Evaluation of four pretreatment procedures for MALDI-TOF MS yeast identification in the routine clinical laboratory

CAROLE CASSAGNE, ANNE-LAURE CELLA, PIERRE SUCHON, ANNE-CECILE NORMAND, STEPHANE RANQUE & RENAUD PIARROUX

Laboratoire de Parasitologie-Mycologie, CHU Timone, Université de la Méditerranée, Marseille, France

MALDI-TOF MS-based yeast identification requires a pretreatment step for which four are described in the literature, i.e., direct smear, fast formic acid and two complete formic acid/acetonitrile extractions. In this study we compared the impact of these procedures on the performance of MALDI-TOF MS-based yeast identification of samples from colonies grown on Sabouraud or chromogenic media. A total of 103 yeast isolates recovered from clinical samples were identified in parallel using the four pretreatment procedures. The proportions of both correct identifications (regardless of LogScore values) and of reliable identifications (i.e., correct identifications with a LogScore ≥2, as recommended by the manufacturer) obtained with the four techniques were compared. Even if the proportion of correct identifications exceeded 85% independent of the pretreatment procedure, results obtained with complete formic acid/acetonitrile extractions of colonies grown on Sabouraud media were significantly superior to those with smear and fast formic acid extraction procedures. If one considers only reliable identifications, then both smear and fast formic acid extraction procedures yielded lower (<40%) correct identification rates than the use of the two complete extraction procedures (>77%) of portions of colonies on both Sabouraud and chromogenic media. The data would indicate that the direct smear and fast formic acid procedures cannot be recommended due to the LogScore values which were continually below those recommended by the manufacturer for biological validation. Thus, complete extraction methods are better suited for MALDI-TOF MS-based yeast identification in the clinical laboratory setting although they are more labor-intensive.

Keywords spectrometry, mass, matrix-assisted laser desorption-ionization, clinical laboratory techniques, fungi

Introduction

The rapid and accurate identification of yeast species is of particular importance in developing the appropriate treatment regimen. The five main Candida species accounting for about 95% of yeast infections, i.e., C. albicans, C. glabrata, C. tropicalis, C. parapsilosis and C. krusei, have distinct antifungal susceptibility profiles [1,2]. If antifungal drug resistance is rare in C. albicans clinical isolates, C. krusei is inherently resistant and C. glabrata is resistant or less susceptible to fluconazole. In addition, C. parapsilosis has elevated echinocandin minimal inhibitory concentrations (MICs) [1]. Timely initiation of an adequate treatment protocol has been associated with decreased attributable mortality in cases of candidemia [3]. In clinical laboratories, yeast identification mainly relies on conventional methods based on morphological features, latex agglutination and/or biochemical assays. These methods are labor-intensive, time-consuming (48–72 h) and sometimes lead to erroneous identifications because they are unable to differentiate species within complexes such as C. parapsilosis or C. glabrata [4,5].
While DNA sequence analysis is the gold standard for accurate species identification, it is relatively expensive, requires a turnaround of several days and is not widely available in clinical laboratories [6]. Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a powerful tool to rapidly and accurately identify bacteria and yeast. This technique analyzes cell contents to generate a mass spectral fingerprint of microorganisms. Using this technique, the identification of an unknown isolate is made by comparing its spectrum to that in a spectra reference library [7].

MALDI-TOF MS-based identification of yeast requires a pretreatment of the yeast sample before the acquisition of the spectra. The procedure recommended by the manufacturer (Bruker Daltonics GmbH, Germany) involves a complete extraction of the fungal material using formic acid and acetonitrile. In a previous work, we slightly modified this pretreatment procedure in order to adapt it to batch processing of fungal isolates to obtain identifications in the routine laboratory [8]. Other authors developed faster and simpler pretreatment procedures. Pinto et al. and Seyfarth et al. used a ‘smear procedure’ consisting in applying pieces of yeast colonies directly on the MALDI-TOF target [9,10]. Bille et al. proposed a fast formic acid extraction method consisting of covering the direct application of yeast colonies onto the MALDI-TOF target with 1 μl of formic acid [11]. Yet, the impact of pretreatment procedures on the performance of MALDI-TOF MS-based yeast identifications has not been assessed. This study aimed at comparing the performance of these four pretreatment procedures.

