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Comparative study of *Candida* spp. isolates: Identification and echinocandin susceptibility in isolates obtained from blood cultures in 15 hospitals in Medellín, Colombia

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ABSTRACT

Objectives: Invasive candidiasis has a high impact on morbidity and mortality in hospitalised patients. Accurate and timely methods for identification of *Candida* spp. and determination of echinocandin susceptibility have become a priority for clinical microbiology laboratories.
Methods: This study was performed to compare matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) identification with sequencing of the D1/D2 region of the rRNA gene complex 28 subunit in 147 *Candida* spp. isolates obtained from patients with candidaemia. Antimicrobial susceptibility testing was performed by broth microdilution (BMD) and Etest. Sequencing of the *FKS1* and *FKS2* genes was performed.
Results: The most common species isolated were *Candida albicans* (40.8%), followed by *Candida parapsilosis* (23.1%) and *Candida tropicalis* (17.0%). Overall agreement between the results of identification by MALDI-TOF/MS and molecular identification was 99.3%. Anidulafungin and caspofungin susceptibility by the BMD method was 98.0% and 88.4%, respectively. Susceptibility to anidulafungin and caspofungin by Etest was 93.9% and 98.6%, respectively. Categorical agreement between Etest and BMD was 91.8% for anidulafungin and 89.8% for caspofungin, with lower agreements in *C. parapsilosis* for anidulafungin (76.5%) and *C. glabrata* for caspofungin (40.0%). No mutations related to resistance were found in the *FKS* genes, although 54 isolates presented synonymous polymorphisms in the hotspots sequenced.
Conclusions: MALDI-TOF/MS is a good alternative for routine identification of *Candida* spp. isolates. DNA sequencing of the *FKS* genes suggested that the isolates analysed were susceptible to echinocandins; alternatively, unknown resistance mechanisms or limitations related to antifungal susceptibility tests may explain the resistance found in a few isolates.

1. Introduction

Invasive candidiasis represents >70% of fungal infections in hospitalised patients [1], and 90% of invasive candidiasis is caused by five species, namely *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Candida krusei* [2,3]. However, there are significant differences in the distribution of *Candida* spp. and their antifungal susceptibility according to geographic region [4,5].

Conventional methods for identification of yeasts to species level, based on macroscopic and microscopic characteristics, biochemical profile by manual or automated systems as well as

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chromogenic agar media are widely used for identification of yeasts and offer efficiency and accuracy to facilitate the diagnosis of fungal infections [6]. Molecular identification of *Candida* spp. is considered the gold standard [7], however this technology has high costs and is time consuming and technically demanding.

Recently, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) has been used in routine diagnosis for the identification of micro-organisms by analysing their protein spectra [8]. This technology allows rapid identification of different types of micro-organisms, including common bacteria, mycobacteria, anaerobes, yeasts and moulds [9]. This identification technique has been evaluated compared with conventional methods and has been found to be more accurate for identification of *Candida* spp. [10,11].

Mutations in the *Candida FKS* genes, which encode the enzyme targeted by echinocandins, result in elevated minimum inhibitory concentrations (MICs) and have been linked to therapeutic failures [12]. Patients infected with *C. glabrata* isolates harbouring *FKS* mutations are more likely to fail echinocandin therapy than those infected with isolates without *FKS* mutations [13]. In addition, in patients with prior echinocandin exposure, treatment failure with echinocandins was frequent, as well as *FKS* mutation rates; in particular, caspofungin MICs were significantly higher and caspofungin resistance was more common compared with patients without prior exposure to echinocandins [14].

Echinocandin resistance is an emerging phenomenon in severely ill patients receiving longer-term therapy. Results of a population-based laboratory surveillance for candidaemia in four metropolitan areas in the USA, conducted by the US Centers for Disease Control and Prevention (CDC), demonstrated that the proportion of non-susceptible *C. glabrata* rose from 4.2% in 2008 to 7.8% in 2014 and the proportion of non-susceptible isolates at each hospital ranged from 0% to 25.8% [15,16].

