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Development and Validation of an In-House Database for Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry-Based Yeast Identification Using a Fast Protein Extraction Procedure

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In recent studies evaluating the usefulness of the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)-based identification of yeasts for the routine diagnosis of fungal infections, preanalytical sample processing has emerged as a critical step for reliable MALDI-TOF MS outcomes, especially when the Bruker Daltonics Biotyper software was used. In addition, inadequate results often occurred due to discrepancies between the methods used for clinical testing and database construction. Therefore, we created an in-house MALDI-TOF MS library using the spectra from 156 reference and clinical yeast isolates (48 species in 11 genera), which were generated with a fast sample preparation procedure. After a retrospective validation study, our database was evaluated on 4,232 yeasts routinely isolated during a 6-month period and fast prepared for MALDI-TOF MS analysis. Thus, 4,209 (99.5%) of the isolates were successfully identified to the species level (with scores of ≥2.0), with 1,676 (39.6%) having scores of >2.3. For the remaining 23 (0.5%) isolates, no reliable identification (with scores of <1.7) was obtained. Interestingly, these isolates were almost always from species uniquely represented or not included in the database. As the MALDI-TOF MS results were, except for 23 isolates, validated without additional phenotypic or molecular tests, our proposed strategy can enhance the rapidity and accuracy of MALDI-TOF MS in identifying medically important yeast species. However, while continuous updating of our database will be necessary to enrich it with more strains/species of new and emerging yeasts, the present in-house MALDI-TOF MS library can be made publicly available for future multicenter studies.

To date, literature-based evidence has accumulated with respect to the reliability of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the identification of yeast isolates in diagnostic clinical microbiology laboratories (1–10). As already shown with bacteria, MALDI-TOF MS identifies yeasts with rapidity, accuracy, and superiority over conventional phenotypic methods (for review, see references 11–15).

As an alternative to the ethanol/formic acid-based procedure, also referred to as complete tube extraction, recommended for use only with the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany), the on-plate extraction or fast formic acid method (4, 16, 17), which consists of covering the smeared yeast colony with a formic acid solution, was proposed. Using the Bruker Biotyper 3.0 database and spectral scores of ≥1.7 as cutoffs for species-level identification, Theel et al. found that the formic acid-based direct on-plate method yielded identification percentages that were similar to those obtained with the more complex tube-based extraction method (18). Nonetheless, Van Herendael et al. showed that MALDI-TOF MS analysis using the short extraction method is suitable for the rapid identification of yeast isolates but that its use necessitates the identification threshold to be lower (<1.7) to account for the lower scores originating from the method (19). Therefore, it was speculated that the highest concordance between acquired spectra and those included in the reference library—in terms of high-confidence (percentage or score) identification—is achievable only if the sample preparation procedure employed for the MALDI-TOF MS system at hand does not differ from that employed to construct the system's reference library (11, 12).

To address this issue, we used a previously established protocol for fast fungal protein extraction (20)—a slight modification from the formic acid-based method—to develop a spectral database for the rapid and unambiguous identification of medically important yeasts by MALDI-TOF MS. First, we retrospectively evaluated the performance of this database by comparing it with the Bruker Biotyper library (V3.2.1.1) and using a clinical collection of well-characterized yeast isolates ($n = 100$) that underwent the fast preparation method. Second, we evaluated our database prospectively by testing freshly collected yeast isolates which were recovered during 6 months of routine laboratory workflow ($n = 4{,}232$) and prepared with the above method. Results for the isolates successfully identified (with scores of ≥2.0) were validated without

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additional phenotypic tests; for only a minority of them (with scores of <1.7) was further molecular analysis undertaken.

