Comparison of four reading methods of broth microdilution based on the Clinical and Laboratory Standards Institute M27-A3 method for *Candida* spp.

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This study aimed to compare the susceptibilities of 5 reference strains and 28 isolates of *Candida* spp., to micafungin, amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and miconazole, obtained by visually determined minimum inhibitory concentration (MIC) using the agitation method (V-A), as described in the Clinical and Laboratory Standards Institute M27-A3 document; visual determinations without agitation (V-NA); and spectrophotometric determinations for the presence or absence of agitation (SP-A and SP-NA, respectively). Our results indicate that when the V-NA, SP-A, and SP-NA—the 3 alternative microdilution procedures for MIC endpoint determinations—were compared with the V-A, excellent agreements were observed between the V-NA and V-A rather than with the spectrophotometric methods (between the SP-A or SP-NA, and V-A). Furthermore, many errors occurred while using the SP-A method in the presence of agitation and some isolates showed major errors. Three of 5 isolates that showed very major errors between the spectrophotometric SP-A or SP-NA, and the reference V-A method were trailing isolates. Therefore, it was suggested that the MICs of *Candida* spp. obtained by the V-NA method were more precise than those by the conventional SP-A method.

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Introduction

The increasing incidence of human immunodeficiency virus-1 infection, the use of therapeutic modalities for advanced life support, and certain surgical procedures, such as organ transplantation and the implantation of prosthetic devices, have had considerable influences on the expanding incidence of *Candida* infections[1]. *Candida* is the most frequently implicated agent in invasive fungal infections, now ranking as the fourth most common cause of nosocomial bloodstream infections and accounting for 8% of all hospital-acquired bloodstream infections in the United States of America. Candidemia is associated with an extremely high rate of motility[2]. Therefore, antifungal drug susceptibility testing, which offers a guide to the selection and follow-up of treatment for accurate and reproducible results, has become more important due to the rise in serious fungal infections and the concomitant emergence of resistance to antifungal agents[3–5]. The Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) has published an approved reference method for antifungal susceptibilities of *Candida* species. In 2008, CLSI developed an approved reference procedure (document M27-A3) through which a new level of reproducibility was achieved[6]. Recent efforts have been directed to the problem of accurate determination of minimum inhibitory concentration (MIC) endpoints, particularly with the azole antifungal agents tested against *Candida* spp[7]. According to the CLSI M27-A3, the microdilution MIC endpoints are visually determined by the agitation of microdilution trays prior to the reading of the MICs. Recently, conducting the reading after agitation facilitates the use of a spectrophotometer for determining MIC endpoints. Since it easily lends itself to automation[7], the spectrophotometric approach to MIC endpoint determination provides an objective, rapid MIC reading and eliminates the subjective judgments concerning minimal turbidity, which confounds antifungal susceptibility testing[8]. However, the description of MIC endpoint determination for the azoles in CLSI is overly complex and somewhat ambiguous for the microdilution procedure [3].

Therefore, the present study aimed to evaluate the performance of visual and spectrophotometric MIC readings for the presence or absence of agitation by determining the susceptibilities of 33 isolates of *Candida* spp., including 28 *Candida albicans* isolates.

Materials and Methods

Isolates

Thirty-three isolates of *Candida* spp. were tested. Five of these were American Type Culture Collection strains (*C. parapsilosis* ATCC 90018, *C. krusei* ATCC 6258, *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, and *C. tropicalis* ATCC 750). Ten *C. albicans* isolates from the respiratory and urogenital systems were derived from a hospital in Tokyo, Japan. Seven *C. albicans* isolates (TIMM 3163-3166, 3209, 3309, and 3960) were kindly provided by the Teikyo University

Institute of Medical Mycology and 9 isolates (IFM 46910, IFM 54354, IFM 57376, IFM 57378, IFM 57388, IFM 57389, and IFM 57408-57410) by the Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses. Sixteen isolates of *C. albicans* putatively indicated resistance to all azoles. Two putative azole-trailing strains, *C. tropicalis* KYTE1 and KYTE2 (kindly provided by Kyokuto Chemical, Tokyo, Japan), were also tested. Confirmation of species identification was performed with API products (bioMérieux, Tokyo, Japan) as recommended by the manufacturer. Each isolate was maintained in sterile water and subcultured in antimicrobial-free medium to ensure viability and purity prior to testing.

