

Modified Protocol for Yeast Identification Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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Abstract Advances in Matrix-assisted Laser Desorption Ionization–time of Flight Mass Spectrometry (MALDI-ToF MS) identification methods have greatly facilitated the rapid identification of yeasts. We validate the use of a shorter on-plate extraction method as well as a lower score cutoff value on the Bruker Biotyper. Overall, when the on-plate extraction method was used together with a score cutoff of > 1.7 , a total of 151 of 187 (81%) *Candida* isolates were correctly identified. At species level, almost 100% of *C. krusei* and *C. albicans* isolates were correctly identified. We find the revised protocol to be effective for routine identification of yeasts.

Keywords: protein extraction, *Candida*, identification scores

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1. Introduction

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-ToF MS) has become the workhorse in clinical microbiology laboratories for the rapid identification of microorganisms [1]. The instrument identifies microbes by analyzing proteins released from the cells against its reference database of profiles. Identification of Gram-negative bacteria is achieved fairly effortlessly through the direct method of analysis where colonies are smeared as a thin layer of cells onto the target plate followed by overlaying matrix onto the cell layer whereas the identification of yeast and Gram-positive bacteria is more onerous. Yeast cells require a harsher pre-processing step for the release of cellular proteins [2]. The standard tube-based method of protein extraction, regarded as the ‘gold-standard’ for MALDI-ToF MS extractions, employs formic acid-acetonitrile treatment and multiple centrifugation steps in an Eppendorf tube before finally transferring processed sample onto the target plate. This method achieves close to 100% correct identification of yeasts [3,4] but takes about 25 minutes or 6-fold more time than the direct method. The standard method of extraction is too tedious, demanding excessive hands-on time for a clinical laboratory. A shorter (15 minutes) and uncomplicated alternative to the standard extraction method described as on-plate extraction has been found to be comparable to the standard method for providing correct identifications [5,6,7].

Several validation studies have indicated that it is acceptable to revise the manufacturer-recommended

interpretive criteria for yeasts. The Bruker MALDI Biotyper system, for example, recommends an organism score of greater than 2.0 to report a species identification. Scores of between 1.7 and 1.99 provide confidence to the genus level, and scores below 1.7 are not reliable. This criterion has been found to be overly conservative for certain microbes groups including the yeasts [4,8]. Studies have found that scores > 1.7 for *Candida* spp. were accurate for diagnosis to the species level [4,6,9,10].

In this study, we wanted to validate for yeasts: (i) the on-plate method of extraction and (ii) the lowering of the interpretative cutoff for species level to > 1.7 , instead of the manufacturer’s (Bruker Daltonik) recommended score of > 2 .

2. Materials and Methods

2.1. *Candida* Isolates and Phenotypic Identification

The *Candida* isolates were obtained from patients with *Candida* infections and cultured onto Sabouraud’s dextrose agar (Difco, MI, USA). A total of 187 clinical isolates from 5 different species were studied (Table 1). *Candida* spp. were identified phenotypically by inoculating all isolates onto BBL CHROMagar *Candida* (Becton Dickinson, Heidelberg, Germany). Colony growth and color development was noted on CHROMagar *Candida*. Green and blue colonies were identified as *C. albicans* and *C. tropicalis*, respectively. Colonies producing colors apart from green and blue were

identified on the automated system for microorganism identification using VITEK 2 YST card (bioMérieux, Marcy l'Etoile, France). Inconclusive results were

resolved by identification with API 20 C AUX (bioMérieux).

