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Identification and antifungal susceptibility of a large collection of yeast strains isolated in Tunisian hospitals

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In this study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used as a rapid method to identify yeasts isolated from patients in Tunisian hospitals. When identification could not be exstablished with this procedure, sequencing of the internal transcribed spacer with 5.8S ribosomal DNA (rDNA) (ITS1–5.8S–ITS2) and D1/D2 domain of large-subunit (LSU rDNA) were employed as a molecular approach for species differentiation. *Candida albicans* was the dominant species (43.37% of all cases), followed by *C. glabrata* (16.55%), *C. parapsilosis* (13.23%), *C. tropicalis* (11.34%), *C. dubliniensis* (4.96%), and other species more rarely encountered in human diseases such as *C. krusei*, *C. metapsilosis*, *C. lusitaniae*, *C. kefyr*, *C. palmioleophila*, *C. guilliermondii*, *C. intermedia*, *C. orthopsilosis*, and *C. utilis*. In addition, other yeast species were obtained including *Saccharomyces cerevisiae*, *Debaryomyces hansenii* (anamorph known as *C. famata*), *Hanseniaspora opuntiae*, *Kodamaea ohmeri*, *Pichia caribbica* (anamorph known as *C. fermentati*), *Trichosporon* spp. and finally a novel yeast species, *C. tunisiensis*. The *in vitro* antifungal activities of fluconazole and voriconazole were determined by the agar disk diffusion test and Etest, while the susceptibility to additional antifungal agents was determined with the Sensititre YeastOne system. Our results showed low incidence of azole resistance in *C. albicans* (0.54%), *C. tropicalis* (2.08%) and *C. glabrata* (4.28%). In addition, caspofungin was active against most isolates of the collection with the exception of two *K. ohmeri* isolates. This is the first report to describe caspofungin resistant isolates of this yeast.

Keywords *Candida* spp., MALDI-TOF mass spectrometry, ITS, disk diffusion, fluconazole, voriconazole

Introduction

The incidence of fungal infections has dramatically increased in the past several decades. Invasive infections caused by yeasts have become a major cause of morbidity and mortality in immunodeficient patients and those receiving immunosuppressive chemotherapy for cancer and organ transplantation [1,2]. In the United States, *Candida* species are the fourth leading cause of nosocomial bloodstream infections [2]. And although *C. albicans* remains the leading and the most widespread pathogenic yeast species causing a variety of infections, the incidence rates of non-*C. albicans Candida* infections has increased in recent years, including those with intrinsic or acquired resistance to azole antifungals [1–3]. More recently, infections caused by less common yeast species such as *Pichia*, *Rhodotorula*, *Trichosporon*, and *Saccharomyces* spp. [4–8] and other rarely encountered species have been reported [1,9,10]. Rapid identification of yeast isolates from clinical samples is particularly important to initiate appropriate therapy given their variable antifungal susceptibility profiles. However, this task is complicated by the increasing number of emerging pathogenic fungal species that are not included

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in the repertoires of commercially available conventional identification kits [11,12] and their identification by conventional methods may be difficult and sometimes inconclusive. Most often, the widely available methods in routine use in clinical laboratories are chromogenic agar media, immunological kits and biochemical or enzymatic tests, e.g., ID 32C strip or VITEK YST card (bioMérieux) [13–15]. Due to limited databases, misidentifications have been reported [10,16,17], thus necessitating more discriminatory technologies. Species identification is also important for understanding the epidemiology of fungal infections, including trends in species distribution and antifungal susceptibility patterns. Thus, the identification, taxonomy and epidemiological analysis of fungal pathogens are increasingly dependent on modern molecular techniques based on PCR amplification of conserved regions of the genome and on sequencing the resulting PCR products [10,11,17–19]. These methods provide high sensitivity and specificity, but their efficiency is limited by the complexity of the culture and isolation procedures. Moreover, they are completely dependent on the known genetic sequences of the target microorganisms. Additionally, molecular biology techniques require a high level of technical expertise, remain expensive, and are therefore not suitable for routine identification. New approaches are required for the rapid analysis of microorganisms in clinical microbiology laboratories to initiate early and appropriate treatment to improve patient care. Among recently developed rapid techniques for identification of microorganisms encountered in clinical settings, the use of protein profiles obtained by MALDI-TOF MS directly from growing fungal colonies has been successful. The method analyzes the profiles of macromolecules that are obtained from whole microorganisms [20]. This new proteomic approach allows rapid and accurate identification of bacteria as well as fungi [12,20–27].

