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Identification of Pathogenic Rare Yeast Species in Clinical Samples: Comparison between Phenotypical and Molecular Methods[▽]

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Species identification using both phenotypic and molecular methods and antifungal susceptibility tests was carried out with 60 uncommon clinical yeasts. Our data show that phenotypic methods were insufficient for correct identification (only 25%) and that most of the wrongly identified strains showed a resistant antifungal profile.

Although *Candida* and *Aspergillus* species are the most common causes of invasive fungal infection (IFI) in debilitated individuals, almost all yeasts are potential pathogens, causing great morbidity and mortality in those patients (11). More than 90% of infections due to yeasts are attributed to only six species—*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Cryptococcus neoformans*—but the list of reported species continues to grow as laboratories are pushed to provide identification to the species level as an aid to optimize the treatment of *Candida* and other yeast infections (19). Some of these new pathogens (*Candida orthopsilosis*, *Candida metapsilosis*, *Candida nivariensis*, or *Candida bracarensis*) have been well characterized recently thanks to molecular methods such as PCR-based procedures and sequencing (1, 5, 25). Several reports have addressed the difficulty of identifying yeast strains to the species level by conventional methods, since they are highly dependent on variables such as growth medium and temperature. In addition, databases are limited only to common species, and in general terms, their use is time-consuming. On the other hand, molecular methods based on DNA sequencing have been shown to improve strain characterization (15, 16), which is critical to ensure early and effective antifungal therapy, since differences in susceptibility have been reported between members of the same genus. This paper compares two methods of yeasts identification, molecular and conventional, in a collection of rare yeast isolates from clinical samples. The susceptibility profiles of nine antifungal agents against these isolates were also evaluated in order to provide some insight into their clinical management.

A total of 60 uncommon yeasts were included in this study. They were defined as strains belonging to species which account for less than 1% of the total number of isolates in the yeast collection of the Spanish Mycology Reference Laboratory (SMRL). These strains were received at our institution over a period of 17 years, from 1992 to 2009. They were obtained from clinical samples, most of them from deep sites (Table 1), and identified by using physiological and morphological tests, including some of the following: morphology on CMA (cornmeal agar), assimilation of sugars commercial kits (AuxaColor; Bio-Rad, Madrid, Spain, and API 20 AUX ID32 C galleries; bioMérieux, Madrid, Spain), fermentation of several carbon sources, growth on nitrogen sources, growth at various temperatures, and ability to hydrolyze urea (14).

For molecular identification, genomic DNA was prepared directly from a single yeast colony (17). DNA fragments, comprising the internal transcribed spacer 1 (ITS1) and ITS2 regions, were amplified and sequenced using universal primers (26). For these analyses, we used the sequence database designed by the SMRL, which holds 5,000 strains belonging to 270 different fungal species and contains a large number of sequences from the reference database (Table 2). All phylogenetic analyses were conducted with InfoQuest FP software version 4.50 (Bio-Rad Laboratories, Madrid, Spain), using maximum parsimony clustering methodology. An identity of 96 to 100% to the respective type/validated strain has been proposed for species identification in the study (2). In some particular species (*Candida ciferri*, *Candida rugosa*, and *Issatchenkia terricola*), the nearest CBS or GenBank match was used for final identification. Susceptibility testing followed the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) for fermentative yeasts (23). The antifungal agents used were amphotericin B (AMB), flucytosine (5FC), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), caspofungin (CAS), micafungin (MCF), and anidulafungin (AND). Interpretative breakpoints proposed by EUCAST for FLC and VRC were used (20, 21). For AMB, ITC, and POS, the breakpoints were defined based on the wild-type distribution of MICs determined by a EUCAST method based on preliminary studies of correlation *in vitro*/*in vivo* with strains causing oropharyngeal candidosis in AIDS patients and on pharmacokinetic/pharmacodynamic (PK/PD) bibliographic data (AMB, >1.0 mg/liter; ITC, >0.125 mg/liter; and POS, >0.125 mg/liter) (6, 7, 22). In the case of echinocandins, breakpoints proposed by the CLSI were used to interpret susceptibility results (18).

All strains were easily identified by molecular methods.

