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Original Article

Routine identification and mixed species detection in 6,192 clinical yeast isolates

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Abstract

The clinical laboratory methods used to diagnose yeast infections should be rapid, reliable, and capable of detecting mixed infections with species exhibiting a distinct antifungal susceptibility profile. In this study, we report the performance of a procedure combining the detection of mixed yeast cultures with a chromogenic medium and MALDI-TOF identification of the colonies. We then evaluated the impact that (i) the isolation medium and (ii) lowering the identification log score (LS) threshold value have on yeast identification performance in the routine laboratory.

Among 15,661 clinical samples analyzed, 5,671 tested positive and 6,192 yeasts of 42 distinct species were identified. Overall, 6,117 isolates (98.79%) were identified on the first or second MALDI-TOF Mass Spectrometry (MS) attempt, yielding an average yeast species identification turnaround time of 0.346 days (95% CI [0.326 to 0.364]). The 75 remaining isolates were identified via nucleotide sequencing. Mixed infections accounted for 498 (8.78%) of the positive samples. The MALDI-TOF MS identification procedure performed well, regardless of the culture media tested. Lowering the recommended 2.0 LS threshold value to 1.8 would reduce the number of required (i) second MALDI-TOF MS identification attempts (178 vs. 490) and (ii) ITS2 and D1-D2 sequence-based identifications (17 vs. 75), while achieving an adequate identification rate (6,183/6,192, 99.85%).

In conclusion, we propose applying a 1.8 LS threshold combined with chromogenic medium subculture to optimize the yeast identification workflow and detect mixed infection in the clinical laboratory.

Key words: yeast, identification, clinical laboratory, MALDI-TOF Mass Spectrometry, identification threshold, chromogenic media, mixed fungal infection, fungi, diagnostic.

Introduction

A growing number of patients are at risk of developing yeast infections with high morbid-mortality rates. A recent French population-based study estimated candidemia incidence to be 2.5 /100,000 person per year, with a 40% case fatality rate.[1] The incidence of candidemia increased by 7.8% each year from 2001 to 2010. In contrast, the fatality rate decreased by 1.6% per year.[1] Due to distinct patterns of antifungal susceptibility among yeast species, rapid and accurate identification of such pathogens is critical for patient care. While most *Candida albicans* strains are susceptible to available systemic antifungal drugs, *C. glabrata*, *C. krusei*, and other emerging species, such as *C. inconspicua*, *C. rugosa*, and *C. norvegensis*, exhibit decreased susceptibility to azoles.[2] *Candida parapsilosis* and rarer species/genera such as *Trichosporon, Rhodotorula, Saccharomyces,* and *Geotrichum* display elevated minimum inhibitory concentrations to echinocandins.[2,3] Mycological analysis of clinical samples should also reliably detect mixed fungal species, especially those involving yeasts species with a particular antifungal resistance profile. Indeed, mixed yeast infections have been detected in 2% to 9.3% of fungemia episodes[4,5] and in approximately 13% of vaginal samples.[6] Among mixed fungemia, *C. albicans* is commonly associated with *C. glabrata* and *C. parapsilosis*.[4] If a mixed yeast infection remains undetected, the patient may be exposed to inappropriate antifungal treatment.

MALDI-TOF Mass Spectrometry (MS) technology improves both reliability and rapidity of fungal identification.[7] The efficiency of chromogenic media for mixed culture detection has been demonstrated in blood culture[4] or vaginal[6] samples. However, the efficiency of combining both techniques for the detection and identification of mixed fungal culture has never been evaluated on a comprehensive set of clinical samples. Furthermore, no data are available on the impact of various culture media commonly used in the clinical laboratory practice, including chromogenic media, on the performance of MALDI-TOF MS yeast identification. Since 2012, we have implemented a routine identification procedure combining the use of chromogenic medium subculture and MALDI-TOF MS-based identification. We evaluated this approach on a prospective series of 6200 yeasts prospectively isolated from clinical samples analyzed over the course of one year.

Methods

Clinical specimens and yeast strains

All clinical specimens and yeast strains submitted for fungal culture and/or identification and/or susceptibility testing to the Mycological Laboratory of Marseilles were prospectively included from April 14, 2012, to April 14, 2013. The identification procedure was performed using the MALDI-TOF-based identification procedure as described below and summarized in Figure 1. The clinical samples were plated on Sabouraud gentamicin chloramphenicol (SGC) agar (Oxoid, Dardilly, France) and incubated at 30°C for at least 5 d and up to 28 d for deep-seated specimens. Cultures were examined daily for fungal growth. As soon as fungal colonies were detected, they were subjected to MALDI-TOF MS-based identification as described below. *In vitro* antifungal susceptibility testing was carried out if necessary. The clinical yeast strains received from other laboratories were immediately identified via MALDI-TOF MS, regardless of the culture medium. Each time that at least 10 colonies grew on the primary culture (SGC or bacteriological media), we sampled several colonies from the primary culture and cultured this mix on a chromogenic medium to detect mixed infections. Each colony exhibiting distinct morphology and/or color on the chromogenic medium was identified via MALDI-TOF MS and further subjected to *in vitro* susceptibility testing.

