Detection of triazole resistance among *Candida* species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)

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**Abstract**

MALDI-TOF MS can rapidly identify microorganisms to the species level and may be able to detect antimicrobial resistance. We evaluated the ability of this technology to detect triazole resistance in *Candida* species. 35 *C. albicans*, 35 *C. glabrata*, and 37 *C. tropicalis* strains were exposed to fluconazole, voriconazole, or posaconazole at two different concentrations plus a drug-free control: a midrange concentration (CLSI clinical breakpoint or epidemiologic cut-off value), and a high concentration (fluconazole 64 \(\mu g/ml\), voriconazole & posaconazole 16 \(\mu g/ml\)). The MALDI-TOF MS spectra at these concentrations were used to create the individual composite correlation index (CCI) matrices for each isolate. When the CCI of the midrange/highest concentration was lower than that of the midrange/null concentration, the strain was classified as resistant. These results were then compared to the classifications for susceptible or resistant obtained by measuring the MICs according to the CLSI M27-A3 antifungal susceptibility testing (AFST) method.

The MALDI-TOF MS assay was able to classify triazole susceptibility against all strains. Overall, essential agreement between MALDI-TOF MS and AFST varied between 54% and 97%, and was highest for posaconazole against *C. glabrata*. The reproducibility of the MALDI-TOF MS assay varied between 54.3 and 82.9% and was best for fluconazole against *C. albicans* and posaconazole against *C. glabrata*. Reproducibility was also higher for *C. glabrata* isolates compared to *C. albicans* and *C. tropicalis*.

These results demonstrate that MALDI-TOF MS may be used to simultaneously determine the *Candida* species and classification as susceptible or resistant to triazole antifungals. Further studies are needed to refine the methodology and improve the reproducibility of this assay.

**Key words:** MALDI-TOF MS, susceptibility testing, *Candida*, resistance.
Introduction

Delayed diagnosis and initiation of appropriate therapy against invasive fungal infections contribute significantly to high mortality rates, whereas early intervention with antifungal treatment may result in improved clinical outcomes in high-risk patients [1,2]. Despite new antifungal agents such as the highly active triazoles and the echinocandins, invasive candidiasis is associated with excessive morbidity and mortality, as well as a higher burden on healthcare resources [3]. While C. albicans is still the major species associated with candidemia, the frequency of isolation of non-albicans Candida species differs with geographical location [4]. Candida glabrata has emerged as a major cause of mucosal and invasive fungal infection in the United States, second only to C. albicans [5]. Candida glabrata is also one of the five most commonly isolated Candida species both in intensive care unit (ICU) and non-ICU settings [6,7]. Fluconazole (FLU) and other azoles have proven effective for the management of Candida infections; however, many C. glabrata isolates exhibit low susceptibility to the azole antifungals [8]. Moreover, C. tropicalis has been associated with higher mortality in some studies, particularly in neutropenic and oncology patients than other Candida species including C. albicans [7,9,10].

Antifungal susceptibility testing (AFST) is based on measuring growth in the presence of different drug concentrations so as to determine the minimum inhibitory concentration (MIC). The MIC value helps to predict the likelihood of therapeutic efficacy of the tested agents. The reference susceptibility methods for Candida spp. are broth dilution assays devised by the European Committee on Antibiotic Susceptibility Testing (EUCAST) and by the Clinical Laboratory Standards Institute (CLSI) [11,12]. The CLSI M27-A3 methodology requires reading MIC endpoints for azoles visually in order to identify the lowest concentration with a prominent reduction in growth as compared with the drug-free control. Both reference methods are robust and reproducible. However, each requires additional time following the initial subculture of the organisms. Additionally, variations might be observed in end-points between laboratories because reading the end-points can be somewhat subjective [13,14]. Spectrophotometric reading end-points on microplates with a microdilution plate reader may decrease subjective reading as recommended by EUCAST definitive document for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts [11]. Given these limitations, there is a clear need for the development of an equally robust methodology, with faster turn-around times and where endpoint determination is objective.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF MS) mass spectrometry relies on the generation of a microorganism’s “protein fingerprint.” Identification of a microorganism by MALDI-TOF MS is based on comparing its protein fingerprint to reference spectra deposited in a database of well-characterized isolates [15]. This technology has also been evaluated as a tool for drug susceptibility testing against bacteria and Candida species [16–20]. It has been previously observed that the protein composition of Candida spp. will vary when strains are subjected to antifungal drugs and may be part of compensatory responses that occur in response to environmental stresses. The objective of this study was to evaluate MALDI-TOF MS as a method of determining triazole antifungal resistance in C. albicans, C. tropicalis, and C. glabrata.