Several studies have indicated that resistance to echinocandins in Latin America could be higher than that reported in other populations. A study performed from 2008–2010 in 21 tertiary care hospitals in Latin America showed resistance to anidulafungin in 2/42 (4.8%) *C. glabrata* isolates and 1/253 (0.4%) *C. albicans* isolates. Intermediate susceptibility was found in 2/118 (1.7%) *C. tropicalis* isolates [17]. More recently, Grupo GERMEN reported 7.3% and 7.7% resistance to anidulafungin and caspofungin, respectively, and found higher MICs for these echinocandins in almost all species [18] compared with those reported in previous studies involving isolates from North America, the Asia-Pacific Region, Latin America and Europe [5].

In a recent study conducted in 15 hospitals in Medellín (Colombia), Grupo GERMEN, a local network addressing antimicrobial resistance surveillance in Colombia, analysed 300 isolates causing invasive candidiasis and found variable resistance to fluconazole according to the species: 20% for *C. parapsilosis*; 7.6% for *C. albicans*; and 7.4% for *C. tropicalis* [18]. Azole-resistant *Candida* infections represent therapeutic challenges because treatment options are limited [19]. Therefore, it is essential to make a rapid and accurate identification of isolates and to determine their susceptibility to other antifungals, particularly echinocandins, which are the first-line therapy in patients with severe sepsis caused by azole-resistant *Candida* spp. (*C. glabrata*) or for those with prior exposure to azoles [20].

Owing to the importance of echinocandins as first-line antifungals in severe *Candida* infections, it becomes crucial to know the epidemiology of resistant isolates as well as the accuracy of laboratory microbiological methods available to identify *Candida* spp. and their susceptibility to these antifungals. In the present study, using a collection of isolates from patients with candidaemia obtained and typed in a previous report [18], the MICs for echinocandins determined by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) method and Etest were compared. In addition, the presence of *FKS* gene mutations was sought in all isolates. Species identification was performed comparatively using MALDI-TOF/MS and sequencing of the D1/D2 region of the rRNA gene complex 28 subunit.

2. Materials and methods

2.1. Sample

The sample consisted of 147 *Candida* spp. isolates obtained from patients with candidaemia collected as part of a previous study that included hospitalised patients recruited from 15 hospitals located within the Metropolitan Area of Medellín, Colombia, during a 15-month period (August 2010–November 2011) [18]. Preserved isolates were recovered using different methodologies according to their storage conditions and were subsequently cultured on Sabouraud dextrose agar (Becton Dickinson & Co., Sparks, MD). All yeasts were incubated for 48–72 h at room temperature (25 °C) and their growth and purity were confirmed.

2.2. Molecular identification

The D1/D2 region of the rRNA gene complex 28 subunit was amplified following international guidelines for the molecular identification of fungi for *Candida* identification. Genomic DNA was extracted from isolated colonies grown on Sabouraud dextrose agar using a QIAamp® DNA Mini Kit (QIAGEN, Germantown, MD) following the manufacturer's recommendations. The molecular markers were amplified using the primers and protocols previously described for the D1/D2 region [7,21]. Amplified products from the D1/D2 region (ca. 600 bp) were sent to Macrogen (Rockville, MD) for Sanger bidirectional sequencing. Sequencher 5.0 software (Gene Code Corp., Ann Arbor, MI) was used for editing and aligning the sequences. A search was then made in the following databases for each sequence to establish similarity with known strains: the NCBI databases (BLAST) (National Center for Biotechnology Information, Washington, DC); CBS-KNAW (Fungal Biodiversity Centre); and MycoBank database (International Mycological Association).

