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SUSCEPTIBILITY OF *Candida* spp. ISOLATED FROM BLOOD CULTURES AS EVALUATED USING THE M27-A3 AND NEW M27-S4 APPROVED BREAKPOINTS

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SUMMARY

The high mortality rates associated with candidemia episodes and the emergence of resistance to antifungal agents necessitate the monitoring of the susceptibility of fungal isolates to antifungal treatments. The new, recently approved, species-specific clinical breakpoints (SS-CBPs)(M27-S4) for evaluating susceptibility require careful interpretation and comparison with the former proposals made using the M27-A3 breakpoints, both from CLSI. This study evaluated the susceptibility of the different species of *Candida* that were isolated from candidemias based on these two clinical breakpoints. Four hundred and twenty-two isolates were identified and, among them, *C. parapsilosis* comprised 46.68%, followed by *C. albicans* (35.78%), *C. tropicalis* (9.71%), *C. glabrata* (3.55%), *C. lusitaniae* (1.65%), *C. guilliermondii* (1.65%) and *C. krusei* (0.94%). In accordance with the M27-A3 criteria, 33 (7.81%) non-susceptible isolates were identified, of which 16 (3.79%) were resistant to antifungal agents. According to SS-CBPs, 80 (18.95%) isolates were non-susceptible, and 10 (2.36%) of these were drug resistant. When the total number of non-susceptible isolates was considered, the new SS-CBPs detected 2.4 times the number of isolates that were detected using the M27-A3 interpretative criteria. In conclusion, the detection of an elevated number of non-susceptible species has highlighted the relevance of evaluating susceptibility tests using new, species-specific clinical breakpoints (SS-CBPs), which could impact the profile of non-susceptible *Candida* spp. to antifungal agents that require continuous susceptibility monitoring.

KEYWORDS: Resistance; SS-CBPs; CLSI breakpoints; Antifungals; Candidemia.

INTRODUCTION

Candidemia occupies a prominent place among the invasive fungal infections[1,2,3,6,20]. This infection is among the most common fungal infections in hospitalized patients and leads to a long hospital stay, increased hospital costs[14] and a mortality rate reaching 61% in Brazil[10].

Several conditions are associated with the development of candidemia, such as the following: a) the use of antibacterial agents; b) the presence of a central venous catheter; c) the use of type 2 histamine receptor blockers (H_2); d) total parenteral nutrition; e) admission at an intensive care unit (ICU); f) the use of corticosteroids; g) surgeries; h) previous hospitalization; and i) colonization by *Candida*[2,3,9,16].

Among the causes of high mortality rates that are observed for candidemia is the failure of antifungal therapies, which is often due to the emergence of resistance[10].

Similar to the resistance phenomena that are observed when using antibacterial agents, antifungal resistance can be innate or secondary to the antifungal agents that are used[13]. Additionally, the limited number of systemic antimycotics that are available has contributed to resistance, first against flucytosine and then against fluconazole. Furthermore, multiple studies have shown that cross-resistance may cover other azole class antifungals[18].

The spectrum of *Candida* species isolated from hospitals is directly linked to the type of patient, geographic location and most commonly used procedures; and, overall, it can define the susceptibility profile to antifungal agents[6,23].

From a temporal perspective, antifungal agents used for candidemia have included amphotericin B, flucytosine associated with amphotericin B, fluconazole and, more recently, voriconazole and echinocandins[15].

Since 1997, susceptibility tests of *Candida* to antifungal agents have been in use due to the standardization developed by the National Committee for Clinical Laboratory Standards (NCCLS-M27-A)[13]. The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (EUCAST)[25] also has a

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reference method for antifungal susceptibility testing that is a modification of the CLSI method[4]. Isolates derived from important clinical situations or sterile sites should be evaluated so that their susceptibility agents can be determined[4]. However, CLSI[4] and EUCAST[25] standardized antifungal susceptibility tests define resistance or susceptibility based on different breakpoints.