Table 1. Breakdown of result scores obtained for yeast isolates subjected to three different extraction methods (direct colony, on-plate extraction and standard tube extraction method)

	No. of isolates producing a score of (%)			
	≥ 2	1.7 – 1.99	< 1.7	Invalid identifications
<i>Candida albicans</i> (n = 49)				
Direct	1 (2)	21 (42.9)	5 (10.2)	22 (44.9)
On-plate	29 (59.2)	19 (38.8)	0	1 (2)
Standard	40 (81.6)	9 (18.4)	0	0
<i>Candida tropicalis</i> (n = 50)				
Direct	3 (6)	1 (2)	10 (20)	36 (72)
On-plate	33 (66)	11 (22)	3 (6)	3 (6)
Standard	35 (70)	9 (18)	5 (10)	1 (2)
<i>Candida glabrata</i> (n = 50)				
Direct	0	1 (2)	26 (52)	23 (46)
On-plate	0	28 (56)	18 (36)	4 (8)
Standard	44 (88)	1 (2)	3 (6)	2 (4)
<i>Candida parapsilosis</i> (n = 30)				
Direct	0	4 (13.3)	9 (30)	17 (56.7)
On-plate	5 (16.7)	18 (60)	4 (13.3)	3 (10)
Standard	16 (53.3)	7 (23.3)	4 (13.3)	3 (10)
<i>Candida krusei</i> (n = 8)				
Direct	0	0	3 (37.5)	5 (62.5)
On-plate	2 (25)	6 (75)	0	0
Standard	8 (100)	0	0	0

2.2. MALDI-ToF Extraction and Identification

Yeast identification on the Microflex LT instrument (Bruker Daltonik GmbH, Bremen, Germany) was performed using 3 different extraction protocols. All extraction procedures were performed as according to the manufacturer's protocol. Details of sample preparation can be found on the manufacturer's website at http://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/InstructionForUse/IFU_268711_267615_226413_MALDI_Biotarget_48_Rev1.pdf. Direct colony method involved smearing a small amount of the yeast colony onto the MSP 96 target plate (Bruker Daltonik) using a 1 µL disposable inoculation loop. The sample was overlaid with 1 µL of HCCA matrix (a-cyano-4-hydroxycinnamic acid [Vitek MS-CHCA 41107] in 50 % acetonitrile and 1.5 % trifluoroacetic acid) and dried at room temperature. The on-plate extraction method involved the smearing of a thin layer of isolate onto the target plate followed by the addition of 1 µl of 70 % formic acid which was allowed to dry, 1 µl of acetonitrile was overlaid and permitted to dry before applying 1 µl of HCCA matrix. In the standard tube-extraction protocol, colonies were thoroughly resuspended in 300 µl of distilled water and 900 µl of absolute ethanol was added. The cell suspension was vortexed and centrifuged at 13,000 rpm for 2 min. The supernatant was removed and 50 µl of formic acid was added and the mixture was vortexed. Acetonitrile (50 µl) was added and vortexed vigorously and the tube was centrifuged at 13,000 rpm for 2 min. One µl of the supernatant was spotted onto the target plate and allowed to dry before overlying with 1 µl of HCCA matrix.

The samples prepared by each method were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik), and the results were analyzed by MALDI Biotyper 3.0 software (Bruker Daltonik) in automatic mode at manufacturer's setting. If the score identification was low (< 1.7) then the sample analysis was repeated. In our study,

an isolate had a correct identification when results showed that the first and second species identification was the same, with a score of at least ≥ 1.7 . The following were considered as invalid when (i) the first and second identification result were different (ii) the misidentification was obvious, for example, a bacterial identification is suggested (iii) no spectral peaks were generated. American type culture collection (ATCC) isolates of *C. albicans* 14053 and *C. glabrata* 15126 were used as positive controls for the analysis. These isolates were correctly identified to species level (score values >1.8) but were not included in the final analysis.

3. Statistical Analysis

Comparisons of extraction methods for species level identifications were performed using McNemar's test for paired samples. *P* values less than 0.05 were considered statistically significant.

4. Results and Discussion

In this study, we validate the use of a shorter on-plate extraction method as well as a lower score cutoff value on the Bruker MALDI-ToF Biotyper. The identification rates and breakdown of score categories using the 3 (see above) different extraction methods are shown in Table 1. In the score category of ≥ 2 , the standard extractions produced the greatest number of correct identifications. This was 100% for *C. krusei* followed by 88% for *C. glabrata* and 81% for *C. albicans*. Compared to standard extractions, the on-plate method yielded fewer correct identifications whilst the direct method generated the poorest results for this score category, yielding no identifications for *C. krusei*, *C. parapsilosis* and *C. glabrata* and < 6% successful identifications for the other species (Table 1).