Antifungal agents and microdilution panels

The antifungal susceptibilities of *Candida* spp. isolates were determined by using microdilution panels (Yeast-like fungi DP, Eiken kit; Eiken Chemical Company, Ltd., Tokyo, Japan) containing two-fold serial dilutions of micafungin, amphotericin B, 5-fluorocytosine (flucytosine), fluconazole, itraconazole, voriconazole, and miconazole. The tested drugs concentrations ranged from 0.015 to 16 μg/mL for micafungin, 0.03 to 16 μg/mL for amphotericin B and miconazole, 0.12 to 64 μg/mL for flucytosine and fluconazole, and 0.015 to 8 μg/mL for itraconazole and voriconazole. The microdilution panels were shipped frozen and stored at −70°C until testing.

Antifungal susceptibility test methods

Inoculum suspensions of *Candida* spp. isolates were obtained from 24 h cultures on Sabouraud dextrose agar at 35°C. The turbidity of each yeast suspension was adjusted by the spectrophotometric method to match the turbidity of a 0.5 McFarland standard and diluted to a concentration of 0.5×10^3 to 2.5×10^3 CFU/mL with the final testing inoculum in RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan), buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS; Kanto Chemical, Tokyo, Japan), and an aliquot of 100 μL was added to each well of the microdilution panels. The panels were incubated in ambient air at 35°C and MICs determined at 24 h and 48 h for all species.

Determination of MICs

MIC results for micafungin were read following a 24 h of incubation, whereas MIC results for amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and miconazole were read after 48 h of incubation. Optical densities of each microplate well before and after agitation were measured either visually using inverted reading mirror or spectrophotometrically with the Multiskan FC Microplate Photometer (Thermo Scientific, Yokohama, Japan) set at 640 nm. The visual and spectrophotometric MIC values for the azoles, micafungin, and flucytosine were defined as the lowest drug concentration that resulted in a reduction of approximately ≥50% in turbidity in comparison with the drug-free growth control well. For amphotericin B, MIC values

were defined as the lowest drug concentration for which the well was optically clear. The visual and spectrophotometric MICs for the presence or absence of agitation were represented as V-A, V-NA, SP-A, and SP-NA, respectively.

Analysis of results

Reproducibility was ensured by testing each of the five reference strains a total of two times by each of microdilution methods. CLSI-determined MICs (the V-A determined MICs) for these quality control strains were within the recommended ranges. The CLSI interpretative breakpoints for micafungin (susceptible [S], ≤2 μg/mL; nonsusceptible [NS], >2 μg/mL), amphotericin B (S, ≤1 μg/mL), flucytosine (S, ≤4 μg/mL; intermediate [I], ≥8–16 μg/mL; resistant [R], ≥32 μg/mL), fluconazole (S, ≤8 μg/mL; susceptible-dose dependent [S-DD], ≥16–32 μg/mL; R, ≥64 μg/mL), itraconazole (S, ≤0.12 μg/mL; S-DD, ≥0.25–0.5 μg/mL; R, ≥1 μg/mL), and voriconazole (S, ≤ 1 μg/mL; S-DD, 2 μg/mL; R, ≥4 μg/mL) (CLSI subcommittee, 2008 meeting) were used to obtain categorical agreement percentages among V-A, V-NA, SP-A, and SP-NA. The V-A was defined as the reference broth microdilution method in the CLSI M27-A3 document. The MIC range, MIC_{50}, and MIC_{90} (the lowest drug concentrations that inhibited 50% and 90% of isolates, respectively) were determined for each drug tested. An agreement was reported when the MIC results among the 4 methods were in exact agreement or within 1 two-fold dilution. Very major errors were identified when R was indicated by the reference (V-A) MIC and S by one of the others (V-NA, SP-A, and SP-NA). Major errors were identified when the isolate was classified as S by the reference method and R by the other methods. Minor errors were identified when the result of the reference readings was either S or R, and that of the others was S-DD or I.