Azole antifungals are often the preferred first line agents for treatment of invasive fungal infections and have good but variable activity against *Candida* spp. [28,29]. They inhibit an enzyme required for the production of ergosterol, which is an essential component of the fungal cytoplasmic membrane [28–30]. Fluconazole has been widely used for the treatment of candidiasis as it is available in intravenous and oral formulations, and is inexpensive, but some species of *Candida* such as *C. krusei* and *C. glabrata* can exhibit resistance to fluconazole [31–35].

In this study, we first demonstrated the suitability of MALDI-TOF MS for identification of clinical fungal isolates originating from Tunisian hospitals. Second, we determined azole susceptibility profiles of this collection to estimate the occurrence of resistance. Some isolates atypical for their susceptibility profiles were further evaluated with different antifungal susceptibility methods and different antifungal agents.

Materials and methods

Organisms and growth conditions

In this study, a total of 423 yeast isolates were collected between September 2006 and July 2010 from three different hospitals in Tunisia (Hospital Habib Thameur, Hospital La Rabta (Tunis, the capital) and Hospital Ibn El Jazzar of Kairouan in central Tunisia). The yeasts were sequentially isolated from various clinical specimens of non-AIDS patients in hospitals and outpatient locations during the study period, including blood, other normally sterile body fluids, oral cavity, gastrointestinal tract, respiratory tract, biomedical devices, skin, and soft tissue. The various specimens were obtained and processed by standard microbiological procedures by inoculating them onto Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin and incubating at 35°C for 24–48 h. CHROMagar Candida plates (CHROMagar Candida, Paris, France) were employed for primary identification of four *Candida* species using the following criteria; *C. albicans* and *C. dubliniensis* = green colonies, *C. tropicalis* = metallic blue colonies and *C. krusei* = pink to light mauve colonies. To differentiate *C. dubliniensis* from *C. albicans*, we investigated the isolate's ability to grow on YPDA (1% Bacto peptone [Difco Laboratories, Basel, Switzerland]), 0.5% yeast extract [Difco], 2% glucose [Fluka, Buchs, Switzerland] and 2% agar [Difco]) at 42°C. *Candida albicans* ATCC 90028, *C. dubliniensis* CBS 7987, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258 were employed as controls and it should be noted that for some isolates, the ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France) was used and the results were interpreted according to manufacturer's instructions.

Identification by MALDI-TOF MS

Identification of the yeast collection by MALDI-TOF MS was carried out using the Microflex LT (Bruker Daltonics GmbH, Leipzig, Germany) with FlexControl (version 3.0) software (Bruker Daltonics) as described previously [27]. Briefly, 1.5 µl of the protein extracts were placed on steel target plates and allowed to dry in air, after which, each sample was overlaid with 1.5 µl of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid: CHCA, in 50% acetonitrile and 2.5% trifluoracetic acid; Bruker Daltonik) and again air dried at room temperature. For standardization purposes, positive controls were prepared from 11 reference isolates (*C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. dubliniensis* ATCC 2118, *C. dubliniensis* ATCC 2119, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018,

C. tropicalis ATCC 750 and *Cryptococcus neoformans* ATCC 90112) and included in the set of analysis. The data were processed by the associated software and the sample spectra were compared to reference spectra in the provided database for microorganism identification. Each sample was tested in duplicate to ensure reproducibility of the spectra. A characterization score cut-off value was attributed to each sample and was interpreted as recommended by the manufacturer.

Molecular species identification

DNA of yeast cells grown overnight at 30°C in YPD broth was isolated by mechanical breakage with glass beads as described previously [36]. Polymerase chain reaction (PCR) for ITS1–5.8S–ITS2 and D1/D2 domains of the large subunits (LSU) ribosomal RNA (28S rRNA) using fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and 26S-1 (5′-GGTGAGTTGTTACACACTCC-3′) [27]. PCR was carried out in a total reaction volume of 50 µl consisting of 200 µM concentrations of each dNTP, 250 nM of each primer, 1.5 mM $MgCl_2$, 2.6 U of Expand High Fidelity PCR System (Roche, Switzerland), and 1–5 ng of total genomic DNA, according to the following conditions; initial denaturation at 94°C for 5 min followed by 35 thermal cycles of 94°C (denaturation), 54°C (annealing) for 60 s and 72°C (extinction) for 4 min and a final elongation at 72°C for 10 min on a Primus-HT PCR Systems Dualblock thermocycler (MWG BioTech, Ebersberg, Germany). A negative control was introduced by replacing the template DNA with sterile water in the PCR mixture. The purified PCR products were sequenced with primers listed in Table 1, using an ABI prism 3130 XL automated DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems). Final sequences were assembled and edited using the software package Sequencer 3.0 (Gene Codes Corp., Ann Arbor, MI, USA). The sequences of the ITS1–5.8S–ITS2 region and D1/D2 domain of the 28S rRNA were submitted to the NCBI GenBank database and the accession numbers are given in Table 2.