2.3. FKS sequencing

Genomic DNA was extracted from yeast cells grown overnight in yeast extract–peptone–dextrose (YPD) broth medium using a QIAamp® DNA Mini Kit. PCR and sequencing primers were designed to amplify an ca. 3-kb region of the echinocandin drug target genes *FKS1* and *FKS2* as previously described [22–24]. PCR amplification was carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA) in a 50 μL reaction volume containing 50 ng of genomic DNA, 0.2 μM of each primer and 25 μL of EmeraldAmp® Master Mix (Takara Bio Inc., Mountain View, CA). PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). Automated fluorescent sequencing was performed in both 5′ and 3′ directions by Macrogen Corp. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene 12.0; DNASTAR, Inc., Madison, WI).

2.4. MALDI-TOF/MS identification

Using a 1 μL plastic loop, a small quantity of a single colony was deposited on a spot of the target slide (disposable 48-well target slide; bioMérieux, Durham, NC) and was processed according to the manufacturer's recommendations in a MALDI-TOF mass spectrometer (bioMérieux). Resulting spectra were analysed using the IVD database v.2.0 included in the MYLA® software

(bioMérieux). A quantitative value (% probability) was calculated, which relates to how well the observed spectrum compares with the typical spectrum of each organism, so that values closer to 99.9% indicate a closer match; in this study, a cut-off of 90.0% was used. Calibration was performed using the reference strain *Escherichia coli* ATCC 8739, and uninoculated matrix was included in each run as a negative control. All isolates were analysed in duplicate.

2.5. Antimicrobial susceptibility testing

The BMD method for echinocandins was performed in duplicate in accordance with CLSI guidelines [25]. Caspofungin and anidulafungin powders were obtained from Sigma-Aldrich (cat. SML0425-25MG; Merck KGaA, Darmstadt, Germany) and Pfizer (Peapack, NJ), respectively. Etest (bioMérieux, Marcy-l'Étoile, France) was performed in accordance with the manufacturer's recommendations. MICs were interpreted by applying the clinical interpretive breakpoints defined by the CLSI [26]. The isolates were classified as susceptible (S), intermediate or resistant (R) to both echinocandins according to the following breakpoints: for *C. albicans*/*C. dubliniensis*, *C. tropicalis* and *C. krusei*, S, ≤0.25 μg/mL, R, ≥1 μg/mL; for *C. parapsilosis* complex and *Candida guilliermondii*, S, ≤2 μg/mL, R, ≥8 μg/mL; and for *C. glabrata*, S, ≤0.12 μg/mL, R ≥0.5 μg/mL. Quality control was performed by testing *C. krusei* ATCC 6258 and *C. parapsilosis* 22019.

2.6. Agreement analysis

For each species, percent agreement was calculated from the results of identification to species level obtained by MALDI-TOF/MS and molecular identification of the D1/D2 region of the rRNA gene complex 28 subunit. Likewise, results of the MICs by both susceptibility testing methods were compared and overall categorical agreement was calculated. Categorical agreement was defined as susceptible, intermediate and resistant results that matched between the two methods. Results were considered to be in categorical agreement when the Etest results and reference MICs were within the same interpretive category. Percent categorical agreement was calculated by dividing the number of tests with no category discrepancy by the number of organisms tested.

The percentage error was ranked into three categories: very major error (VME), consisting of false-susceptible result by Etest; major error (ME), consisting of false-resistant result produced by Etest; and minor error (mE), consisting of intermediate result by Etest method and a resistant or susceptible category for the reference BMD method. According to the CLSI, acceptable levels recommended are <1.5% for VME, <3% for ME and 10% for mE [25].

2.7. Ethics statement

This study, based on national and international ethical considerations, was a retrospective study limited to laboratory analysis of *Candida* spp. isolates from hospitalised patients obtained as part of their clinical care. No additional procedures were performed and there were no risks associated with this research. The original investigation received approval from the Ethics Committee of the Corporación para Investigaciones Biológicas (Medellín, Colombia) and included the review and approval of hospitals participating in the selection of patients.

