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ANTIFUNGAL SUSCEPTIBILITY PROFILE OF *TRICHOSPORON* ISOLATES: CORRELATION BETWEEN CLSI AND ETEST METHODOLOGIES

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ABSTRACT

The aim of the present study was to evaluate the antifungal susceptibility profile of *Trichosporon* species isolated from different sources employing the Clinical and Laboratory Standards Institute (CLSI) method and E-test method. Thirty-four isolates of *Trichosporon* spp. and six CBS reference samples were tested for their susceptibility to Amphotericin B, 5-flucytosine, Fluconazole, Itraconazole, Voriconazole and Terbinafine. All species showed high Minimun Inhibitory Concentrations (MIC) for Itraconazole and susceptibility to Fluconazole, The comparison among the results obtained by the CLSI method and E-test revealed larger discrepancies among 5-flucytosine and Itraconazole. The present work provides epidemiological data that could influence therapeutic choices. Furthermore, the comparison between different methodologies could help to analyze results obtained by different laboratories.

Key Words: *Trichosporon* spp., antifungal drugs, CLSI, E-test

INTRODUCTION

Systemic fungal infections occur with increasing frequency in hospitalized patients. Although *Candida* species account for the majority of fungal systemic diseases, the number of yeasts that can cause infection continues to increase. In recent years, several reports of trichosporonosis have appeared (3, 6, 11, 16) *Trichosporon* infections are associated with a wide spectrum of clinical manifestations, ranging from superficial cutaneous involvement in immunocompetent individuals to severe systemic disease in immunocompromised patients (19).

Trichosporonosis is mainly caused by six species: *T. asahii* and *T. mucoides* are causative agents of deep-seated infection, *T. cutaneum* and *T. asteroides* cause superficial infections, and *T. ovoides* and *T. inkin* are involved in white piedra of the head and genital area, respectively (4, 17). Kustimur *et al.* (6) reported the first case of disseminated infection due to *T. asteroides* in an intensive care patient.). Carvalho *et al.* (2) reported a systemic infection involving T. cutaneum in a child with Wilm's tumour and Neves *et al.* (9) isolated T. pulullans from the oral cavity of an HIV positive patient.

The increase in the incidence and morbidity of fungal infections has caused interest in the development of new appropriate therapeutics (13). The CLSI method (8) for

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antifungal susceptibility testing includes the genera *Candida* and *Cryptococcus*, but not *Trichosporon* spp. According to Arikan and Hasçelik (1), it is unknown whether there is any species-related variation in the antifungal susceptibility profile of *Trichosporon* isolates. Additionally, the concordance between the methodologies used to delineate antifungal susceptibility profiles has not been extensively accessed for *Trichosporn* spp.

The present study was undertaken to determine the in vitro antifungal susceptibility profile of different species of *Trichosporon* isolates by using the CLSI reference method and E-test. The epidemiological data obtained could be helpful in the development of therapeutic strategies. Besides, the comparison between different methodologies is important to analyze results obtained by different laboratories.

MATERIAL AND METHODS

Isolates

Thirty-four isolates belonging to the collection of the laboratory of Mycology of the department of Microbiology of the Federal University of Minas Gerais were tested. The isolates were obtained from different sources: urine (12), oropharynx (8), blood (4), nail (3) skin (3), hair (1), bronchoalveolar lavage (1), fingers (1) and environment (1). The isolates were previously identified using morphological, physiological and biochemical proofs according with Kurtzman and Fell (4). The tests employed for identification were macroscopic appearance of the giant colony, microscopic features, susceptibility to cycloheximide, growth temperature, diazonium Blue color reaction, urease production and carbohydrate and nitrogen assimilation profiles. Six CBS reference samples (CENTRAALBUREAU VOOR SCHIMMELCULTURES, Baarn, The Netherlands) were included in susceptibility testing: *T. asahii* (CBS – 2479), *T. coremiiforme* (CBS -2482), *T. asteroides* (CBS - 3481), *T. inkin* (CBS - 5585), *T. mucoides* (CBS - 7625) and *T. ovoides* (CBS – 7556). All the strains were maintained at 4° C on Sabouraud Dextrose Agar (SDA, Difco, Detroit, MI, USA) with 300 µg/ml of chloramphenicol and BHI broth (Brain Heart Infusion, Difco), until susceptibility tests were carried out. Transfers were done at 3-month intervals. The isolates were stored for three months.