Results

1. Comparison between the V-A, V-NA, SP-A, and SP-NA for the seven antifungal agents against *Candida* spp. isolates

Table 1 summarized MICs of 33 *Candida* spp. isolates as determined V-A as described in CLSI M27-A3. For FLCZ, three isolates were classified as S-DD and 7 isolates were classified as R. For ITCZ, five isolates were classified as S-DD and 7 isolates were classified as R. For VRCZ, eight isolates were classified as R and S-DD isolates were no observed.

Table 2 summarizes MICs of the 5 CLSI reference isolates to micafungin, amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and miconazole as determined by the 4 reading methods following incubation for 48 h (only 24 h for micafungin). MICs that fell outside of the CLSI recommended range included the fluconazole MICs for *C. albicans* ATCC 90028 and ATCC 24433, generated by SP-A and SP-NA; fluconazole MIC for *C. tropicalis* ATCC 750, generated by SP-NA; and flucytosine MIC for *C. krusei* ATCC 6285, generated by SP-A. Although

Table 1. Susceptibilities of *Candida* spp. to seven antifungal agents as determined with the reference CLSI M27-A3 method following a 48 h incubation (*n*=33).

Agent[1]	No. of isolates with MIC (μg/mL) of:													
	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	≥128
micafungin	27	4		1		1								
amphotericin B						6	24	3						
flucytosine				24	5	1	1		1					1
fluconazole				9	7	2	3	1	1		2	1	1	6
itraconazole		10	7	4	2	3	1				6			
voriconazole	15	3	1	1	5				1		7			
miconazole		6	6	3	2	3	5	4	2	2				

[1] Micafungin MICs were determined following a 24 h incubation.

Table 2. Comparison of broth microdilution MICs read by four methods for seven antifungal agents against five reference strains following a 48 h incubation.

Organism	Agent[1]	Reference MIC range (μg/mL)[2]	Modal MIC (μg/mL) determined			
			V-A	V-NA	SP-A	SP-NA
C. albicans ATCC 90028	micafungin	NA	≤0.015	≤0.015	≤0.015	≤0.015
	amphotericin B	0.5-2	1	1	1	1
	flucytosine	0.5-2	0.5	0.5	1	2
	fluconazole	0.25-1	0.25	0.5	>64	>64
	itraconazole	NA	0.06	0.06	>8	0.06
	voriconazole	NA	≤0.015	0.06	>8	>8
	miconazole	NA	0.12	0.12	8	4
C. albicans ATCC 24433	micafungin	NA	≤0.015	≤0.015	≤0.015	≤0.015
	amphotericin B	0.25-1	1	1	1	1
	flucytosine	1-4	1	0.5	1	4
	fluconazole	0.25-1	0.25	0.25	>64	>64
	itraconazole	NA	0.03	0.03	>8	0.5
	voriconazole	NA	≤0.015	≤0.015	>8	0.06
	miconazole	NA	0.06	0.06	4	4
C. tropicalis ATCC 750	micafungin	NA	0.03	≤0.015	0.03	0.03
	amphotericin B	0.5-2	1	1	1	1
	flucytosine	≤0.12-0.25	≤0.12	≤0.12	≤0.12	≤0.12
	fluconazole	1-4	1	1	4	64
	itraconazole	NA	0.25	0.25	0.25	>8
	voriconazole	NA	0.25	0.12	0.25	2
	miconazole	NA	1	0.5	1	4
C. parapsilosis ATCC 90018	micafungin	NA	0.5	0.25	0.5	0.5
	amphotericin B	0.5-2	1	1	1	1
	flucytosine	≤0.12-0.25	≤0.12	≤0.12	≤0.12	≤0.12
	fluconazole	0.25-1	1	0.5	1	1
	itraconazole	NA	0.12	0.06	0.12	0.12
	voriconazole	NA	≤0.015	≤0.015	≤0.015	≤0.015
	miconazole	NA	0.5	0.25	0.5	0.25
C. krusei ATCC 6258	micafungin	NA	0.12	0.12	0.06	0.06
	amphotericin B	0.25-2	2	2	2	2
	flucytosine	4-16	4	4	0.5	8
	fluconazole	16-64	16	16	32	32
	itraconazole	0.12-0.5	0.12	0.12	0.06	0.25
	voriconazole	NA	0.25	0.12	0.25	0.12
	miconazole	NA	1	1	1	0.5