Table 1 Primers used for sequencing of (ITS1–5.8S–ITS2–D1–D2) regions of nuclear ribosomal DNA.

Primers	Sequence	Reference
ITS3	5′-GCATCGATGAAGAACGCAGC-3′	[66]
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	[66]
NL1	5′-GCATATCAATAAGCGGAGGAAAAG-3′	[66]
NL4	5′-GGTCCGTGTTTCAAGACGG-3′	[66]
26S-5	5′-AGCAGAACTGGCGATGCG-3′	[27]
26S-F	5′-GTACAGTGATGGAAAGATGA-3′	[27]

Table 2 GenBank accession numbers of the sequences of ITS1–5.8S–ITS2 region and D1/D2 domain of LSU rRNA for clinical isolates not identified by conventional methods.

Accession numbers	Isolate references	Species
JQ612155[a]	JEY63	*Candida tunisiensis*
KC111442	JEY379	*Candida palmioleophila*
KC111443	JEY380	*Candida palmioleophila*
KC111444	JEY420	*Debaryomyces hansenii*
KC111445	JEY258	*Hanseniaspora opuntiae*
KC111446	JEY269	*Hanseniaspora opuntiae*
KC111447	JEY270	*Hanseniaspora opuntiae*
KC111448	JEY182	*Kodamaea ohmeri*
KC111449	JEY234	*Kodamaea ohmeri*
KC111450	JEY267	*Pichia caribbica*

[a]All rDNA includes SSU rDNA, ITS1–5.8S–ITS2 and LSU rDNA [27].

Susceptibility testing

Disk diffusion *in vitro* susceptibility tests to fluconazole (FLC) and voriconazole (VRC) was performed as described by Hazen *et al*. [37] and in accordance with Clinical and Laboratory Standards Institute (CLSI) document M44-A [38]. Mueller-Hinton agar supplemented with 2% of glucose and 0.5 µg/ml of methylene blue was prepared in plates to a depth of 4.0 mm. Each isolate was cultured overnight at 35°C to ensure purity and viability. The inoculum was adjusted to the turbidity of a 0.5 McFarland standard (approximately $1–5 \times 10^6$ CFU/ml) in sterile saline (0.85%) and streaked onto plates by using a cotton swab. FLC (25 µg) and VRC (1 µg) disks (Becton Dickinson, Sparks, MD, USA) were placed aseptically on the agar surface. After 24 h of incubation in air at 35°C, zone diameter endpoints were evaluated at 80% growth inhibition by the BIOMIC image analysis plate reader system (Giles Scientific, Santa Barbara, CA, USA) [37,39]. The interpretive criteria for the FLC and VRC disk diffusion tests are described in Table 3 [40,41].

Quality controls were performed with *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018 and *C. tropicalis* ATCC 750.

The Etest (bioMérieux) for FLC and VRC and Sensititre YeastOne colorimetric (SYO) plates (TREK Diagnostic Systems, East Grinstead, UK) were used according to the manufacturer's instructions. These two methods were only performed with isolates that were categorized as resistant by disk diffusion. The minimum inhibitory concentration (MIC) interpretive guidelines for *in vitro* susceptibility testing for *Candida* species are mentioned in Table 3.

Table 3 Clinical breakpoints for fluconazolel (FLC) and voriconazole (VRC) as recommended by Clinical and Laboratory Standards Institute (CLSI) [40,41].

