Microbiol Immunol 2008; **52**: 435–439
doi:10.1111/j.1348-0421.2008.00056.x

ORIGINAL ARTICLE

Comparative evaluation of *Trichosporon asahii* susceptibility using ASTY colorimetric microdilution and CLSI M27-A2 broth microdilution reference methods

Ayse Kalkanci[1,4], Nanthawan Mekha[2,4], Natteewan Poonwan[2], Koichi Makimura[3] and Takashi Sugita[4]

[1]Gazi University Faculty of Medicine, Department of Microbiology, Besevler, Ankara, 06500 Turkey, [2]Mycology Laboratory, National Institute of Health, Department of Medical Sciences, Nonthaburi, 11000 Thailand, [3]Department of Molecular Biology and Gene Diagnosis, Institute of Medical Mycology and Genome Research Center, Graduate School of Medical Science and Faculty of Medicine, Teikyo University, Hachioji, Tokyo 192-0395 and [4]Department of Microbiology, Meiji Pharmaceutical University, Kiyose, Tokyo 2004-8588 Japan

ABSTRACT

The *in vitro* activity of AMPH-B, 5-FC, FLCZ, MCZ, ITCZ, and VRCZ against 50 isolates of *T. asahii* was determined using CLSI M27-A2 microdilution and ASTY colorimetric methods. Observed agreement ranged from 96 to 100% according to the drug. Overall, the agreement between two methods was 97.7%. The ASTY colorimetric method was thus determined to be comparable to the CLSI reference method when testing the susceptibility of *T. asahii* to a variety of antifungal agents.

Key words ASTY, CLSI, susceptibility, *Trichosporon asahii*.

Deep-seated infection with *Trichosporon* species is an emerging mycosis in neutropenic patients, particularly those who are being treated for hematological malignancies with cytotoxic and immunosuppressive therapy. The prognosis for deep-seated trichosporonosis with persistent neutropenia is very poor, with a high mortality rate (1, 2). This infection is caused mainly by *T. asahii*. In recent years, trichosporonosis has also been recognized as a breakthrough infection after treatment with candin derivatives, such as micafungin or caspofungin (3, 4). Therefore, the frequency of deep-seated trichosporonosis may be gradually increasing, although data on the antifungal susceptibilities of *T. asahii* are limited. In addition, azole-resistant *T. asahii* have been isolated from patients (5, 6).

Commercial colorimetric methods are available to test antifungal agents *in vitro*. Comparative evaluation studies of the Sensititre YeastOne Panel (Trek Diagnostic Systems, Westlake, OH, USA) have found close agreement between the methods for both yeasts and molds (7–9). A second commercial colorimetric panel is ASTY (Kyokuto Pharmaceutical Industry, Tokyo, Japan), which uses an oxidation–reduction colorimetric indicator to aid in determining the antifungal MIC endpoint. However, only one comparative study testing *Candida* spp. has been reported using this method (10), and only a few studies have included MIC data for antifungal agents against *Candida* spp. and *Geotrichum capitatum* using the ASTY panel (11–13).

To the best of our knowledge, no study has examined the performance of these colorimetric microdilution panels for testing the *in vitro* susceptibility of *T. asahii* isolates. Therefore, we evaluated the ASTY method with AMPH-B, 5-FC, FLCZ, MCZ, ITCZ, and VRCZ for 50 isolates of *T. asahii*, the major causative agent of deep-seated trichosporonosis. MIC obtained using ASTY were compared

Correspondence
Dr. Takashi Sugita, Department of Microbiology, Meiji Pharmaceutical University, 2-552-1 Noshio, Kiyose, Tokyo 204-8588 Japan.
Fax. +81-424-95-8762; e-mail: sugita@my-pharm.ac.jp

Received 21 January 2008; revised 17 March 2008; accepted 30 April 2008

List of Abbreviations: AMPH-B, amphotericin B; CLSI, Clinical Laboratory Standards Institute; 5-FC, 5-flucytosine; FLCZ, fluconazole; *G. capitatum*, *Geotrichum capitatum*; IGS region, intergenic spacer; ITCZ, itraconazole; MCFG, micafungin; MCZ, miconazole; MIC, minimal inhibitory concentration; *T. asahii*, *Trichosporon asahii*; *T. mucoides*, *Trichosporon mucoides*; VRCZ, voriconazole

© 2008 The Societies and Blackwell Publishing Asia Pty Ltd

to those obtained using the CLSI (formerly National Committee for Clinical Laboratory Standards) M27-A2 method.

MATERIALS AND METHODS

Fungal isolates

We evaluated 50 *T. asahii* strains. These were isolated from clinical specimens (blood, urine, and lung) from immunocompromised hosts in Thailand and Japan between 1996 and 2006. The isolates were identified using the DNA sequence from the IGS region of 26S rDNA according to the method of Sugita *et al.* (14).

Antifungal susceptibility

We assessed the antifungal susceptibility of *T. asahii* strains in two ways. First, we performed susceptibility testing with 96-well plates, as described in the M27-A2 procedure for yeast susceptibility testing (15). AMPH-B (Wako Pure Chemical, Tokyo, Japan), 5-FC (Wako Pure Chemical), FLCZ (ICN Biochemical, Cleveland, Ohio, USA), ITCZ (Janssen Pharmaceutical, Tokyo, Japan), MCZ (Wako Pure Chemical) and VRCZ (Pfizer Japan, Tokyo, Japan) were used. Briefly, the final concentrations of the antifungal agents were 0.03–16 μg/ml for AMPH-B, 0.12–64 μg/ml for 5-FC, 0.12–64 μg/ml for FLCZ, 0.03–16 μg/ml for MCZ, 0.01–8 μg/ml for ITCZ, and 0.01–8 μg/ml for VRCZ. The plates were incubated at 35°C for 48 hr and then read visually. For AMPH-B, the MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition. For the azoles and 5-FC, 50% inhibition in growth compared to that of the drug-free growth control was used as the MIC endpoint.