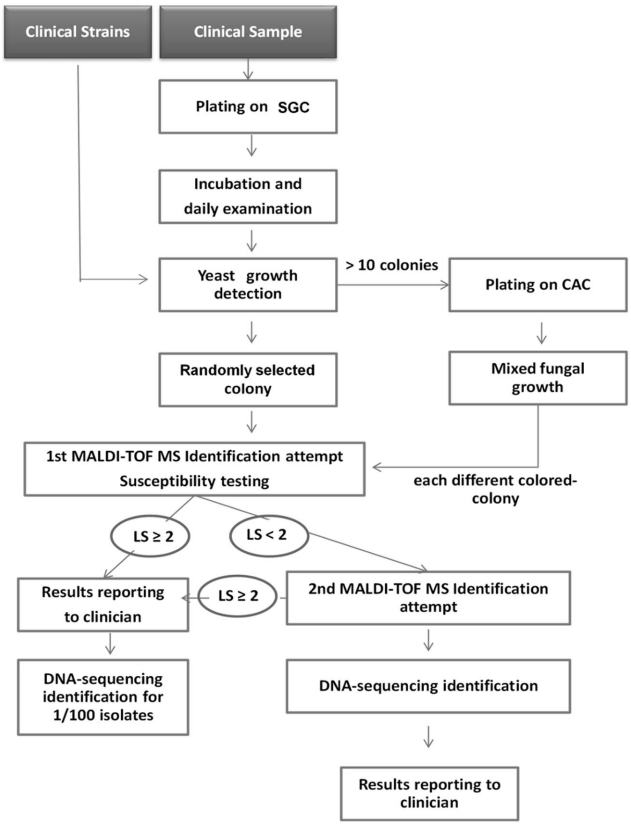

Figure 1. Identification procedure applied on prospectively collected clinical samples over the course of one year. (SGC: Sabouraud gentamycin chloramphenicol agar; CAC: CHROMAgar *Candida* agar; LS: MALDI Biotyper log score.)

MALDI-TOF MS identification

For each sample, one yeast colony was gently scraped with an inoculation loop and suspended in a 1.5-ml sterile tube containing 300 μl of sterile water (Water HPLC, Prolabo BDH, Fontenay-sous-Bois, France) and 900 μl of anhydrous ethyl alcohol (Carlo Erba SDS, Val de Reuil, France). After a 2-min centrifugation step at 13,000 rpm, the pellet was resuspended in 10 μl of 70% formic acid (Sigma-Aldrich, Lyon, France). After approximately 5 minutes of incubation, 10 μl of 100% acetonitrile (Prolabo BDH) was added. The suspension was then centrifuged at 13,000 rpm for 2 min, and 1 μl of supernatant was deposited onto the target and air-dried. One microliter of matrix, a solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid (solution prepared daily) (Sigma-Aldrich, Saint Quentin Fallavier, France), was deposited onto each spot and air-dried. MALDI-TOF MS spectra were then acquired using a Microflex LT^{TM} Instrument (Bruker Daltonics GmbH, Bremen, Germany) after 240 shots in linear mode with the ion-positive mode and a 337 nm nitrogen laser. The following adjustments were used: delay, 170 ns; ion source1 voltage, 20 kV; ion source2 voltage, 18.5 kV; and mass range, 2–20 kDa. The data were automatically acquired using the AutoExecute application of the FlexControl v3.3 software (Bruker Daltonics GmbH). The data were then exported into the MALDI Biotyper v3.0 software (Bruker Daltonics GmbH) containing the 4111-entry reference version of the Bruker Daltonics database and supplemented with our in-house yeast reference spectra for identification. The in house-database included 162 reference spectra acquired from 96 isolates belonging to 33 different yeast species, of which 25 reference spectra were acquired from 17 isolates belonging to 10 different species that were not represented in the Bruker Database. The remaining 23 species were represented in the Bruker Database but required reference spectra supplementation to improve the quality of identification (Table 1). Each run included a negative extraction control and the Bruker Bacterial Standard Test calibrator (Bruker Daltonics GmbH). Calibration was verified daily using the Bruker Bacterial Standard Test, and the MS instrument was recalibrated only when necessary as described by the manufacturer. Each unknown sample spectrum was compared with the reference database, and the corresponding log score (LS) were automatically computed using the MALDI Biotyper software (Bruker Daltonics GmbH), which then recorded the identification results. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as MALDI-TOF MS identification internal quality controls.

