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Reagent-Free Identification of Clinical Yeasts by Use of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

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ABSTRACT Invasive fungal infections by opportunistic yeasts have increased concomitantly with the growth of an immunocompromised patient population. Misidentification of yeasts can lead to inappropriate antifungal treatment and complications. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy is a promising method for rapid and accurate identification of microorganisms. ATR-FTIR spectroscopy is a standalone, inexpensive, reagent-free technique that provides results within minutes after initial culture. In this study, a comprehensive spectral reference database of 65 clinically relevant yeast species was constructed and tested prospectively on spectra recorded (from colonies taken from culture plates) for 318 routine yeasts isolated from various body fluids and specimens received from 38 microbiology laboratories over a 4-month period in our clinical laboratory. ATR-FTIR spectroscopy attained comparable identification performance with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). In a preliminary validation of the ATR-FTIR method, correct identification rates of 100% and 95.6% at the genus and species levels, respectively, were achieved, with 3.5% unidentified and 0.9% misidentified. By expanding the number of spectra in the spectral reference database for species for which isolates could not be identified or had been misidentified, we were able to improve identification at the species level to 99.7%. Thus, ATR-FTIR spectroscopy provides a new standalone method that can rival MALDI-TOF MS for the accurate identification of a broad range of medically important yeasts. The simplicity of the ATR-FTIR spectroscopy workflow favors its use in clinical laboratories for timely and low-cost identification of life-threatening yeast strains for appropriate treatment.

KEYWORDS ATR-FTIR, Fourier transform infrared spectroscopy, MALDI-TOF mass spectrometry, attenuated total reflectance, clinical yeasts, identification, rapid, reagent-free, routine

Invasive fungal infections (IFIs) by opportunistic yeasts in humans have increased over the years largely due to the concomitant growth of an immunocompromised patient population (1). Although *Candida albicans* is the leading cause of IFIs, emerging rare and nonalbicans *Candida* (NAC) species are on the rise (1, 2). *C. albicans* accounts for approximately 50% of all IFIs, followed by NAC, namely, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*, in decreasing order of infection frequency (1, 3, 4). Other yeasts, such as *Cryptococcus* spp., *Rhodotorula* spp., *Trichosporon* spp., and *Saccharomyces* spp., are also increasingly reported to cause infections but are far less common (5). The problem of IFIs is further compounded with the advent of multidrug-resistant (MDR) yeasts, such as *C. auris*, which has emerged rapidly worldwide and now poses a threat to public health (6–8). The correct identification of

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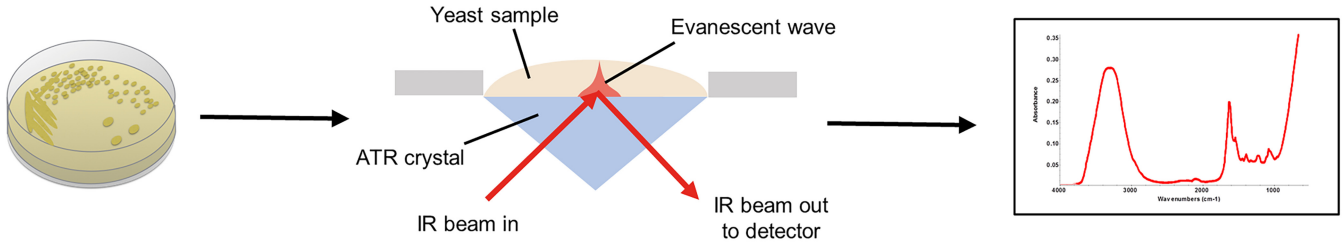

FIG 1 Experimental workflow of the ATR-FTIR spectroscopy-based method for yeast analysis. A single colony is directly transferred from the agar plate (without prior treatment) onto the ATR sampling surface of the ATR-FTIR spectrometer. An infrared (IR) beam is directed into the ATR crystal, resulting in the generation of an evanescent wave perpendicular to the propagating IR beam within the crystal. Attenuation of the evanescent wave by the sample yields the ATR-FTIR spectrum in ~1 minute. Identification of the sample is based on its spectral similarity to the infrared spectrum of an isolate in the ATR-FTIR spectral reference database.

rare and emerging yeast species is necessary for adequate antifungal therapy but can be challenging with the conventional identification systems used in most clinical microbiology laboratories.