V-A: visual-agitation, V-NA: visual-no agitation, SP-A: spectrophotometric-agitation, SP-NA: spectrophotometric-no agitation

[1] Micafungin MICs were determined following a 24 h incubation. [2] NA, not available.

both flucytosine MIC for *C. albicans* ATCC 24433 by V-NA and itraconazole MIC for *C. krusei* ATCC 6258 by SP-A were outside of the recommended range, each of those MICs was similar to the reference (V-A) MIC (within 1 two-fold dilution).

2. Categorical agreement between the V-A, V-NA, SP-A, and SP-NA

Table 3 summarizes the categorical agreement among the V-A, V-NA, SP-A, and SP-NA for 33 isolates of *Candida* spp. against the 6 antifungals tested, except for miconazole which did not have a defined breakpoint by CLSI M27-A3. All the *Candida* spp. isolates were classified as S for

Table 3. Distribution of 33 organisms tested according to methods and susceptibility profile following a 48 h incubation.

Agents	Method	No. (%) of isolates			% of errors			% Categorical agreement
		S	S-DD[1]	R[2,3]	very major	major	minor	
micafungin	V-A	33 (100)	NA	0 (0)	NA	NA	NA	NA
	V-NA	33 (100)	NA	0 (0)	0 (0)	NA	0 (0)	100.0
	SP-A	33 (100)	NA	0 (0)	0 (0)	NA	0 (0)	100.0
	SP-NA	33 (100)	NA	0 (0)	0 (0)	NA	0 (0)	100.0
amphotericin B	V-A	30 (90.9)	NA	3 (9.1)	NA	NA	NA	NA
	V-NA	30 (90.9)	NA	3 (9.1)	0 (0)	NA	0 (0)	100.0
	SP-A	29 (87.9)	NA	1 (3.0)	0 (0)	NA	1 (3.0)	97.0
	SP-NA	30 (90.9)	NA	3 (9.1)	0 (0)	NA	0 (0)	100.0
flucytosine	V-A	32 (97.0)	0 (0)	1 (3.0)	NA	NA	NA	NA
	V-NA	32 (97.0)	0 (0)	1 (3.0)	0 (0)	0 (0)	0 (0)	100.0
	SP-A	32 (97.0)	0 (0)	1 (3.0)	0 (0)	0 (0)	0 (0)	100.0
	SP-NA	31 (93.9)	1 (3.0)	1 (3.0)	0 (0)	0 (0)	1 (3.0)	97.0
fluconazole	V-A	23 (69.7)	3 (9.1)	7 (21.2)	NA	NA	NA	NA
	V-NA	18 (54.5)	4 (12.1)	11 (33.3)	0 (0)	5 (15.2)	1 (3.0)	81.8
	SP-A	24 (72.7)	3 (9.1)	6 (18.2)	5 (15.2)	4 (12.1)	0 (0)	72.7
	SP-NA	18 (54.5)	6 (18.2)	9 (27.3)	2 (6.0)	5 (15.2)	3 (9.1)	69.7
itraconazole	V-A	21 (63.6)	5 (15.2)	7 (21.2)	NA	NA	NA	NA
	V-NA	17 (51.5)	5 (15.2)	11 (33.3)	0 (0)	5 (15.2)	2 (6.0)	78.8
	SP-A	22 (66.7)	4 (12.1)	7 (21.2)	4 (12.1)	3 (9.1)	1 (3.0)	75.8
	SP-NA	18 (54.5)	8 (24.2)	7 (21.2)	1 (3.0)	2 (6.0)	8 (24.2)	66.7
voriconazole	V-A	25 (75.8)	0 (0)	8 (24.2)	NA	NA	NA	NA
	V-NA	21 (63.6)	1 (3.0)	11 (33.3)	1 (3.0)	5 (15.2)	1 (3.0)	78.8
	SP-A	26 (78.8)	1 (3.0)	6 (18.2)	4 (12.1)	3 (9.1)	1 (3.0)	75.8
	SP-NA	25 (75.8)	1 (3.0)	7 (21.2)	3 (9.1)	2 (6.0)	1 (3.0)	81.8