The MALDI-TOF MS-based identification of a strain was considered final if the LS measured $\geq$2, that is, the species identification threshold recommended by the manufacturer (Bruker Daltonics GmbH). Samples with an identification LS < 2 were extracted and subjected to a second extraction and MALDI-TOF identification. For each isolate, we analyzed the influence on the LS value of the culture media, the presence of mixed species in the clinical sample, and the identification time (defined as the lag time from growth detection to final identification).

Table 1. Composition of the in-house database.

Species	Nbr. of references	Nbr. of strains	Present in the Bruker database
Arxula adeninivorans	3	2	yes
Candida albicans	47	26	yes
Candida blankii	2	2	no
Candida bracarensis	6	3	no
Candida ciferrii	2	1	yes
Candida dubliniensis	7	4	yes
Candida famata	2	2	no
Candida glabrata	40	20	yes
Candida inconspicua	2	2	yes
Candida nivariensis	2	1	yes
Candida orthopsilosis	6	3	yes
Candida palmioleophila	1	1	yes
Candida parapsilosis	2	1	yes
Candida pararugosa	2	1	yes
Candida sorboxilosa	1	1	no
Candida tropicalis	2	1	yes
Cryptococcus albidus	4	4	yes
Cryptococcus difluens	1	1	no
Debaryomyces hansenii	1	1	yes
Gallactomyces geotrichum	3	2	yes
Geotrichum capitatum	1	1	yes
Hanseniaspora guilliermondii	2	1	yes
Kluyveromyces marxianus	4	2	yes
Kodamaea ohmeri	2	2	no
Pichia caribbica	5	3	no
Pichia fabianii	2	1	no
Pichia jadinii	1	1	no
Pichia manshurica	1	1	yes
Pichia rhodanensis	1	1	yes
Saccharomyces cerevisiae	2	1	yes
Sporopachydermia lactativora	2	1	no
Torulaspora delbrueckii	1	1	yes
Trichosporon asahii	2	1	yes
Total	162	96	–

Note: Nbr.: number.

Nucleotide sequence-based identification

Nucleotide sequence-based identification was systematically performed for isolates displaying an LS < 2 at the second identification attempt. From each set of 100 isolates identified via MALDI-TOF MS with a LS >2, one

isolate was randomly selected for internal quality control nucleotide sequence-based identification confirmation. Nucleotide sequence-based identification was performed by analyzing the ITS2 and the D1-D2 variable region of the 28S unit of the rRNA gene as previously described.[8] DNA was extracted using the QIAamp DNA kit (QIAGEN, Courtaboeuf, France), and sequencing reactions were carried out using a ABI3130 × l Genetic Analyzer (Applied Biosystems, Villebon Sur Yvette, France). The resulting sequences were then queried against the—CBS-KNAW Fungal Biodiversity Centre—Yeast database and the ISHAM-ITS reference DNA barcoding[9] databases, both accessed via the MycoBank website (www.mycobank.org). In cases of discrepancy between MALDI-TOF MS and DNA identification results, the latter was considered the gold standard.

Statistical analysis

All statistical tests were computed using R software (http://www.r-project.org) using two-sided tests with a 0.05 significance level. The mixed culture proportions, according to the type of clinical sample, were tested using the equality of independent proportions test (prop.test function). The influence of culture medium and mixed culture on the MALDI-TOF MS LS value was tested using the non-parametric Kruskal-Wallis test.

Determination of the optimal log score threshold (LS-T)

The optimal LS threshold (LS-T) was determined from the results of the first MALDI-TOF MS identification attempt. Therefore, we tested the influence of increasing the LS threshold by units of 0.1, from 1.5 to 2, on the rates of correct identification, misidentification, and lack of MALDI-TOF identification. We then calculated the positive predictive value of the identification results, that is, the proportion of correctly identified isolates among those that yielded a LS>LS-T. These indicators are better suited than other diagnostic indices, such as specificity and sensitivity, to evaluate the microorganism identification process in the routine clinical laboratory. The proportion of correct identifications was calculated as the percentage of isolates that yielded a LS>LS-T and were correctly identified. The proportion of misidentification was calculated as the percentage of isolates that both yielded a LS>LS-T and exhibited discrepancies, compared to the DNA identification results. The percentage of no MALDI-TOF identification was calculated as the percentage of isolates yielding a LS < LS-T. In such cases, nucleotide sequence-based identification was required, according to the evaluated identification procedure.