Materials and methods

Strains and AFST by CLSI reference method

A total of 35 C. albicans, 35 C. glabrata, and 37 C. tropicalis strains were included in this study. The selected strains were clinical isolates identified between years 2003 and 2013 and deposited in the culture collection of Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio. The number of susceptible (S), susceptible-dose dependent, or resistant (R) isolates for each species against fluconazole (FLU), voriconazole (VOR), or posaconazole (POS) as determined by CLSI were given in Table 1. The MICs were confirmed by broth microdilution following the CLSI M27-A3 guidelines, using prominent growth inhibition as the endpoint [12]. Microdilution plates were incubated at 35 °C, and the MICs were read after 24 and 48 hours of incubation. The CLSI-recommended quality control strains (Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258) were not included in AFST by MALDI-TOF MS, but were run on each day of CLSI AFST. The isolates were categorized as susceptible, susceptible-dose dependent (SDD), or resistant on the basis of the CLSI breakpoints, or on the basis of accepted Epidemiological Cut-off Values (ECVs) described in the literature, as shown in Table 2 [21].

AFST by MALDI-TOF MS

AFST by MALDI-TOF MS was performed similarly as described originally by De Carolis et al. and simplified later by this group to facilitate the discrimination of susceptible and resistant isolates in the presence of “breakpoint” level drug concentrations [16,22]. The inoculum of each strain was adjusted first to a density between 1.1 and 1.3
Table 1. Distribution of susceptible, susceptible dose-dependent and resistant isolates.

<table>
<thead>
<tr>
<th></th>
<th>Susceptible</th>
<th>Susceptible Dose-dependent</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLU</td>
<td>VOR</td>
<td>POS</td>
</tr>
<tr>
<td>C. albicans (n:35)</td>
<td>8</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>C. glabrata (n:35)</td>
<td>NA</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>C. tropicalis (n:37)</td>
<td>28</td>
<td>28</td>
<td>33</td>
</tr>
</tbody>
</table>

Note: NA: not applicable, FLU: Fluconazole, VOR: Voriconazole, POS: Posaconazole.

Table 2. Clinical and Laboratory Standards Institute breakpoints (or, Epidemiological Cut-off values) and induction concentrations (μg/ml) of the triazoles for Candida albicans, Candida glabrata, and Candida tropicalis.

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Species</th>
<th>CLSI breakpoints (or, ECVs)</th>
<th>Induction concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible</td>
<td>SDD</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>C. albicans</td>
<td>≤2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>C. glabrata</td>
<td>NA</td>
<td>≤32</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>≤2</td>
<td>4</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>C. albicans</td>
<td>≤0.12</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td></td>
<td>C. glabrata</td>
<td>≤0.5</td>
<td>(&gt;0.5)</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>≤0.12</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>C. albicans</td>
<td>≤0.06</td>
<td>(&gt;0.06)</td>
</tr>
<tr>
<td></td>
<td>C. glabrata</td>
<td>≤2</td>
<td>(&gt;2)</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>≤0.12</td>
<td>(&gt;0.12)</td>
</tr>
</tbody>
</table>

Note: CLSI: Clinical and Laboratory Standards Institute, ECVs: Epidemiological Cut-off values, SDD: Susceptible-dose dependent, NA: Not applicable. Prepared from the reference [21].