3. Results

3.1. Identification of Candida spp.

In this study, the most common species were *C. albicans* (40.8%), followed by *C. parapsilosis* (23.1%) and *C. tropicalis* (17.0%). Overall agreement between the results of identification by MALDI-TOF/MS and molecular identification by sequencing the D1/D2 region of the rRNA gene complex 28 subunit was 99.3%. For most species, agreement between both identification methods was 100%, except for *Candida auris*, for which no identification result was obtained by MALDI-TOF/MS (Table 1).

3.2. Susceptibility results to anidulafungin and caspofungin

Overall susceptibility to anidulafungin and caspofungin determined by using BMD method was 98.0% and 88.4%, respectively, whilst susceptibility to both echinocandins by Etest was 93.9% and 98.6%.

Differences in anidulafungin susceptibility between the two methods were observed in *C. parapsilosis* (97.1% by BMD and 79.4% by Etest). In contrast, susceptibility to caspofungin in *C. glabrata* was higher by Etest compared with the reference BMD method (100% and 20.0%, respectively) (Table 2).

Overall categorical agreement between BMD and Etest methods for anidulafungin and caspofungin was 91.8% and 89.8%, respectively. However, differences in agreement were observed according to species and antifungal used; *C. parapsilosis* showed 76.5% categorical agreement and *Meyerozyma guilliermondii* complex showed 80.0% categorical agreement for anidulafungin; and *C. krusei* and *C. glabrata* showed 33.3% and 40.0% categorical agreement, respectively, for caspofungin. Etest showed higher MICs than the BMD method for anidulafungin against *C. parapsilosis* and *M. guilliermondii* complex (MIC_{50} = 1 μg/mL by Etest vs. 0.5 μg/mL by BMD) (Table 3).

By Etest, one (0.7%) VME, two ME (1.4%) and nine (6.1%) mE were found for anidulafungin. Two (1.4%) VME and 16 (10.9%) mE

Table 1
Performance of MALDI-TOF/MS versus molecular identification by rRNA sequencing (region D1/D2) of *Candida* spp. isolates.

Candida spp. identified by sequencing	No. (%) of isolates	MALDI-TOF/MS identification	No. of isolates	% agreement
C. albicans	60 (40.8)	*C. albicans*	60	100
C. parapsilosis	34 (23.1)	*C. parapsilosis*	34	100
C. tropicalis	25 (17.0)	*C. tropicalis*	25	100
C. glabrata	10 (6.8)	*C. glabrata*	10	100
C. guilliermondii	7 (4.8)	*C. guilliermondii*	7	100
C. krusei	3 (2.0)	*C. krusei*	3	100
Meyerozyma guilliermondii complex (*Meyerozyma guilliermondii*/*M. caribbica*)	3 (2.0)	*C. guilliermondii*	3	100
C. lusitaniae	2 (1.4)	*C. lusitaniae*	2	100
C. intermedia	1 (0.7)	*C. intermedia*	1	100
C. auris	1 (0.7)	Without identification	1	0.00
C. duobushaemulonii/*pseudohaemulonii* group	1 (0.7)	*C. haemulonii*	1	100
Overall agreement	147		146	99.3

MALDI-TOF/MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry.

Table 2
Comparative antimicrobial susceptibility results for anidulafungin and caspofungin by the broth microdilution (BMD) and Etest methods.