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TABLE 1. DNA-based identities, conventional identification, and clinical sources of 60 uncommon yeasts analyzed

Strain	Identification		Clinical sample type
	Conventional method[a]	Sequencing	
CL-4637	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-4710	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-5198	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-5362	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-5372	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-6822	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-6823	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-5720	*Candida parapsilosis*	*Candida orthopsilosis*	Blood
CL-4438	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-5144	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-7098	Unidentifiable	*Candida metapsilosis*	Skin wound
CL-6329	*Candida parapsilosis*	*Candida metapsilosis*	Unknown
CL-5221	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-7106	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-4886	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-4926	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-4638	*Candida parapsilosis*	*Candida metapsilosis*	Blood
CL-5897	*Candida sake*	*Candida dubliniensis*	Bronchial secretion
CL-7124	*Candida sake*	*Candida dubliniensis*	Urine
CL-7022	*Candida albicans*	*Candida dubliniensis*	Oropharyngeal exudate
CL-7028	*Candida dubliniensis*	*Candida dubliniensis*	Blood
CL-6838	*Candida dubliniensis*	*Candida dubliniensis*	Bronchoalveolar lavage fluid
CL-5390	*Candida dubliniensis*	*Candida dubliniensis*	Blood
CL-5418	Unidentifiable	*Candida dubliniensis*	Sputum
L06-390	Unidentifiable	*Debaryomyces hansenii*	Skin
CL-6240	*Candida sake*	*Lodderomyces elongisporus*	Bronchial secretion
CL-6877	Unidentifiable	*Lodderomyces elongisporus*	Urine
L07-121	*Candida pelliculosa* (*Pichia anomala*)	*Pichia anomala*	Nail
CL-343	*Pichia anomala*	*Pichia anomala*	Nail
CL-6620	Unidentifiable	*Pichia fermentans*	Unknown
CL-7027	*Candida rugosa*	*Pichia fermentans*	Sputum
CL-6542	Unidentifiable	*Pichia membranifaciens*	Sputum
CL-7074	*Pichia jadinii*	*Pichia fabianii*	Blood
CL-6710	*Candida pelliculosa*	*Pichia fabianii*	Blood
L06-338	Unidentifiable	*Candida ciferri*	Nail
CL-7030	*Candida glabrata*	*Candida bracarensis*	Unknown
CL-3905	*Candida kefyr*	*Kluyveromyces lactis*	Blood
CL-6301	*Kluyveromyces lactis* (*Candida sphaerica*)	*Kluyveromyces lactis*	Retropharyngeal abscess
CL-6194	*Candida intermedia*	*Candida intermedia*	Bronchial biopsy
CL-6800	Unidentifiable	*Candida haemulonii*	Skin Wound
CL-4640	Unidentifiable	*Candida haemulonii*	Unknown
CL-4641	Unidentifiable	*Candida haemulonii*	Unknown
CL-7073	*Candida sake*	*Candida haemulonii*	Cutaneous exudate
CL-4642	Unidentifiable	*Candida haemulonii*	Unknown
CL-6149	*Candida rugosa*	*Candida rugosa*	Vaginal exudate
L06-34	*Candida rugosa*	*Candida rugosa*	Blood
CL-6932	*Candida norvegensis*	*Candida inconspicua*	Unknown
CL-6946	*Candida inconspicua*	*Candida inconspicua*	Sputum
CL-6150	*Candida rugosa*	*Candida inconspicua*	Bronchial secretion
CL-6867	Unidentifiable	*Candida inconspicua*	Blood
CL-7156	Unidentifiable	*Candida inconspicua*	Urine
CL-6598	*Kodamaea ohmeri*	*Kodamaea ohmeri*	Blood
CL-6272	*Kodamaea ohmeri*	*Kodamaea ohmeri*	Blood
CL-7143	*Kodamaea ohmeri*	*Kodamaea ohmeri*	Tracheal aspirate
CL-6744	Unidentifiable	*Kodamaea ohmeri*	Bile
CL-7006	Unidentifiable	*Issatchenkia terricola*	Blood
CL-6878	Unidentifiable	*Issatchenkia terricola*	Blood
CL-6574	*Pichia membranifaciens*	*Issatchenkia terricola*	Blood
CL-2026	*Kloeckera apiculata* (*Hanseniaspora uvarum*)	*Hanseniaspora uvarum*	Blood
CL-6749	*Kloeckera apiculata* (*H. uvarum*)	*Hanseniaspora uvarum*	Nail

[a] Conventional identification includes physiological and morphological testing. Unidentifiable, inconclusive results by biochemical and morphological identification.