V-A: visual-agitation, V-NA: visual-no agitation, SP-A: spectrophotometric-agitation, SP-NA: spectrophotometric-no agitation

[1] intermediate interpretative category for flucytosine only.

[2] Micafungin is classified as nonsusceptible and MICs were determined following a 24 h incubation.

[3] There is no official breakpoint for amphotericin B MIC.

NA, not available.

micafungin by the 4 methods. For amphotericin B, 87.9% of *Candida* spp. isolates were classified as S, as determined by the SP-A and 90.9% by each of other methods. As for the result with flucytosine, the categorical agreement between the SP-NA-determined MICs and the other 3 methods MICs was low (3.0%) due to a minor error resulting from an isolate shifting from S to S-DD. For the azoles, 21.2–24.2% of *Candida* spp. isolates were classified as R by the V-A. However, these isolates determined by SP-A and SP-NA were classified as S and showed a very major error (from 12.1% for itraconazole and voriconazole to 15.2% for fluconazole, and 3.0% for itraconazole to 9.1% for voriconazole, respectively). For voriconazole, categorical agreement of SP-NA was the highest (81.8%). Although only 1 isolate determined by V-NA for voriconazole exhibited a very major error (3.0%), the highest categorical agreements were observed with V-NA for fluconazole and itraconazole rather than through the determination by the other spectrophotometric method.

3. The percentages of agreement between the V-A, V-NA, SP-A, and SP-NA for seven antifungal agents

Table 4 summarizes the percentages of agreement between the V-A, V-NA, SP-A, and SP-NA for all organism-drug combinations following a 48 h incubation (only 24 h for micafungin). Complete agreement (100%) noted within 1 two-fold dilution between the 4 methods was obtained for the micafungin and amphotericin B susceptibilities of 33 *Candida* spp. isolates. The agreements among the 4 methods for flucytosine were very strong and varied from 93.9% (between the V-A and SP-NA) to 100% (between the V-A and V-NA). When the azoles were tested, the agreements within 1 two-fold dilution among the 4 methods were as follows: 69.7–72.7%, 72.7–81.8%, 69.7–72.7%, and 66.7–69.7% for fluconazole, itraconazole, voriconazole, and miconazole, respectively. The poorest agreement was observed between the V-A and SP-A for all the azoles.

Discussion

The recently published CLSI standard method for susceptibility testing of yeasts[6] specifies visual endpoints after agitation of microdilution plates, as this method has good-to-excellent reproducibility in a multilaboratory comparison[9] and resulted in clear-cut endpoints that were reproducible[7,10]. However, in the present study, we showed that it was difficult to determine the MICs of the azole-high resistant isolates by the CLSI method due to significant turbidity in all the wells. Moreover, some laboratories often determine MICs by spectrophotometry after constantly agitating microplates in a shaker, due to the simplicity of this procedure[11–16]. In the present study, we compared antifungal susceptibilities of 33 *Candida* spp. to the 7 antifungal agents by the broth microdilution method (the V-A) based on the CLSI M27-A3 to the MICs obtained by the

Table 4. Percents agreement between the V-A, V-NA, SP-A and SP-NA for seven antifungal agents following a 48 h incubation (n=33).