Routine identification of clinical yeasts most often relies on the use of manual or automated commercial carbon-assimilation-based identification systems, such as the API 20 C gallery, Vitek 2, and Phoenix, which are used in conjunction with conventional biochemical and phenotypic assays. While gene sequencing methods are considered the gold standard and the future of infection control in clinical microbiology, they are costly, time-consuming, and not readily available for routine identification at most clinical sites (9). More recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has proven to be the most rapid and cost-efficient method for the identification of rare and cryptic yeast species (5, 10, 11).

Fourier transform infrared (FTIR) spectroscopy is a well-established analytical technology that has been used in various fields of study, including chemistry, forensics, ecology, medicine, astronomy, and more (12–14). In microbiology, the infrared (IR) spectrum of a microorganism is acquired directly from intact cells taken from a culture plate and is representative of their biochemical composition. As such, IR spectroscopy is referred to as a whole-organism fingerprinting technique (15).

While infrared spectra of microorganisms are commonly acquired in the conventional transmission mode, sample preparation can be greatly simplified by using the attenuated total reflectance (ATR) mode of spectral acquisition. In the ATR mode, the IR beam from the IR source is launched into an IR-transparent ATR crystal (made of a high-refractive-index material, such as zinc selenide, germanium, or diamond) at a defined angle (exceeding the critical angle for internal reflection) whereby total internal reflection occurs within the crystal. Under these conditions, an evanescent wave (perpendicular to the propagating IR beam) forms at the surface of the ATR crystal and decays exponentially with distance from the surface. Interaction of the evanescent wave with a sample placed on the surface of the crystal results in partial attenuation of the totally internally reflected IR beam at the wavelengths at which the sample absorbs IR energy (Fig. 1). An IR spectrum of the sample is then obtained by measuring the intensity of the totally internally reflected beam reaching the detector as a function of wavelength (14).

A number of studies have demonstrated the capabilities of FTIR spectroscopy for clinical bacterial identification with promising results to the species and strain levels, including having the potential for discrimination between antibiotic-resistant and -susceptible strains (16–20). Previous studies on fungal identification utilizing FTIR spectroscopy have mostly been restricted to applications in food and environmental microbiology rather than clinical microbiology (21, 22). A few studies have investigated the use of FTIR spectroscopy for the discrimination of yeast species, but those studies have relied on limited numbers of clinical isolates and species and were mostly aimed toward a general exploration of FTIR technology for identification purposes (13, 15, 23, 24).

The objective of this prospective study was to evaluate an ATR-FTIR spectroscopy-based method for routine identification of medically important clinical yeasts. This necessitated construction of a comprehensive ATR-FTIR spectral reference database of clinically relevant yeasts. The ATR-FTIR spectroscopy-based method was validated prospectively by acquiring ATR-FTIR spectra of clinical yeasts obtained from 38 hospitals. Identification of the routine yeast samples was based on matching the ATR-FTIR spectra of the clinical isolates against the reference spectra in the spectral database. The overall performance of our ATR-FTIR spectroscopic method was then compared with that of MALDI-TOF MS identification of the same clinical isolates.

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MATERIALS AND METHODS

Construction of the ATR-FTIR spectral reference database of clinically relevant yeast species. A total of 263 pure yeast isolates representing 65 species belonging to 12 genera were employed in construction of the spectral reference database (see Table S1 in the supplemental material). All isolates were from clinical specimens and had been previously identified at the Laboratoire de Santé Publique du Québec (LSPQ) and stored in 10% glycerol at −80°C. The identification of all isolates was confirmed at LSPQ by MALDI-TOF MS and/or gene sequencing of the ribosomal DNA (rDNA) D1/D2 or internal transcribed spacer (ITS) regions (using NL1-NL4 or ITS1-ITS4 primers, respectively) and comparing sequence similarity to that of reference sequences in GenBank, International Society of Human and Animal Mycology (ISHAM) ITS, and the Westerdjik Fungal Biodiversity Institute nucleotide databases. Each sample was thawed and subcultured onto Sabouraud dextrose agar (SAB; BD Difco, Franklin Lakes, NJ) and incubated at 30°C for 48 h followed by subculturing using the same parameters prior to spectral acquisition. Samples that displayed various colony morphologies or colors on culture plates were rejected to prevent incorporation of contaminated or mixed isolates in spectral database construction. An initial spectral database of commonly obtained routine opportunistic yeast species (e.g., *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*) was constructed using a minimum of 8 isolates per species. For uncommon species (e.g., *Candida duobushaemulonii*, *Cryptococcus laurentii*, and *Trichosporon inkin*), 1 to 6 isolates per species were obtained and added to the initial spectral database. Subsequently, an expanded spectral reference database was created comprising a total of 789 ATR-FTIR spectra, corresponding to triplicate spectra of the 263 isolates referred to above.