MATERIALS AND METHODS

Yeasts used in this study. A total of 156 yeast isolates (representing 48 species of 11 genera) comprising 14 reference strains obtained from the American Type Culture Collection (ATCC) and the National Institutes of Health (NIH) and 142 clinical strains mostly obtained from the stock collections of the Università Cattolica del Sacro Cuore (UCSC) (Rome, Italy), the Università degli Studi di Milano (Milan, Italy), and the New Jersey Medical School (Newark, NJ) were used to construct the UCSC yeast database (Table 1; see also Table S1 in the supplemental material). Except for the reference strains, the isolates included in the library were selected, randomly whenever possible, to cover the diversity of the yeast species routinely encountered in many hospital settings and were identified as described below. For the retrospective part of the study, a collection of 100 medically important yeast and yeast-like isolates obtained from UCSC patients was established to include 14 *Candida* species, *Cryptococcus neoformans*, and 4 species in the genera *Geotrichum*, *Rhodotorula*, and *Saccharomyces* (Table 2). For the second part of the study, a set of 4,232 yeasts isolated in a 6-month period (March 2012 to August 2012) from UCSC patients were prospectively analyzed, as described below. All patients' isolates were recovered from clinical specimens obtained from deep sites (blood, cerebrospinal fluid, and peritonea), from the respiratory, gastrointestinal, and genitourinary tracts, and from the skin or superficial lesions. Except for 4,232 isolates that were tested fresh from clinical cultures, all other isolates were retrieved from frozen (−70°C) storage. In both cases, isolates were subcultured on Sabouraud dextrose agar (SDA) (Kima, Padua, Italy) to ensure viability, purity, or sufficient growth before testing; if a pure culture was obtained from the primary SDA plate, it was analyzed directly. All isolates were processed for mass spectrometric analysis within 24 to 48 after visible growth at 35°C.

MALDI-TOF MS sample preparation, spectral analysis, and database generation. For MALDI-TOF MS identification, isolates were prepared using a short extraction method as previously described (20). Briefly, cells from a single colony on an SDA plate were suspended in 50 μl of 10% formic acid and vortexed, and 1 μl of the lysate was used for the analysis. Alternatively, the complete formic acid/acetonitrile extraction procedure was performed as described by the manufacturer. Following preparation, each sample was analyzed, in duplicate, with the MALDI Biotyper software package (version 3.0) on a Microflex LT mass spectrometer (Bruker Daltonics) as previously described (20).

To create the UCSC yeast database, spectra were generated from the 156 reference isolates processed with the short extraction method, and for each isolate, 10 replicate spectra were required for the entry of that isolate into the database and for the creation of a mass spectral profile (MSP). The relationships between the replicates of each set of spectra were estimated by the composite correlation index analysis, where values of around 1 represent a high conformance of the spectra and values near 0 indicate clear diversity of the spectra (21). Indeed, each database entry was generated as a composite of 10 spectra, which were imported into the MALDI Biotyper software to produce a list of the most significant peaks in terms of average masses, average intensities, and relative frequencies (1). As approximately 10 min per each database entry was needed, the entire library construction process was completed within 3 working days.

By means of the pattern-matching process resulting in logarithmic scores of 0 to 3, the spectra of 100 challenge isolates were analyzed against the Bruker Biotyper V3.2.1.1 database alone (4,110 entries), the UCSC yeast database alone, and the UCSC and Bruker databases combined (4,266 entries). The supplemented database was challenged against the spectra of 4,232 isolates from the prospective study. According to the manufacturer, a score of ≥2.0 was recorded as identification to the species level, and a score of ≥1.7 was recorded as identification to the genus level. However, in our final evaluation, only species identifications with scores

TABLE 1 Fungal isolates used to construct the UCSC yeast database

Genus	Species	Total no. of isolates included (no. of reference strains)[a]
Blastobotrys	*adeninivorans*	2
Candida	*albicans*	14 (1)
	bracarensis	2
	catenulata	1
	dubliniensis	4
	glabrata	6
	guilliermondii	3
	inconspicua	3
	kefyr	1
	krusei	8 (1)
	lambica	1
	lusitaniae	5
	metapsilosis	5 (1)
	nivariensis	3
	norvegensis	2
	orthopsilosis	5 (1)
	palmioleophila	1
	parapsilosis	11 (1)
	pararugosa	2
	pelliculosa	2
	rugosa	2
	sorbosa	1
	sphaerica	1
	tropicalis	9
	utilis	1
Cryptococcus	*neoformans*	17 (2)
	gattii	8 (7)
Exophiala	*dermatitidis*	2
Geotrichum	*candidum*	2
	capitatum	2
	gigas	1
	silvicola	1
Lodderomyces	*elongisporus*	1
Pichia	*caribbica*	1
	manshurica	1
	membranifaciens	1
	onychis	1
	terricola	1
Rhodotorula	*dairenensis*	1
	mucilaginosa	1
Trichomonascus	*ciferrii*	1
Trichosporon	*asahii*	2
	asteroides	1
	coremiiforme	1
	debeurmannianum	1
	inkin	4
	mucoides	1
Saccharomyces	*cerevisiae*	9