Materials and methods

Fungal strains

A panel of 103 clinical yeast isolates, including 27 species of eight genera, was studied. These strains were selected from the isolates recovered in 2011 at Marseille’s University Hospital and were initially identified by conventional methods (see below). Only those test isolates that were represented by at least one reference spectrum in the MaldiBioTyper (Bruker Daltonics, GmbH, Germany) reference database were included in the investigation. While between seven and 13 strains of the most frequently recovered yeast species, i.e., Candida albicans, Candida glabrata, Candida guillermondii, Candida kefyr, Candida krusei, Candida lusitaniae and Candida parapsilosis were randomly chosen for this investigation, less than five isolates of uncommon species (see Table 1) were also evaluated. Altogether, these 27 species corresponded to more than 99% of the yeast species routinely identified in our laboratory.

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>SGC medium</th>
<th>Chromogenic medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthrographis karae</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Candida catenulata</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Candida guillermondii</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Candida intermedia</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Candida kefyr</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Candida lambica</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida lipolytica</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Candida norvegensis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida orthopsilosis</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Candida palmioleophila</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Candida pararugosa</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida pelliculosa</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pichia cactophila</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Geotrichum capitatum</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Geotrichum silvicola</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rhodotorula</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>mucilaginosa</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Trichosporon inkin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>88</td>
</tr>
</tbody>
</table>

Culture

Strains were grown in parallel on Sabouraud-Gentamicin-Chloramphenicol (SGC) agar plates (Biomerieux, Marcy l’Etoile, France) and a chromogenic medium (BBL CHROMagar Candida, Beckton Dickinson, France) and incubated for 48 h at 30°C.

Conventional identification and DNA sequence-based identification of clinical strains

The conventional identification was based on phenotypic characteristics of yeast colonies on both SGC and chromogenic media. Depending on the colonies’ appearance (color, form, surface appearance), one or more latex agglutination tests (Fumouze Diagnostics, France) were
performed, i.e., BichrolatexTM albicans, Bichro-DubliTM and Krusei-ColorTM. When agglutination tests were negative, isolates were identified using biochemical assays (API ID32C, bioMérieux, France or/and AUXACOLOR 2, Bio-Rad, France). There were eight mismatches between conventional and MALDI-TOF MS-based identifications which were subjected to DNA sequencing. The DNA sequence-based identifications were performed by analyzing the ITS1-5.8-ITS2 and the D1-D2 variable regions of the 28S unit of the rRNA gene as previously described [12]. DNA extraction was performed using the QIAmp DNA kit (QIAGEN, France) and sequence reactions were run with a 3130 Genetic Analyzer (Applied Biosystems, France). Resulting sequences were then compared using the Medical Fungi pairwise sequence alignment tool (http://www.cbs.knaw.nl/Medical/BioloMICSSequences.aspx). DNA sequencing led to the re-identification of five of these eight isolates that were initially identified as C. parapsilosis, C. inconspicua and C. rugosa, as three strains of Candida orthopsilosis, one of Pichia cactophila and one strain of C. pararugosa, respectively. One strain of Geotrichum silvicola and two strains of Rhodotorula mucilaginosa could not be identified to the species level by conventional methods.

MALDI-TOF mass spectrometry

Pretreatment procedures. Four different pretreatment procedures (referred to as E1 to E4) were performed in parallel on each yeast strain after at least 48 h of growth of the colonies on both SGC and CHROMagar media. The yeast pretreatment procedure E1 corresponded to the ‘smear procedure’ described by the manufacturer (Bruker Daltonics GmbH, Germany). A thin layer of a colony was spotted onto a 96-plate polished steel target and allowed to air-dry. The E2 technique consisted of the fast formic acid extraction described by the manufacturer. A thin layer of a colony was applied on a spot of the 96-plate polished steel target and allowed to air-dry. Then the sample spot was covered by 1 μl of 70% formic acid and again allowed to air-dry. The pretreatment procedure E3 matched the complete formic acid/acetonitrile extraction procedure described by the manufacturer. A single yeast colony, gently scraped from the plate of media with a sterile plastic device, was suspended in a 1.5 ml sterile tube containing 300 μl of sterile water (HPLC Water, ProLabo BDH, France), to which was added 900 μl of anhydrous ethyl alcohol (Carlo Erba SDS, France). After 2-min centrifugation at 13,000 rpm, the pellet was resuspended in 10 μl of 70% formic acid (Sigma-Aldrich, France) and then 10 μl of 100% acetonitrile (ProLabo BDH) was immediately added. After 2-min centrifugation at 13,000 rpm, 1 μl of the supernatant was deposited onto a single spot of the target and air-dried. Procedure E4 was a modified version of the E3 technique used by Cassagne et al. to identify moulds [8]. The E4 procedure was identical to E3 except that the pellet was incubated 5 min with 70% formic acid before acetonitrile was added and incubated 5 min with acetonitrile before the centrifugation step. Finally, regardless of the procedure used, each spot was covered by 1 μl of a daily prepared solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetic acid (TFA) (Sigma-Aldrich, France) matrix.