	Zone diameter (mm)						MIC (μg/ml)					
	S		SDD		R		S		SDD		R	
Species	FLC	VRC	FLC	VRC	FLC	VRC	FLC	VRC	FLC	VRC	FLC	VRC
C. albicans	≥17	≥17	14–16	15–16	≤13	≤14	≤2	≤0.125	4	0.25–0.5	≥8	≥1
C. parapsilosis	≥17	≥17	14–16	15–16	≤13	≤14	≤2	≤0.125	4	0.25–0.5	≥8	≥1
C. tropicalis	≥17	≥17	14–16	15–16	≤13	≤14	≤2	≤0.125	4	0.25–0.5	≥8	≥1
C. krusei	–	≥15	–	13–14	–	≤12	–	≤0.5	–	1	–	≥2
C. glabrata[a]	–	–	≥15	–	≤14	–		–	≤32	–	≥64	–

S, susceptible; SDD, susceptible dose-dependent; R, resistant; [a]Breakpoint was not defined for voriconazole.

Results

Conventional species identification

Of the total of 423 yeast isolates investigated in this study, 206 were identified with CHROMagar Candida after 24–48 h of incubation as *C. albicans* and/or *C. dubliniensis* (48.7% of all isolates). The lack or highly restricted growth of *C. dubliniensis* in 42°C on YPD allowed us to differentiation 4.96% of all isolates in this group. Forty-eight isolates (11.34%) were identified as *C. tropicalis*, eight isolates as *C. krusei* (1.89%), while the rest of the collection (161 isolates, 38.06%), was found to be atypical in terms of color formation and of morphologies.

MALDI-TOF MS identification

In order to identify all isolates of our collection, we systematically screened them using the MALDI-TOF MS. Using a standard protein extraction protocol, correct species level identification according to the manufacturer's criteria (cut-off score ≥ 2.00) was obtained for all reference strains (*C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. dubliniensis* ATCC 2118, *C. dubliniensis* ATCC 2119, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018, *C. tropicalis* ATCC 750 and *Cryptococcus neoformans* ATCC 90112) [27]. Correct species level identifications were obtained for 97.63% (413) of the clinical isolates (423; for more information about level scores see Eddouzi et *al*. [27]). Among the identified isolates, 84.16% showed a cut-off score ≥ 2.3, while the rest had a cut-off score < 2.299. MALDI-TOF MS distinguished between *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* with spectral scores between 2.176 and 2.4. The distribution of yeast species identified by MALDI-TOF MS has been already reported by Eddouzi et *al*. [27].

MALDI-TOF failed to identify 10 isolates given their low spectral scores (< 1.7) and neither could they be identified by ID 32 gallery tests. Since appropriate reference strains were not included in the MALDI-TOF MS instrument database, we concluded that these isolates were species rarely encountered in clinical samples or were not yet described. However, the ribosomal DNA of these isolates could used to identify nine strains distributed in five species including *Hanseniaspora opuntiae* (three isolates), *Candida palmioleophila* (two isolates), *Kodamaea ohmeri* (two isolates), *Debaryomyces hansenii* (anamorph form *C. famata*, one isolate) and *Pichia caribbica* (anamorph form *C. fermentati*, one isolate) (see Table 2) [27]. The remaining isolate was identified as a newly described yeast species: *Candida tunisiensis* [27].

FLC and VRC susceptibilities of the yeast collection

The species distribution and *in vitro* susceptibilities to FLC and VRC determined by CLSI disk diffusion of 405 *Candida* isolates are summarized in Table 4. The results of *in vitro* susceptibility studies showed that FLC was most active against *C. albicans*, *C. parapsilosis sensu lato*, *C. tropicalis*, *C. dubliniensis*, *C. lusitaniae*, *C. kefyr*, *C. guilliermondii*, *C. utilis* and *C. intermedia* in 99.07% of cases. Decreased susceptibility to FLC was observed with *C. glabrata*, *C. krusei*, *C. palmioleophila* and *C. guilliermondii*. Thus, despite the fact that overall 95.56% of clinical *Candida* isolates were susceptible to FLC, these data demonstrate that the rare species (*C. palmioleophila*) exhibited decreased susceptibility in the range known for resistant species such as *C. krusei* and *C. glabrata*. Only one *C. albicans* isolate (JEY355) was resistant to FLC. The zone of FLC growth inhibition was 13 mm and 32 mm for VRC.