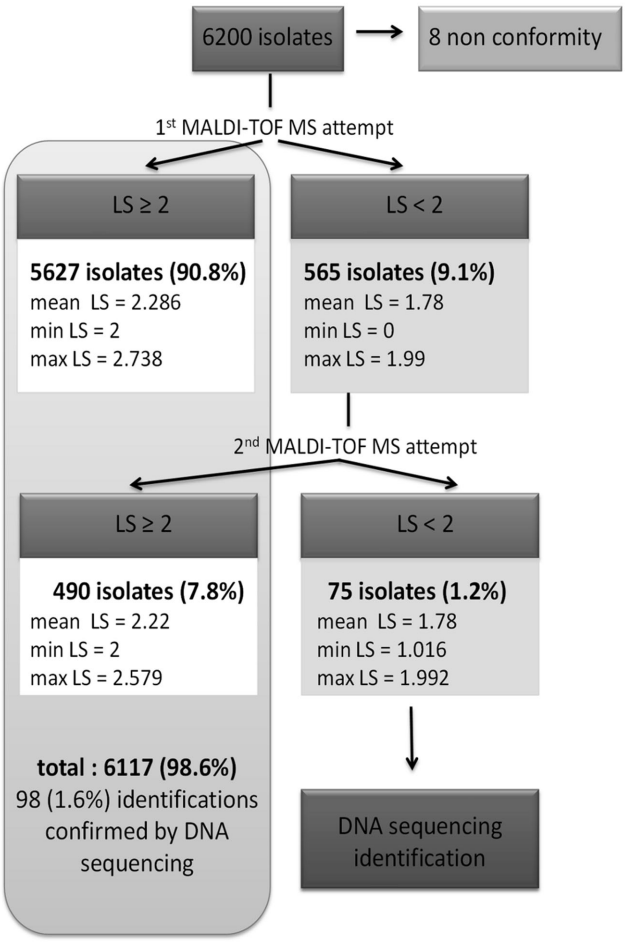

Figure 2. Flow chart of the routine MALDI-TOF MS-based clinical yeast identification results obtained over the course of one year. (LS: MALDI Biotyper log score.)

Results

Study flow

During the one-year study period, our laboratory received 15,661 clinical samples, from which 6,200 yeasts were isolated from 5,679 (36.2%) samples. These 6,200 yeast isolates were subjected to our identification procedure (Figure 2). As the procedure was not entirely respected for eight isolates, which were therefore excluded from the study, a total of 6,192 yeast isolates recovered from 5,671 samples were included in the study. The details of these non-conformities were (i) absence of nucleotide sequence-based identification for seven isolates for which the MALDI-TOF MS identification was $1.9 \leq LS < 2$ for the two sequential MALDI-TOF MS attempts) and (ii) lack of a second MALDI-TOF MS attempt in one *Rhodotorula mucilaginosa* isolate identified via nucleotide sequence analysis. The 6,192 yeast isolates were grouped into 42 different yeast species (Table 2). *Candida albicans* was the most frequently recovered species (68.6%), followed by *C. glabrata*

Table 2. Summary of the identification results obtained using our procedure.

		First MS[a] identification attempt		After two MS identification attempts		DNA-I[d]	
Species	N	LS1[b]>2	Mean LS1 [min–max]	LS2[c]>2	Cumulative%	QC[e]	R[f]
Arxula adeninivorans	7	4	1.884 [0.8252.308]	2	85.71	0	1
Candida albicans	4251	3990	2.263 [0–2.738]	253	99.81	37	8
Candida bracarensis	4	4	2.334 [2.123–0.626]	0	100.00	1	0
Candida ciferrii	2	0	1.845 [1.828–1.861]	2	100.00	2	0
Candida famata	1	1	2.413 [2.413–2.413]	0	100.00	0	0
Candida metapsilosis	5	2	1.918 [1.703–2.259]	0	40.00	2	3
Candida nivariensis	1	0	1.706 [1.706–1.706]	0	0.00	0	1
Candida palmioleophila	1	1	2.185	0	100.00	1	0
Candida pruinosa	1	0	1.945	0	0.00	0	1
Candida solani	1	1	2.246	0	100.00	0	0
Candida catenulata	3	3	2.210 [2.079–2.318]	0	100.00	2	0
Candida colliculosa	1	1	2.249	0	100.00	1	0
Candida dubliniensis	134	74	2.015 [0–2.616]	50	92.54	4	10
Candida glabrata	631	609	2.358 [0–2.718]	22	100.00	10	0
Candida guilliermondii	24	14	1.955 [0–2.331]	6	83.33	2	4
Candida inconspicua	7	7	2.313 [2.043–2.430]	0	100.00	2	0
Candida intermedia	1	1	2.162	0	100.00	1	0
Candida kefyr	85	76	2.208 [1.723–2.555]	7	97.65	3	2
Candida krusei	154	145	2.24 [1.647–2.56]	9	100.00	2	0
Candida lipolytica	3	1	2.054 [1.883–2.366]	2	100.00	0	0
Candida lusitaniae	54	43	2.06 [0.863–2.381]	9	96.30	2	2
Candida norvegensis	2	2	2.22 [2.187–2.254]	0	100.00	2	0
Candida orthopsilosis	27	17	2.03 [1.424–2.334]	6	85.19	1	4
Candida parapsilosis	310	252	2.099 [0–2.424]	47	96.45	5	11
Candida rugosa	2	0	1.453 [1.051–1.854]	1	50.00	1	1
Candida tropicalis	331	282	2.135 [0.759–2.458]	38	96.68	6	11
Galactomyces geotrichum	21	9	1.981 [1.638–2.214]	10	90.48	0	2
Geotrichum candidum	1	0	1.943	0	0.00	0	1
Geotrichum capitatum	21	18	2.07 [1.182–1.317]	3	100.00	1	0
Geotrichum silvicola	15	14	2.111 [1.834–2.34]	1	100.00	0	0
Pichia cactophila	10	6	1.987 [1.303–2.298]	3	90.00	6	1
Pichia caribbica	4	1	1.543 [1.19–2.137]	1	50.00	2	2
Pichia fabianii	1	1	2.29	0	100.00	1	0
Pichia manshurica	2	1	1.792 [1.545–2.04]	0	50.00	1	1
Pichia rhodanensis	2	1	1.579 [0.882–2.275]	0	50.00	0	1
Rhodotorula dairenensis	1	0	1.778	0	0.00	0	1
Rhodotorula mucilaginosa	5	3	1.99 [1.387–2.334]	1	80.00	0	1
Saccharomyces cerevisiae	50	30	2.015 [1.38–2.385]	16	92.00	0	4
Trichosporon asahii	12	12	2.241 [2.021–2.403]	0	100.00	0	0
Trichosporon famata	1	0	1.913	1	100.00	0	0
Trichosporon inkin	1	0	1.663	0	0.00	0	1
Trichosporon faecalis	1	0	0.899	0	0.00	0	1