TABLE 2. Reference strains used for comparison of ITS regions and their GenBank accession numbers

Yeast species	CBS strain	ATCC strain[a]	GenBank accession no.
C. orthopsilosis		ATCC 96139^T	AJ698048
C. metapsilosis		ATCC 96144^T	AJ698049
L. elongisporus	CBS 2606		AY391845
P. anomala		ATCC 8168	U96720.1
P. fabianii	CBS 5640		AF335967
C. bracarensis	CBS 1054		M589573.2
C. sphaerica	CBS 6170		AY338967
C. intermedia	CBS 572^T		AF218968
C. haemulonii	CBS 5149^T		DQ898168
C. rugosa		ATCC 10571^T	AF335927
P. fermentans		ATCC 24750	AF336843.1
P. membranifaciens	CBS 5516		DQ198964.1
K. ohmeri	CBS 9452		EF196810
D. hansenii	CBS 161		AF210327
H. uvarum	CBS 2584		AJ512428
I. terricola	CBS 5259		
C. dubliniensis	CBS 7987		AB035589
C. inconspicua	CBS 180^T		AB179767
C. ciferri	CBS 5295		AY493435

[a] T, type strain.

However, 44 could be identified by biochemical means (16 out of 60 were classified as unidentifiable since they were not properly discriminated by phenotyping), and only 15 of those 44 matched the molecular identification (Table 1).

It should be noted that conventional identification was not discriminatory enough to characterize the most recently described species, such as *C. orthopsilosis*, *C. metapsilosis*, *C. bracarensis*, and *Lodderomyces elongisporus*. Notably, most *Candida haemulonii* isolates (4 out 5 isolates) and most *Issatchenkia terricola* isolates (2 out of 3 included) were not classified by conventional methods. MIC distributions are shown in Table 3. Most strains were considered susceptible *in vitro* to AMB, as defined by a MIC of <1 mg/liter. It is worth noting that 4 out 5 of the *C. haemulonii* strains showed high MICs to AMB (1 to 4 mg/liter).

The azole agents showed a broad range of activity against these isolates. A total of 33.34% (19/57) of the strains were resistant to FLC (MIC of >4 mg/liter). In addition, ITC (MIC of >0.125 mg/liter), VRC (MIC of >0.125 mg/liter), and POS (MIC of >0.125 mg/liter) showed reduced activity for 10.52% (6/57), 19.29% (11/57), and 17.54% (10/57) of the isolates, respectively. *Candida ciferri*, *C. haemulonii*, *Pichia anomala*, *Pichia membranifaciens*, and *Pichia fermentans* showed high MICs or resistance *in vitro* to all azole compounds tested.

Most strains revealed patterns of susceptibility to echinocandins (MIC range of between 0.015 and 2 mg/liter). Three out of four strains of *Kodamaea ohmeri* showed high MICs to CAS (4 to >16 mg/liter) but showed different susceptibility profiles to AND (0.03 to 4 mg/liter) and MCF (0.03 to 16 mg/liter). One of two *C. rugosa* strains had a CAS MIC value of 16 mg/liter but showed a MIC value of <2 mg/liter for MCF and AND. It should be noted that only 8 out of 27 of the antifungal-resistant isolates were correctly classified by phenotyping, as follows: *Candida inconspicua*, 1 out of 5 strains; *K. ohmeri*, 3 out of 4 strains; *C. rugosa*, 2 out of 2 strains, and *P. anomala*, 2 out of 2 strains.

In our study, a high percentage of uncommon yeasts were

TABLE 3. MIC ranges and geometric means (GMs) of MICs for 57 isolates included in the study