VRC was considerably more active (98.51%) than FLC (95.56%) against all tested *Candida* species, although it was not particularly active against isolates of *C. glabrata* and *C. tropicalis* that were already resistant to FLC. Only four isolates, including *C. glabrata* (JEY4, JEY16 and JEY314) and *C. tropicalis* (JEY162), exhibited cross-resistance between FLC and VRC. JEY16 and JEY314 showed

Table 4 *Candida* species distribution and *in vitro* susceptibilities to fluconazolel (FLC) and voriconazole (VRC) as determined by Clinical and Laboratory Standards Institute (CLSI) disk diffusion method.

		Susceptibility of isolates (%)							
		FLC				VRC			
Species	No. of isolates (%) tested	Range of inhibition zone diameters (mm)	S	SDD	R	Range of inhibition zone diameters (mm)	S	SDD	R
Candida albicans	185 (45.67)	13–41	184 (99.46)		1 (0.54)	24–46	185 (100)		
C. glabrata	70 (17.28)	NIZ–32	64 (91.42)	3 (4.28)	3 (4.28)	NIZ–39	66 (94.28)	1 (1.44)	3 (4.28)
C. parapsilosis	56 (13.82)	17–33	56 (100)			23–41	56 (100)		
C. tropicalis	48 (11.85)	NIZ–34	47 (97.92)		1 (2.08)	NIZ–35	47 (97.92)		1 (2.08)
C. dubliniensis	21 (5.18)	27–44	21 (100)			31–48	21 (100)		
C. krusei	8 (1.97)	7–13			8 (100)	18–22	8 (100)		
C. metapsilosis	5 (1.23)	19–23	5 (100)			28–31	5 (100)		
C. lusitaniae	4 (1)	30–41	4 (100)			38–46	4 (100)		
C. kefyr	2 (0.5)	34–38	2 (100)			37–41	2 (100)		
C. palmioleophila	2 (0.5)	6–8			2 (100)	18–21	1 (50)	1 (50)	
C. orthopsilosis	1 (0.25)	21	1 (100)			27	1 (100)		
C. guilliermondii	1 (0.25)	17	1 (100)			23	1 (100)		
C. utilis	1 (0.25)	30	1 (100)			33	1 (100)		
C. intermedia	1 (0.25)	36	1 (100)			40	1 (100)		
Total	405 (100)	–	387 (95.56)	3 (0.74)	15 (3.7)	–	399 (98.51)	2 (0.49)	4 (0.98)

S, **s**usceptible; SDD, **s**usceptible dose dependent; R, resistant; NIZ, No inhibition zone.

no growth inhibition zone for the two drugs and the same result was observed for the *C. tropicalis* isolate JEY162. The apparent intrinsic resistance of *C. krusei* to FLC was not observed for VRC. VRC was active against 73.33% of FLC-resistant *Candida* isolates.

Etest and Sensititre YeastOne colorimetric methods

The isolates that exhibited cross-resistance or susceptibility dose-dependent (SDD) profiles with FLC and VRC were verified by two commercial methods including Etest and Sensititre YeastOne (SYO) plates. The MIC values for control and clinical strains are shown in Table 5. Etest showed that *C. albicans* isolate JEY355 was still susceptible to FLC and VRC (MIC values of 8 μg/ml and 0.032 μg/ml, respectively). Using SYO plates, JEY355 was SDD to FLC and resistant to VRC (MIC values of 16 and 4 μg/ml, respectively). High MIC values for other azoles were observed for JEY355 (posaconazole: >8 μg/ml; itraconazole: 16 μg/ml and ketoconazole: 8 μg/ml). Further

Table 5 Minimum inhibitory concentrations (MICs) for yeast isolates determined by Etest and Sensititre YeastOne.

	Etest (μg/ml)		Sensititre YeastOne (μg/ml)							
Isolate	FLC	VRC	FLC	VRC	PCZ	ITR	KCZ	5-FC	AMB	CAS
JEY355[a]: *Candida albicans*	**8**[b]	0.032	**16**	4	**>8**	**16**	**8**	0.5	1	0.03
ATCC 90028: *Candida albicans*	0.25	0.012	-	-	-	-	-	-	-	-
JEY4: *Candida glabrata*	**>256**	4	**128**	4	**>8**	**>16**	2	0.12	1	0.25
JEY16: *Candida glabrata*	**>256**	**>32**	**>256**	**16**	**>8**	**>16**	**8**	0.12	1	0.25
JEY314: *Candida glabrata*	**>256**	**>32**	**>256**	**16**	**>8**	**>16**	**8**	0.12	1	0.25
ATCC 90030: *Candida glabrata*	14	0.15	-	-	-	-	-	-	-	-
JEY162: *Candida tropicalis*	**>256**	**>32**	**>256**	**>16**	**>8**	**>16**	**16**	0.12	**8**	0.25
ATCC 750 *Candida tropicalis*	0.75	0.064	-	-	-	-	-	-	-	-
JEY182: *Kodamaea ohmeri*	**24**	0.25	**32**	0.25	0.25	1	0.12	0.06	0.5	**>16**
JEY234: *Kodamaea ohmeri*	**24**	0.25	**16**	0.06	0.12	0.25	0.06	0.12	0.5	**>16**
JEY379: *C. palmioleophila*	**>256**	1	**128**	1	0.5	1	4	0.5	0.5	0.25
JEY380: *Candida palmioleophila*	**256**	0.75	**128**	0.5	0.5	1	4	0.5	0.5	0.12