Candida spp.	Interpretative category	*n* (%) susceptible			
		Anidulafungin		Caspofungin	
		BMD	Etest	BMD	Etest
C. albicans (*n* = 60)	S	59 (98.3)	60 (100)	56 (93.3)	60 (100)
	I	0 (0.0)	0 (0.0)	4 (6.7)	0 (0.0)
	R	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)
C. parapsilosis (*n* = 34)	S	33 (97.1)	27 (79.4)	34 (100)	34 (100)
	I	1 (2.9)	4 (11.8)	0 (0.0)	0 (0.0)
	R	0 (0.0)	3 (8.8)	0 (0.0)	0 (0.0)
C. tropicalis (*n* = 25)	S	25 (100)	25 (100)	23 (92.0)	25 (100)
	I	0 (0.0)	0 (0.0)	2 (8.0)	0 (0.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
C. glabrata (*n* = 10)	S	9 (90.0)	10 (100)	2 (20.0)	10 (100)
	I	1 (10.0)	0 (0.0)	6 (60.0)	0 (0.0)
	R	0 (0.0)	0 (0.0)	2 (20.0)	0 (0.0)
Meyerozyma guilliermondii complex (*Meyerozyma guilliermondii*/*M. caribbica*)[a] (*n* = 10)	S	10 (100)	8 (80.0)	10 (100)	10 (100)
	I	0 (0.0)	2 (20.0)	0 (0.0)	0 (0.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
C. krusei (*n* = 3)	S	3 (100)	3 (100)	2 (66.7)	2 (66.7)
	I	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other *Candida* spp.[b] (*n* = 5)	S	5 (100)	5 (100)	3 (60.0)	4 (80.0)
	I	0 (0.0)	0 (0.0)	2 (40.0)	1 (20.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total *Candida* spp. (*n* = 147)	S	144 (98.0)	138 (93.9)	130 (88.4)	145 (98.6)
	I	2 (1.4)	6 (4.1)	15 (10.2)	2 (1.4)
	R	1 (0.7)	3 (2.0)	2 (1.4)	0 (0.0)

S, susceptible; I, intermediate; R, resistant.
[a] Includes 7 *C. guilliermondii* and 3 *Meyerozyma guilliermondii* complex (*Meyerozyma guilliermondii*/*M. caribbica*).
[b] Includes *C. lusitaniae* (*n* = 2), *C. intermedia* (*n* = 1), *C. auris* (*n* = 1) and *C. duobushaemulonii*/*pseudohaemulonii* group (*n* = 1).

Table 3
MIC_{50} and MIC_{90} values (in μg/mL) and percent categorical agreement (%CA) between susceptibility results by the Etest and broth microdilution (BMD) methods for anidulafungin and caspofungin.

	Anidulafungin			Caspofungin		
	MIC_{50}	MIC_{90}	%CA	MIC_{50}	MIC_{90}	%CA
C. albicans (*n* = 60)						
BMD	0.03	0.064	98.3	0.125	0.25	93.3
Etest	0.002	0.004		0.012	0.064	
C. parapsilosis (*n* = 34)						
BMD	0.5	2	76.5	0.5	1	100
Etest	1	3		0.5	0.5	
C. tropicalis (*n* = 25)						
BMD	0.03	0.03	100	0.125	0.25	92.0
Etest	0.012	0.016		0.023	0.094	
C. glabrata (*n* = 10)						
BMD	0.03	0.03	90.0	0.25	0.5	40.0
Etest	0.004	0.008		0.023	0.094	
Meyerozyma guilliermondii complex (*Meyerozyma guilliermondii*/*M. caribbica*)[a] (*n* = 10)						
BMD	0.5	1	80.0	0.5	0.5	100
Etest	1	3		0.38	1	
C. krusei (*n* = 3)						
BMD	0.064	0.064	100	0.25	0.5	33.3
Etest	0.012	0.023		0.25	0.5	
Other *Candida* spp.[b] (*n* = 5)						
BMD	0.032	0.032	100	0.25	0.5	80.0
Etest	0.012	0.023		0.25	0.38	
Total *Candida* spp. (*n* = 147)						
BMD	0.03	1	91.8	0.25	1	89.8
Etest	0.008	2		0.064	0.5	

$MIC_{50/90}$, minimum inhibitory concentration for 50% and 90% of the isolates, respectively.
[a] Includes 7*C. guilliermondii* and 3 *Meyerozyma guilliermondii* complex (*Meyerozyma guilliermondii*/*M. caribbica*).
[b] Includes *C. lusitaniae* (*n* = 2), *C. intermedia* (*n* = 1), *C. auris* (*n* = 1) and *C. duobushaemulonii*/*pseudohaemulonii* group (*n* = 1).

were observed for caspofungin. A false-susceptible result was obtained by Etest (VME) in one isolate of *C. albicans* for anidulafungin and in two isolates of *C. glabrata* for caspofungin.

