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Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry to detect emerging pathogenic *Candida* species☆

Cledir Santos[a], Nelson Lima[a], Paula Sampaio[b], Célia Pais[b,*]

[a]*IBB–Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal*
[b]*Centre of Molecular and Environmental Biology (CBMA), University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal*

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry (MALDI-TOF-ICMS) was used to differentiate pathogenic *Candida* species, difficult to identify by traditional methods such as growth and biochemical reactions. Results showed that species complexes like *C. parapsilosis, C. orthopsilosis*, and *C. metapsilsosis*, and very closely related species like *C. glabrata* and *C. bracarensis*, and *C. albicans* and *C. dubliniensis* could be clearly separated. MALDI-TOF-ICMS stands out as a promising tool for the rapid detection of emerging pathogens.

Keywords: *Candida*; *C. parapsilosis* complex; MALDI-TOF ICMS

The clinical impact of fungal infections has greatly increased in the last years, particularly in immunocompromised hosts. Yeasts belonging to the genus *Candida* have emerged as the major opportunistic pathogens in these patients and currently constitute the fourth most common cause of nosocomial infections in intensive care units (Perlroth et al., 2007; Rueping et al., 2009). *Candida albicans* is the species most commonly isolated, although others, such as *C. tropicalis*, *C. krusei, C. parapsilosis,* and *C. glabrata*, have increasingly been recognized as pathogens with a wide distribution (Pfaller and Diekema 2007, Perlroth et al., 2007). Additionally, new species have been described, for example, *C. dubliniensis* related to *C. albicans* (Sullivan et al., 1995); *C. orthopsilosis* and *C. metapsilosis*, very closely related to *C. parapsilosis* (Tavanti et al., 2005); and *C. bracarensis*, phenotypically similar to *C. glabrata* (Correia et al., 2006), which are emerging as potential pathogens.

Early identification of the species causing infection is crucial for adequate treatment since the antifungal susceptibility profile differs greatly among species. Moreover, the time needed for the identification of the pathogen is an important determinant of infection-related mortality rates in hospitalised patients. The methods currently used for yeast identification in clinical laboratories are not able to distinguish emerging pathogenic species and, additionally, are either time consuming and/or require considerable expertise.

Several reports have shown that matrix-assisted laser desorption/ionisation time-of-flight intact cell mass spectrometry (MALDI-TOF ICMS) is suitable for the fast and reliable identification of pathogenic microorganisms (Barbuddhe et al., 2008; Claydon et al., 1996; Degand et al 2008; Mellman et al., 2008), including filamentous fungi (Santos et al., 2010) and yeasts (Marklein et al., 2009; Qian et al., 2008; Putignani et al., 2011; Stevenson et al., 2010; van Veen et al., 2010). The performance and cost analysis of MALDI-TOF ICMS for routine identification of yeast were recently evaluated by Dhiman et al. (2011) who concluded that this is an accurate, rapid, and cost-effective technique that could be applied in a medical microbiology laboratory for identification of yeast clinical isolates. Therefore, the aim of the present work was to use MALDI-TO-ICMS for the identification of *Candida* sp.

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* Corresponding author. Tel.: +351-253604312; fax: +351-253678987.
E-mail address: cpais@bio.uminho.pt (C. Pais).

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clinical isolates and to evaluate its ability for differentiating newly described species impossible to detect by the conventional phenotypic methods used in clinical laboratories.

Sixty-seven clinical isolates, belonging to 9 *Candida* species, namely, *C. albicans, C. tropicalis, C. kruzei, C. parapsilosis, C. orthopsilosis, C. matapsilosis, C. lusitaniae, C. glabrata,* and *C. bracarensis*, were analysed. Two strains of *Lodderomyces elongisporus* and 4 *Saccharomyces cerevisiae* isolates were also included in the study. *Candida* clinical isolates were obtained from several hospitals and health institutions and collected from blood, bronchial secretions, urine, and vaginal exudates. All isolates were identified by identification cards (BioMerieux, France) at the institutions of origin, and most were confirmed by 26S rDNA and ITS sequencing at the Department of Biology, University of Minho. The isolates were cryopreserved at −80 °C. Yeast cells were grown on YEPD agar medium plates for 24 h, at 37 °C. A loopful of yeast cells was directly transferred from the culture medium onto each position of the 48-well flex target plate, and 0.5 μL of 25% formic acid was immediately mixed with the yeast. After evaporation, 0.5 μL matrix solution (75 mg/mL 2,5-dihydroxybenzoic acid in ethanol/water/acetonitrile [1:1:1] with 0.03% trifluoroacetic acid) was added and gently mixed. All sample mixtures were air dried at room temperature. Each isolate was spotted in triplicate.