Datasets that correlate CLSI and EUCAST MICs with outcomes revealed lower response rates when MICs were > 4 µg/mL for *C. albicans, C.tropicalis* and *C. parapsilosis* and > 16 µg/mL for *C. glabrata*. These findings led to the CLSI Subcommittee on Antifungal Susceptibility testing in an effort to harmonize the CLSI and EUCAST breakpoints for some *Candida* species. The new approved breakpoints (SS-CBPs) provide consistency with the EUCAST breakpoints and should be more sensitive in the detection of emerging resistance among *Candida* spp[8,22].

Here, these two criteria for *Candida* spp. isolates were compared in cases of candidemia that occurred between 1995 and 2009 and were registered at the University Hospital of Santa Maria (HUSM), Santa Maria, RS, Brazil.

METHODS

1. Period of evaluation: This study evaluated the susceptibility of *Candida* species isolated from episodes of candidemia over 15 years (1995 to 2009).

2. Microorganisms: Four hundred and twenty-two *Candida* spp. strains isolated from episodes of candidemia that occurred at the University Hospital of Santa Maria (HUSM); Santa Maria, Rio Grande do Sul, Brazil. HUSM is a public, tertiary care teaching hospital with 328 beds and 10300 admissions, including adults and children, per year. The samples (blood) were collected from different wards, including adult, pediatric and neonatal intensive care units (ICU). Only one isolate of a candidemia case was included in this study.

3. Identification: Blood cultures were performed using an automated BACTEC 9120 (Becton Dickinson). *Candida* spp. isolated from blood agar plates were identified using standard methods, such as chlamydospore production, germ tube assays, micro-morphology studies in corn-meal-Tween 80 agar and biochemical tests using the commercial system ID32C (bioMérieux Marcy l'Etoile, France). The isolates were stored in BHI with 20% glycerol and 0.2% agar suspensions and frozen at -70 ºC until processed for the study. Before testing, each isolate was cultured on Sabouraud dextrose agar and CHROMagar to ensure purity and viability.

4. Susceptibility tests to antifungal agents: Assays for susceptibility were performed using broth microdilution according to the M27-A3 protocol of the Clinical and Laboratory Standards Institute (CLSI)[4]. All isolates were tested against amphotericin B (Sigma Chemical Co., St Louis, Mo), fluconazole (Sigma Chemical Co, St Louis, Mo), itraconazole (Janssen-Cilag Pharmaceutica; Belgium), flucytosine (Sigma Chemical Co), voriconazole (Pfizer, Inc) and caspofungin (Merck, Rahway, NJ), which were obtained from pure powder and prepared at the indicated concentrations according to the procedures of protocol M27-A3[4]. The tests were performed in microdilution plates in which 0.1 mL of antifungal 2X concentrate was used. The microplates were then sealed with Parafilm and frozen at -70 °C until use. The inocula were prepared from 24-48 h cultures in Sabouraud dextrose agar by suspending the fungal cells in sterile, distilled water, and the turbidity was spectrophotometrically fixed according to M27-A3. Lastly, the antifungal drugs were diluted in RPMI-1640 buffered with morpholinepropanesulfonic acid (MOPS). On the day of testing, microdilution plates containing 100 µL of RPMI-1640 with different concentrations of antifungals were inoculated with 100 µL of diluted culture, resulting in 0.5 x 10^3 to 2.5 x 10^3 cells/mL in each well. The addition of this volume resulted in the required final concentration of the antifungal agents and, at the same time, the correct number of cells that were recommended in each well. The plates were closed and incubated at 35 ºC for 24-48 h, and the MIC endpoint was determined according to document M27-A3. The susceptibility tests were interpreted according to two criteria: a) CLSI (M27-A3) and b) SS-CBPs (CLSI M27-S4)[5,24] as shown in Table 1. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included for quality control tests, sterility control of the medium and control of the medium with antifungals[5].

5. Statistical analysis: The Wilcoxon nonparametric test was used to compare two variables. Values of $p \leq 0.05$ were considered significant.

