Original Article

Effects of Culture Conditions (Microplate and Solvent) on *in vitro* Antifungal Activity of Caspofungin Against *Candida* Species

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ABSTRACT

Effects of the type of microplates and solvent for preparation of caspofungin (CPFG) on antifungal susceptibility testing of CPFG against clinical isolates of *Candida albicans*, *Candida glabrata*, and *Candida krusei* (20 strains each) by broth microdilution method according to the Clinical and Laboratory Standards Institute were evaluated. When CPFG was dissolved in water, MICs against the three *Candida* species decreased 3.1-6.0-fold in surface-untreated microplates compared to those in treated microplates. When CPFG was dissolved in dimethyl sulfoxide, MICs against the three *Candida* species decreased 1.3-2.5-fold in surface-untreated microplates compared to those in treated microplates. Differences in MICs according to the type of solvent did not exceed the difference for one dilution interval (0.5-2-fold MIC ratio) regardless of whether the microplate surface was treated or not. These findings suggest that differences in CPFG MICs may depend mainly on the type of surface treatment of assay microplates.

Key words : Antifungal susceptibility, *Candida*, Caspofungin, Dimethyl sulfoxide, Microplate

I. Introduction

Echinocandin antifungal agents are a highly effective treatment option for candidiasis (candidemia, disseminated candidiasis, etc.) when disseminated fungal infection is suspected in association with febrile neutropenia and azole resistant infection¹⁾. Reports of the increasing resistance of *Candida glabrata* to echinocandin globally indicate that correct antifungal susceptibility testing of *Candida* species against echinocandins is crucial for the success of treatment, and that antifungal stewardship is needed to reduce the resistance selection pressure in clinical settings^{2,3)}.

In our analysis of an interim report of a post-marketing survey for caspofungin (CPFG) susceptibility testing against *Candida* isolates in Japan, we found no resistant strain in a total of 510 strains of 6 *Candida* species isolated between 2012 and 2014, based on clinical breakpoints (CBPs) according to the Clinical and Laboratory Standards Institute (CLSI) M27-S3^{4,5)}. Based on the analysis of CBPs according to CLSI M60 1st ed.⁶⁾ (M27-S4⁷⁾), however, many strains of *C. glabrata* and *Candida krusei* were found to be resistant to

CPFG⁵⁾. Similar results were obtained with *Candida* species isolated between 2015 and 2016⁸⁾.

Espinel-Ingroff et al.⁹⁾ and Arendrup et al.¹⁰⁾ pointed out that there was a significant variation in CPFG MICs between institutions and that this may result in errors in susceptibility testing based on CBPs revised in CLSI M27-S4⁷⁾. Therefore, the CLSI standard method in M60 1st ed.⁶⁾ recommends that susceptibility testing to other echinocandins or genetic screening in resistant mutations should be conducted if a strain is found to be resistant or have intermediate resistance to CPFG because resistance to echinocandins is mainly induced by mutation of *FKS* genes, and cross-resistance has been reported in the echinocandin antifungal agent class.

Recently, Fothergill et al. showed that surface-treated microplates were associated with higher CPFG MICs than non-surface-treated microplates in susceptibility against *Candida* species¹¹⁾. Different range and variability of CPFG MICs were also reported between the solvents for stock solution¹²⁾.

Taking all these findings into account, we evaluated the effects of surface treatment of microplates and the solvent for preparation of CPFG solution using *Candida albicans*, *C.*

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glabrata, and *C. krusei* collected in the post-marketing survey for CPFG to investigate these two factors responsible for differences in CPFG MICs determined by the CLSI broth microdilution method.

II. Materials and methods

1. Fungal strains

Twenty strains each of *C. albicans*, *C. glabrata*, and *C. krusei* isolated from specimens collected from patients suspected to have fungal infections at medical institutions in Japan (mainly Kanto District) between January 2012 and December 2016 were used. Strains were isolated generally from blood and respiratory samples, including sputum. In accordance with CLSI M60 1st ed.⁶⁾ (M27-S4⁷⁾) CBPs, all strains were found to be susceptible to micafungin.

All the strains were collected in compliance with the ethical guidelines for epidemiological research of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare. Privacy of patients was strictly protected in data correction¹³⁾.

2. Drug

CPFG (MSD K.K. Tokyo, Japan; Lot No.: 003M041) was used.

3. Susceptibility testing

The broth microdilution assay was performed according to CLSI M27-A3¹⁴⁾.

Either purified water or dimethyl sulfoxide (DMSO: Kanto Chemicals Co., Inc.) was used as the solvent for the preparation of stock CPFG solution. When CPFG was dissolved in purified water, CPFG solution was diluted with the medium before the assay. When DMSO was used, CPFG solution was diluted with DMSO so that the final concentration of DMSO in all the assay media was 1%. CPFG MICs assayed in surface-treated (treated; Code No. 3870-096; plate was treated using discharge apparatus to increase hydrophobicity) and surface-untreated (untreated; Code No. 3875-096) U-shaped bottom microplates manufactured by IWAKI (AGC TECHNO GLASS Co., Ltd.) were compared¹¹⁾.