Agents[1]	Model MIC compared	≤-3	-2	-1	0	1	2	≥3	±0	±1
micafungin	V-A/V-NA			9.1	87.9	3.0			87.9	100.0
	V-A/SP-A			3.0	97.0				97.0	100.0
	V-A/SP-NA			6.1	93.9				93.9	100.0
	mean			6.1	92.9	1.0			92.9	100.0
amphotericin B	V-A/V-NA				93.9	6.1			93.9	100.0
	V-A/SP-A				84.8	15.2			84.8	100.0
	V-A/SP-NA				90.9	9.1			90.9	100.0
	mean				89.9	10.1			89.9	100.0
flucytosine	V-A/V-NA			3.0	97.0				97.0	100.0
	V-A/SP-A	3.0			87.9	9.1			87.9	97.0
	V-A/SP-NA				87.9	6.1	6.1		87.9	93.9
	mean	1.0		1.0	90.9	5.1	2.0		90.9	97.0
fluconazole	V-A/V-NA		3.0	6.1	57.6	9.1	9.1	15.2	57.6	72.7
	V-A/SP-A	15.2		6.1	54.5	9.1	3.0	12.1	54.5	69.7
	V-A/SP-NA	6.1		3.0	51.5	18.2		21.2	51.5	72.7
	mean	7.1	1.0	5.1	54.5	12.1	4.0	16.2	54.5	71.7
itraconazole	V-A/V-NA		3.0	6.1	63.6	12.1		15.2	63.6	81.8
	V-A/SP-A	15.2		6.1	57.6	9.1		12.1	57.6	72.7
	V-A/SP-NA	9.1	3.0	6.1	48.5	18.2	3.0	12.1	48.5	72.7
	mean	8.1	2.0	6.1	56.6	13.1	1.0	13.1	56.6	75.8
voriconazole	V-A/V-NA	6.1		9.1	48.5	12.1	9.1	15.2	48.5	69.7
	V-A/SP-A	18.2	3.0	6.1	54.5	9.1		9.1	54.5	69.7
	V-A/SP-NA	9.1		9.1	51.5	12.1	9.1	9.1	51.5	72.7
	mean	11.1	1.0	8.1	51.5	11.1	6.1	11.1	51.5	70.7
miconazole	V-A/V-NA	6.1	3.0	6.1	48.5	12.1	3.0	21.2	48.5	66.7
	V-A/SP-A	21.2	6.1	18.2	42.4	6.1		6.1	42.4	66.7
	V-A/SP-NA			18.2	27.3	24.2	6.1	24.2	27.3	69.7
	mean	9.1	3.0	14.1	39.4	14.1	3.0	17.2	39.4	67.7

V-A: visual-agitation, V-NA: visual-no agitation, SP-A: spectrophotometric-agitation, SP-NA: spectrophotometric-no agitation

[1] Micafungin MICs were determined following a 24 h incubation.

0: Modal MICs (V-NA, SP-A, SP-NA) were equal to V-A MICs.

1: Modal MICs (V-NA, SP-A, SP-NA) were 1 dilution, 2-fold, higher than V-A MICs.

−1: Modal MICs (V-NA, SP-A, SP-NA) were 1 dilution, 2-fold, lower than V-A MICs.

2: Modal MICs (V-NA, SP-A, SP-NA) were 2 dilutions, 4-fold, higher than V-A MICs.

−2: Modal MICs (V-NA, SP-A, SP-NA) were 2 dilutions, 4-fold, lower than V-A MICs.

other 3 methods: V-NA, SP-A, and SP-NA.

In the present study, the highest agreement for almost all antifungal agents was observed between V-NA and the CLSI reference method (the V-A) rather than among the SP-A, SP-NA, and V-A, as Pfaller *et al.* and Lozano-Chiu *et al.* have reported[7,17]. However, the spectrophotometric approach was noted to provide precise, objective MIC endpoints, particularly for the azole antifungal agents[18,19], and determination of MICs spectrophotometrically has shown good agree-

ment with the standardized visual reading for *Candida* spp., as previously reported by other researchers[7,12,20–22]. Moreover, NISHIYAMA *et al.* demonstrated the Japanese Society for Medical Mycology (JSMM) method, in which the endpoint was to read as 50% inhibitory concentration (IC_{50}) and use a spectrophotometer, was superior in both stability and reproducibility, as compared to the CLSI method in which growth was assessed by visual observation[23]. Additional studies will be required to compare between V-NA method and JSMM method.