[a]MS: MALDI-TOF MS.
[b]LS1: log score recorded on the first MALDI-TOF MS attempt.
[c]LS2: log score recorded on the second MALDI-TOF MS attempt.
[d]DNA-I: DNA sequence-based identification.
[e]QC: quality control.
[f]R: required.

Table 3. Effect of the culture media on the MALDIBiotyper log(score) values.

Culture media	MALDIBiotyper log(score) values			
	Range	Mean	Median	Interquartile range
Sabouraud (SGC)	0.000 to 2.673	2.204	2.245	2.084 to 2.365
Chocolate agar	0.000 to 2.656	2.271	2.288	2.188 to 2.398
Blood agar	0.759 to 2.667	2.268	2.271	2.170 to 2.389
CHROMagar *Candida*	0.000 to 2.738	2.244	2.260	2.128 to 2.386
Other miscellaneous media	1.190 to 2.624	2.230	2.257	2.111 to 2.389

(10.2%), *C. tropicalis* (5.3%), *C. parapsilosis* (5%), and *C. krusei* (2.5%). These five species represented more than 90% of the yeast isolates. Furthermore, the average time lag from growth detection to species identification was 0.346 days (standard deviation = 0.77 days; 95%CI [0.326 to 0.364]).

Mixed fungal culture detection

Mixed yeast samples were cultured from 498 (8.78%) of the 5,671 culture-positive samples. Of the 498 samples, 476 contained two species, 21 contained three species, and one contained four species. Finally, a total of 1,019 yeasts grew in the mixed yeast cultures. The most common species combinations were either *C. albicans* combined with *C. glabrata* (36.95%), *C. tropicalis* (7.83%), *C. krusei* (6.02%), or *C. parapsilosis* (5.42%) or rather *C. glabrata* combined with *C. tropicalis* (5.22%). Considering only bloodstream, implanted material, and deep-seated samples, 16 distinct yeast species combinations were observed, among which *C. albicans*-*C. glabrata* and *C. albicans*-*C. krusei* represented 69% of all combinations. The clinical sample type significantly ($p < 0.001$) influenced the occurrence of mixed yeast cultures. Mixed cultures occurred more frequently in respiratory and stool samples (13.2%), followed by ear, nose, and throat (ENT) samples (12.5%), deep-seated samples (7.5%), urinary samples (5.5%), implanted material samples (4.4%), vaginal samples (2.4%), and blood culture samples (1.4%).

Effect of media on MALDI-TOF MS identification

Overall, 6,192 isolates were identified via MALDI-TOF MS on various solid culture media, including SGC (n = 2,412), Columbia blood agar (n = 1,667), chocolate agar (n = 1,365), CHROMagar™ Candida (n = 654), and other miscellaneous culture media (n = 94). The culture medium significantly influenced the LS values ($P < .005$) as detailed in Table 3. Although the spectra included in the spectral reference library had been acquired on SGC, the LS values acquired from colonies grown on SGC (mean = 2.204) were significantly ($P < .007$) lower than those grown on chocolate agar, blood agar or CHROMAgar.

Procedure evaluation

Of the 6,192 isolates, 5,627 (90.7%) were identified at the species level (LS ≥ 2) on the first MALDI-TOF attempt. A total of 490 additional isolates were identified on the second attempt. Ninety-eight (1.6%) of these isolates were subjected to nucleotide sequence-based identification for MALDI-TOF MS identification quality control; both identifications were concordant in 100% of the samples (Table 4). For 75 (1.21%) of the 6,192 isolates, the LS values remained <2 on the second MALDI-TOF MS attempt. These 75 isolates were identified via DNA sequence-based identification according to the evaluated identification procedure. All these isolates were ultimately identified, with a mean identification time of 19.1 days (standard deviation [SD] = 9.83 days, 95% confidence interval [CI] [16.9 to 21.4]) (Table 4).

