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Comparison of microdilution method and E-test procedure in susceptibility testing of caspofungin against *Candida* non-*albicans* species

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SUMMARY

It is widely known that systemic and mucosal candidiasis caused by *Candida* non-*albicans* strains endangers the lives of hospitalised patients since these pathogens are extremely difficult to defeat by commonly used antifungal agents. The present study determined the *in vitro* activities of a novel antimicotic drug - caspofungin - against 76 *Candida* non-*albicans* isolates by means of the CLSI reference method for broth dilution antifungal susceptibility testing of yeasts and the E-test procedure for comparison. Caspofungin was efficacious against the majority of strains tested, with the average $MICs_{90}$ evaluated by the microdilution method and E-tests amounting to 1 mg/l and 0.5 mg/l, respectively. Since the agreement between MICs within ±2 dilutions obtained by these two techniques was 92% (Kappa coefficient of 0.92), the E-test procedure seems to be a reliable alternative to the broth microdilution method and may provide another choice for clinical laboratories.

KEY WORDS: Caspofungin, *Candida* non-*albicans*, Broth microdilution method, E-test

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INTRODUCTION

Recently, the yeasts belonging to *Candida* non-*albicans* species have been frequently isolated from mucous membranes of both hospitalised and ambulatory patients. These microorganisms are often known to be more resistant to conventional antifungal therapy than *Candida albicans* strains. Thus, the infections caused by *Candida* non-*albicans* are usually more difficult to treat. The candidiasis problem especially concerns immunocompromised patients (Cybulski *et al*., 2003).

Caspofungin is a representative of a novel antifungal echinocandin family with a unique mode of action. Being an inhibitor of (1,3)-beta-glucan synthesis, caspofungin possesses an excellent safety profile since the mammalian cells are deprived of this compound by nature (Ullmann, 2003; Letscher-Bru and Herbrecht, 2003).

A number of *in vitro* and *in vivo* studies have described caspofungin as a potent anti-*Candida* drug that destroys both planktonic and sessile cells of the yeasts. Caspofungin, which exerts fungicidal activity on *C. albicans* strains as well as on non-*albicans* isolates (Espinel-Ingroff, 1998; Marco *et al*., 1998; Pfaller *et al*., 1999a; Kuhn *et al*., 2002; Serefko *et al*., 2006) has proved to be highly effective as a salvage therapy for the treatment of mucosal and invasive candidiasis (Ullmann, 2003).

Broth microdilution method for assessing *in vitro* activity of antifungals is the one recommended by CLSI (Clinical Laboratory Standards Institute) and widely employed in clinical laboratories. It is a highly time-absorbing and labour-consuming procedure despite being not difficult to perform. According to literature (Laverdiere *et*

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al., 2002; Chryssanthou and Cuenca-Estrella, 2002), the E-test technique has proven a simple straightforward and reliable alternative to the CLSI method for determining *in vitro* susceptibility of fungal pathogens.

The aim of the present study was to:

1. estimate the *in vitro* activity of caspofungin against yeasts of *Candida* non-*albicans* species;
2. evaluate the agreement between MICs of caspofungin achieved by the CLSI reference broth microdilution method and the E-test procedure.

MATERIALS AND METHODS

Microorganisms

A collection of 76 isolates belonging to 12 different species of *Candida* non-*albicans* was selected for this study: *C. glabrata* (24), *C. tropicalis* (16), *C. kefyr* (10), *C. famata* (8), *C. parapsilosis* (4), *C. dubliniensis* (3), *C. maris* (3), *C. sake* (2), *C. krusei* (2), *C. lusitaniae* (2), *C. inconspicua* (1), *C. norvengensis* (1). The isolates were cultured from the nasopharynx of hospitalised and ambulatory adults. All microorganisms were identified by standard methods and stored on Sabouraud dextrose agar until the study was performed. Before the experiment, each isolate was passaged onto fresh agar to ensure purity and optimal growth characteristics.

Caspofungin

Standard antifungal powder of caspofungin acetate was examined (Merck & Co., Inc., USA). Stock solution containing 16 mg/ml was prepared in sterile distilled water and stored frozen at -20ºC until usage. The E-test strips with caspofungin were purchased from AB Biodisk, Sweden.

Susceptibility testing *in vitro*.