Antifungal susceptibility testing

CLSI method- Susceptibility testing was performed according to the M27-A2 document of the CLSI (8). Amphotericin B (Sigma Chemical Co., St. Louis, USA), 5-flucytosine (Hoffman La Roche, Bale, Switzerland), Fluconazole (Pfizer São Paulo, Brazil), Itraconazole (Janssen Pharmaceutica, São Paulo, Brazil), Voriconazole (Pfizer, São Paulo, Brazil), and Terbinafine (Novartis Biociências S.A., São Paulo, Brazil) were obtained as reagent grade powders from their respective manufacturers. Dilutions were made in RPMI 1640 medium (Sigma, St Louis, Mo, USA) buffered to pH 7 with 0,165M (3[N-morpholino] propanesulfonic acid) buffer (Sigma). The inoculum was prepared in a concentration of 1-5 x 10^6 cells/ml. The final concentration of the inoculums was 1.0 × 10^3 to 1.5 × 10^3 cell/ml. The final concentration of the antifungal agents was 0.03 to 16 µg/ml for Itraconazole, 0.12 to 64.0 µg/ml for Amphotericin B, Voriconazole, Terbinafine, and Fluconazole and 0.25 to 128.0 µg/ml for 5-flucytosin. Trays were incubated at 35°C and MIC (Minimum Inhibitory Concentration) endpoints were read after 48h of incubation. Drug free and yeast controls were included.

Following incubation, the MICs of Fluconazole, Terbinafine, Voriconazole and Itraconazole were read as the lowest concentration at which prominent decrease in turbidity relative to the growth control was observed (decrease of 80% in turbidity). For Amphotericin B and 5-flucytosine, MIC was considered as the complete inhibition of growth. Quality control was ensured by testing the CLSI recommended strain *C. parapsilosis* ATCC 22019. The isolate was considered to be susceptible if the MIC value was 2-8µg/ml for Fluconazole, ≤ 0,125µg/ml for Itraconazole, 8-16µg/ml for 5-flucytosine, and ≤1µg/ml for Amphotericin B, as suggested by Wolf *et al* (19) and ≤1µg/ml for Voriconazole, as suggested by Pfaller *et al* (10). The isolate was considered to be susceptible if the MIC value for Terbinafine was ≤ 1.4 µg/ml, as considered for

Candida albicans by Ryder *et al* (13).

E-test

All the samples were tested by E-test for susceptibility profile. The test was performed according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). Briefly, the inoculum concentration was adjusted to a 0.5 McFarland standard for *Candida* species (1-5 x 10^6 cell/ml). Then, 0.5 ml of this suspension was inoculated onto plates containing RPMI 1640 agar (1.5%) with 2% glucose using a cotton swab. After a period of 15 minutes, the E-test strips were applied. The antifungal drugs Amphotericin B, Itraconazole, Fluconazole and 5-flucytosine were tested. The plates were incubated at 35° C and read after 24 and 48 hours.

RESULTS

MICs of Voriconazole, Terbinafine, Amphotericin B, 5 - flucytosine, Fluconazole and Itraconazole for six CBS *Trichosporon* spp. strains are represented in Table 1. Amphotericin B, 5 - flucytosine, Fluconazole and Itraconazole were tested by both, CLSI and E-test methods.