Identification of routine clinical yeast isolates. Three hundred eighteen fresh routine clinical isolates (from skin, blood, urine, and other specimens) were prospectively collected from 38 clinical microbiology laboratories over a 4-month period and sent to LSPQ for identification by MALDI-TOF MS (Vitek MS; bioMérieux, Marcy-l'Étoile, France) using the clinical knowledge database (v3.0). These samples are independent from those used to create our spectral reference database and were identified by the ATR-FTIR-based method developed in this study in parallel with MALDI-TOF MS identification by taking colonies from the same agar plate. All samples that were suspected of being contaminated were subcultured and their ATR-FTIR spectra were reacquired in triplicate; in addition, colonies from the same agar plate were identified by MALDI-TOF MS for confirmation. In the case of discordant results between the ATR-FTIR and the MALDI-TOF MS identification, the samples were further analyzed by rDNA D1/D2 or ITS sequencing using the same primers as mentioned above.

ATR-FTIR spectral acquisition. A single colony was isolated using a sterile disposable loop and deposited directly onto the ATR sampling surface of an ATR-FTIR spectrometer (Cary 630; Agilent Technologies, Santa Clara, CA) (Fig. 1). A spectrum was immediately collected by coaddition of 64 scans in the spectral range between 4,000 and 650 cm^{-1} with a spectral resolution of 8 cm^{-1} and ratioed against a background spectrum previously collected from the clean sampling surface. Following spectral acquisition, disinfection of the ATR sampling surface was achieved by wiping it with lint-free paper moistened with 70% ethanol. Triplicate spectra were acquired from three individual colonies per agar plate to verify spectral reproducibility and sample purity, as described below. The triplicate spectra were employed for the construction of the spectral reference database or the identification of routine clinical yeast isolates.

Spectral quality assessment, processing, and analysis. The reproducibility of the triplicate ATR-FTIR spectra acquired for each isolate was examined as a spectral quality check (prior to incorporation into the spectral reference database or prior to identification) by employing their proximity in a dendrogram produced by hierarchical cluster analysis (HCA) of spectral data as a measure of spectral similarity. The spectra were then subjected to vector normalization and their 1st derivatives computed by an in-house-written software or with commercially available spectral analysis software (OMNIC; Thermo Fisher Scientific, Madison, WI). Principal-component analysis (PCA) was used in conjunction with a forward region selection algorithm to discriminate among classes (genera and species) based on selection of specific spectral regions in the ATR-FTIR spectra for each classification (25, 26).

ATR-FTIR spectroscopy-based identification strategy. The spectral reference database was constructed with 789 spectra acquired from 65 species belonging to 12 genera. A hierarchical identification strategy was employed, whereby identification is performed in two stages, namely, (i) at the genus level and (ii) at the species level. Differentiation among genera and among the species within each genus is based on spectral differences (reflecting differences in the biochemical and metabolic profiles) of the

TABLE 1 Identification of routine clinical yeasts by the ATR-FTIR method using the preliminary and expanded spectral databases

Microorganism	No. (%) of isolates by database						
		Preliminary			Expanded		
	Total	Identified	Unidentified	Misidentified	Identified	Unidentified	Misidentified
Candida spp.							
C. albicans	130	128 (98.5)	0	2[a]	130 (100)	0	0
C. dubliniensis	13	13 (100)	0	0	13 (100)	0	0
C. glabrata	68	67 (98.5)	1	0	68 (100)	0	0
C. guilliermondii	2[b]	0 (0.00)	2[b]	0	1 (100)	0	0
C. kefyr	2	1 (50.0)	1	0	2 (100)	0	0
C. krusei	8	8 (100)	0	0	8 (100)	0	0
C. lipolytica	1	0 (0.00)	1	0	1 (100)	0	0
C. lusitaniae	16	16 (100)	0	0	16 (100)	0	0
C. orthopsilosis	4	4 (100)	0	0	4 (100)	0	0
C. parapsilosis	32	32 (100)	0	0	32 (100)	0	0
C. pararugosa	1	0 (0.00	1	0	1 (100)	0	0
C. pelliculosa	1	0 (0.00)	1	0	1 (100)	0	0
C. tropicalis	23	21 (91.3)	1	1[c]	22 (95.7)	0	1[c]
C. utilis	3	0 (0.00)	3	0	3 (100)	0	0
Cryptococcus neoformans	7	7 (100)	0	0	7 (100)	0	0
Meyerozyma caribbica[b]	NA[d]				1 (100)[b]	0	0
Rhodotorula mucilaginosa	1	1 (100)	0	0	1 (100)	0	0
Saccharomyces cerevisiae	5	5 (100)	0	0	5 (100)	0	0
Trichosporon spp.	1	1 (100)[e]	0	0	1 (100)[e]	0	0
Total	318	304 (95.6)	11 (3.5)	3 (0.9)	317 (99.7)	0	1 (0.3)