Species (no. of isolates)[a]	MIC (GM)[b]							
	AMB	5FC	FLC	ITC	VRC	POS	CAS	MCF	AND
C. orthopsilosis (8)	0.03–0.12 (0.077)	0.12 (0.12)	0.5 (0.5)	0.03–0.06 (0.038)	0.015–0.03 (0.021)	0.03–0.06 (0.032)	0.12–0.5 (0.24)	0.12–0.5 (0.22)	0.12–1 (0.38)
C. haemulonii (5)	0.5–4 (1.14)	0.12–2 (0.49)	8–>64 (42.22)	0.06–>8 (1.712)	0.12–>8 (4.55)	0.5–>8 (4.59)	0.12–16 (0.65)	0.03–0.06 (0.042)	0.06 (0.06)
C. inconspicua (5)	0.25–0.5 (0.42)	1–16 (2.82)	32–>64 (90.50)	0.12 (0.12)	0.12–1 (0.24)	0.06–0.12 (0.071)	0.25 (0.25)	0.03 (0.03)	0.03 (0.03)
C. metapsilosis (9)	0.06–0.12 (0.088)	0.12–0.25 (0.13)	0.5–8 (1.16)	0.015–0.06 (0.034)	0.015–0.06 (0.03)	0.015–0.12 (0.018)	0.015–0.3 (0.33)	0.12–1 (0.24)	0.06–1 (0.18)
C. dubliniensis (7)	0.03–0.06 (0.036)	0.12 (0.12)	0.12–0.5 (0.163)	0.015 (0.015)	0.015 (0.015)	0.015 (0.015)	0.03–0.5 (0.174)	0.03 (0.03)	0.03 (0.03)
K. ohmeri (4)	0.03–0.12 (0.05)	0.12–1 (0.29)	2–8 (3.36)	0.03–0.06 (0.035)	0.03–0.06 (0.030)	0.015–0.06 (0.02)	0.5–>16 (4)	0.03–16 (0.144)	0.03–4 (0.144)
I. terricola (3)	0.03–0.06 (0.037)	0.5–1 (0.629)	8–16 (12.69)	0.015–0.03 (0.018)	0.03–0.12 (0.06)	0.015–0.03 (0.018)	1 (1)	0.03–0.12 (0.047)	0.03 (0.03)
L. elongisporus (2)	0.03–0.12	0.12–0.25	0.12	0.015–0.03	0.015	0.06	0.06	0.03	0.03
P. anomala (2)	0.03	0.12	4	0.12	0.12	0.5	0.12	0.03	0.03
P. fermentans (2)	0.03	0.5–2	>64	0.25–0.5	1–4	0.25–0.5	0.12–0.5	0.03	0.03
P. membranifaciens	0.25	16	>64	1	1	0.5	0.25	0.03	0.03
P. fabianii (2)	0.06–0.25	0.12	0.5–1	0.03–0.12	0.015	0.03–0.06	0.12–0.5	0.03	0.03
C. ciferri	0.25	2	>64	0.5	0.25	0.5	0.12–0.5	0.03	0.03
C. bracarensis	0.25	0.5	2	0.06	0.03	0.12	0.25	0.03	0.03
C. sphaerica (2)	0.06–0.12	0.12–0.25	0.25–0.5	0.03–0.06	0.015–0.03	0.015	0.12	0.03	0.03
C. intermedia	0.03	0.12	0.25	0.015	0.015	0.015	0.12	0.03	0.03
C. rugosa (2)	0.25	0.12–0.25	1–2	0.015	0.015	0.015	1–>16	0.03	0.03–0.25

[a] *Hanseniaspora uvarum* (2) and *Deberyomyces hansenii* (1) isolates were not include because these isolates were not able to grow on RPMI media.

[b] The GM was not calculated if the number of isolates was <3. MICs are measured in mg/liter. AMB, amphotericin B; 5FC, 5-flucytosine; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; CAS, caspofungin; MCF, micafungin; AND, anidulafungin.

not identified using some commercial kits (44 out of 60; 73%), and only a few of them showed some correlation between conventional and molecular methods (15 out of 60; 25%). In general terms, commercial kits are designed to identify common yeasts in the clinical laboratory; however, they usually fail to characterize those less frequent strains (15). Recently described yeast species such as *C. orthopsilosis*, *C. metapsilosis*, *C bracarensis*, *C. haemulonii*, and *C. nivariensis* have been identified and classified only by molecular methods (1, 5, 13, 25), since they are not included in the currently available commercial databases. However, sequencing of the ITS region is an effective tool for differentiating the rare species most frequently confused, bearing in mind that reliable sequence databases should be used. Indeed, given the inability of standard phenotypic methods to distinguish some of these rare yeasts species, it is possible that molecular methods may ultimately become the primary means of identification of clinically important yeast isolates.

In addition, our study stated that most of the uncommon yeasts wrongly identified using conventional identification methods showed a resistant antifungal profile (*C. haemulonii*, *C. ciferri*, *P. anomala*, *P. membranifaciens*, *P. fermentans*, *K. ohmeri*, and *C. rugosa*), as well as the fact that their mistaken identification could imply inappropriate treatment and clinical management (3, 4, 6, 8, 10, 12, 24). In other cases, although different susceptibility profiles among species have not been demonstrated to be clinically relevant yet, there is no doubt that a precise standard of species identification is necessary to monitor changes in fungal infection epidemiology and antifungal susceptibility (9).

For labs with no experience using molecular identification methods, or where this service is unavailable, susceptibility testing must be performed, since some of the antifungal agents available are inactive against most of these species (12, 13, 24). At least susceptibility results may provide valuable information to physicians for patient management.

We suggest submitting strains to reference laboratories as a cost-effective alternative to using currently available tests for the identification of problem cases and rare yeast species which cannot be easily identified using biochemical tests.

In summary, we have presented data demonstrating that sequencing the ITS region is a robust procedure, identifying many clinically relevant yeast isolates. This is a quick and accurate method for better definition of both the epidemiology of the fungal infection and the prevalence of antifungal-resistant species of yeasts. In conclusion, because of the emergence of rare yeast pathogens and their resistant susceptibility patterns, it is of paramount importance that the identification methods available provide the highest possible degree of precision.

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