[a] A total of 156 isolates were represented in the database. Details about the isolates, including the reference strains, are listed in Table S1 in the supplemental material.

TABLE 2 MALDI-TOF MS identification scores for 100 clinical *Candida* and non-*Candida* isolates challenged against the UCSC yeast and the Bruker Biotyper databases

Organism	No. (%) of isolates identified with the indicated MALDI-TOF MS database[a]					
		UCSC by scores of[b]:		Bruker by scores of:		
	Total	≥2.0	≤1.99 and ≥1.7	≥2.0	≤1.99 and ≥1.7	<1.7
C. albicans	24	24 (100)	0 (0)	1 (4.2)	19 (79.2)	4 (16.6)
C. glabrata	21	21 (100)	0 (0)	4 (19.0)	9 (42.9)	8 (38.1)
C. guilliermondii	2	2 (100)	0 (0)	1 (50.0)	1 (50.0)	0 (0)
C. inconspicua	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. kefyr	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. krusei	10	10 (100)	0 (0)	2 (20.0)	6 (60.0)	2 (20.0)
C. lusitaniae	2	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)
C. nivariensis	2	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)
C. norvegensis	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
C. metapsilosis	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
C. orthopsilosis	3	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)
C. parapsilosis	10	10 (100)	0 (0)	0 (0)	8 (80.0)	2 (20.0)
C. pelliculosa	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
C. tropicalis	13	12 (92.2)	1 (7.8)	1 (7.8)	6 (46.1)	6 (46.1)
C. neoformans	2	2 (100)	0 (0)	0 (0)	1 (50.0)	1 (50.0)
G. capitatum	2	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)
G. silvicola	1	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)
R. mucilaginosa	1	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)
S. cerevisiae	2	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)
Total	100	99 (99.0)	1 (1.0)	11 (11.0)	54 (54.0)	35 (35.0)

[a] For identification, isolates were all treated with the fast extraction protocol prior to MALDI-TOF MS analysis.
[b] No isolates yielded spectral scores of <1.7 with the UCSC yeast database.

of >2.0 were regarded as correct, whereas the proposed genus-level identifications were refused.

Molecular identification. All 156 isolates included in the UCSC yeast database, the 100 isolates from the retrospective study, and any other study isolates for which a definitive species identification needed to be achieved or randomly confirmed were characterized molecularly by sequencing the ITS1-5.8S-ITS2 ribosomal DNA (rDNA) region and, when necessary, the 28S ribosomal subunit gene region, as previously described (22).

RESULTS

Construction of in-house database for fast MALDI-TOF MS yeast identification. First, we evaluated the reliability of a short extraction formic acid-based method for MALDI-TOF MS sample preparation that was previously employed to identify *Cryptococcus* isolates to the species and subspecies levels (20). By ensuring the removal of extracellular and other potentially inhibiting compounds (11), this method generated a good number of spectral peaks for fungal species discrimination and, in the meantime, decreased the time for MALDI-TOF MS identification compared to that for the complete extraction method (data not shown).