Analyses were performed on an Axima LNR system (Kratos Analytical, Shimadzu, Manchester, UK) equipped with a nitrogen laser (337 nm). The mass range from 2,000 to 20,000 Da was recorded by using the linear mode. *Escherichia coli* ribosomal proteins were used for external calibration of the spectra. Spectra were exported to the SARAMIS™ software package (Spectral Archiving and Microbial Identification System, AnagnosTec, Postdam-Golm, Germany, http://www.anagnostec.eu) where the final identifications were achieved. Cluster analysis of the MALDI-TOF ICMS mass spectral data was performed using the SARAMIS™ database by comparing database peak lists of individual samples with SuperSpectra and/or reference spectra.

The mass spectra obtained presented peaks ranging from 2,000 to 12,000 Da, with the most intense within the range of 5,000 to 7,000 Da. Mass spectra of the different *Candida* species analyzed showed distinctive features that could be used for species differentiation, and examples of distinctive spectra are shown in Fig. 1.

Initially, the isolates of *C. metapsilosis*, *C. orthopsilosis*, and *C. bracarensis* could not be identified since spectra for appropriate reference strains were not available in the database. As the identity of the isolates analysed in this study had been previously confirmed by D1/D2 26S rDNA and ITS sequencing, a reference spectrum for each was introduced into the database and subsequent comparison with the unidentified species resulted in a matching profile for all the isolates.

The species presenting the highest number of peaks were the *C. glabrata* and *C. bracarensis* isolates, which both showed an additional intense peak at around 11,000 Da. *C. parapsilosis* and *C. metapsilosis* isolates were the ones yielding a lower number of peaks. Additionally, the closely related species, *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*, and *C. glabrata* and *C. bracarensis*, presented similar but yet distinguishable profiles (Fig. 1).

L. elongisporus, a species hypothesized to be *C. parapsilosis* teleomorph (James et al., 1994) and considered very closely related by sequence analysis (Butler et al., 2009), presented a completely distinct profile (Fig. 1). This observation is particularly interesting since *L. elongisporus* has been recently described in cases of bloodstream infection misidentified as *C. parapsilosis* (Lockhart et al., 2008a).

C. glabrata and *C. bracarensis* presented similar spectra. The *S. cerevisiae* profile was distinctive, although peaks in the same spectral region as for *C. glabrata* and *C. bracarensis* (between 4,000 and 6,600 Da) could be observed. These data confirm the close genomic and proteomic relation between *S. cerevisiae* and *C. glabrata,* as described in the literature (Roetzer et al., 2011), but still the 3 species appear as distinct.

Cluster analysis of the MALDI-TOF ICMS mass spectral data included all peaks obtained from 2,000 to 20,000 Da.