A false-resistant result by Etest (ME) to anidulafungin occurred in two isolates of *C. parapsilosis*. Six of the nine mE that occurred for anidulafungin were in *C. parapsilosis*, of which five had intermediate susceptibility by Etest and being susceptible by the BMD method. Of the 16 mE that occurred in caspofungin, 14 correspond to false-susceptibility results by Etest, corresponding to an intermediate susceptibility result by the BMD method, especially in *C. glabrata*.

3.3. FKS sequencing

DNA sequencing of the two hotspot (HS) regions of the drug target gene *FKS1* was performed in all isolates of *C. albicans*, *C. tropicalis*, *C. krusei*, *Candida lusitaniae* and *M. guilliermondii* complex. For *C. glabrata and C. parapsilosis*, DNA sequencing of the two HS regions of the drug target genes *FKS1* and *FKS2* was performed.

No mutations leading to amino acid changes were found in the *FKS* gene(s) in the 147 *Candida* spp. isolates analysed. However, 54 of the 147 isolates presented single nucleotide polymorphisms in the hotspots sequenced, of which 46 (85%) had silent mutations mainly in the *FKS1* HS1 and 8 (15%) presented silent mutations in *FKS1* HS2.

The majority of the *FKS1* HS1 synonymous mutations were found in *C. albicans* (82%). Mutations in the *FKS1* HS2 were found only in *C. tropicalis* isolates. The following strains were not evaluated as no reference sequences are available for these specific species: *Candida haemulonii* ($n = 1$); *C. auris* ($n = 1$); and *C. intermedia* ($n = 1$).

4. Discussion

Identification of *Candida* spp. in clinical isolates is important owing to differences in the antifungal susceptibility profiles depending on the species, with direct consequences in the selection of antifungal treatment for these infections. Thus, rapid identification of species will narrow the spectrum of therapeutic options, reduce costs and improve outcomes [1,2,5,27].

Agreement between MALDI-TOF/MS identification for 147 isolates of *Candida* spp. compared with molecular identification by DNA sequencing obtained in this study was 99.3%. This agreement was higher than that reported by Galán et al. (96.7%) [8] and Lacroix et al. (98.3%) [28]. According to this, MALDI-TOF/MS could be an alternative for routine identification of *Candida* clinical isolates in diagnostic microbiology laboratories, taking advantage of the limited hands-on of the method and the rapid results.

The only species that could not be identified by the MALDI-TOF method was *C. auris* because this species is not included in the IVD database v.2.0 coupled to the MYLA® program. Recently, the company has released a new version that includes *C. auris* in the RUO module. However, experience about the performance of the system in identifying *C. auris* with this database addition is unknown. This species may be misidentified as *C. haemulonii* by VITEK®2 [29]. Proper identification of *C. auris* has become particularly important since it is an emerging nosocomial pathogen that often exhibits resistance to fluconazole, as has been described recently in isolates from India and Venezuela [30,31].

No discrepancies in the identification of other species were found; however, identification using sequencing showed three yeast isolates belonging to the *M. guilliermondii* complex in this study. The genus *Meyerozyma* includes *Candida guilliermondii* (*Meyerozyma guilliermondii*) and *Candida fermentati* (*Meyerozyma caribbica*) [32]. These results confirm those reported by Romi et al. who showed that species which are closely related in the *M. guilliermondii* complex are difficult to differentiate in vitro and it may be necessary to use the internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) method for this purpose [33]. Difficulty in identification using phenotype-based methods is also found in related species such as *Candida famata* and *C. haemulonii*; for this reason, Brandt and Lockart recommend identification in these cases using either molecular or MALDI-based methods [32].

