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# Rapid and reliable MALDI-TOF mass spectrometry identification of *Candida* non-*albicans* isolates from bloodstream infections

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) fingerprinting has recently become an effective instrument for rapid microbiological diagnostics and in particular for identification of micro-organisms directly in a positive blood culture. The aim of the study was to evaluate a collection of 82 stored yeast isolates from bloodstream infection, by MALDI-TOF MS; 21 isolates were identified also directly from positive blood cultures and in the presence of other co-infecting micro-organisms. Of the 82 isolates grown on plates, 64 (76%) were correctly identified by the Vitek II system and 82 (100%) by MALDI-TOF MS; when the two methods gave different results, the isolate was identified by PCR. MALDI-TOF MS was unreliable in identifying two isolates (*Candida glabrata* and *Candida parapsilosis*) directly from blood culture; however, direct analysis from positive blood culture samples was fast and effective for the identification of yeast, which is of great importance for early and adequate treatment.



## 1. Introduction

*Candida* is an opportunistic pathogen that lives as a commensal of the gastrointestinal tract in individuals but can cause mucocutaneous candidiasis and disseminated infections in immunocompromised and hospitalized hosts (Trofa et al., 2008). *Candida albicans* is the most common fungal pathogen in humans, although recently a shift towards systemic infections by non-*albicans* species has been reported especially in hematological, transplanted and intensive care unit (ICU) patients (Bassetti et al., 2006). Some non-*albicans* species are intrinsically resistant to the available antifungal agents used as first-line antifungal therapy (Bassetti et al., 2009; Rodriguez-Tudela et al., 2008); others show an increased biofilm forming ability that influences antifungal susceptibility (Pulcrano et al., 2012b). For these reasons, the correct identification of the infecting organism has become essential to guide antifungal therapy.

Blood cultures remain the gold standard in the microbiological diagnosis of bacterial or fungal bloodstream infection (BSI): a presumptive identification can be suggested by microscopic observations as soon as growth of micro-organisms is detected by semi-automated incubation systems, while final identification was performed by phenotypic methods. Thus, final results are ready very after empiric therapy is started. MALDI-TOF MS has recently been introduced into routine laboratory procedures and is used as a new approach for rapid identification of bacteria as well as yeast (van Veen et al., 2010; Putignani et al., 2011). Many studies have been performed to evaluate the correct identification of *Candida* clinical isolates at the species level (Marklein et al., 2009; Dhiman et al., 2011) and recently also below the species level to make epidemiological analysis of clinical samples (Dieckmann et al., 2008; Pulcrano et al., 2012a). Other studies used MALDI-TOF MS for differentiation of pathogens directly from positive blood cultures (La Scola and Raoult, 2009; Prod'hom et al., 2010; Spanu et al., 2012; Christner et al., 2010; Ferroni et al., 2010).

The aim of this study was to assess the performance of the Bruker Biotyper MALDI-TOF MS system for the correct identification of *Candida* non-*albicans* isolates from bloodstream infection by comparing identifications from plate culture with those directly from blood culture (BC) bottles.

## 2. Materials and methods

### 2.1. Yeast strains

Eighty-two isolates of *Candida* non-*albicans* were obtained from blood culture of patients admitted to the University hospital Federico II, Naples, between October 2007 and October 2011. All isolates were

*Abbreviations:* ICU, intensive care unit; BSI, bloodstream infection; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; BC, blood culture; ITS, internal transcribed spacer.

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recovered from each patient prior to antifungal drug exposure. The identification of species was performed by an automatic Vitek II system (bioMerieux, Marcy L'Etoile, France). The micro-organisms were routinely subcultured aseptically onto Sabouraud Dextrose agar plates (bioMerieux, Marcy l'Etoile, France) and maintained as glycerol stocks at −80 °C. *Candida parapsilosis* ATCC 22019 and *C. albicans* ATCC 10231 were included as quality control strains.

### 2.2. MALDI-TOF MS species identification

The identification of yeast isolates from culture plates was performed as described by Pulcrano et al. (2012a). Briefly, a loop full of colonies, grown over night on Sabouraud agar, was suspended in 300 μl of de-ionized water to which 900 μl of absolute ethanol was added. After centrifugation at 12,000 ×*g* for 2 min, the supernatant was discarded and the pellet was air-dried. The pellet was then dissolved in 20 μl of 70% formic acid and 20 μl of acetonitrile and mixed. The samples were centrifuged at 12,000 ×*g* for 2 min, and 1 μl of the clear supernatant was spotted in duplicate onto the MALDI target (MSP 96 target polished steel (MicroScoutTarget) plate; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature.