FLC, fluconazole; VRC, voriconazole; PCZ, posaconazole; ITR, itraconazole; KCZ, ketoconazole; 5-FC, 5-flucytosine; AMB, amphotericin B; CAS, caspofungin. [a]For strain references see Eddouzi *et al*. [27]. [b]Values equal or above resistance breakpoints are indicated in bold letters.

investigations revealed that these high MICs were due to overexpression of *MDR1* encoding for the major facilitator superfamily (MFS) membrane transporter as the result of a new gain-of-function mutation in the zinc cluster transcription factor Mrr1 (data not shown).

Three *C. glabrata* isolates (JEY16, JEY314 and JEY4) showed cross-resistance to FLC and VRC with high MIC values obtained by Etest (>256 μg/ml). Among these strains, JEY16 and JEY314 exhibited high MIC values for VRC (>32 μg/ml), while only 4 μg/ml for JEY4. Both JEY16 and JEY314 appeared to be also more resistant to azole drugs as compared to JEY4 using the SYO plate (Table 5).

Among *C. tropicalis* isolates, only JEY162 showed cross-resistance between FLC and VRC by disk diffusion assays, which was confirmed by Etest and SYO plates (Etest MICs >256 μg/ml for FLC and >32 μg/ml for VRC). Surprisingly, JEY162 was also resistant to amphotericin B (SYO MIC: 8 μg/ml). Using molecular analysis, we demonstrated that JEY162 displayed alterations in genes involved in ergosterol biosynthesis (data not shown). Two clinical isolates identified by molecular methods as *C. palmioleophila* were resistant to FLC (Etest and SYO MICs: 256 μg/ml and 128 μg/ml, respectively). The disk diffusion test showed that two *K. ohmeri* isolates were resistant to FLC but SDD to FLC as determined by Etest and SYO, respectively. In this study, we observed that *K. ohmeri* was resistant to caspofungin (SYO MIC >16 μg/ml), which so far has not been reported. The two isolates were still susceptible to remaining agents.

Discussion

In this work, 423 yeasts isolated from different specimens of Tunisian hospital patients were characterized morphologically and then identified by MALDI-TOF MS which is recommended as a reference method for yeast identification [27]. CHROMagar Candida is one of the most widely used media in the mycology laboratory that is advertised as able to identify *C. albicans*, *C. krusei*, and *C. tropicalis*. With the increasing incidence of human disease being produced by the less common *Candida* species, we were interested in testing the performance of this medium by identifying the distribution of this species in our collection. While MALDI-TOF MS could resolve up to 97% of the species in the investigated collection, this proportion was however only 65% for CHROMagar Candida [27], thus highlighting the power of MALDI-TOF MS. The results obtained by the chromogenic medium after 24–48 h of incubation showed correct identifications for *C. krusei* and *C. tropicalis* [42–44]. CHROMagar Candida was however unable to differentiate between the two related species *C. albicans* and *C. dubliniensis*, as already mentioned by Sahand et *al*. [44]. Our findings are in accordance with previous reports that *C. dubliniensis* shows different green color tones that are difficult to differentiate from the green color produced by *C. albicans* [42,44,45]. Growth at 42°C shows that no or highly restricted growth of all *C. dubliniensis* isolates as compared to *C. albicans* [45]. Other studies also considered CHROMagar Candida as reliable for the presumptive identification of *C. glabrata* [43,46], although other researchers did not agree [42,47]. Purple, dark pink and violet colonies, could only be interpreted with precaution as presumptive *C. glabrata* isolates, since other yeasts also grow as purple, dark pink and violet colonies on CHROMagar Candida [42,47,48].