McFarland standard (approximately 1.1–1.3 × 10⁷ cfu/ml) using a DensiChek (bioMérieux SA, Marcy l’Etoile, France), followed by a 1:10 dilution in RPMI medium, and then mixed with 2-fold concentrations of the antifungal drug in a 1 ml volume. Concentrations included the highest drug concentration that is routinely tested according to CLSI M27-A3 and a mid-range drug concentration, which was the CLSI breakpoint for susceptibility or the published ECVs for each antifungal, as shown in Table 2. A FLU concentration of 32 μg/ml was used as the midrange concentration for C. glabrata. Additionally, each strain was grown in 1 ml of drug-free RPMI broth as a null concentration. After incubating at 35°C without agitation for approximately 16 hours, fungal cells were collected by centrifugation, washed twice with sterile deionized water, and resuspended in 30% (vol/vol) formic acid. One μl of this cell suspension was directly spotted onto a target slide (bioMérieux) for each testing condition, covered with 1 μl of a saturated CHCA (α-cyano-4-hydroxycinnamic acid) matrix solution (bioMérieux) and then allowed to dry completely. Reading of the slides was performed by using a VITEK® MS MALDI-TOF MS instrument (bioMérieux). Each strain was tested for each drug concentration and drug-free control in duplicate in different runs. As recommended by the manufacturer, the Escherichia coli ATCC 8739 strain was used as a calibrator. The CLSI QC strains, Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258), were not included in AFST by MALDI-TOF MS, but were tested by the CLSI method on each day of antifungal susceptibility testing.

Data analysis
The relative differences and similarities between the spectra obtained for each strain against each drug concentration and drug-free control was analyzed by using Spectral ARchive And Microbial Identifications System software (SARAMIS™, bioMérieux). The procedure was similar to the composite correlation index (CCI) method previously described by Arnold and Reilly [23] and was adapted for MALDI-TOF MS-based AFST by De Carolis et al. for Candida spp. [22] and simplified by Vella et al. for C. albicans [16]. The spectrum obtained at mid-range drug concentrations (see Table 2) was compared with the spectrum obtained with both the highest and null concentrations. An individual composite correlation index (CCI) matrix was prepared for each strain under each test condition. Categorical classification of each isolate by the MALDI-TOF MS based AFST assay was performed according to predefined criteria. A strain was classified as susceptible to the drug
when the CCI value derived from the correlation of spectra at the mid-range and the highest drug concentrations was higher than the CCI value derived from the midrange and null concentrations. Conversely, it was classified as resistant when the CCI value derived from the correlation of spectra at the mid-range and the highest drug concentrations was lower than the CCI value derived from the mid-range and null concentrations. Figure 1 shows an example of CCI-based evaluation of a FLU-resistant, and a FLU-susceptible C. glabrata strain.

Classification of each isolate by the MALDI-TOF MS based assay was compared then with its CLSI-based AFST classification. The categorical placement of a strain according to the CLSI breakpoint or ECV was used as a gold standard to evaluate the categorical essential agreement (EA) of the MALDI-TOF MS based susceptibility assay. Strains assigned as susceptible dose-dependent by the CLSI method were accepted as susceptible in these comparisons due to the lack of definition of this category with MALDI-TOF MS based AFST. Very major errors (VMEs) were recorded when a resistant isolate by MALDI-TOF MS but susceptible by the CLSI method was classified as susceptible by MALDI-TOF MS assay. Major errors (MEs) were recorded when a susceptible isolate by CLSI method was classified as resistant by MALDI-TOF MS assay. Minor errors were not recorded because a SDD category was not available by the MALDI-TOF MS assay. The MALDI-TOF MS based AFST assay was considered reproducible when the same categorical evaluation was obtained after repeat testing regardless of its agreement with CLSI categorical assignment.

Additionally, we evaluated how a 5% tolerance for CCI ratios affected reproducibility, EA, VME, and ME percentages. This tolerance was applied as follows; isolates classified as resistant by MALDI-TOF MS but susceptible by the CLSI method with a CCI ratio value between 0.95 and 0.99 were recorded as susceptible while isolates classified as susceptible by MALDI-TOF MS but resistant by the CLSI methods with a CCI ratio value between 1.0 and 1.05 were recorded as susceptible.