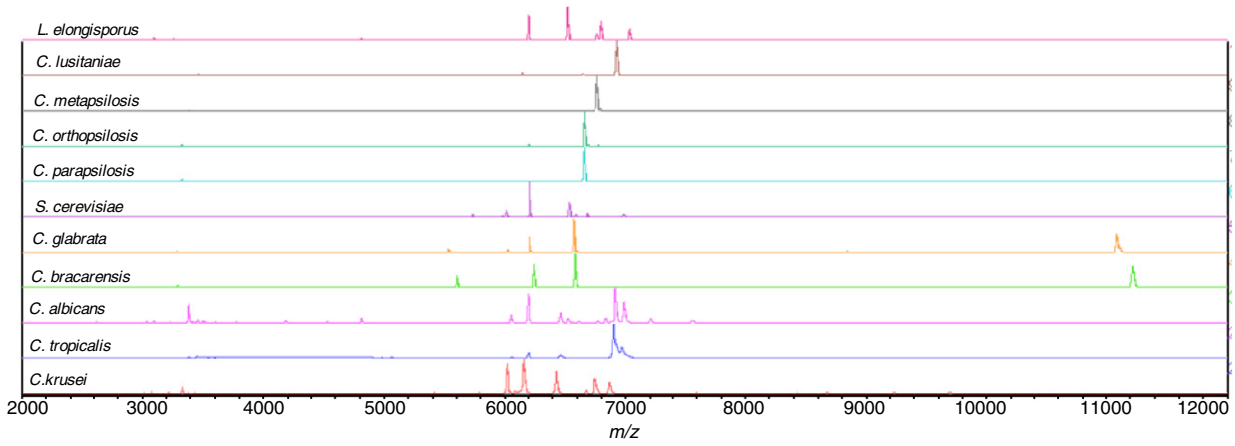
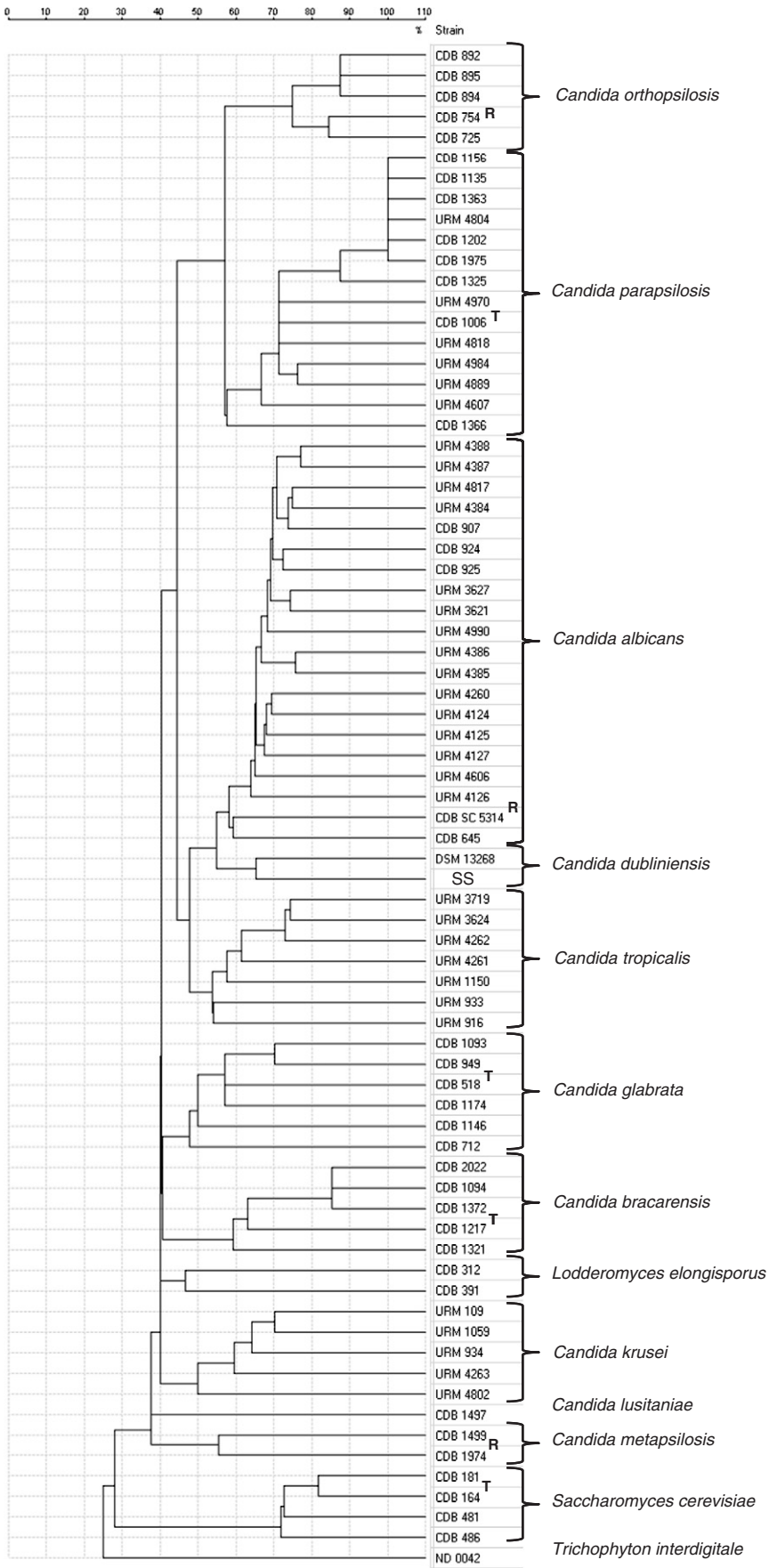