Candida parapsilosis ATCC 22019 and *C. krusei* ATCC 6258 were assayed as quality control strains. CPFG MICs obtained in the assays with these strains were found to be within the permitted range.

4. Data analysis

MIC₅₀, MIC₉₀, and geometric mean MIC were calculated from the susceptibility of each strain. Ratios of MICs obtained with the same strain under each assay condition and their arithmetic means were calculated.

Susceptibility of each strain to CPFG was evaluated based

on the CLSI M60 1st ed.⁶⁾ (M27-S4⁷⁾) CBPs. CBPs of *C. albicans* and *C. krusei* to CPFG in CLSI M60 1st ed. were classified as susceptible (S) at MIC of 0.25 µg/mL or lower, intermediate (I) at 0.5 µg/mL, and resistant (R) at 1 µg/mL or higher, while CBPs of *C. glabrata* were classified as S at 0.12 µg/mL or lower, I at 0.25 µg/mL, and R at 0.5 µg/mL or higher. CBPs of *Candida* species to CPFG in CLSI M27-S3 were classified as susceptible at 2 µg/mL or lower, and non-susceptible at more than 2 µg/mL.

III. Results

1. Effect on susceptibility of various *Candida* species to CPFG

Results of effect on CPFG MICs of the microplate surface treatment (treated or untreated) and solvent used to dissolve CPFG (water or DMSO) are shown in Table 1.

1) Treated or untreated microplates

When CPFG was dissolved in water, CPFG MICs for *C. albicans*, *C. glabrata*, and *C. krusei* in untreated microplates were 3.1-, 6.0-, and 3.7-fold lower than the respective MICs in treated microplates. The effect of surface treatment on CPFG MICs assay was greatest for *C. glabrata*. When CPFG was dissolved in DMSO, CPFG MICs for *C. albicans*, *C. glabrata*, and *C. krusei* in untreated microplates were 1.3-, 1.7-, and 2.5-fold lower than the respective MICs in treated microplates. The effect of surface treatment on CPFG MICs assay was greatest for *C. krusei*. The difference in CPFG MICs between treated and untreated microplates was less when dissolving in DMSO than in water.

2) Solvent

CPFG MICs against *C. albicans*, *C. glabrata*, and *C. krusei* in treated microplates were 1.6-, 2.0-, and 1.6-fold lower when CPFG was dissolved in water than when it was dissolved in DMSO. CPFG MICs against these *Candida* species in untreated microplates were 0.63-, 0.6-, and 1.0-fold lower when CPFG was dissolved in water than when it was dissolved in DMSO. Effects of both DMSO and water against each species did not exceed the difference for one dilution interval (0.5-2 fold MIC ratio).

2. Susceptibility evaluation based on CBPs

Based on CLSI M27-S3⁴⁾ CBPs, all strains tested in this study were susceptible to CPFG⁵⁾. Results of susceptibility to CPFG based on CLSI M60 1st ed.⁶⁾ (M27-S4⁷⁾) are shown in Table 1. Ten strains (50%) of *C. albicans* were classified as I when CPFG was dissolved in water and treated microplates were used. Under the other assay conditions wherein CPFG was dissolved in water and untreated microplates were used, and CPFG was dissolved in DMSO and both treated and untreated microplates were used, however, all *C. albicans* strains were classified as susceptible to CPFG. In case of *C.*

Table 1. Effect of solvent and microplate for caspofungin MIC determined by CLSI standard method

Organism ¹⁾	Test method		No. of isolates and MICs (μg/mL)						MIC (μg/mL)		Category ³⁾ (%)		
	Solvent	Microplate	0.06	0.12	0.25	0.5	1	2	Geometric mean	MIC ratio (mean) ²⁾	S	I	R
<i>Candida albicans</i>	D.W.	treated		3	7	10			0.32	3.1 1.6 0.63	50	50	0
	D.W.	untreated	4	16					0.11		100	0	0
	DMSO	treated	1	2	17				0.22		100	0	0
	DMSO	untreated	2	5	13				0.18		100	0	0
<i>Candida glabrata</i>	D.W.	treated				7	13		0.78	6.0 2.0 0.6	0	0	100
	D.W.	untreated		17	3				0.14		85	15	0
	DMSO	treated			6	14			0.41		0	30	70
	DMSO	untreated			20				0.25		0	100	0
<i>Candida krusei</i>	D.W.	treated				2	16	2	1.0	3.7 1.6 1.0	0	10	90
	D.W.	untreated		1	15	4			0.28		80	20	0
	DMSO	treated				12	8		0.66		0	60	40
	DMSO	untreated			17	3			0.28		85	15	0

D.W.: distilled water, DMSO: dimethyl sulfoxide

1): Twenty strains isolated in Japan from 2012 to 2016 were tested.