Table 1. Minumun inhibitory concentration (µg/ ml) for CBS (Centraalbureau Voor Schimmelcultures) strains obtained by CLSI and E-test methodologies

	AMB	**5-FC**	**FLU**	**ITR**	**TER**	**VOR**
T. asahii (CBS 2479)						
CLSI	0.25	2	32	2	0.03	0.5
E-test	0.25	8	32	1	-	-
T. asteroides (CBS 3481)						
CLSI	1	32	0.5	0,5	1	0.5
E-test	0.125	32	0.75	0.75	-	-
T. coremiiforme (CBS 2482)						
CLSI	0.5	0.5	1	2	2	1
E-test	0.38	0.75	256	0.75		
T. inkin (CBS 5585)						
CLSI	64	2	64	64	8	4
E-test	32	0.006	256	32	-	-
T. mucoides (CBS 7625)						
CLSI	0.12	64	0.5	2	0.25	0.25
E-test	0.38	32	8	0.75	-	-
T. ovoides (CBS 7556)						
CLSI	0.125	2	2	2	2	0.25
E-test	0.25	0.38	0.38	0.38	-	-

- = Test not performed
AMB= Amphotericin B; 5-FC= 5 flucytosine; FLU = Fluconazole; ITR = Itraconazole; TER= Terbinafine; VOR= Voriconazole.

Results shown in table 2 regarding MIC_{50} and MIC_{90} (MIC for 50 % and 90 % of the strains tested respectively) reveal that *T. mucoides* was susceptible to Amphotericin B (1/ 1µg/ ml), Terbinafine (0.5/ 1µg/ ml), and Voriconazole (0.25/ 0.5 µg/ml), while the other species were resistant to these drugs (8, 11, 16) All species showed resistance to Itraconazole and susceptibility to Fluconazole, according to the parameters suggested by Wolf *et al* (16). *T. ovoides* showed susceptibility to Fluconazole and 5-flucytoosine only.

The samples of *Trichosporon* spp. were submitted to comparison between the two methods (CLSI and E-test). Only one sample (*T. ovoides*) showed resistance to Amphotericin B

by the CLSI method (2 µg/ ml) and susceptibility to this drug by E-test (0,75 µg/ ml). When the samples were compared for 5-flucytosine, two (*T. mucoides*, $MIC_{50/\ 90}$ > 250 / > 32 µg/ ml and *T. asahii*, $MIC_{50/\ 90}$ 16/ >32 µg/ ml) showed resistance in both methods. Regarding Itraconazole, two isolates showed susceptibility by the E-test and resistance by CLSI method. The parameters for resistance and susceptibility are those proposed by Wolf *et al* (16) and Pfaller *et al* (8).

Table 2. *In vitro* antifungal susceptibility profile of 34 isolates of *Trichosporon* spp

	MIC range		MIC_{50} (24/48h)	MIC_{90} (24/48h)
Species (n)	**24 hours**	**48 hours**		
T. asahii (26)				
Amphotericin B	0.06 - 64	0.06 - 64	2/ 4	64/ 64
5-flucytosine	0.25 - 32	0.25 - 64	2/ 4	8/ 16
Fluconazole	0.5 - 8	0.5 - 16	2/ 4	8/ 8
Itraconazole	0.5 - 32	0.5 – 64	2/ 4	8/ 16
Terbinafine	0.03 - 4	0.03 - 8	0.5/ 2	4/ 8
Voriconazole	0.03 - 4	0.03 - 4	0.5/ 1	1/ 2
T. asteroides (5)				
Amphotericin B	0.5 - 2	1 - 4	0.5/ 2	2/ 64
5-flucytosine	0.25 - 64	4 - 64	2/ 8	64/ 64
Fluconazole	0.5 - 2	1 - 8	1/ 2	2/ 8
Itraconazole	2 - 4	2 - 16	2/ 8	4/ 16
Terbinafine	0.25 - 4	2 - 8	1/ 4	4/ 8
Voriconazole	0.062 - 1	0.5 - 2	0.5/ 1	1/ 2