Then, samples from 156 yeast isolates of reference and clinical strain collections were similarly processed to create an in-house MALDI-TOF MS "fast" yeast library, namely, the UCSC yeast database (Table 1). For each isolate, an MSP was generated through the accumulation of ≥5,000 laser shots from 10 technical replicates of the same spectrum, and each MSP was checked for specificity before entry into the database. Thus, while composite correlation index analysis provided high similarity values (≥0.9) (data not shown) between the spectra composing each isolate's MSP, the specificities of individual MSPs were assessed by matching all spectra from the 156 isolates against those in our user-modified Bruker-UCSC database, i.e., the Biotyper database V3.2.1.1 supplemented with in-house reference spectra generated in this study (for a total of 4,266 MSPs). The 156 spectra matched to an excellent degree (with scores of ≥2.5) with their own corresponding MSPs, whereas no cross-identifications were observed at a score of ≥2, and scores between 1.7 and 1.9 resulted from the matching of newly created MSPs with those from the unmodified (original) Bruker database with respect to the same isolates (data not shown).

Validation of the fast yeast database. To validate the UCSC yeast database, 100 isolates from the clinical strain collection of the UCSC Microbiology Institute (Table 2) were prepared for MALDI-TOF MS analysis using the short (fast) and the complete extraction methods, as detailed above. Overall, two series of samples from 14 *Candida* species (26% *Candida albicans* and 74% non-*albicans Candida* species) and eight non-*Candida* species (from the genera *Cryptococcus*, *Geotrichum*, *Rhodotorula*, and *Saccharomyces*) were analyzed by MALDI-TOF MS. When isolates were used to challenge the UCSC yeast database (Table 2), the fast method yielded correct identifications to the species level (with scores of ≥2.0) for 99 (99%) of the isolates; while one isolate (*Candida tropicalis*) was acceptably identified to the genus level (with a score of ≥1.7 but <2.0), 50 (50%) of these isolates had scores of >2.3, and 49 (49%) of them had scores ranging from 2.0 to 2.3 (data not shown). In contrast, when the same 100 spectra were analyzed against the Bruker database alone (Table 2), only 11 isolates (11%) were identified to the species level (with scores of ≥2.0), and another 54 isolates (54%) were identified to the genus level; the remaining 35 isolates (35%) failed to provide reliable identification (with scores of <1.7), despite having representative spectra in the Bruker library. As expected, using the Bruker data-

base, the complete extraction applied to the 100 isolates increased the average score by 0.502 (from 1.745 to 2.247) score units compared to the fast method, and this resulted in 90% correct identifications to the species level, with 43% of 90 isolates having scores of >2.3 and 47% scores between 2.0 and 2.3, whereas 10% of the isolates were identified to the genus level (data not shown).

Prospective evaluation of the fast yeast database for routine MALDI-TOF MS analysis. During the 6 months of this prospective study, 4,232 clinical isolates of yeasts were routinely identified by MALDI-TOF MS using the fast method and the UCSC yeast database. By applying a species-level identification cutoff score of 2.0, we obtained 4,043 (95.5%) correct identifications and 189 (4.5%) nonidentifications (Table 3). Among the isolates identified correctly, 39.6% had scores of >2.3. Spectra that did not yield a score of ≥2.0 were reanalyzed after they were manually acquired, or a second acquisition of spectra was performed with the same isolates allowed to grow for another 24 h prior to being retested. In particular, the spectra yielding scores in the range of ≥1.7 to <2.0 were from isolates that, in the first run, were mostly identified as *Candida parapsilosis* (47 isolates), *C. albicans* (42 isolates), *C. tropicalis* (27 isolates), *Saccharomyces cerevisiae* (21 isolates), *Candida krusei* (10 isolates), or *Candida norvegensis* (10 isolates) (Table 3). After the second run, 166 out of 189 correct identifications were obtained, leaving only 23 (0.5%) cases with no reliable identification (with scores of <1.7) (Table 3). When identified by rDNA sequencing, these isolates were almost shown to belong to species having unique representative spectra in the UCSC yeast database (Table 1) or to species not included in the database (11 isolates) (Table 3). After the spectra from the 11 isolates were subsequently added to the UCSC yeast database, they were found to match, as expected, with their own corresponding spectra contained in the database. Overall, 99.5% of the yeasts were identified (4,209/4,232) (Table 3), and this percentage increased to 99.7% (4,209/4,221) when the species included only in the UCSC yeast database were considered.