2): MIC ratio was calculated based on the MIC for the same isolate.

3): Frequency of susceptible (S), intermediate (I), and resistant (R) strains were calculated according to CLSI M60 1st ed. (M27-S4).

glabrata and *C. krusei*, all strains were either I or R regardless of the type of solvent when treated microplates were used. No strain was classified as S. However, all strains were classified as either S or I when surface-untreated microplates were used, with no strain classified as R.

IV. Discussion

There are several issues in determination of CPFG MICs by broth microdilution standard method due to the fact of having significant differences among assay laboratories⁹⁾. It may therefore be difficult to evaluate susceptibility based on the current CBPs. In addition, these issues may affect revisions of the future CBPs based on accurate and stable susceptibility surveillance data. No clear single factor—such as CPFG lot, stock solution solvent, CPFG powder storage method, medium, or evaluation method—was found to influence CPFG MICs⁹⁾, while there were some reports of the effects of the surface treatment of microplates and the solvent for preparation of drug substance^{11, 12)}. The present study focused on the surface treatment of the microplates (treated or untreated) and the solvent used to dissolve the drug (water or DMSO) as factors that may affect the susceptibility of three *Candida* species to CPFG.

Microplates used in susceptibility testing have generally been developed to culture animal cells *in vitro*. They are as transparent as glass and composed of polystyrene resins with easy processing characteristics. Because they have little affinity to animal cells without surface treatment due to their

surface hydrophobicity, microplates should be hydrophilized by plasma treatment, corona discharge treatment, etc. to adhere to culture cells¹⁵⁾. In our study, culture yeast cells were macroscopically observed on the bottom of surface-treated microplates with U-shaped wells with all the three *Candida* species, while they were observed as a uniform cell suspension in each well in untreated microplates. Agitating the microplates did not cause any apparent difference in turbidity (cell growth) between the two types of microplates (data not shown). These findings suggest that all three *Candida* species may grow as uniformly suspended (planktonic) cells without sedimentation in untreated microplates, while they may grow as cells attached to the bottom of wells in treated microplates.

In the present study, MICs of CPFG dissolved in water were lower with a 3-6-fold difference in untreated microplates compared to those in treated microplates. It was reported that culturing *Candida* strains for 24 hours in treated microplates at high cell concentrations resulted in the formation of biofilms at the bottom of the microplate well and a decrease in activities of antifungal agents¹⁶⁻¹⁸⁾, which might partially explain the results in this study. Further studies are needed to determine whether biofilm formation of yeast cells contributes to differences in CPFG MICs between treated and untreated microplates. As in the present study, the study conducted by Fothergill et al.¹¹⁾ showed that CPFG MICs for echinocandin-susceptible strains without mutation of *FKS* genes were lower in untreated microplates compared to those in treated microplates. The CLSI standard method revised in 2017 (CLSI M27 4th ed.¹⁹⁾) recommends the use of untreated

polystyrene microplates based on these findings. However, treated microdilution plates are used in the EUCAST antifungal MIC method for yeasts (EUCAST E DEF 7.3.1)²⁰.

CPFG is readily soluble in water because it has a cyclic hexapeptide structure with amphiphilic acyl side chains²¹. However, the solvent of echinocandin stock solution in CLSI M27-S4 was changed from water to DMSO⁷. When CPFG was dissolved in DMSO, CPFG MICs for various *Candida* species were decreased and became less variable⁷. This is thought to be attributable to the fact that the electric permittivity of DMSO is lower than that of water, and it results in better solubility of echinocandin antifungal agents, better dispersion in the medium, and better distribution to target enzymes in the cell¹². Using the EUCAST standard method, Alastruey-Izquierdo et al. reported that DMSO as a solvent for CPFG stock solution produced lower MICs than when water alone was used¹². In the present study, we evaluated the effects of water and DMSO on CPFG MICs and found that differences in CPFG MICs did not deviate from possible errors due to manual reading (within one dilution interval or 0.5-2-fold MIC ratio) regardless of the assay microplates. Unlike previous reports, therefore, our findings showed that the effects of solvent on CPFG's MICs are limited.

In conclusion, our findings suggest that differences of CPFG MICs for *Candida* may greatly depend on the treatment of assay microplates. An assay method that gives reliable results with no inter-laboratory variability needs to be developed to adequately evaluate CPFG susceptibility based on CBPs.

Conflict of interest

Yuhei Higashitsuji, Rie Ueno, Masami Ogawa, and Daniel Ruzicka are employees of MSD K.K.

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