The identification of yeast isolates from aerobic bottles (PLUS Aerobic/F, BD, Erembodegem-Aalst, Belgium) was performed from aliquots (1.8 ml) of positive blood cultures (BACTEC 9240 system, BD, Erembodegem-Aalst, Belgium) and supplemented with SDS 0.5%. The mixture was vortexed for 1 min and then centrifuged at 12,000 ×*g* for 2 min. The pellet was washed twice with 1 ml of de-ionized water, centrifuged and suspended in 300 μl of de-ionized water plus 900 μl of absolute ethanol. Samples were processed similarly to yeast isolates from culture plates.

Then each spot was overlaid with 1 μl of HCCA (a-cyano-4-hydroxy cinnamicacid) matrix solution saturated with organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried completely before MALDI-TOF MS measurement. MALDI-TOF MS was carried out with a MALDI Microflex LT (Bruker Daltonics, Bremen, Germany). Peptide mass fingerprint product ion spectra were acquired in a linear positive mode at a laser frequency of 20 Hz within a mass range from 2,000 to 20,000 Da. For each spectrum, 240 laser shots in 40 shot steps from different positions of the target spot (random walk movement) were automatically acquired with AutoXecute acquisition control software (Flex control 3.0; Bruker Daltonics, Bremen, Germany). The spectra were externally calibrated using the standard calibrant mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin; Bruker Daltonics). Calibration masses were as follows: RL36, 4365.3 Da; RS32, 5096.8 Da; RS34, 5381.4 Da; RS33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10300.1 Da; RNase A, 13683.2 Da; and myoglobin, 16952.3 Da.

A total of 168 spectra representing the 82 isolates of *Candida* and ATCC strains were generated with the automated MALDI-TOF MS measurements.

Raw spectra were processed using the MALDI BIOTYPER software, version 2.0, (Bruker Daltonics, MC, Italy) with default settings, and every peak list generated was matched directly against reference libraries (4,502 species) using the integrated patterns matching algorithm of BIOTYPER, version 2.0 (Bruker Daltonics MC, Italy). Results were classified using modified score values proposed by the manufacturer: a score ≥ 2 indicated species identification, and a score in the range 1.7–1.99 indicated genus identification and a score <1.7 denoted no identification.

### 2.3. Phylogenetic data analysis and MSP construction

For phylogenetic analysis, spectra were acquired for each isolate and MSP creation was carried out with the default setting of the Biotyper software (Desired mass error for the MSP: 200 Da; minimum desired peak frequency: 25%; maximum desired peak number for the MSP: 70). Each MSP was then assigned to its specific node on the taxonomy tree. In order to visualize the relationship between the MSPs, dendrogram clustering was carried out with the standard settings of Biotyper 2.0 (distance measure: correlation; linkage: average).

### 2.4. Sequence data

When MALDI-TOF MS and Vitek II system reported different identification at the species level, the identification was confirmed by PCR and sequence analysis using specific primers directed against the internal transcribed spacer (ITS) region as previously described (Pulcrano et al., 2012a).

## 3. Results

The study included two reference strains (*C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 10231) and 82 clinical isolates of *Candida* non-*albicans* collected from bloodstream infections of immunocompromised, ICU or otherwise critically ill patients hospitalized at the University Hospital Federico II of Naples, during a period of four years. Of these, 61/82 were isolated from October 2007 to October 2010, were stored at −80 °C and were identified only from culture plate; the other 21/82 were isolated from November 2010 to October 2011 and were identified directly from blood culture and from culture plate after 24 h. All 82 isolates were identified at the species level by the Vitek II system giving identification with a confidence of 99% for 74/82 isolates. The Vitek II failed six identifications giving three potential species identification (*Candida guillermondii*/*Candida famata*/*Candida glabrata*) and other two identifications giving no results also on 24-h plate culture.

MALDI Biotyper identified all 82 isolates at the species level using scores of ≥2.0.