Raw mass spectra acquisition and MALDI-TOF MS-based identification. The spectra were acquired after 240 shots in linear mode with a Microflex LT (Bruker Daltonics GmbH, Germany) 337 nm nitrogen laser in the ion-positive mode. The following adjustments were used: delay, 170 ns; ion source1 voltage, 20 kV; ion source2 voltage, 18.5 kV; mass range, 2–20 kDa. The data were automatically acquired by the AutoXecute v3.3 software (Bruker Daltonics GmbH, Germany) and exported into MaldiBiotyper v3.0 software (Bruker Daltonics GmbH, Germany) containing a 4111 entries reference database for identification. Each run included a negative extraction control and the Bruker Bacterial Standard Test calibrator (Bruker Daltonics GmbH, Germany). A calibration was performed before and after each run to detect a shift during operation. The identification result was obtained by comparing each unknown sample spectrum with the reference database, with the recorded corresponding LogScore (LS) automatically computed by the MaldiBiotyper software. The MALDI-TOF MS-based identification of a strain was considered correct if it matched with the conventional and/or the sequencing-based identification regardless the value of the LogScore, otherwise the MALDI-TOF MS-based identification was considered false. The MALDI-TOF MS identification of a strain was considered reliable if it matched with the conventional and/or the sequencing-based identification and was associated with a LogScore above 2, the threshold recommended by the manufacturer (Bruker Daltonics GmbH, Germany).

Comparison of the four yeast pretreatment procedures

The MALDI-TOF MS identifications of 103 clinical isolates using the four pretreatment procedures were compared. More specifically, the comparisons were based on: (i) the proportion of correct identifications; (ii) the proportion of reliable identification (correct identification associated with a LS value ≥2); and (iii) the LS value obtained when the identification was correct.

The proportions of correct identifications and the proportions of reliable identifications were first assessed
by the Cochran Q test. When the result of the Cochran’s Q test indicated a significant difference between the proportions obtained by the four pretreatment procedures, a post hoc analysis was conducted. This consisted of comparing, two by two, the ratios of correct identification obtained with the four pretreatment procedures using the non-parametric MacNemar’s test with Bonferroni’s adjustment for multiple comparisons.

The LS values associated with correct identification results were compared using the non-parametric Friedman’s test. When the result of the Friedman’s test indicated a statistical difference between the values of the LS obtained by the four different pretreatment procedures, a post hoc statistical analysis was conducted. The post hoc analyses consisted of pairwise comparisons of the LS values obtained with each four pretreatment procedures using the pairwise Wilcoxon’s test with Bonferroni’s adjustment.

**Statistical analysis**

All the statistical analyses were performed with the free R software (http://www.r-project.org/). All statistical tests were two-sided with a $p \leq 0.05$ significance level.

**Results**

*Comparison of the proportions of correct identifications and reliable identifications between the four pretreatment procedures*

When analyzing yeast colonies grown on the SCG medium, the proportions of correct identifications were 85.4%, 89.3%, 98%, and 97.1% with pretreatment procedures E1, E2, E3 and E4, respectively (Fig. 1A and Table 1) and differed significantly depending on the pretreatment procedure used ($p < 10^{-15}$). The post hoc pairwise comparison indicated that the performances of procedures E3 and E4 were similar ($p > 0.05$) and significantly better than the performances of E1 and E2 methods ($p < 10^{-8}$) (Fig. 1A).

The proportions of correct identifications among samples grown on the chromogenic media were 94.2, 92.2, 99 and 97.1% when using pretreatment procedures E1, E2, E3 and E4, respectively (Fig. 1B and Table 1). In this case, the Cochran’s Q test for dependent samples could not be used because expected values were <5 in some categories.