Results

A preliminary experiment demonstrated that a minimum inoculum density of above 1 McFarland standard was needed to obtain data counts high enough to be evaluated by the MALDI-TOF MS assay (data not shown). Thus, the starting inoculum was adjusted to a density between 1.1 and 1.3 McFarland’s standard (approximately 1.1–1.3 × 10⁷ cfu/ml), as a significant change in CCI ratios change was not observed between these two higher inocula and categorical placement. Incubation duration and conditions described in CLSI M27-A3 were followed in order to enable a better comparison between CLSI and MALDI-TOF MS based AFST. Using the reference CLSI AFST as the gold standard, the percentages of EA, and reproducibility values for each species and triazole drug combination were calculated (Table 3). Essential agreement varied between 54 and 97%. The best EA was observed for C. glabrata strains. The reproducibility of the MALDI-TOF MS based AFST also varied between 54–83%, with the best reproducibility observed for FLU among the drugs tested and for C. glabrata among the species tested. When a 5% tolerance for CCI ratio based evaluation was applied, up to 20% improvement in reproducibility was noted with a range between 63.0 and 94.4%. The poorest reproducibility was observed for the posaconazole/C. albicans combination with and without 5% tolerance, while reproducibility was best for posaconazole/C. glabrata combination. The very major and major errors are presented in Table 4. Error values were high among C. albicans strains for posaconazole (n:14/22), among C. glabrata strains for voriconazole (n:14/31), and among C. tropicalis strains for fluconazole (n:16/28), except for the conditions where the strain number is low to make a meaningful comparison (Table 4). When a 5%
Table 3. Essential agreement, essential agreement with 5% tolerance, and reproducibility values.

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug</th>
<th>Fluconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EA</td>
<td>EA 5%T</td>
<td>ΔEA</td>
<td>EA</td>
</tr>
<tr>
<td>C. albicans (N = 35)</td>
<td>No. 29</td>
<td>31</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Percent</td>
<td>82.9%</td>
<td>88.6%</td>
<td>5.7%</td>
<td>74.3%</td>
</tr>
<tr>
<td>C. glabrata (N = 35)</td>
<td>No. 27</td>
<td>30</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Percent</td>
<td>77.1%</td>
<td>85.7%</td>
<td>8.6%</td>
<td>60%</td>
</tr>
<tr>
<td>C. tropicalis (N = 37)</td>
<td>No. 20</td>
<td>24</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Percent</td>
<td>54.1%</td>
<td>64.9%</td>
<td>10.8%</td>
<td>64.9%</td>
</tr>
</tbody>
</table>

Note: EA: Essential agreement, EA 5%T: Essential agreement with 5% tolerance, ΔEA: Change in essential agreement.

Table 4. Very major error and major error values of Matrix Assisted Laser Desorption/Ionization-Time of Flight assay based antifungal susceptibility testing in comparison to Clinical and Laboratory Standards Institute-based antifungal susceptibility testing.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fluconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>5%T</td>
<td>Total</td>
</tr>
<tr>
<td>Candida albicans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VME</td>
<td>5/24</td>
<td>3/24</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>1/8</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VME</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>7/34</td>
<td>4/34</td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VME</td>
<td>1/7</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>16/28</td>
<td>12/28</td>
<td></td>
</tr>
</tbody>
</table>

Note: 5%T: when 5% tolerance applied, VME: Very major errors, ME: Major errors. The denominators show the number of resistant strains for very major error calculations and the number of susceptible strains for major error calculations for a given drug/species combination. Minor errors were not calculated because SDD category was not available by the MALDI-TOF assay.

tolerance was applied, error percentages were decreased by up to 33.3%.

Discussion

Because a MALDI-TOF MS assay allows comparison of reference spectra deposited in a well-characterized library [15], variations between proteome composition of resistant and susceptible strains can be detected by MALDI-TOF MS when the strains are challenged with antifungal drugs. However, the difference in protein expression level, or the amount of the modified protein should be abundant enough to be discriminated from other “background” proteins expressed in each cell.