DISCUSSION

Although *Candida albicans* remains the leading cause of invasive fungal infections from yeasts (23), infections due to non-*albicans Candida* species or other unusual opportunistic pathogens have been increasingly reported (24, 25) as a result of growing populations of immunocompromised and otherwise compromised patients (26, 27). Such a diversified landscape of emerging fungi poses a diagnostic challenge when traditional methods are used, since they often fail to identify uncommon fungal species (28). Over the past few years, MALDI-TOF MS appeared to modify this scenario (15), leading to substantial improvement of fungal diagnostics (29) and patient treatment (30).

In the present study, we sought to further enhance the rapidity and accuracy of the Bruker MALDI-TOF MS system in identifying common and uncommon yeast species. First, to decrease the time needed for the preanalytical sample processing (in part shared by other investigators [18, 19, 31]) but to also preserve the quality of the mass spectra for subsequent MALDI-TOF MS analysis, we adopted a preparation method that is a just compromise between two extreme procedures, complete protein extraction and direct colony transfer. Then, the same method was utilized during the in-house database construction process.

After a validation step using 100 well-characterized relevant fungal organisms, we found that 4,209 of 4,232 (99.5%) yeasts, consecutively isolated under routine clinical conditions, were successfully identified by MALDI-TOF MS, with 1,676 isolates yielding scores much higher than the reliability cutoff score recommended by Bruker Daltonics for species-level identification

TABLE 3 Performance of the UCSC yeast database for *Candida* and non-*Candida* clinical isolates prospectively tested by MALDI-TOF MS analysis[a]

Organism	No. of isolates tested	No. of isolates with score of:		
		>2.0[b]	≥1.7 but <2.0[c]	<1.7[d]
Species included in the database				
C. albicans	2,924	2,882	42	0
C. glabrata	642	639	3	0
C. parapsilosis	210	163	47	0
C. tropicalis	203	176	27	0
S. cerevisiae	178	157	21	0
C. krusei	18	8	10	0
C. norvegensis	12	2	10	0
C. dubliniensis	5	5	0	0
C. kefyr	5	2	2	1
C. neoformans	5	4	1	0
C. guilliermondii	3	2	0	1
C. nivariensis	3	2	0	1
T. inkin	3	1	1	1
C. lusitaniae	2	0	1	1
C. lambica	1	0	0	1
C. pararugosa	1	0	0	1
C. utilis	1	0	0	1
G. candidum	1	0	0	1
L. elongisporus	1	0	0	1
P. caribbica	1	0	0	1
P. membranifaciens	1	0	0	1
T. asteroides	1	0	1	0
Total	4,221	4,043	166	12
Species not included in the database				
Arxiozyma telluris	1	0	0	1
Candida aaseri	1	0	0	1
Candida blankii	1	0	0	1
Candida famata	1	0	0	1
Candida lipolytica	1	0	0	1
Candida sloffiae	1	0	0	1
Malassezia pachydermatis	1	0	0	1
Pichia fabianii	1	0	0	1
Rhodotorula glutinis	1	0	0	1
Rhodotorula sloffiae	1	0	0	1
Williopsis sp.	1	0	0	1
Total	11	0	0	11
Overall species	4,232	4,043	166	23
ID (%) at the first run		95.5	4.0	0.5
ID (%) at the second run		99.5	0	0.5

[a] Isolates were treated with the fast extraction protocol prior to MALDI-TOF MS analysis for identification.
[b] A total of 1,676 (39.6%) isolates with scores of ≥2.3 (high-probable species identification by Bruker Daltonics) were from *C. albicans* (1,046 isolates), *C. glabrata* (491 isolates), *C. parapsilosis* (35 isolates), *C. tropicalis* (46 isolates), *S. cerevisiae* (32 isolates), *C. krusei* (8 isolates), *C. norvegensis* (2 isolates), *C. dubliniensis* (5 isolates), *C. kefyr* (2 isolates), *C. neoformans* (4 isolates), *C. guilliermondii* (2 isolates), and *C. nivariensis* (2 isolates).
[c] Isolates were all identified to the species level after the second run of MALDI-TOF MS analysis.
[d] Isolates were all identified by the rDNA sequence-based method.