All *C. krusei* (eight isolates, 1.9%) and *C. tropicalis* (48 isolates, 11.34%) identified by CHROMagar Candida were confirmed by MALDI-TOF MS [27]. MALDI-TOF MS shows the ability to easily differentiate species that are morphologically and phylogenetically similar to each other. In this study, it was not difficult to distinguish between the species of *C. parapsilosis sensu lato*. Among the *C. parapsilosis sensu lato* isolates, 56 (90.32%) were identified as *C. parapsilosis sensu stricto*, 5 (8.06%) as *C. metapsilosis* and 1 (1.61%) as *C. orthopsilosis* [27]. These data were in agreement with previously published reports [21,23,26]. The specificity of MALDI-TOF MS for identifying unusual yeast isolates was over 99%. In most cases, organisms not identified by MALDI-TOF MS were not included in the instrument database. Consequently, MALDI-TOF MS did not produce any misidentifications, providing that spectra for the appropriate reference strains were present in the database [21,22,49]. A novel finding was the ability of MALDI-TOF MS to identify *C. glabrata* (69/70, 98.57%), *C. parapsilosis sensu lato* (57/62, 91.93%), *C. krusei* (8/8, 100%) and *C. lusitaniae* (4/4, 100%) with high spectral scores ≥2.3 [27]. Several studies have reported the efficiency of MALDI-TOF MS for fast and accurate identification of bacterial, yeast and mold species [12,20–24,26,49]. From 10 isolates not identified by MALDI-TOF MS, the sequencing of their ITS regions including 5.8S as well as D1/D2 domains of LSU rRNA allowed to resolve nine isolates to the species level (see Table 2). Only the isolate, JEY63, was identified as novel species and was named *C. tunisiensis* [27].

The results of the identification of our collection showed that the species distribution of 406 *Candida* isolates was in agreement with published studies [31,37,50]. The five most common *Candida* species including *C. albicans* (45.56%), *C. glabrata* (17.24%), *C. parapsilosis* (13.79%), *C. tropicalis* (11.82%) and *C. dubliniensis* (5.17%), accounted for 93.59% of all isolates. For non-*C. albicans Candida* species, we observed that *C. parapsilosis* preceded *C. tropicalis*. The same results were observed in North America in a study conducted between 2001 and

2007 and in an epidemiology study in China [50,52]. Recently, Diekema *et al*. [33] and Lockhart *et al*. [34] found the same epidemiology profiles for the four major *Candida* species in healthcare-associated bloodstream infections.

In this work, the *in vitro* susceptibility of *Candida* species showed that the incidence of FLC resistance was higher than for VRC. Indeed, only one isolate of *C. albicans* was resistant to FLC. This was due to a new gain-of-function mutation in the zinc cluster transcription factor Mrr1 [53,54], which results in the overexpression of *MDR1*. In addition, the decreased susceptibility to FLC was most pronounced in *C. glabrata* and *C. krusei*, which was consistent with broad surveillance studies [31,33,34,37,50–52]. It is known that *C. glabrata* can develop azole resistance at a relatively high frequency [35]. Recent findings suggest that gain-of-function mutations in the transcription factor CgPdr1 are involved in the constitutive high expression of ABC-transporter genes (*CgCDR1*, *CgCDR2* and *CgSNQ2*) [55]. Interestingly, the azole-resistant *C. glabrata* isolates of this study showed mitochondrial dysfunctions with the typical absence of growth on non-fermentable carbon sources such as glycerol (data not shown). Mitochondrial deficiency is one of the mechanisms by which azole resistance can occur in *C. glabrata* [35,55–58]. *C. glabrata* isolates with mitochondrial dysfunctions, even if they exhibit strong *in vitro* fitness decrease [56], can still be obtained from clinical samples. Interestingly, such isolates may exhibit no decrease of *in vivo* fitness as compared to wild type isolates as suggested by Ferrari *et al*. [58], which could explain why they can be still recovered from patients.