Even though most identification results were correct, they were often associated with low LogScores (i.e., <2) that did not ensure reliable identifications. Only 10.7% and 21.3% reached the reliable identification LS threshold (i.e., $\geq 2$) using E1 and E2 procedures when colonies were grown on SCG medium and 31.1% and 38.8% when they were grown on chromogenic medium. By contrast, E3 and E4 procedure displayed respectively, 79.6% and 77.7% reliable identifications on SCG medium and 77.7% and 79.6% on chromogenic medium. The proportion of reliable identifications differed statistically ($p < 10^{-15}$) between the pretreatment procedures whatever the culture medium used. The post-hoc analysis demonstrated that the performances of E3 and E4 procedures were similar ($p > 0.05$) and better than those of both E1 and E2 procedures ($p < 10^{-8}$) (Fig. 2).

Because LS $\geq 1.7$ or 1.8 has been considered as sufficiently reliable when identifying yeasts [9,16,17], the four pretreatment procedures were also compared using these thresholds. Again, procedures E3 and E4 were found similar and more reliable than E1 and E2 ($p < 10^{-10}$).

---

![Fig. 1](image-url) Barplots showing the number of correct identifications of yeasts strains cultured on Sabouraud medium (A) and on chromogenic medium (B) in function of the pretreatment procedures used. Stars indicate statistically different pairs. E1, smear procedure; E2, fast formic acid procedure; E3, complete extraction procedure; E4, modified complete extraction procedure.
The results of our study showed the usefulness of the 1.7 threshold as all LS values associated with a false MALDI-TOF MS-based identification were below 1.7, regardless of medium and pretreatment procedure.

**LogScores associated with a correct identification for each pretreatment procedure**

As shown in Fig. 3, the E3 and E4 procedures had the highest LS median values, i.e., 2.225 and 2.166 on SCG medium and 2.223 and 2.172 on chromogenic medium, respectively. The median LS values obtained with E1 and E2 procedures on SGC medium were 1.714 and 1.876, and 1.828 and 1.941 on chromogenic medium, respectively. The global Friedmann’s test used to detect differences in pretreatment procedures was statistically significant ($p < 10^{-15}$) on both chromogenic and SCG media. Thus, the LS values differed statistically between pretreatment procedures, on both SGC and chromogenic media. The post-hoc statistical analysis, used to detect differences among pretreatment procedures, demonstrated that E3 and E4 did not statistically significantly differ ($p > 0.05$) but both yielded statistically significantly better results ($p < 10^{-13}$) than those obtained with E1 and E2 procedures on both SCG and chromogenic media (Fig. 3).

**Discussion**

The results of this study clearly demonstrate that using adequate pretreatment procedures significantly enhances the results obtained with MALDI-TOF MS-based yeast identification. Indeed, the reliability of yeast identification results significantly improved by using pretreatment extraction procedures involving complete formic acid/acetonitrile methods (E3 and E4) as compared to those without (E1) or incomplete extraction (E2). The correct identifications obtained with E1 and E2 procedures were associated with significantly lower LS values than with complete extraction procedures. Remarkably, LS values above 2, as recommended by the manufacturer, were significantly more often obtained with E3 and E4 than with E1 and E2 procedures. Indeed, if one was using yeasts grown on SCG medium in a routine laboratory setting, only 10.7% or 21.3% of the identifications would have been biologically validated with the E1 or E2 procedures, respectively. In contrast, more than 77% of the isolates would have been biologically validated when using complete extraction procedures. Another problem is that E1 and E2 failed to identify all *Cryptococcus neoformans* strains, a yeast species causing severe infections in immunocompromised hosts [18]. One explanation might be that incomplete extraction methods are ineffective to lyse this encapsulated yeast.

In this study, we tested the four pretreatment procedures with a panel of yeasts that represents more than 99% of the species recovered from clinical samples in the routine laboratory setting. However, we would like to point out that the proportions between species in the panel greatly differed from those in the routine setting. For example, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* accounted for only 41.4% of the test.
isolates in the panel but these five species accounts for about 95% of the yeasts recovered from clinical specimens in a routine laboratory [1]. Rare species, such as Arthrogryphis kalrae, C. lambica or G. silvicola, were deliberately over-represented in this study to better challenge MALDI-TOF MS-based identification performances.