Isolates with three potential species identification (*C. guillermondii*/*C. famata*/*C. glabrata*) were identified by sequencing of the noncoding ribosomal region internal transcribed spacer (ITS1-4) resulting *C. glabrata* (1/6) and *C. guillermondii* (5/6). Other nine *Candida* isolates identified by the Vitek II with a confidence of 99%, but differently identified by MALDI-TOF MS, were identified by sequencing and the discordant results were resolved in favor of the MALDI Biotyper (Table 1). Finally, as reported in Table 1, Vitek II misidentified *Lodderomyces elongisporus* as *C. parapsilosis* and *Saccharomyces cerevisiae* as *Candida tropicalis*. Instead, MALDI-TOF MS gave all correct identifications at the species level, in particular for the identification of *L. elongisporus* where no other biochemical methods could distinguish it from *C. parapsilosis*.

For twenty-one isolates, MALDI-TOF MS yeast identifications were also performed directly from positive blood cultures, and results obtained were compared with the ones obtained by MALDI-TOF MS identification from plate culture. When MALDI-TOF identifications were performed using the colony grown overnight on agar plates, the correct identifications were reported for 21/21 isolates (Table 2). On the contrary, when MALDI-TOF was used directly on blood cultures, it gave the wrong specie identification for one isolate; it failed to identify *C. parapsilosis* when there were co-infections with *E. coli*.

For the 19 isolates that were correctly identified, the median ID log (score) was 1.71. Lower ID log(score) values were observed mainly with *C. glabrata* isolates.

## 4. Discussion

*C. albicans* is the most common species causing invasive candidiasis, although the frequency of other non-*albicans* species, including *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *Candida krusei*, has been increasing (Bille et al., 2005). Fungal infections affect the duration of hospitalization and increase treatment costs (Arnold et al.,

**Table 1**
Performance of MALDI-TOF vs Vitek II for identification of *Candida* species from agar plates. PCR was used for identification of species when MALDI-TOF and Vitek II gave different results.

| Species identification | Vitek II identification | MALDI-TOF MS identification |
|---|---|---|
| *Candida parapsilosis* (53) | *C. parapsilosis* (47)<br>*Candida famata* (4)<br>No result (2) | *C. parapsilosis* (53) |
| *Candida tropicalis* (8) | *C. tropicalis* (5)<br>*C. famata* (1)<br>*Candida glabrata* (1)<br>*C. parapsilosis* (1) | *C. tropicalis* (8) |
| *C. glabrata* (11) | *C. glabrata* (8)<br>*Candida guillermondii*/<br>*C. famata*/*C. glabrata* (1)<br>*Candida krusei* (1)<br>*C. parapsilosis* (1) | *C. glabrata* (11) |
| *C. guillermondii* (6) | *C. guillermondii* (1)<br>*C. guillermondii*/*C. famata*/<br>*C. glabrata* (5) | *C. guillermondii* (6) |
| *Lodderomyces elongisporus* (1) | *C. parapsilosis* (1) | *L. elongisporus* (1) |
| *Saccharomyces cerevisiae* (1) | *C. tropicalis* (1) | *S. cerevisiae* (1) |
| *C. krusei* (1) | *C. krusei* (1) | *C. krusei* (1) |
| *Candida lipolytica* (1) | *C. lipolytica* (1) | *C. lipolytica* (1) |

2010). Thus, it is quite important to identify correctly fungi causing infection and give a prompt and effective treatment to improve patient outcome.

In particular, the correct identification of *Candida* species could find a survival benefit when early, appropriate antifungal therapy is administered. In a recent study on *C. krusei*, it was demonstrated that early fluconazole therapy did not affect the mortality rate due to BSI (Grim et al., 2012).

Many diagnostic approaches capable of identifying a large number of *Candida* species are available: the standard phenotypical assays, based on substrate assimilation, which often are time consuming and incomplete for the limited spectrum of specific biochemical patterns discriminatory of species; the new and more accurate molecular methods, such as genotypic identification based on sequence of ITS regions, which are expensive, require considerable expertise and are unsuitable for routine diagnostics; and the Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), which emerged several years ago as a powerful tool for improving the quality of microbiological identification and reduces the time needed for determination (Putignani et al., 2011).