Cryptic yeast species such as *C. orthopsilosis* and *C. metapsilosis* within the *C. parapsilosis* complex were unambiguously differentiated. However, only two of the five *C. metapsilosis* samples were correctly identified via MALDI-TOF MS, while the other three isolates required nucleotide sequencing for definitive identification. Within the *C. glabrata* complex, *C. glabrata* and *C. bracarensis* identification was successful in 96.5% and 100% of samples, respectively; a single *C. nivariensis* isolate was not identified. The biochemically similar *C. guilliermondii*, *C. famata*, and *C. palmioleophila* were unambiguously identified via MALDI-TOF MS in 83.3%, 100%, and 100% of respective cases. The closely related species *C. albicans* and *C. dubliniensis* were the more difficult to distinguish. Indeed, 7.46% of the *C. dubliniensis* isolates required nucleotide sequencing for proper identification due to low LS value associated with the *C. albicans* identification results. Six isolates of six different species could not be identified via MALDI-TOF MS. Three of these six species, namely, *C. pruinosa*, *Rhodotorula dairenensis*, and *Trichosporon faecale*, were not included in the Bruker Daltonics database, and only one or two references of the three

Table 4. Identification results of the 173 sequenced isolates.

	MALDI-ToF MS identiffication failure (LS<2)				IQC with MALDI-ToF MS LS≥2		
Species	n	LS1 range	Concordant MS and DNA based identification	Discordant identification LS1 values	n	LS1 range	Concordant MS and DNA based identification
Arxula adeninivorans	1	[1.871–1.871]	1 (100%)	–	0	–	–
Candida albicans	8	[1.233–1.998]	8 (100%)	–	37	[2.006–2.592]	100%
Candida bracarensis	0	–	–	–	1		100%
Candida catenulata	0	–	–	–	2	[2.234–2.318]	100%
Candida ciferii	0	–	–	–	2	[2.464–2.54]	100%
Candida colliculosa	0	–	–	–	1	[2.249–2.249]	100%
Candida dubliniensis	10	[0.878–1.982]	8 (80%)	1.449; 0.878	4	[2.034–2.425]	100%
Candida glabrata	0	–	–	–	10	[2.043–2.618]	100%
Candida guilliermondii	4	[0–1.735]	0 (0%)	0; 1.514; 1.66; 1.735	2	[2.128–2.293]	100%
Candida inconspicua	0	–	–	–	2	[2.043–2.165]	100%
Candida intermedia	0	–	–	–	1	[2.162–2.162]	100%
Candida kefyr	2	[1.723–1.943]	2 (100%)	–	3	[2.053–2.307]	100%
Candida krusei	0	–	–	–	2	[2.253–2.309]	100%
Candida metapsilosis	3	[1.703–1.9]	3 (100%)	–	2	[2.018–2.259]	100%
Candida nivariensis	1	[1.706–1.706]	1 (100%)	–	0	–	–
Candida norvegensis	0			–	2	[2.187–2.254]	100%
Candida orthopsilosis	4	[1.841–1.998]	4 (100%)	–	1	[2.028–2.028]	100%
Candida palmioleophila	0	–	–	–	1	[2.185–2.185]	100%
Candida parapsilosis	11	[1.772–1.997]	11 (100%)	–	5	[2.069–2.261]	100%
Candida prunicola	1	[1.945–1.945]	1 (100%)	–	0	–	–
Candida rugosa	1	[1.051–1.051]	1 (100%)	–	1	[2.196–2.196]	100%
Candida tropicalis	11	[1.416–1.996]	11 (100%)	–	6	[2.05–2.27]	100%
Clavispora lusitaniae	2	[0.863–1.673]	2 (100%)	–	2	[2.106–2.117]	100%
Galactomyces geotrichum	2	[1.638–1.886]	2 (100%)	–	1	[2.138–2.138]	100%
Geotrichum candidum	1	[1.943–1.943]	1 (100%)	–	0	–	–
Pichia cactophila	1	[1.992–1.992]	1 (100%)	–	6	[2.092–2.206]	100%
Pichia caribbica	2		1 (50%)	1.19	2	[2.137–2.183]	100%
Pichia fabianii	0	–	–	–	1	[2.29–2.29]	100%
Pichia manshurica	1	[1.545–1.545]	1 (100%)	–	1	[2.04–2.04]	100%
Pichia rhodanensis	1		0	0.882	0	–	–
Rhodotorula dairensis	1	[1.778–1.778]	0	1.778	0	–	–
Rhodotorula mucilaginosa	1	[1.387–1.387]	1 (100%)	–	0	–	–
Saccharomyces cerevisiae	4	[1.38–1.953]	4 (100%)	–	0	–	–
Trichosporon faecale	1	[0.899–0.899]	0	0.899	0	–	–
Trichosporon inkin	1	[1.663–1.663]	1 (100%)	–	0	–	–

Note: IQC: internal quality control; LS: MALDI Biotyper log score; MS: mass spectrometry; n: number; LS1: MALDI Biotyper log score obtained at the first MALDI-TOF MS identification attempt.

others species, *C. nivariensis*, *Geotrichum candidum*, and *Trichosporon inkin*, were included in the Bruker Daltonics database.