[a]Both misidentified isolates identified as *C. dubliniensis*.
[b]One of the 2 unidentified isolates of *C. guilliermondii* was later identified as *Meyerozyma caribbica* by gene sequencing. This isolate was omitted from calculation and was later correctly identified with the use of the expanded database.
[c]Same isolate of *C. tropicalis* was misidentified as *C. lusitaniae* with the use of both the preliminary and the expanded database.
[d]NA, not applicable.
[e]*Trichosporon* spp. are underrepresented with less than 4 isolates per species. The isolates were correctly identified at the genus level and later identified as *Trichosporon mycotoxinivorans* by rDNA sequencing and the expanded database.

microorganisms. A multitier spectral database structure was established to allow for pairwise classification of genera or of species within a genus with the use of classification models developed with the appropriate subsets of database spectra with the use of PCA. The classification model development process entails the application of a forward region selection algorithm to maximize the separation between the two classes in a principal-component space. The algorithm searches for spectral regions containing discriminatory spectral features in two stages, namely, a grid search followed by a "greedy" search, with the grid search filtering through large spectral regions and providing starting points for the greedy search. The algorithm was implemented in data analysis software written in-house as previously described (27).

In the identification step, the ATR-FTIR spectrum of a routine isolate is assigned to either group within a given tier in the multitiered spectral database structure or flagged as an outlier based on spectral dissimilarity to the spectra in the pairwise group and is not identified. This process is iterated in a stepwise fashion for all tiers until identification (or no identification) of the isolate at the species level is achieved. Comparable results can also be achieved with the use of the discriminate analysis routines in the commercially available multivariate statistical software JMP Pro (SAS, Cary, NC).

RESULTS

Identification of clinical yeasts by ATR-FTIR spectroscopy. The initial ATR-FTIR spectral reference database contained the spectra of 199 isolates belonging to 5 genera and 14 species; identification of 318 routine clinical yeast isolates with the use of this database yielded 95.6% correct species identification ($n = 304$) (in concordance with MALDI-TOF MS and rDNA D1/D2 sequencing). One-hundred-percent correct species identification was achieved for all routine samples belonging to *Candida dubliniensis*, *C. krusei*, *Candida lusitaniae*, *Candida orthopsilosis*, *C. parapsilosis*, *Candida neoformans*, *Rhodotorula mucilaginosa*, and *Saccharomyces. cerevisiae*. The remaining 4.4% ($n = 14$) of the routine isolates were either unidentified ($n = 11$) due to the lack of representation in the spectral reference database or misidentified ($n = 3$) (Table 1).

Expansion and revalidation of the ATR-FTIR spectral reference database. To address the lack of representation of certain genera and species noted above, the

ATR-FTIR spectral reference database was expanded by acquiring the spectra of isolates belonging to 7 additional genera and 51 additional species (Table S1). Reanalysis of the spectra collected from the 318 isolates with the use of the expanded spectral database yielded 99.7% correct species identification (Table 1). It should be noted that an isolate identified by ATR-FTIR spectroscopy as *Meyerozyma caribbica* was identified by MALDI-TOF MS as *Candida guilliermondii* with 99.9% confidence. This sample was recultured a second time and yielded the same discordant results upon identification by ATR-FTIR spectroscopy and MALDI-TOF MS analysis. The isolate was identified by rDNA D1/D2 sequencing (NL1-NL4 primers) as *Meyerozyma caribbica*. Ultimately, only one isolate was misidentified by ATR-FTIR spectroscopy (misidentification of *C. tropicalis* as *C. lusitaniae*, confirmed by rDNA D1/D2 sequencing), resulting in an overall misidentification rate of 0.3%.