RESULTS

The study of the susceptibility of *Candida* spp. isolated from episodes of candidemia that occurred between 1995 and 2009 included 422 isolates that were distributed as follows: *C. parapsilosis* (197/46.68%), *C. albicans* (151/35.78%), *C. tropicalis* (41/9.71%), *C. glabrata* (15/3.55%), *C. lusitaniae* (7/1.65%), *C. guilliermondii* (7/1.65%) and *C. krusei* (4/0.94%). Two hundred and seventy-one (64.21%) isolates were grouped as *Candida* non-*albicans* species. The parameters for evaluating antifungal susceptibility (range: of susceptibility, MIC50 and MIC90) as well as the percentage of resistance that was obtained using the CLSI criteria according to M27-A3 and M27-S4 (SS-CBPs) are found in Tables 2 and 3.

When the susceptibility of all *Candida* isolates that were obtained from candidemia cases over 15 years was evaluated based on the CLSI M27-A3 criteria[4], 16 resistant isolates were identified: itraconazole, n = 9 (*C. albicans* = 3; *C. glabrata* = 4; *C. tropicalis* = 1; *C. krusei* = 1); fluconazole, n = 5 (*C. glabrata* = 1; *C. krusei* = 4); and voriconazole, n = 2 (*C. glabrata* = 2). Using the same criteria, 17 susceptible-dependent-dose (SDD) or intermediate (I) isolates were found: flucytosine, n = 5 (*C. parapsilosis* = 1 and *C. glabrata* = 4); itraconazole, n = 6 (*C. glabrata* = 3 and *C. krusei* = 3); and fluconazole, n = 6 (*C. tropicalis* = 1 and *C. glabrata* = 5). The number of non-susceptible (SDD or Intermediate plus Resistant) strains was 33 (Tables 2 and 3).

Based on the SS-CBPs (CLSI M27-S4)[5], the total number of resistant isolates was 10: fluconazole, n = 8 (*C. tropicalis* = 3, *C. glabrata* = 1 and *C. krusei* = 4); and voriconazole, n = 2 (*C. tropicalis*). Of the susceptible dose-dependent (SDD) or Intermediate (I) isolates, 70 (16.58%) were detected and distributed as follows: fluconazole, n = 51 [*C. albicans* (15), *C. parapsilosis* (14), *C. tropicalis* (8) and *C. glabrata* (14)]; voriconazole, n = 16 [*C. albicans* (4), *C. parapsilosis* (7) and *C. tropicalis* (5)], and caspofungin (n = 3) [*C. glabrata* (3)]. The number of non-susceptible (SDD or Intermediate plus Resistant) strains was 80 (Tables 2 and 3).

Table 1
Interpretative breakpoints according to the MICs (µg/mL) from the M27-S4 and M27-A3 CLSI documents

Candida Species	Antifungal agents*	M27-S4 breakpoints			M27-A3 breakpoints		
		S	**SDD**	**R**	**S**	**SDD**	**R**
C. albicans	FLZ	≤ 2.0	4.0	≥ 8.0	≤ 8.0	16-32	≥ 64
	VOR	≤ 0.12	0.25-0.5	≥ 1.0	≤ 1.0	2.0	≥ 4.0
	CAS	≤ 0.25	0.5	≥ 1.0	≤ 2.0	--	--
C. glabrata	FLZ	--	≤ 32	≥ 64	≤ 8.0	16-32	≥ 64
	VOR	--	--	--	≤ 1.0	2.0	≥ 4.0
	CAS	≤ 0.12	0.25	≥ 0.5	≤ 2.0	--	--
C. krusei	FLZ	--	--	--	≤ 8.0	16-32	≥ 64
	VOR	≤ 0.5	1.0	≥ 2.0	≤ 1.0	2.0	≥ 4.0
	CAS	≤ 0.25	0.5	≥ 1.0	≤ 2.0	--	--
C. parapsilosis	FLZ	≤ 2.0	4.0	≥ 8.0	≤ 8.0	16-32	≥ 64
	VOR	≤ 0.12	0.25-0.5	≥ 1.0	≤ 1.0	2.0	≥ 4.0
	CAS	≤ 2.0	4.0	≥ 8.0	≤ 2.0	--	--
C. tropicalis	FLZ	≤ 2.0	4.0	≥ 8.0	≤ 8.0	16-32	≥ 64
	VOR	≤ 0.12	0.25-0.5	≥ 1.0	≤ 1.0	2.0	≥ 4.0
	CAS	≤ 0.25	0.5	≥ 1.0	≤ 2.0	--	> 2.0
C.guilliermondii	CAS	≤ 2.0	4.0	≥ 8.0	≤ 2.0	--	> 2.0

(*)FLZ = fluconazole; VOR = voriconazole; CAS = caspofungin; (--) breakpoints not provided by CLSI documents M27-S4 and M27-A3.