In our study, data collected in 4 years showed a higher proportion (6% of total) of fungemia episodes occurring in patients admitted to a neonatal ICU or surgical ward, according to other studies concerning hospital fungemia (Neu et al., 2009; Zaoutis et al., 2004). In our study, the predominant non-*albicans* species isolated is *C. parapsilosis*, according to a Spanish study where *C. parapsilosis* caused 46.8% of bloodstream infection episodes (Peman et al., 2011), although species distribution varies according to patients' comorbidities.

The Vitek II system correctly identified 76% of species, misidentifying most of *C. guillermondii* isolates, likely due to the high similarity in biochemical profiles generated by *C. famata* and *C. parapsilosis* species.

Instead, 100% of isolates growth on plate were identified at the species level using MALDI-TOF scores of 2.0; our results are comparable with those obtained in previously published studies, where 92.5% species were identified using a score of 2.0 (Marklein et al., 2009). A score-oriented MSP dendrogram clustered the isolates into five main groups defined by species. As expected, *L. elongisporus* grouped with the *C. parapsilosis* cluster (Fig. 1).

To identify yeasts directly from blood cultures without plate subculture, the threshold was set up at scores of 1.8; Dinhman showed that spectral scores between 1.8 and 2.0 gave accurate identification to the species level with no misidentifications; a threshold of 1.8 for species identification could suitable in a clinical laboratory (Dhiman et al., 2011).

Fungemia was reliably detected by MALDI-TOF MS in 20/21 prospective cases; a correct identification was obtained in 19/20 cases. In contrast with our data, Ferreira reported a correct identification only for 1/18 isolate (Ferreira et al., 2011). The unlike results are probably due to the different protocol used: the initial lysis with detergent and subsequent centrifugation allowed us to collect fungal cells from BC, as reported also by Marinach-Patrice (Marinach-Patrice et al., 2010), who used a similar protocol on simulated BC.

Our failed identifications occurred for *Candida lipolytica*, incorrectly identified as *C. glabrata*, and for *C. parapsilosis*, which remained unidentified. In both cases, if yeast cells were less than $10^6$ CFU/ml, spectra resulted indistinguishable from those of sterile blood cultures and yield low-scoring incorrect IDs due to arbitrary matching of non-bacterial peaks. Furthermore, polymicrobial bloodstream infections, reported for one of them, could hide the detection of fungal micro-organisms covert by the faster growth of Gram negative bacterial agents, present at the same time in the blood of patients. On the contrary, it was noted that the co-infections of Gram positive bacteria did not affect the identification of yeast (Klotz et al., 2007; Pulimood et al., 2002; Ferroni et al., 2010).

Finally, the use of SDS 0.5% to disrupt red blood cell and centrifugation were much faster than other procedures commonly described for direct MALDI-TOF identification from BC (Juiz et al., 2012; Marinach-Patrice et al., 2010) and probably more useful to recover fungi in the case of multiple infection with bacteria.

Direct analysis from positive blood culture samples by MALDI-TOF MS might give correct results earlier than conventional procedures and allow an early species- or genus-oriented empirical treatment, both on the basis of the knowledge of the species that show intrinsic resistance to antifungal agents (Pappas et al., 2009), or strong biofilm forming ability (Pulcrano et al., 2012b), or other virulence factor, both by the analysis of the peaks in resistant species as recently evidenced by De Carolis et al. (2012).

This new approach could improve yeast diagnostics by saving time, reducing costs of patient hospitalization and minimizing the selection of