The study population was designed to encompass highly resistant, susceptible dose-dependent and susceptible C. albicans, C. glabrata, and C. tropicalis strains as determined by the CLSI reference M27-A3 AFST method. We investigated the influence of inoculum density on obtaining an acceptable spectrum for evaluation by the MALDI-TOF MS AFST assay and found that an inoculum density above McFarland standard 1.0 was needed to obtain sufficient spectra for Candida species, especially when using higher drug concentrations. We also tested variations in inoculum density between McFarland standards 1.1 and 1.3 to determine if this might be a cause of possible evaluation error. Categorical changes within this range were not observed. Duplicate testing of each isolate on different runs against each drug was performed to evaluate the reproducibility of the assay. Reproducibility of MALDI-TOF MS based AFST ranged between 54.3% and 82.9% with best performance by C. glabrata, followed by C. albicans and for FLU (see Table 3). It is important to note that a reproducibility value of 50% percent can lead to the misclassification of isolates as resistant or susceptible. Poorest reproducibility was observed with C. tropicalis. This was interesting as testing of this species is also somewhat problematic by CLSI based AFST methods. An explanation for poor categorical agreement may be the lack of a gray-zone in MALDI-TOF MS based AFST. A single strain could be evaluated as resistant with a CCI ratio of 0.99, and then susceptible with a CCI ratio of 1.01. A larger number of isolates needs to be tested
to determine what this zone should be, or if a 5–10% tolerance for CCI ratios is appropriate and may allow for more reproducible values. By using a 5% tolerance for the CCI ratio obtained by MALDI-TOF MS, up to a 17% increase in EA values was noted, with a corresponding 33% decrease in error values (Tables 3 and 4). CLSI and EUCAST based microdilution AFST uses doubling concentrations, and requires the use of quality control (QC) strains with each run to ensure that the assays are performing within standards. Because we followed the simplified methodology described by Vella et al. [16], we did not test a full range of antifungal concentrations for the isolates. If we had determined CCI ratios obtained from a full range of concentrations, this might help us describing a more robust tolerance value. Additionally, this simplified study with MALDI-TOF MS based AFST by using three-point drug concentrations (zero, midrange, and the highest drug concentration) lacked an established QC procedure. Slightly lower drug concentrations in induction conditions than that required may have caused categorical changes between experiments. In addition, ECVs were used for antifungal/organisms for which CLSI breakpoints have not been set, and this may be critical in the three-point MALDI-TOF MS AFST methodology. In a recent study, lower ECVs have been suggested for VOR and POS for C. glabrata strains than the midrange drug concentration we used for the induction [24]. Thus, the use of higher ECVs may have led to categorical disagreements since inadequate induction of resistance may result in misevaluation or misclassification of a strain by the AFST MALDI-TOF MS assay.

On the other hand, we preferred here CLSI clinical breakpoints to induce resistance and to evaluate categorical agreement between MALDI-TOF MS based and CLSI based AFST methodologies when CLSI breakpoint values were available. However, it should be remembered that clinical breakpoints are designed to guide therapy; they do not distinguish between isolates with or without resistance mechanisms. In this respect, use of ECVs might be more suitable for MALDI-TOF MS based AFST.

It is also important to note that the isolates used in this study were chosen irrespective of the type of drug resistance mechanisms. As expected, the CLSI-based AFST can detect resistance of an isolate regardless of resistance mechanism; however, resistance detected by MALDI-TOF MS may only be based on a proteomic change, and the degree of proteomic changes needs to be sufficiently high enough to be detected by the MALDI-TOF MS assay. This restriction might be an important reason at least for some of VMEs, where resistant strains by CLSI-based AFST were found susceptible by MALDI-TOF MS. On the other hand, major errors might be caused by preliminary proteomic changes which have not resulted a phenotypically observable resistance pattern. However, this speculative explanation needs to be confirmed by molecular studies.

The MALDI-TOF MS based AFST method did not result in significant time savings (overnight vs 24 h) over the CLSI method. We have not tested shorter incubation periods, however, Vella and colleagues showed that a 3-h induction might be enough for testing resistance of C. albicans strains against caspofungin [16]. Other advantages of MALDI-TOF MS based AFST may be elimination of subjective visual endpoint determination, allowance of automated determination of resistance, and an opportunity for concomitant antifungal susceptibility testing for those mycology laboratories that routinely use MALDI-TOF MS for identification of clinical isolates.

In conclusion, further studies are needed to truly develop MALDI-TOF MS assays as an alternative to traditional AFST methods for the detection of triazole resistance among C. albicans, C. glabrata, and C. tropicalis strains. Although some promising results were observed using a three-point drug concentration assessment, there is a need for improving the methodology, especially in respect of quality control procedures and the definition of a gray-zone for evaluation of the results.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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