Fig. 1. Examples of representative MALDI-TOF ICMS spectra of the yeast species analysed in this study.


0 10 20 30 40 50 60 70 80 90 100 110
% Strain
CDB 892
CDB 895
CDB 894
CDB 754 R
CDB 725
Candida orthopsilosis
CDB 1156
CDB 1135
CDB 1363
URM 4804
CDB 1202
CDB 1975
CDB 1325
URM 4970
CDB 1006 T
URM 4818
URM 4984
URM 4889
URM 4607
CDB 1366
Candida parapsilosis
URM 4388
URM 4387
URM 4817
URM 4384
CDB 907
CDB 924
CDB 925
URM 3627
URM 3621
URM 4990
URM 4386
URM 4385
URM 4260
URM 4124
URM 4125
URM 4127
URM 4606
URM 4126
CDB SC 5314 R
CDB 645
Candida albicans
DSM 13268
SS
Candida dubliniensis
URM 3719
URM 3624
URM 4262
URM 4261
URM 1150
URM 933
URM 916
Candida tropicalis
CDB 1093
CDB 949
CDB 518 T
CDB 1174
CDB 1146
CDB 712
Candida glabrata
CDB 2022
CDB 1094
CDB 1372
CDB 1217 T
CDB 1321
Candida bracarensis
CDB 312
CDB 391
Lodderomyces elongisporus
URM 109
URM 1059
URM 934
URM 4263
URM 4802
Candida krusei
CDB 1497
Candida lusitaniae
CDB 1499
CDB 1974 R
Candida metapsilosis
CDB 181
CDB 164 T
CDB 481
Saccharomyces cerevisiae
CDB 486
ND 0042
Trichophyton interdigitale

Mass spectral signatures allowed the grouping of all isolates into clusters according to their species designation (Fig. 2). These results are in accordance with the ones reported previously (Bader et al., 2010; Marklein et al., 2009; Qian et al., 2008; Stevenson et al., 2010). At a threshold of about 48% similarity, all different species were clearly distinguished except the pairs *C. parapsilosis/C. orthopsilosis* and *C. albicans/C. dubliniensis,* which could only be distinguished at a threshold of 57% and 52%, respectively. *C. dubliniensis* isolates were not analysed in the present work but spectra, obtained from the SARAMIS database, were included in the analysis. Spectra obtained for all isolates belonging to the same species grouped together, presenting a very close profile.

All of the closely related species, *C. albicans* and *C. dubliniensis*; *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*; as well as *C. glabrata* and *C. bracarensis*, could be clearly distinguished. Also, *S. cerevisiae* and *L. elongisporus* species were distinctly separated. Due to their close phylogenetic relationship with the species most commonly found, the new species mentioned above are often misidentified and MALDI-TOF ICMS analysis can be a valuable tool for their early and unequivocal detection. This is particularly important since in recent *C. parapsilosis* surveys a growing importance of *C. orthopsilosis* has been reported (Gomez-Lopez et al., 2008; Lockhart et al., 2008b).

MALDI-TOF ICMS appears to be a powerful high-throughput proteomic technique to discriminate closely related *Candida* species, which is feasible otherwise only by molecular biology techniques. The equipment is straightforward to operate and inexpensive on an individual sample basis. The rapid identification of uncommon yeast species that are emerging as human pathogens may be accurately achieved provided that a complete and quality-controlled database is available.

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Fig. 2. MALDI-TOF ICMS spectra-based dendrogram of the yeast isolates studied in this work. The spectrum of the dermatophyte fungus *Trichophyton interdigitale* was used as outgroup. CDB = Collection Department of Biology; T = Type strain; R = reference strain; SS = Super septrum.

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