**Table 2**
Performance of MALDI-TOF for identification of *Candida* species from agar plates vs positive blood culture.

| Isolate | Identification with MALDI-TOF MS from colonies | Identification with MALDI-TOF MS from blood | Co-infection |
|---|---|---|---|
| 1 | *C. glabrata* | *C. glabrata* | |
| 2 | *C. parapsilosis* | — | *Escherichia coli* |
| 3 | *C. glabrata* | *C. glabrata* | |
| 4 | *C. parapsilosis* | *C. parapsilosis* | |
| 5 | *C. glabrata* | *C. glabrata* | |
| 6 | *C. tropicalis* | *C. tropicalis* | |
| 7 | *C. parapsilosis* | *C. parapsilosis* | |
| 8 | *C. parapsilosis* | *C. parapsilosis* | *Staphylococcus haemolyticus* |
| 9 | *C. parapsilosis* | *C. parapsilosis* | |
| 10 | *C. parapsilosis* | *C. parapsilosis* | |
| 11 | *C. glabrata* | *C. glabrata* | *Staphylococcus epidermidis* |
| 12 | *C. parapsilosis* | *C. parapsilosis* | |
| 13 | *C. tropicalis* | *C. tropicalis* | |
| 14 | *C. glabrata* | *C. glabrata* | |
| 15 | *C. parapsilosis* | *C. parapsilosis* | *S. epidermidis* |
| 16 | *C. glabrata* | *C. glabrata* | |
| 17 | *C. parapsilosis* | *C. parapsilosis* | |
| 18 | *C. glabrata* | *C. glabrata* | *Staphylococcus hominis* |
| 19 | *C. parapsilosis* | *C. parapsilosis* | |
| 20 | *C. parapsilosis* | *C. parapsilosis* | *S. epidermidis* |
| 21 | *C. lipolytica* | *C. glabrata* | |

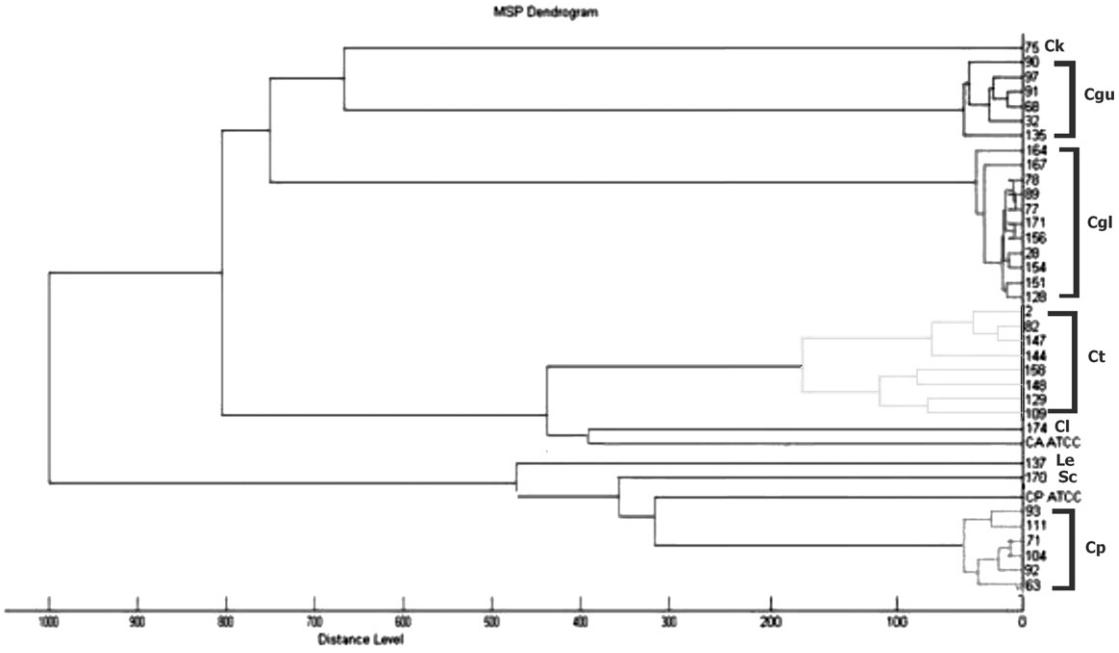


**Fig. 1.** Cluster analysis of matrix-assisted laser desorption ionization time-of-flight mass spectrometry spectra of reference strains (CA ATCC: *Candida albicans*; CP ATCC: *Candida parapsilosis*) and clinical isolates. For *C. parapsilosis*, only the six misindentified isolates were reported compared to the ATCC strain. Distance is displayed in relative units. Ck: *Candida krusei*; Cgu: *C. guillermondi*; Cgl: *Candida glabrata*; Ct: *Candida tropicalis*; Cl: *Candida lipolytica*; Le: *Lodderomyces elongisporus*; Sc: *Saccharomyces cerevisiae*; Cp: *C. parapsilosis.* The score-oriented MSP dendrogram was generated with the default setting in Biotyper 2.0.

drug-resistant fungal pathogens with the deletion of harmful empiric therapy.

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## Conflict of Interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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