DISCUSSION

To our knowledge, no prior study has utilized ATR-FTIR spectroscopy for routine identification of clinical yeasts. Our group is the first to create a comprehensive clinical yeast ATR-FTIR spectral reference database, comprising of 263 reference strains (or 789 spectra) of 65 species belonging to 12 genera and encompassing both rare and emerging strains, such as *C. auris* and *Trichosporon asahii*. As with any method used for fungal identification for diagnostic purposes, misidentification is a major concern as it may impede selection of appropriate species-specific antifungal therapy; it is, thus, desirable to obtain an "unidentified isolate" result as opposed to a false identification. Indeed, a higher rate of unidentified (3.5%) than misidentified (0.9%) isolates was observed in our initial validation study, which employed a spectral database that did not include sufficient representation of rare species, such as *Candida utilis*, *Candida pararugosa*, and *Trichosporon mycotoxinivorans*. This latter limitation was addressed by expanding the spectral database to encompass a larger number of species and additional genera. By employing the expanded ATR-FTIR database for the reanalysis of 318 routine clinical isolate spectra, 100% and 99.7% correct identification at the genus level and the species level, respectively, was achieved. This performance coupled with the single-step protocol employed for the analysis of clinical isolates by ATR-FTIR spectroscopy makes this technology both superior and more cost-effective than any currently available identification platform. For example, rates of correct identification of medically important yeasts reported for Vitek 2, API ID32C, Phoenix ID, AuxaColor, and Vitek MS range from 72.7% to 97.1% with misidentification rates ranging from 0.4% to 33.3% (28–32). MALDI-TOF MS has overcome difficulties of identifying closely related yeast complexes, such as *C. albicans/C. dubliniensis* and *C. parapsilosis*/*C. orthopsilosis*, compared with conventional biochemical techniques (30). Likewise, ATR-FTIR-based identification results from this study also demonstrated that the IR spectra contained sufficient information to allow discrimination among *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. orthopsilosis*.

Overall, among the 318 routine clinical isolates identified by ATR-FTIR spectroscopy in this study, only 1 *C. tropicalis* isolate was misidentified (as *C. lusitaniae*) when the expanded spectral database was employed. It should be noted that one isolate identified as *Meyerozyma caribbica* by rDNA sequencing was misidentified as *C. guilliermondii* (with 99.9% confidence) by the Vitek MS system. As both species are part of the same species complex and are genetically similar (33), the MALDI-TOF MS misidentification error is considered minor. This error may be attributed to the absence of a mass-spectrometric spectral representation of *M. caribbica* in the Vitek MS database (clinical knowledge database v3.0). Similarly, the initial ATR-FTIR spectral database did not include IR spectra representative of *M. caribbica*; however, unlike the MALDI-TOF MS result, the sample was unidentified rather than misidentified. Inclusion of IR spectra of *M. caribbica* in the expanded IR spectral database of clinical yeast isolates resulted in correct identification of this sample by ATR-FTIR spectroscopy. Additionally, two isolates identified as *C. lusitaniae* by rDNA sequencing were unidentified by MALDI-TOF MS, whereas they were both correctly identified by ATR-FTIR spectroscopy. ATR-FTIR

spectroscopy offers several advantages over MALDI-TOF MS, being an inexpensive and reagent-free method that employs a one-step procedure, obviating the need for consumables or the need for an extraction step with the use of harsh acids (e.g., formic acid). In addition, the method is free from interruption associated with time delays related to drying of the chemical matrix (and acid) and achieving a high vacuum prior to MS spectral acquisition.

The minimum number of reference strains required for adequate representation of a given species in the IR spectral reference database may be species specific and dependent on intraspecies and interspecies variability of the biochemical composition of the microorganism. For example, the spectral differences between *C. pelliculosa* and the other *Candida* species were substantial and spectral representation from only two *C. pelliculosa* isolates in the spectral database was sufficient for complete discrimination. Other species, such as *C. albicans* and *C. dubliniensis*, were more difficult to discriminate from each other and required 13 reference strains per species for complete discrimination between the two species. The inclusion of ATR-FTIR spectra of newly discovered and emerging species in the spectral reference database is expected to enhance the predictive performance of the ATR-FTIR spectroscopy-based method. The continuous addition of spectra of clinical isolates to the spectral database will aid in the empirical determination of the minimum number of reference strains required to achieve 100% correct identification of a given species.

Overall, our centralized study demonstrates a strong potential for ATR-FTIR spectroscopy-based identification in rapid routine analysis of clinical yeasts. In addition to achieving 99.7% correct species-level identification, ATR-FTIR spectroscopy demonstrated advantages over current conventional biochemical, MALDI-TOF MS, and gene-sequencing identification methods. ATR-FTIR spectroscopy requires no sample preparation after incubation, and as little as a single colony is sufficient for analysis. It is a standalone and reagent-free method, and complete data acquisition and analysis for the identification at the species level is completed in less than 2 minutes per sample. These advantages make our ATR-FTIR-based technology the fastest and lowest-cost platform technology developed to date for microbial identification.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01739-18.

SUPPLEMENTAL FILE 1, PDF file, 0.02 MB.

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