when only two out of three methods yielded identification, ITS rDNA and/or D1-D2 sequencing was performed and was considered as the final species identification. For the remaining *Candida* species, the final identification of isolates was by molecular identification, regardless of the results obtained by the three methods.

## Results

#### Final species identification of isolates

We analysed 1383 isolates obtained from the eight diagnostic laboratories during routine practice. For 950 isolates (68.7%), the use of conventional methods and the two MALDI-TOF systems resulted in the accurate identification of 836 isolates of *C. albicans*, 31 of *C. dubliniensis*, 44 of *C. krusei* and 39 of *C. tropicalis*. For the 433 (31.3%) remaining isolates, final identification was obtained by rDNA sequencing. In all, final identification at the species level was obtained for all the 1383 isolates that encompassed 20 *Candida* species (Table 1).

#### Performance of each identification method

Biochemical methods could accurately identify 1336 of the isolates (96.5%). Forty-four isolates (3.5%) were misidentified and three isolates were not identified (two *C. albicans* and one *C. bracarenensis*). Isolates belonging to the species complexes were misidentified or not identified by conventional biochemical methods. Similarly, none of the uncommon species, *C. fabianii* ( $n = 17$ ), *C. sphaerica* ( $n = 1$ ), *C. fermentati* ( $n = 3$ )

**TABLE 1.** Discrepancies and errors in conventional methods and MALDI TOF MS systems (Andromas™ and Bruker Biotyper™) for identification of *Candida* species

Final identification	No. of isolates		MALDI-TOF identification			
	Conventional identification		Andromas™		Biotyper™	
	No identification	Misidentification	No identification	Misidentification	No identification	Misidentification
<i>C. albicans</i> (n = 838)	2	0	0	0	0	0
<i>C. dubliniensis</i> (n = 32)	0	0	1	0	0	0
<i>C. glabrata</i> (n = 176)	0	0	0	1	0	0
<i>C. bracarensis</i> (n = 1)	1	0	0	0	1	0
<i>C. tropicalis</i> (n = 81)	0	1	0	0	0	0
<i>C. parapsilosis</i> (n = 78)	0	3	0	0	0	0
<i>C. orthopsilosis</i> (n = 3)	0	3	0	0	0	0
<i>C. krusei</i> (n = 44)	0	0	0	0	0	0
<i>C. kefyr</i> (n = 60)	0	1	0	0	0	0
<i>C. lusitanae</i> (n = 21)	0	4	0	0	0	0
<i>C. inconspicua</i> (n = 12)	0	8	0	0	0	0
<i>C. guilliermondii</i> (n = 9)	0	0	0	0	0	0
<i>C. fabianii</i> (n = 17)	0	17	17	0	13	4
<i>C. intermedia</i> (n = 3)	0	2	0	0	0	0
<i>C. magnoliae</i> (n = 1)	0	0	1	0	0	0
<i>C. sphaerica</i> (n = 1)	0	1	0	0	1	0
<i>C. fermentati</i> (n = 3)	0	3	2	1	2	1
<i>C. ethanolica</i> (n = 1)	0	1	0	1	1	0
<i>C. utilis</i> (n = 1)	0	0	0	0	0	0
<i>C. lipolytica</i> (n = 1)	0	0	0	0	0	0
Total (n = 1383)	3	44	21	3	18	5

and *C. ethanolica* (n = 1), was identified correctly by biochemical methods.

The MALDI-TOF MS Andromas™ system was able to accurately identify 1359 isolates (98.2%). Three isolates (0.24%) were misidentified: one *C. parapsilosis* instead of a *C. glabrata*, one *C. albicans* instead of a *C. fermentati*, and one *C. guilliermondii* instead of a *C. ethanolica*. Twenty-one isolates belonging to four species were not identified because these species were not included in the database (*C. fabianii* and *C. magnoliae*) or because of no spectral acquisition (one *C. dubliniensis* and two *C. fermentati*).