1. Broth microdilution method. The broth microdilution testing was performed according to CLSI directions. Serial two fold dilutions of caspofungin were made in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). Final concentrations of caspofungin ranged from 0.03 to 32 mg/l. First, stock inoculum suspensions of yeasts were adjusted to optical density corresponding to 0.5 Mc Farland standard, i.e. 1×10^6 to 5×10^6 cells/ml in sterile 0.85% NaCl, and then suitably diluted to a concentration of 0.5×10^3 to 2.5×10^3 cells/ml in RPMI 1640 medium. Unexpectedly, it appeared that 0.5 McFarland standard was insufficient for conducting assay by the broth microdilution method for *C. maris* isolates, due to very poor growth of all 3 strains. The studies of *C. maris* isolates were conducted on stock inoculum suspension matching 2 McFarland standard since their suspensions are non-homogenous. After 48 h of incubation at 35ºC, the minimal inhibitory concentrations (MICs) were visually evaluated and defined as the lowest drug concentration that showed complete growth inhibition. Drug-free and yeast-free controls were included. All experiments were done in triplicate. The representative data are presented.
2. E-test procedure. The E-test method was performed according to the manufacturer's guidelines. The strips of caspofungin possess continuous concentration gradients of 0.002 to 32 mg/l. The yeast inocula were prepared in sterile 0.85% NaCl to match the turbidity of 0.5 McFarland standard. The E-test procedures for *C. maris* strains remained unchanged. 90 mm-diameter plates containing RPMI 1640 agar medium supplemented with 2% glucose and MOPS were used. The RPMI agar was inoculated by using of a sterile swab dipped in a yeast suspension and the plates were left at room temperature for about 15 min in order to allow moisture to be absorbed completely. Then, an E-test strip was applied onto each plate. The plates were incubated at 35ºC for 48 h. MIC values were read at the point of intersection between inhibition ellipse edge and the scale on the E-test strip. Microorganisms inside the inhibition zone were not taken into account. All experiments were done in triplicate. The representative data are presented.

Correlation coefficient

The Kappa test was used to calculate the degree of agreement. We converted our results for statistical analysis requirements and treated the MIC values obtained for each isolate by both methods within two dilutions as compatible. Kappa coefficients greater than 0.75 represent excellent agreement, coefficients below 0.4 represent poor

agreement and coefficients between 0.4 and 0.75 represent fair to good agreement.

Quality control

Quality control was performed for both broth microdilution and E-test methods by using *C. parapsilosis* ATCC 22019. The MICs of caspofungin obtained for the quality control isolate were within the control range (1 mg/l evaluated by the microdilution method and 0.5 mg/l assessed by the E-test strips) established by Barry *et al.* (2000).

RESULTS

Tables 1 and 2 summarize the susceptibilities of the examined yeasts to caspofungin, depending on the species and the method. The MICs of caspofungin for all *Candida* sp. isolates ranged from 0.06 to 4 mg/l as determined by the broth microdilution method and from 0.06 to 1 mg/l, when the E-test strips were used.

In the course of examinations by the broth microdilution method, the so-called "Eagle effect" -

TABLE 1 - *In vitro activity of caspofungin against Candida sp. isolates determined by the broth microdilution method.*

Organism	*Number of isolates tested*	*MIC (mg/l)*		
		Range	*50%*	*90%*
C. glabrata	24	0.06-2	0.5	2
C. tropicalis	16	0.06-1	0.25	0.5
C. kefyr	10	0.06-0.5	0.25	0.5
C. famata	8	0.12-1	0.5	1
C. parapsilosis	4	1-4	1	4
C. dubliniensis	3	0.12-0.5	0.25	0.5
C. maris	3	0.25-0.5	0.5	0.5
C. sake	2	0.25	0.25	0.25
C. krusei	2	0.5-2	0.5	2
C. lusitaniae	2	0.5-1	0.5	1
C. inconspicua	1	1	nd	nd
C. norvengensis	1	0.25	nd	nd
All	76	0.06-4	0.5	1

nd - not determined

TABLE 2 - *In vitro activity of caspofungin against Candida sp. isolates determined by the E-test method.*

Organism	*Number of isolates tested*	*MIC (mg/l)*		
		Range	*50%*	*90%*
C. glabrata	24	0.06-0.5	0.25	0.5
C. tropicalis	16	0.06-0.25	0.12	0.25
C. kefyr	10	0.06-0.25	0.12	0.25
C. famata	8	0.06-0.5	0.12	0.5
C. parapsilosis	4	0.25-1	0.5	1
C. dubliniensis	3	0.12-0.25	0.12	0.25
C. maris	3	0.5	0.5	0.5
C. sake	2	0.12-0.25	0.12	0.25
C. krusei	2	0.5	0.5	0.5
C. lusitaniae	2	0.5	0.5	0.5
C. inconspicua	1	0.25	nd	nd
C. norvengensis	1	0.12	nd	nd
All	76	0.06-1	0.25	0.5

nd - not determined

TABLE 3 - *The percentages of agreement between E-test and reference caspofungin MICs for Candida sp. isolates tested on RPMI 1640.*

Organism	*Number of isolates tested*	*% agreement*		
		The same dilution	*Within 1 dilution*	*Within 2 dilutions*
C. glabrata	24	12	67	92
C. tropicalis	16	31	69	87
C. kefyr	10	50	90	90
C. famata	8	12	62	100
C. parapsilosis	4	25	25	75
C. dubliniensis	3	33	100	100
C. maris	3	67	100	100
C. sake	2	50	100	100
C. krusei	2	50	50	100
C. lusitaniae	2	50	100	100
Other	2	0	50	100
All	76	28	71	92

Other - *C. inconspicua, C. norvengensis*

paradoxical turbidity at the highest drug concentrations imitating fungal growth - was observed for 6 isolates. Based on the literature data (Bartizal *et al*., 2003; Stevens *et al*., 2004) the "Eagle effect" was ignored for the MIC determinations.