Based on the number of *Candida* spp. that were not susceptible to antifungal agents, the new SS-CBPs detected 2.4 times the number of non-susceptible isolates that were detected using the M27-A3 breakpoints ($p < 0.05$) (Table 3).

DISCUSSION

The study's results indicated minor variations in relation to the species of *Candida* involved in the episodes of candidemia: *C. parapsilosis* was the most prevalent, followed by *C. albicans* and then *C. tropicalis*. The prevalence of *C. parapsilosis* could be associated with the number of candidemia episodes that were detected in neonates and pediatric patients. HINRICHSEN *et al.*[10] mainly reported *C. parapsilosis* in these patients. Additionally, the *Candida* non-*albicans* was more prevalent than C. *albicans*. These results were in accordance with those of many Brazilian studies[6,7,10,19] and have been observed in multicentric, international studies[23].

In general, the study's results showed that the detection of resistant isolates was not a common phenomenon. The definition of *in vitro* resistance to antifungal agents is based on the breakpoints that were established by methods standardized to yeasts in a set of CLSI documents named M27. Although the technical recommendations for performing these tests have not been changed, the interpretative breakpoints were redefined. In the CLSI M27-A3 document[4], the breakpoints that define susceptible, SDD, or resistant to each antifungal agent encompass all *Candida* species. Recently the Subcommittee on Antifungal Susceptibility Tests of the CLSI developed new species-specific clinical breakpoints (SS-CBPs) for some *Candida* species (CLSI M27-S4[5]). These new breakpoints are an attempt to balance the CLSI and EUCAST breakpoints. Thus, they took into account the distribution of MICs of the wild-type of each *Candida* species, the molecular mechanism of resistance, the categorical agreement between MICs that were generated by both methods (CLSI and EUCAST), as well as the reassessment of the correlation between MICs and outcome of candidemias[8,24]. Due to these recent advances, in the present study, the susceptibility profiles of *Candida* were compared using both parameters.

Based on CLSI M27-A3[4], the total number of non-susceptible isolates that were detected throughout the study was 33 (7.81%), and, using the new breakpoints (SS-CBPs), 80 (18.95%) isolates were non-susceptible; thus, the number of non-susceptible isolates that were identified by these different criteria differed by two-fold (Table 3).

By comparing the detection of resistance between the two interpretative criteria, a major difference in resistance identification was observed for the M27-A3 breakpoints. However, this difference was due to the presence of itraconazole, which was absent among the new M27-S4 breakpoints. On the other hand, the number of susceptible dose-dependent (SDD) or intermediate (I) isolates was increased 4-fold based on the new SS-CBPs breakpoints. The majority of susceptible dose-dependent (SDD) or intermediate (I) isolates occurred against fluconazole, which was in accordance with previous studies because fluconazole is the most common azole associated with alterations in susceptibility, and its use as an indicator of resistance to azoles has been proposed[21]. In this study,

Table 2
Susceptibility profile of *Candida* spp. isolated from blood cultures in 1995-2009. Detection of non-susceptible isolates based on the breakpoints from CLSI M27-A3 and species-specific clinical breakpoints from CLSI M27-S4