Despite the increase in frequency of fungal infections caused by the yeast *C. tropicalis*, azole resistance has been insufficiently investigated. Furthermore, *C. tropicalis* develops drug resistance in the presence of FLC more rapidly than other *Candida* species [59]. Our results in the present study showed that JEY162 was resistant to several azoles including FLC, VRC, posaconazole, itraconazole and ketoconazole. Interestingly, this isolate exhibited a decreased susceptibility to amphothericin B. Using a quantitative analysis of ergosterol, the main sterol component of cell membrane was typical for both a $\Delta^{5,6}$ desaturase and a lanosterol 14α-demethylase defect. Using molecular analysis, we demonstrated that JEY162 displayed loss function mutations in genes encoding these enzymes *ERG3* and *ERG11* (data not shown).

Resistance to FLC for uncommon *Candida* species was observed in this collection. The major species was *C. palmioleophila*, which was misidentified by conventional methods as *C. famata* or *C. guilliermondii* [60,61]. Recently, Jensen and Arendrup [60], showed that the susceptibility pattern for *C. palmioleophila* was unique with FLC MICs in a range of 8 to > 16 μg/ml. *Candida palmioleophila* was ranked as susceptible or intermediate for itraconazole, posaconazole and VRC but was still susceptible to echinocandins. In this study, the two FLC-resistant isolates were less susceptible to itraconazole (1 μg/ml) and ketoconazole (4 μg/ml) but still susceptible to caspofungin (0.12–0.25 μg/ml). Many authors have found increasing numbers of uncommon *Candida* species resistant to azole drugs [31,50–52]. Thereby, it seems relevant to assess their susceptibility profiles to guide the clinician for therapeutic decisions.

Previous studies have reported that *K. ohmeri* was an emerging fungal pathogen in immunocompromised patients [62–65]. The antifungal susceptibility of the two isolates of this study (JEY182 and JEY234) showed SDD MICs for FLC but were resistant to caspofungin (MIC: > 16 μg/ml). In previous reports, decreased susceptibility to FLC had been reported together with low MICs to other echinocandins (anidulafungin, micafungin and caspofungin) [62–65]. Our study is the first to report caspofungin resistance in *K. ohmeri*. Unfortunately, it cannot be determined from

Table 6 Correlation of susceptibility of yeast clinical isolates to azole drugs determined by three susceptibility methods.

	Zone diameter (mm)		Etest		SYO	
Isolate	FLC	VRC	FLC (μg/ml)	VRC (μg/ml)	FLC (μg/ml)	VRC (μg/ml)
Candida albicans JEY355	13	32	8	0.032	16	4
C. glabrata JEY4	NIZ	12	> 256	4	128	4
C. glabrata JEY16	NIZ	NIZ	> 256	> 32	> 256	16
C. glabrata JEY314	NIZ	NIZ	> 256	> 32	> 256	16
C. tropicalis JEY162	NIZ	NIZ	> 256	> 32	> 256	16
Kodamaea ohmeri JEY182	13	25	24	0.25	32	0.25
K. ohmeri JEY234	14	27	24	0.25	16	0.06
C. palmioleophila JEY379	6	15	> 256	1	128	1
C. palmioleophila JEY380	8	17	256	0.75	128	0.5

SYO, Sensititre YeastOne; FLC, fluconazole; VRC, voriconazole; NIZ, No inhibition zone.

patient records whether or not this resistance profile was due to candin exposure in the patient.

In this work, three different methods were used to assess antifungal susceptibility on specific subset of isolates. Comparisons of MIC values for FLC and VRC obtained by Etest and SYO plate indicated a good correlation between obtained MICs (Table 6). One exception was for JEY355 which was ranked as VRC-susceptible by disk diffusion and Etest methods however as VRC-resistant by SYO system. The results obtained for *C. glabrata* and *C. tropicalis* isolates were in agreement in all three tests. Some discrepancy was observed for *K. ohmeri* isolates, which were FLC-resistant by disk diffusion, while only SDD by Etest and SYO plate.

In conclusion, our study has provided clinically useful data of yeast infections in Tunisia. MALDI-TOF MS was used as a reference method to identify clinically yeast isolates. It is an advanced technique with high potential in the identification of uncommon yeasts and permitted identification of a new yeast species. MALDI-TOF MS can also give a robust phylogenetic approach very similar to that deduced from molecular methods. Examination of susceptibility of clinical isolates to azoles by three methods showed good agreement to confirm antifungal susceptibility profiles and a good practical approach to provide target drugs for therapy. Even if low incidence of antifungal resistance was observed in this work, the constant susceptibility surveillance of clinical isolates is useful since it does not only provide important data for therapeutic decisions, but also provides novel specimens for in-depth molecular studies.

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