MIC: Minimum Inhibitory Concentration
$MIC_{90:}$ Minimum Inhibitory concentration for 90% of the isolates
$MIC_{50:}$ Minimum Inhibitory concentration for 50% of the isolates
T. ovoides and *T. mucoides* were not included in this table due to the small number of isolates tested

DISCUSSION

The comparison of the susceptibility profile of CBS reference samples with the data available in the literature reveals that the results obtained for *T. asahii* (CBS 2479) in the present work were concordant with those obtained by Wolf *et al*. (19) for Amphotericin B and Fluconazole. When we compared the results obtained in the present work by the CLSI method of (2µg/ml), with those obtained by Ghého *et al*. (4), (0,019µg/ ml) the discrepancy among MICs obtained for Itraconazole against *T. mucoides* and *T. ovoides* reached 100 folds the dilution. The comparisons allowed the observation of the differences in MIC values obtained for different groups, even considering reference samples. These differences emphasize the importance of the standardized intra-laboratory conduct, since results obtained in our experiment were compatible with those obtained by Wolf *et al*. (19) that also used a visual system of evaluation, while Guého *et al*. (4) used an automatic system.

The presented data reveal a profile of high resistance of the genus *Trichosporon* to Amphotericin B and Itraconazole, high susceptibility to Fluconazole and moderate resistance to 5-flucytosine. Rodriguez-Tudela *et al*. (12) reported that the majority of the *T. asahii, T. faecali* and *T. coremiiforme* exhibited resistance to Amphotericin B in vitro. Li *et al*. (7) reported resistance to Amphotericin B among *T. asahii, T. cutaneum* and *T. inkii* strains. Differently, Uzun *et al*. (18), analyzing the in vitro susceptibility of eight samples of *Trichosporon* spp. reported that those samples were susceptible to Amphotericin B, but susceptible to Fluconazole. Silva, *et al*., (15) also evaluate the antifungal susceptibility profile of *T. asahii* clinical isolates. The isolates had reduced susceptibility *in vitro* to all drugs, showing 4-6 times higher MICs to itraconazole and 3-4 times to 5-flucytosine compared of MICs

breakpoint values. In this study, Fluconazole exhibited the best activity *in vitro* against the majority of the isolates (90%), with MIC of 16 µg/ml and only one isolate with 32 µg/ml. Three isolates have MIC value for Amphotericin B above 2 µg/ml.

Variable susceptibility to Amphotericin B has been observed with samples of the genus *Trichosporon*, especially among isolates obtained from immunocompromised patients. This observation could result in a better conduct for antifungal therapy. On the other hand, Fluconazole was effective indicating that the azolic can be the valid option in the therapy of this infection, commonly difficult to treat.

Resistance to Itraconazole was observed in all of the *Trichosporon* species, as well as susceptibility to the Fluconazole. Only *T. mucoides* was susceptible to Amphotericin B, Terbinafine and Voriconazole, being the other species resistant to these drugs. The results obtained in this work with Voriconazole, when compared to those obtained by Uzun et al. (17) were surprisingly more than 30 times higher. On the other hand, Serena et al. (14) used a guinea pig model of systemic trichosporonosis to demonstrate a better efficacy of Voriconazole in comparison with Amphotericin B.

Regarding the comparison between the CLSI method and the E-test, the greater divergence from those reported in previous works were obtained for 5-flucytosine, while data obtained for Amphotericin B were considerably in agreement with other researchers. Although the number of samples analyzed is small, the correlation between the two methods for Amphotericin B is significant (1, 4, 19).

Trichosporon species has been increasingly involved in systemic infections and, despite this fact, there are relatively few studies about its antifungal susceptibility profile. Although these data do not correlate the susceptibility "in vitro/in vivo", the present work provides epidemiological data that could influence therapeutic choices. Furthermore, the comparison between different methodologies could help to analyze results obtained by different laboratories.

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