Species	N	ATF*	MIC (µg/mL)			CLSI M27-A3 Breakpoints (n)		CLSI M27-S4 Breakpoints (n)	
			Range	MIC 50%	MIC 90%	R	SDD/I	R	SDD/I
C. parapsilosis	197	AMB	0.06-0.5	0.125	0.25	0	0	--	--
		FLZ	0.125-4.0	1.0	4.0	0	0	0	14
		ITZ	0.03-0.5	0.125	0.5	0	0	--	--
		5FC	0.03-16.0	0.5	2.0	0	1	--	--
		VOR	0.03-0.5	0.125	0.25	0	0	0	7
		CAS	0.03-2.0	0.5	2.0	0	0	0	0
C. albicans	151	AMB	0.06-1.0	0.25	0.5	0	0	--	--
		FLZ	0.125-4.0	0.25	4.0	0	0	0	15
		ITZ	0.015-1.0	0.25	0.5	3	0	--	--
		5FC	0.03-4.0	1.0	4.0	0	0	--	--
		VOR	0.015-0.5	0.125	0.5	0	0	0	4
		CAS	0.03-0.5	0.125	0.25	0	0	0	0
C. tropicalis	41	AMB	0.125-1.0	0.125	0.5	0	0	--	--
		FLZ	0.25-32.0	2.0	8.0	0	1	3	8
		ITZ	0.03-2.0	0.125	0.5	1	0	--	--
		5FC	0.125-4.0	1.0	4.0	0	0	--	--
		VOR	0.03-2.0	0.25	0.5	0	0	2	5
		CAS	0.03-0.25	0.06	0.25	0	0	0	0
C. glabrata	15	AMB	0.125-0.5	0.5	1.0	0	0	--	--
		FLZ	0.5-64.0	8.0	32.0	1	5	1	14
		ITZ	0.03-2.0	0.5	1.0	4	3	--	--
		5FC	0.125-16.0	4.0	16.0	0	4	--	--
		VOR	0.06-4.0	0.5	2.0	2	0	--	--
		CAS	0.06-0.5	0.125	0.25	0	0	0	3
C. guilliermondii	7	AMB	0.125-0.25	0.25	0.25	0	0	--	--
		FLZ	0.5-4.0	2.0	4.0	0	0	--	--
		ITZ	0.03-0.5	0.06	0.5	0	0	--	--
		5FC	0.06-1.0	0.5	1.0	0	0	--	--
		VOR	0.06-0.25	0.06	0.06	0	0	--	--
		CAZ	0.03-0.25	0.06	0.06	0	0	0	0
C. lusitaniae	7	AMB	0.125-0.5	0.5	0.5	0	0	--	--
		FLZ	0.25-4.0	2.0	2.0	0	0	--	--
		ITZ	0.03-0.5	0.5	0.5	0	0	--	--
		5FC	0.25-2.0	0.5	2.0	0	0	--	--
		VOR	0.06-0.25	0.25	0.25	0	0	--	--
		CAS	0.06-0.5	0.125	0.25	0	0	--	--
C. krusei	4	AMB	0.125-0.5	0.25	0.5	0	0	--	--
		FLZ	4.0-8.0	8.0	8.0	4**	0	4**	0
		ITZ	0.25-1.0	0.5	0.5	1	3	--	--
		5FC	0.25-2.0	0.5	2.0	0	0	--	--
		VOR	0.06-0.25	0.25	0.25	0	0	0	0
		CAS	0.06-0.5	0.125	0.25	0	0	0	0

(*) AMB = amphotericin B; FLZ = fluconazole; ITZ = itraconazole; 5FC = flucytosine; VOR = voriconazole; CAS = caspofungin; (**) *C. krusei* are assumed to be intrinsically resistant to fluconazole; (--) breakpoints not provided by CLSI documents M27-S4 and M27-A3.

Table 3
Comparison (number and %) of non-susceptible *Candida* spp. to antifungal agents detected using the CLSI M27-A3 breakpoints and the new species-specific clinical breakpoints from CLSI M27-S4

Antifungal Agents	M27-A3		M27-S4	
	SDD	R	SDD	R
Itraconazole	6	9	--	--
Fluconazole	6	5	51	8
Voriconazole	0	2	16	2
Flucytosine	5	0	--	--
Caspofungin	0	0	3	0
Total	17[a] (4.02%)	16 (3.79%)	70[b](16.58%)	10 (2.36%)
	33[c] (7.81%)		**80[d] (18.95%)**	

(--) antifungal agents not contemplated in M27-S4; (%) referent to the total of isolates evaluated in the period (n = 422); (a ≠ b and c ≠ d) $p < 0.05$.

fluconazole resistance detected by M27-A3 was 1.18%, which was similar to the results obtained by COLOMBO *et al.*[6]; on the other hand, the non-susceptible isolates to fluconazole totaled 11 (2.60%), which was lower than the 5% reported by COLOMBO *et al*.[6], but similar to the 2.1% that was reported by DA MATTA *et al.*[7].