For the score category of 1.7 to 1.99, the majority of identifications made were produced via the on-plate method. For *C. krusei*, *C. parapsilosis*, and *C. glabrata*,

75%, 60% and 56%, respectively, of isolates were correctly identified using the on-plate procedure. The direct colony method identified less than 13.3% of isolates in four different species although *C. albicans* was an exception, having 42.9% of reliable identifications (Table 1).

Low scoring results (< 1.7) and invalid identifications were typically generated by the direct colony method. Misidentifications ranging from between 44.9% to 72% for the different species were produced whereas the on-plate and standard methods had less than 10% misidentifications in this category (Table 1).

For the Bruker MALDI Biotyper system, lowering the species-secure score cutoff to > 1.7 instead of the

manufacturer's recommendation of ≥ 2.0 is now deemed acceptable [4,6,9,10]. In our study, when the cutoff for identification to species level was modified to > 1.7, we find that the level of correct identifications across the different species rises from 66% up to 100% for the on-plate extraction method (Figure 1). Statistically, the on-plate method had a similar rate of identification compared to the standard extraction method ($P = > 0.05$) (Figure 1) with the exception of *C. glabrata*. For this species, the identification using on-plate method of extraction was inferior to the standard method of extraction ($P = 0.0001$). The direct colony method was significantly inferior ($P < 0.0001$) in providing accurate species identification for all *Candida* analyzed (Figure 1).

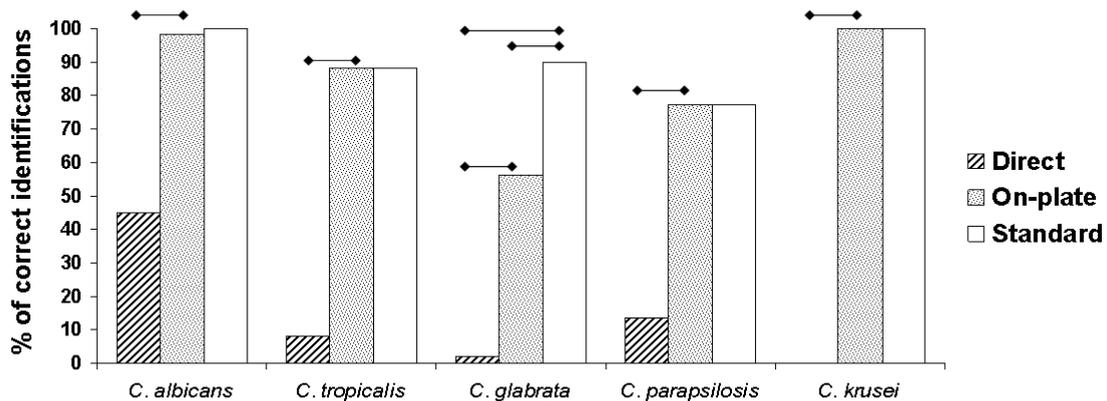


Figure 1. Bar plots showing the percentage of correct yeast identifications using three different extraction methods (direct colony, on-plate and standard-tube extraction methods) and a revised score cutoff of >1.7 for species-level identification. ◆◆ Above the bars indicate statistically significant pairs

5. Concluding Remarks

Prompt and reliable species identification of *Candida* is critical to the treatment of candidiasis. *C. glabrata* has less susceptibility to fluconazole and *C. krusei* is intrinsically resistant to this drug hence differential therapeutic regimens may be required for the various species [11]. Our findings correlate well to investigations performed by other groups where the on-plate method of extraction coupled with a modified species score of >1.7, is non-tedious, fast and reliable approach [4,6,9,10]. It is therefore highly suited for the workflow of the clinical microbiology laboratory for the identification of yeast isolates.

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