The MALDI-TOF MS Biotyper™ system could accurately identify 1360 isolates (98.2%). Five isolates (0.36%) were misidentified: two *C. parapsilosis* instead of *C. fabianii*, one *C. albicans* instead of *C. fabianii*, one *C. kefyr* instead of *C. fabianii*, and one *C. guilliermondii* instead of *C. fermentati*. Eighteen isolates belonging to five species could not be identified because these species were not included in the database (*C. bracarensis*, *C. sphaerica*, *C. ethanolica*, *C. fermentati* and *C. fabianii*).

Discrepancies and errors in the three systems of identification (biochemical methods, and Andromas™ and Biotyper MALDI-TOF MS systems™) are given in Table 1.

## Discussion

This study shows that the MALDI-TOF MS Andromas™ and Biotyper™ systems appear to be fast and powerful techniques

for the accurate identification of *Candida* species routinely isolated in medical laboratories, both providing a high rate of correct identification at the *Candida* species level (98.2% each). Both MALDI-TOF systems appear more reliable than the conventional phenotypic methods that are used routinely (96.5% of correct identification). These findings are in accordance with recent studies showing comparable results, with yeast identification rates of 97.6% with Biotyper 2.0 and 96.1% with Saramis MALDI-TOF systems [11,21].

Importantly, we demonstrated an excellent agreement between identifications by MALDI-TOF MS and those provided by rDNA sequencing. Indeed, only three (0.7%) and five (1.1%) isolates of the 433 requiring molecular identification were misidentified, respectively, by MALDI-TOF MS Andromas™ and Biotyper™ systems, while 44 (10.2%) misidentifications were obtained using conventional methods. Unlike previous studies, in which molecular identification was performed only when discrepant results were noted between the compared methods [11,21], all identifications at the species level (except for *C. albicans*, *C. dubliniensis*, *C. krusei* and *C. tropicalis* with an ID32C score of  $\geq 98\%$ ) have been confirmed by molecular methods in the present study. Therefore, our results strongly highlight the accuracy of two MALDI-TOF systems for *Candida* species identification. Additionally, the two MALDI-TOF MS systems were able to discriminate isolates belonging to species complexes at the species level. However, most of the rare *Candida* species were not identified using MALDI-TOF MS. Indeed, specific spectra were not included in the databases at the time of our study.

Once the spectra were included (on obtaining firm identification of these isolates by sequence analysis), subsequent identification of these species resulted in a perfectly matching profile.

The MALDI-TOF database can be updated when a species is absent from the database; however, the high level of expertise required for molecular identification is sometimes a pitfall. In our study, *C. fabianii* identification was difficult because the genomic databases did not contain the complete ITS region of *C. fabianii* as a single nucleotide sequence and misidentification with other yeast species (e.g. *Lindnera (Pichia) mississippiensis*) could have occurred. Partial sequencing of the *EF1 $\alpha$*  gene facilitated correct identification of this species [22]. One limitation of mycological identification from clinical samples is in the analysis of mixed cultures; in particular, distinct species show similar colours when using chromogenic media. We encountered this situation in our study in the analysis of a mixed culture containing both *C. albicans* and *C. dubliniensis*. This mixture could only be detected by molecular analysis; biochemical and MALDI-TOF MS analysis could only identify one of the two species. One improvement that can be easily incorporated into MALDI-TOF MS would be to screen several colonies presenting the same colour from the same culture plate.

Notably, the two MALDI-TOF MS systems use distinct sample preparation protocols. An initial extraction step of the yeast is necessary for the Biotyper protocol, which is time-consuming; however, when using the Andromas™ system, cell lysis is performed directly on the steel plate. This difference in the extraction steps of the two systems could clearly have an impact on identification speed in routine laboratory practice. However, some studies have recently reported that spectra obtained by on-target lysis with the Bruker system can be sufficient for identification of yeasts [23,24].

In conclusion, the present study indicates that the MALDI-TOF MS systems Andromas and Biotyper are reliable and cost-effective techniques for the identification of clinically relevant *Candida* species. Identification would be faster and more precise than when using conventional non-automated methods. Furthermore, spectral databases should be regularly updated by suppliers to improve identification rates. The availability of MALDI-TOF MS in the clinical laboratory will improve the diagnosis of fungal infections and patient management, allowing appropriate antifungal therapy.

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## Transparency Declaration

XN and MEB are shareholders of Andromas SAS.

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