Several studies that evaluated the performance of routine MALDI-TOF MS yeast identification obtained correct identification rates ranging from 16% for Pinto et al. [9] to 100% [10,11,13–18]. One explanation of such variations in identification rates might be the different pretreatment procedures used in these studies. However, other factors could have played a significant role in these differing results. First, three different MALDI-TOF MS systems composed of a mass spectrometer, an analytical software and a reference database, i.e., Bruker/Biotyper [9,13,14,16–18], Shimadzu/Saramis [10,13], Shimadzu/Andromas [11] were used in the prior investigations. Second, the statistical estimation of the similarity (or similarity score) between the spectrum of a sample and a reference spectrum differ depending on the software (Biotyper, Saramis or Andromas). Third, the thresholds of similarity scores defining a correct identification also differ among studies. For instance, in the study of Dhiman et al., the identification rate increased from 92–96.3% by using a threshold ≥2 or ≥1.8, respectively [17]. Fourth, the reference spectra databases differ from software to software and from version to version of the same software. Moreover, in-house reference databases were created and/or incorporated into the integrated database of the software used in the identification. For instance, Marklein et al. added 241 reference spectra including 131 species to the Biotyper reference database [14], while Seyfarth et al. augmented the Biotyper database with 39 reference spectra [10] and Bille et al. build their own reference database [11]. Fifth, the panels of yeast species differed among investigations, with several [11,17] employing clinical isolates whereas others used a combination of reference and clinical isolates [13,18]. The panel of McTaggart et al. included more than 85% of C. neoformans isolates [18]. Because the comparison between studies is hindered by such technical and methodological heterogeneity, we tested four distinct pretreatment procedures with essentially the same identification process.

Using the direct smear procedure (E1), we obtained low identification rates of 10.7% with colonies grown on SCG medium and 31.1% when cultured on chromogenic medium. This is in keeping with Pinto et al. [9], who identified only 16% of 88 strains to the species level using the smear procedure. In fact, these authors shifted from the smear procedure to a complete extraction method during their study. In contrast, Seyfarth et al. [10] reported 94% correct identifications using the smear procedure, but with a different MALDI-TOF system (Shimadzu/Saramis), matrix (DHB) and reference spectra database.

The identification rates in our study with the fast formic acid treatment (E2) were 21.3% when SCG medium was used and 38.8% with chromogenic medium. Bille et al., with the same pretreatment procedure, obtained 98.8% of correct identifications [11], but they employed the Shimadzu/Andromas MALDI-TOF system, with a specific reference database and similarity score threshold.

Using the complete extraction procedures (E3 or E4) with interpretation criteria recommended by the manufacturer, we obtained with colonies on the SCG medium 79.6% (82/103) and 77.7% (80/103) reliable and correct identifications with E3 and E4, respectively, while on the chromogenic medium 77.7% (80/103) and 79.6% (82/103) with E3 and E4, respectively. The E4 procedure is a slightly modified version of E3 that allows processing samples in batches, and both procedures yielded similar results. The E4 procedure is more suited to the identification process.
of large series of isolates, and can advantageously be used for high-throughput yeast identification in the daily routine of clinical laboratory. The above identifications rates are in line with those of Stevenson et al. of 87.11%, 85.2% found by van Veen et al. and Pinto et al. 80.6%, all using the same interpretation criteria [9,15,16]. Our identification rates are slightly lower than those reported by Marklein et al. (92%) and McTaggart et al. (93.1%) [14,18]. However, in these previous studies, the MALDIBiotyper database had been augmented by an ‘in-house’ databases. Only Bader et al. and Dhiman et al. reported identifications of more than 90% (96.7% and 92%, respectively) without the use of an ‘in-house’ spectra database [13,17]. One explanation of this good performance could be that the tested strains included more than 80% of the five most common yeast species.

In conclusion, complete extraction procedures are better suited to MALDI-TOF-based yeast identification in the routine laboratory because the correct identification validation criteria recommended by the manufacturer are rarely fulfilled by both smear and fast formic acid extraction procedures. Further studies, directed at modifying the reference spectra database and/or the LS validation threshold, are needed to enhance yeast identification results with these fast pretreatment procedures.

Acknowledgments

The authors thank Annie Michel-Nguyen for sharing her technical expertise in yeast strains identification. No specific funding was received for this study.

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

References