Optimization of the log score threshold (LS-T)

Lowering the LS-T from 2 to 1.5 yielded the following results: (i) an increase (from 98.79% to 99.53%) in accepted identification rates, (ii) a 0.35% increase in misidentification rates, and (iii) a decrease (from 75 to 7) in required nucleotide sequence-based identifications. The rates of accepted identification, correct identification, misidentification, positive predictive value, and number of required nucleotide sequence-based identifications are summarized in Table 5.

Using a 1.8-LS-T would result in nine misidentifications. In seven cases, these misidentifications occurred in species that are difficult to differentiate, even on chromogenic media. In particular three *Candida dubliniensis* were misidentified as *C. albicans*, one *C. albicans* was misidentified as *C. dubliniensis*, one *Pichia caribbica w*as misidentified as *C. kefyr*, one *Galactomyces geotrichum* was misidentified as

Table 5. MALDI-TOF MS identification performance according to the log score threshold (LS-T) value tested on 6,192 clinical yeast strains.

	No. of accepted MALDI-TOF MS identifications			Correct identifications	False identifications		
LS-T[a]	1st run	2nd run	Total	(%)	(%)	PPV[b]	DNA-I[c] required
1.5	6,125	60	6,185	6,163 (99.53%)	22 (0.35%)	0.996	7 (0.11%)
1.6	6,106	78	6,184	6,164 (99.55%)	20 (0.32%)	0.996	8 (0.13%)
1.7	6,070	111	6,181	6,165 (99.56%)	16 (0.26%)	0.997	11 (0.18%)
1.8	5,997	178	6,175	6,166 (99.58%)	9 (0.15%)	0.998	17 (0.27%)
1.9	5,878	284	6,162	6,157 (99.43%)	5 (0.08%)	0.999	30 (0.52%)
2	5,627	490	6,117	6,117 (98.79%)	0 (0%)	1	75 (1.21%)

[a]LS-T: log score threshold.
[b]PPV: positive predictive value of a correct identification.
[c]DNA-I: DNA sequence-based identification.

Geotrichum silvicola, and one *Geotrichum silvicola* was misidentified as *Galactomyces geotrichum*. The remaining two misidentifications were one *C. albicans* misidentified as *C. parapsilosis* with a 1.97-LS and one *C. tropicalis* misidentified as *C. dubliniensis* with a 1.83-LS. These two samples displayed less than 10 colonies and were thus not subcultured on chromogenic medium according to the procedure. These two discrepancies are more likely to result from an undetected mixed culture rather than a true misidentification. The relatively high LS values for such obvious misidentifications are in agreement with this hypothesis.

Discussion

To our knowledge, our study involves the identification of the largest clinical yeast series with the widest diversity of 42 distinct species using MALDI-TOF MS. This identification procedure, combining chromogenic media subculture and MALDI-TOF MS technology, was particularly efficacious in detecting mixed yeast cultures in clinical samples. We acknowledge the limitation that misidentifications could not be formally ruled out for the 6,019 isolates for which MALDI-TOF MS identification was not verified via nucleotide sequencing because the LS value was ≥2. Nevertheless, LS values ≥ 2 are widely considered to yield reliable results,[10–12] and an internal quality control procedure was used to confirm MALDI-TOF MS identification accuracy. Furthermore, performing a nucleotide sequence-based identification for each isolate would have drastically increased the study costs.

In this study, mixed cultures occurred in 8.78% of the positive samples. The sample type significantly influenced the mixed yeast culture rate. Mixed cultures occurred more frequently in respiratory, stool, or ENT samples than in blood, deep-seated samples, or implanted material, thereby reflecting the normal fungal flora of human mucous membranes. As the majority of the detected species combinations involved *C. albicans* together with azole-resistant species, such as *C. glabrata* and *C. krusei*, mixed culture rates as high as 1.4% in blood cultures, 4.4% in implanted material, and 7.5% in deep-seated samples is a significant issue in clinical practice. Detecting these mixed infections clearly depends on the type of culture media. In an 11-year study, no mixed fungemia were detected before the implementation of a chromogenic medium culture.[5] Moreover, Okulicz et al.[13] have detected 18.3% mixed cultures in urine samples using chromogenic medium compared with 1.92% without using chromogenic medium. Indeed, mixed culture were detected in 20% of the cultures displaying LS values ≥1.6 and <2 on the first MALDI-TOF MS attempt. In this case, subculturing and re-isolating the yeast colonies, which have grown on the primary culture, on a chromogenic medium should be mandatory. The main pitfall of this approach concerns the discrimination of the closely related species *C. dubliniensis* and *C. albicans*. Indeed, the *C. albicans* identifications that displayed low LS values and were ultimately identified as *C. dubliniensis* via nucleotide sequence-based analysis are likely to be mixed cultures, which were not detected due to the suboptimal differentiation of both species on available chromogenic media.