Similar results were observed for almost all reference isolates; these MICs determined by the V-NA method were clustered within 1 two-fold dilution, compared with MICs of the V-A method, and were within the established reference MIC range, unlike MICs of the SP-A or SP-NA method. However, MICs derived by the SP-NA varied widely among the MICs obtained by the 4 methods.

The V-NA-determined MICs for amphotericin B were 2 two-fold dilutions lower than the V-A MICs for 2 *C. albicans* clinical isolates (data not shown). For amphotericin B the V-A MIC was defined as the lowest drug concentration required for an optically clear agitated well, whereas the V-NA MIC value indicated precipitation in the well without agitation. The discrepancy might be due to the turbidity not being observed optically when a small quantity of the precipitation was agitated in the well of the V-NA MIC. Despite the presence or absence of agitation, spectrophotometric MICs of amphotericin B for these 2 isolates were identical to the V-A MICs. These results indicate that MIC readings determined by the V-NA may be more precise than those determined by the other MIC readings.

For micafungin, amphotericin B, and flucytosine, the rates of errors were low because almost all isolates were classified as S. For the azoles, the susceptibilities of *Candida* spp. isolates were varied due to the variability of each method. The percentage of errors, particularly very major errors, obtained from the SP-A method was higher than that from the other methods. However, by the V-NA method, only 1 isolate for voriconazole showed a very major error. Three of 5 isolates that showed very major errors between the spectrophotometric SP-A or SP-NA, and the reference V-A method were trailing isolates.

In one study[24], the rates of apparent *in vitro* resistance to fluconazole and itraconazole among bloodstream isolates of *C. albicans* and *C. tropicalis*—derived from visual reading of MIC end points after 48 h of incubation—were higher than those based on spectrophotometric MIC endpoints. The results of the present study are similar to those reported by ARTHINGTON-SKAGGS *et al.* [24] Therefore, it was suggested that optical density varied due to the large amount of precipitation in each well because almost all trailing isolates were determined after 48 h incubation. Therefore, our results showed that MIC determinations of the V-NA method at 48 h may be more appropriate than those of the other methods against *Candida* spp., except for trailing isolates. However, for trailing isolates, agitation of the broth in the CLSI method has been shown to reduce the trailing-endpoint effect, although the incubation time in the study by ANAISSIE *et al.*

was 24 h instead of the standard 48 h[25]. In the present study, the reduction of the trailing MIC endpoints by agitation was not observed for most of the isolates at 48 h. When 3 trailing isolates that showed a very major error between the spectrophotometric SP-A and the reference V-A method were determined by V-A and V-NA method after 24 h of incubation, the MICs by V-NA were similar to V-A MICs within 1 two-fold dilution (data no shown). Therefore, our results also showed that trailing MIC determinations of the V-NA method at 24 h may be more appropriate than those of the other methods against *Candida* spp.

In conclusion, the microdilution MIC endpoints for *Candida* spp. based on CLSI M27-A3 are visually determined by the agitation of microdilution trays prior to the reading of the MICs. Because it is difficult to perform the present CLSI method in routine clinical testing, the spectrophotometric approach has recently been adopted by multilaboratories. In the present study, we compared the susceptibilities of *Candida* spp. to 7 antifungal agents obtained by the V-A method described in CLSI M27-A3 document and the V-NA, SP-A, and SP-NA methods. The results showed a highly significant agreement between the V-A and V-NA method regardless of the presence or absence of agitation, whereas many errors occurred while using the SP-A method in the presence of agitation. Moreover, some isolates determined by the SP-A method showed a very major error, which was suspected to be due to the accuracy of MIC determination by spectrophotometry. Therefore, the V-NA method, for which both trailing and no-trailing endpoints are observed for *Candida* spp., may be easier and more precise than the reference V-A method and the conventional SP-A method.

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