The MICs of caspofungin evaluated by the CLSI reference microdilution method at which 50% and 90% of all examined isolates were inhibited (MIC_{50} and MIC_{90}) were 0.5 mg/l and 1 mg/l, respectively. The same values assessed by the E-test procedure amounted to 0.25 mg/l for MIC_{50} and 0.5 mg/l for MIC_{90}. All strains proved to be susceptible to caspofungin. In general, the E-test MICs values were quite easy to read since the inhibition ellipses were mostly sharply defined.

The E-test MICs were compared with the broth microdilution MICs. As the E-test strips possess the continuous gradient of concentrations, the MICs in-between twofold dilutions were elevated to the next twofold level to make them correspond to the dilution schema of the broth microdilution method. The percentages of agreement between the E-test MICs and MICs attained by the reference microdilution method are presented in Table 3. In general, the agreement between MICs within ±1 and ±2 dilutions provided by both techniques employed in this study was 71% and 92%, respectively. All MICs values were read at 48 h.

TABLE 4 - *Agreement between E-test and reference caspofungin MICs for Candida sp. isolates tested on RPMI 1640 measured by Kappa coefficient.*

Organism	*Number of isolates tested*	*Kappa coefficient*
C. glabrata	24	0.91
C. tropicalis	16	0.86
C. kefyr	10	0.88
C. famata	8	1
C. parapsilosis	4	0.73
C. dubliniensis	3	1
C. maris	3	1
C. sake	2	1
C. krusei	2	1
C. lusitaniae	2	1
C. inconspicua	1	1
C. norvengensis	1	1
All	76	0.92

Kappa coefficient calculated for the MICs obtained with the two methods was 0.92. Its values ranged from 0.73 (for *C. parapsilosis*) to 1 (for 8 species) and are shown in Table 4.

DISCUSSION

Caspofungin, like other members of a new echinocandin family disrupts cell wall glucan formation by a non-competitive inhibition of (1,3)-

beta-glucan synthase. This compound is known to possess a potent *in vitro* activity against a wide range of clinically relevant *Candida* isolates (Ullmann, 2003; Letscher-Bru and Herbrecht, 2003).

MICs of caspofungin obtained in our study were consistent with the values reported previously (Marco *et al*., 1998; Pfaller *et al*., 1999a; Ostrosky-Zeichner *et al*., 2003). Caspofungin proved to be efficacious against all strains tested. Our research also provided some data on the susceptibility profile of less common *Candida* sp. - *C. sake*, *C. norvengensis*, *C. maris*. However, the number of isolates was limited.

Notably, caspofungin demonstrated an excellent activity against species that often develop innate (*C. krusei*) or acquired (*C. glabrata, C. dublinien-sis*) resistance (Pfaller *et al*., 1999a; Pfaller *et al*., 1999b) or show decreased susceptibility (*C. inconspicua*) to azoles and thus present a therapeutic problem (Majoros *et al*., 2005). On the other hand, in agreement with other authors (Ostrosky-Zeichner *et al*., 2003), the MICs of caspofungin generated for *C. parapsilosis* were high.

Given that mean plasma concentration of caspofungin well exceeds 1 mg/l when administered daily as intravenous infusion of 70 mg (Ullmann, 2003; Letscher-Bru and Herbrecht, 2003), the majority of assayed *Candida* sp. isolates were inhibited within concentrations therapeutically attainable.

Having compared caspofungin MICs for *Candida* sp. strains obtained by the E-test procedure with those assessed by the CLSI recommended broth microdilution method we observed that the outcomes were highly similar. Besides, Kappa coefficient of 0.92 indicates excellent agreements between the MICs evaluated by both methods for *Candida* non-*albicans* isolates. As in other publications, the agreement between MICs values was slightly poorer for *C. parapsilosis* and *C. tropicalis*. It is noticeable that in most cases when a discrepancy between outcomes was observed the lower caspofungin MICs were obtained by the E-test procedure. Our data are in accordance with those of similar investigations (Laverdiere *et al*., 2002; Chryssanthou *et al*., 2002).

In general, the E-test MICs values were quite easy to read since the inhibition ellipses were mostly sharply defined.

In conclusion, caspofungin showed a potent *in vitro* activity against most *Candida* non-*albicans* isolates, with relatively low MICs values, and appears to be a significant addition to the conventional well-known antifungal armamentarium. The E-test procedure seems to be a feasible and trustworthy alternative to the CLSI recommended broth microdilution method for estimating *in vitro* susceptibility of the yeast of *Candida* species to caspofungin.

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