Interestingly, using this procedure, yeast colonies were identified on any current culture medium, which is of great interest for clinical laboratories. The significantly lower LS values observed with SGC medium is likely to be associated with a confounding factor. Indeed, in this study, the clinical strains cultured on chocolate or blood agar, originated from other microbiology laboratories and were thus more likely to be identified after a longer delay than those directly cultured on SGC medium in our laboratory. A possible explanation, which cannot be assessed in this study, might

be that colonies grow faster on bacteriological media than SGC.

Overall, 98.6% of 6,192 yeast isolates were identified on the first or second MALDI-TOF MS attempt. This result correlates with the correct identification rates, ranging from 97.5% to 98.2%, reported in other large series.[12,14,15] In correlation with previous reports,[14–16] MALDI-TOF MS enabled accurate discrimination between closely related species and species complexes, including the *C. parapsilosis*, *C. glabrata*, and *Meyerozyma guilliermondii* complexes. Using MALDI-TOF MS, we were able to unambiguously identify yeast species that could not be identified via the conventional auxanogram-based methods, such as *Arxula adeninivorans*, *Candida solani*, *C. palmioleophila*, *C. intermedia*, *Pichia cactophila*, *P. caribbica*, *P. fabianii*, *P. manshurica*, or *P. rhodanensis*. MALDI-TOF MS identification failed only when the species was absent or poorly represented in the reference spectra database. In this group of 98.6% isolates that were only identified via MALDI-TOF MS, identification results were mainly available the same day when colony growth was detected. Such reduced turnaround time is in agreement with a one-day shorter identification time using MALDI-TOF MS compared with conventional methods.[17] The improved performance and flexibility of MALDI-TOF MS identification and the reduced turnaround time, 0.346 days compared with at least 2 days using conventional methods, is unquestionably a major step forward for the routine clinical laboratory.[10] In contrast, the observed 19-day average turnaround time of nucleotide sequence-based identification is clearly incompatible with the objectives of a clinical laboratory oriented toward the diagnosis of patients with invasive fungal disease and clearly needs to be improved.

MALDI-TOF matched with the correct species in 65 of the 75 isolates subjected to nucleotide sequence analysis, according to the manufacturer's procedure because they were considered non-identified by MALDI-ToF because the LS was less than 2. Therefore, lowering the LS identification threshold would effectively reduce the number of required nucleotide sequence-based identifications in the routine clinical laboratory and, as a consequence, reduce identification turnaround time. A LS-T value of 1.8 was associated with a very low rate of non-accepted assays, with a lower LS value. Using a 1.8-LS-T, nucleotide sequencing would have only been required for 17 isolates and nine isolates would have been misidentified. Among these misidentifications, two occurred in samples in which less than 10 yeast colonies grew and were thus not subcultured on chromogenic medium according to the procedure (*C. albicans* was misidentified as *C. parapsilosis* with a 1.97 LS, and *C. tropicalis* was misidentified as *C. dubliniensis* with a 1.83 LS). These latter two discrepancies more likely result from an undetected mixed culture rather than a true misidentification. In keeping with this hypothesis, the LS values were relatively high for such gross misidentifications. Nevertheless, none of these nine misidentifications would have negatively impacted patient care or, in particular, antifungal treatment recommendations.

We are aware that misidentifications may have occurred among isolates with a LS >2 that were not sequenced. However, according to the current literature, misidentifications with LS above 2 are unusual.[10–12] Cassagne et al. have correctly identified 102 out of 103 yeasts, and the only misidentification was associated with LS below 1.5. Indeed, the 98 isolates with LS >2, randomly selected for identification verification via sequencing, were all correctly identified.

Importantly, our findings highlight the capacity to identify a yeast colony as soon as it grows, which represents a major advance in diagnostics afforded by MALDI-TOF MS identification methods. Further studies should focus on reducing diagnosis lag time by speeding up and/or by passing the cultivation steps, which remain unacceptably lengthy, particularly for fungal pathogens. The use of non-selective media may result in the non-identification of a mixed yeast culture. Overcoming this pitfall remains a major challenge in the clinical practice. Antifungal therapy targeted against one pathogen may select another, which was already present at the start of therapy and will either remain undetected or detected too late, thus seriously jeopardizing patient outcome.

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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