This study detected 0.47% (n = 2) of isolates that were resistant to voriconazole, which is similar to the 0.2% that was reported by DA MATTA *et al.*[7] in Brazil, but lower than the percentage reported by other authors: LYON *et al.*[11] detected 1.1%, and MESSER *et al*. detected 0.9%[12].

The percentage resistant to itraconazole (2.13%) was greater than that reported by DA MATTA *et al.*[7], but lower than the 11.5% that was reported by MESSER *et al.*[12] and the 30% that was reported by PEMÁN *et al.* in Spain[17].

For flucytosine, five isolates (5/1.18%) were classified as intermediate (I), which is contrary to the results of DA MATTA *et al.*[7], where 2.5% of the isolates exhibited resistance to this agent. On the other hand, most Brazilian studies did not evaluate the susceptibility of flucytosine because it is only used in combination with amphotericin B once monotherapy is no longer recommended[18].

The susceptibility profile intermediate (I) to caspofungin was found for three isolates of *C. glabrata*. Although none of these were classified as resistant, the finding of this study was in accordance with that of PFALLER *et al*.[23,24], where *C. krusei* and *C. glabrata* were the most common species, with resistances to caspofungin of 12.5% and 2.5%, respectively.

In this study, the evaluation of susceptibility allows one to better understand the local epidemiology of candidemia cases that are resistant to treatment. Detecting a higher number of non-susceptible species has highlighted the importance of evaluating susceptibility tests using new SS-CBPs (CLSI M27-S4[5]), which will impact the monitoring, selection and use of antifungal agents in the clinic.

RESUMO

Suscetibilidade de *Candida* spp. isoladas de hemocultivos, avaliadas pelos *breakpoints* dos documentos M27-A3 e M27-S4 do CLSI

As elevadas taxas de mortalidade associadas com episódios de candidemia e a emergência da resistência aos antifúngicos, requerem o monitoramento da suscetibilidade de *Candida* spp., isoladas das candidemias, frente aos agentes antifúngicos. Os novos *breakpoints,* chamados "espécie-específicos," foram recentemente aprovados (M27-S4) requerendo, pois, cuidadosa interpretação e comparações com aqueles até agora utilizados (M27-A3); ambos são propostos pelo *Clinical Laboratory Standard Institute* (CLSI). O presente estudo avaliou a suscetibilidade de espécies de *Candida* isoladas de candidemias baseando-se nestes dois *breakpoints*. Quatrocentos e vinte e dois isolados de *Candida* foram identificados e assim distribuídos: *C. parapsilosis* (48,68%), *C. albicans* (35,78%), *C. tropicalis* (9,71%), *C. glabrata* (3,55%), *C. lusitaniae* (1,65%), *C. guilliermondii* (1,65%), *C. krusei* (0,94%). Com base nos critérios do M27-A3, um total de 33 (7,81%) isolados foram julgados não-sensíveis, dos quais 16 (3,79%) como resistentes aos antifúngicos. De acordo com os *breakpoints* espécie-específicos (M27-S4) um total de 80 (18,95%) isolados foram considerados não-sensíveis, dos quais 10 (2,36%) resistentes a algum dos antifúngicos testados. Com base nos novos *breakpoints* espécie-específicos, o número de isolados não-sensíveis foi 2,4 vezes maior do que o número de não-sensíveis detectado pelos *breakpoints* do documento M27-A3. A detecção de um elevado número de isolados não-sensíveis através dos *breakpoints* propostos pelo M27-S4 destaca a importância dos testes de suscetibilidade, os quais trarão impactos no reconhecimento de isolados de *Candida* spp. não-sensíveis em episódios de candidemias, requerendo, portanto, continua avaliação.

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