(>2.0). To prove the correctness of these identifications, selected isolates were subjected to rDNA sequencing, and in all cases, sequence-based analyses confirmed the MALDI-TOF MS results (data not shown). Thus, our MALDI success rate was superior to that reported by Theel et al. on 90 clinical yeast isolates studied (18). In that study, using the on-target formic acid extraction method and their own laboratory-validated MALDI-TOF cutoff scores (≥1.5 and ≥1.7, respectively), 86 (95.6%) isolates were correctly identified to the genus level, and 73 (81.1%) isolates were identified to the species level (18). Although these percentages were comparable to those found by Dhiman et al. when the same isolates were previously processed by the complete extraction method (3), it is intriguing that only 82.2% and 41.1% correct genus or species identifications were achieved, in that study, when Theel et al. applied the manufacturer's recommended MALDI-TOF cutoff scores (≥1.7 and ≥2.0, respectively) (18).

In line with our data, in a very recent evaluation of the Bruker Biotyper and Andromas (the latest MALDI-TOF MS system commercially available together with the Vitek MS system [11, 12]), both systems accurately identified 98.3% of 1,383 *Candida* isolates routinely collected in French hospital laboratories (7). However, since the fungal cell is lysed directly on a MALDI plate in the Andromas system, the necessity of performing a time-consuming extraction step by the Biotyper protocol might disadvantage the Bruker system in routine laboratory practice (7). To this regard, we showed that the time required for the analysis of a batch of 96 samples (48 yeast colonies tested in duplicate)—i.e., the time calculated from scraping single colonies grown on the SDA plates to species identification results—was <1 h, compared to the ~2 h needed with the Bruker method.

As the UCSC yeast database might not be exhaustive, we plan to continuously update this reference library with more representative isolates by exploiting the intrinsic expandability of the Bruker Biotyper software. This property has been experimented satisfactorily in our previous studies (20, 32, 33), including a very recent study that evaluated comparatively the Bruker Biotyper and the Vitek MS systems (10). Besides the high accuracy shared by these two systems in identifying the most common yeasts isolated in clinical settings, we showed that the Vitek MS system yielded a higher rate of major errors (i.e., false classifications) for rarer *Candida* and non-*Candida* species due to its nonexpandable database (10). Thus, failing to identify or misidentifying new and emerging strains/species due to the lack of corresponding spectra in the database may be resolved without difficulty using the Bruker Biotyper software, provided that an incautious enrichment of databases (e.g., performed in small but not centralized laboratories) does not generate erroneous microbial identifications (34). In addition, utilizing an expanded library would allow laboratories to drastically abate the number of cases for which no spectra are present in the database at the time of analysis. Herein, only 23 isolates (11 from species not included in the database) remained unresolved by MALDI-TOF MS analysis, which made it necessary to resort to more laborious molecular-based identifications.

Lastly, our findings parallel those recently reported by Lau et al. (35) and, unlike those from other studies (4, 36), suggest that a strategy that uses modified cutoff scores should be avoided, given that this also implies the manual validation of the identification outputs after the application of additional criteria, i.e., a certain number of database hits on a single species at the top or a certain difference in the score to the next species (11). In contrast, using the original cutoff scores might ensure that the time saved during sample preparation is not spent later in the analyses manually validating and might prevent the use of interpretative algorithms (36), which require at least three consecutive database hits and then do not work for fungal species (especially rare species) with fewer spectra archived in the database (11).

In summary, our data suggest that the use of a simplified pretreatment protocol coupled with the fast yeast database developed *ad hoc* may represent a powerful strategy for making more accurate and rapid the MALDI-TOF MS-based identification of yeast species of medical importance. Once our database is made publicly available for future multicenter studies to allow its wide-scale adoption, we feel that laboratories with limited mycological expertise will benefit from this database, thereby bolstering the role of the clinical mycology laboratory in assisting clinicians in the diagnosis of fungal diseases.

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