In one isolate, both identification methods used showed a lack of discrimination between species belonging to the *Candida duobushaemulonii/pseudohaemulonii* group. In a study by Cendejas-Bueno et al., it was concluded that *C. haemulonii*, *C. haemulonii* var. *vulnera*, *C. duobushaemulonii*, *C. pseudohaemulonii* and *C. auris* are part of a group of yeasts that presents difficult identification by different methods, suggesting the use of molecular methods based in non-ITS targets [34].

Overall categorical agreement found between the susceptibility results by Etest and the BMD for anidulafungin and caspofungin was 91.8% and 89.8%, respectively. However, lower percentages of categorical agreement were observed in *C. parapsilosis* (76.5%) and *M. guilliermondii* complex (80.0%) for anidulafungin, and in *C. glabrata* (40.0%) and *C. krusei* (33.3%) for caspofungin. Several studies have pointed out lower percentages of agreement between both methods for caspofungin in *C. glabrata* and *C. krusei* [27,35].

Some isolates of *C. parapsilosis* presented decreased susceptibility to anidulafungin by Etest, but this phenomenon was not observed for caspofungin. This situation could have an important clinical impact if Etest is used as a routine susceptibility testing method, since it could lead to consider not utilising anidulafungin as a therapeutic option.

Higher MICs for anidulafungin in *C. parapsilosis* and *M. guilliermondii* complex were obtained by Etest compared with those obtained by the BMD method. This confirms the report by Espinel-Ingroff et al. in which they concluded that Etest is suitable for determination of susceptibility to anidulafungin in *C. albicans*, *C. tropicalis* and *C. glabrata*, but not for *C. parapsilosis* and *C. guilliermondii* [36]. These findings suggest that isolates showing decreased susceptibility to this antifungal by Etest in these two species must be tested by an alternative method.

The categorical agreement obtained for caspofungin by Etest was 40.0% in *C. glabrata* and 33.3% in *C. krusei*, showing more isolates with intermediate susceptibility and resistance by the BMD method. This is consistent with others reports that showed a significantly lower percentage of concordance for caspofungin (47.8%) in *C. glabrata* [37].

Susceptibility to caspofungin has also been questioned when BMD is used since several factors introduce variability in the results according to a recent multicentre study [38]. Therefore, susceptibility results to caspofungin against *C. glabrata* with either method should be interpreted with caution in clinical practice when this echinocandin is used for severe infections.

Despite the discrepancies found when Etest is compared with the standard BMD method, the overall discrepancies found in the present study were lower than the acceptable percentage recommended by the CLSI ($\leq$1.5% for VME and $\leq$3% for ME) [25]. This may support the use of Etest for routine susceptibility testing of *Candida* isolates for echinocandins owing to its easier inclusion in the laboratory workflow; however, caution in interpreting susceptibility results must be applied when *Candida* isolates different from *C. albicans* are predominant.

Sequencing of the two HS regions of the drug target gene(s) *FKS* known to confer echinocandin resistance did not show non-synonymous mutations in the *Candida* spp. isolates that were analysed, even though some isolates turned out to be resistant to caspofungin but susceptible to anidulafungin (Table 2, see *C. glabrata*). This may suggest that isolates analysed in this study are in fact susceptible and that the resistant and intermediate results could be due to the intrinsic limitations of phenotypic susceptibility testing methods. Conversely, these results could suggest other echinocandin resistance mechanisms yet to be described.

In conclusion, MALDI-TOF/MS (VITEK® MS) is an excellent alternative for routine identification of *Candida* spp. isolates in diagnostic microbiology laboratories. Owing to the lack of agreement found for Etest in species different to *C. albicans*, this method should not be considered as a susceptibility testing method in these cases. Prospective studies are necessary to know the evolving resistance of echinocandins in *Candida* isolates since its increasing use could have an impact on resistance.

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Competing interests

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Ethical approval

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