Second, we used ASTY colorimetric panels containing AMPH-B, 5-FC, FLCZ, MCZ, MCFG, ITCZ, and VRCZ according to the manufacturers' instructions. The final concentrations of the antifungal agents were the same as those used in the CLSI reference method (15). Growth in each well was indicated by a color change from blue (no growth) to red (growth). For all drugs, the MIC was defined as the lowest concentration of antifungal agent that prevented the development of a red color.

Data analysis and definitions

We applied standard antimicrobial susceptibility testing definitions (16) and compared the resulting MIC using ASTY versus CLSI methods. First, we determined the distribution of differences in MIC results and calculated the agreement between MIC as follows: if the MIC obtained using the reference method and tested ASTY method were identical, the difference was 0; if the tested method gave a MIC one dilution step larger than the reference method, the difference was +1, and so on; for lower MIC, the differences were negative (17). The results were considered to essentially be in agreement when the test result was within two dilutions of the reference value, that is, the percentage of isolates that gave identical results were between plus and minus one dilution step (18). The percent essential agreement was calculated by using the number of test results in essential agreement as the numerator and the total number of organisms tested as the denominator. The results could not be compared as being in categorical agreement (*i.e.*, susceptible, susceptible dose-dependent, intermediate, or resistant) because of the no interpretative limits or MIC breakpoints stipulated in CLSI documents for *Trichosporon* species.

RESULTS

We used the MIC of six antifungal agents against 50 *T. asahii* strains to evaluate the accuracy of the ASTY method compared to the recommended CLSI broth microdilution method. Micafungin MIC were obtained only with the ASTY panel; the CLSI method was not used because *Trichosporon* species are resistant to echinocandins. Table 1 shows the distribution of differences in MIC using the ASTY method versus the CLSI method. The data in this table show that the growth of almost all *T. asahii* isolates was inhibited by low concentrations of MCZ, ITCZ, and VRCZ, but only by very high concentrations of 5-FC (4–64 μg/ml) and FLCZ (4–64 μg/ml). Moreover, 42 and 40% of the isolates showed MIC greater than 64 μg/ml for 5-FC when using the ASTY and CLSI methods, respectively. The most effective antifungal agent was VRCZ, whereas the least active drug against the *T. asahii* isolates was 5-FC.

Table 2 shows the number of isolates with $\log_2$ MIC differences and the overall essential agreement for each antifungal agent. Of the 50 isolates used in this study, seven had more than two dilution differences. The overall essential agreement ($\pm 1 \log_2$ dilution) was 97.7%, and the essential agreement was 96% for AMPH-B, 98% for 5-FC, 100% for FLCZ, 96% for MCZ, 98% for ITCZ, and 98% for VRCZ. For these drugs, the ASTY method showed good agreement with the reference method, the best essential agreement being observed for FLCZ. The MIC of the six antifungal agents tended to be slightly lower using the ASTY method.

DISCUSSION

The most important requirement for a mycology laboratory for susceptibility testing is the ability to produce accurate results quickly in order to expedite clinical

© 2008 The Societies and Blackwell Publishing Asia Pty Ltd

Table 1 MIC distribution of 50 isolates of *T. asahii* against six antifungal drugs using the CLSI microdilution reference method and the ASTY colorimetric test.

	AMPH-B		5-FC		FLCZ		MCZ		ITCZ		VRCZ	
MIC (μg/mL)	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI
0.01												
0.03											5 (10)	1 (2)
0.06											20 (40)	18 (36)
0.12	1 (2)[a]	3 (6)									15 (30)	18 (36)
0.25	4 (8)	3 (6)					2 (4)		1 (2)	1 (2)	6 (12)	9 (18)
0.5	12 (24)	11 (22)					19 (38)	18 (36)	16 (32)	11 (22)	1 (2)	1 (2)
1	22 (44)	17 (34)					21 (42)	18 (36)	30 (60)	38 (76)	1 (2)	
2	8 (16)	12 (24)					6 (12)	11 (22)	3 (6)		2 (4)	3 (6)
4		1 (2)	1 (2)	1 (2)	8 (16)	5 (10)	2 (4)	3 (6)				
8			5 (19)	8 (16)	29 (58)	28 (56)						
16	3 (6)	1 (2)	14 (28)	10 (20)	9 (18)	13 (26)						
32			7 (14)	6 (12)	1 (2)	2 (4)						
64			2 (4)	5 (10)	3 (6)	2 (4)						
>8												
>16		2 (4)										
>64			21 (42)	20 (40)								

[a]Percentage

Table 2 Distribution of differences in MIC determined using the CLSI microdilution reference method and ASTY colorimetric test.

	n (%) of isolates with $\log_2$ MIC differences of							
	>+2	2	1	0	−1	−2	<−2	% Agreement
AMPH-B		2 (4)	6 (12)	27 (54)	15 (30)			96
5-FC		1 (2)	8 (16)	32 (64)	9 (18)			98
FLCZ			4 (8)	36 (72)	10 (20)			100
MCZ		1 (2)	7 (14)	26 (52)	15 (30)	1 (2)		96
ITCZ		1 (2)	7 (14)	31 (62)	11 (22)			98
VRCZ			2 (4)	33 (66)	14 (28)	1 (2)		98

decisions (19). Before an alternative antifungal susceptibility test device can safely be used for clinical testing in place of the CLSI M27-A2 reference standard, a detailed comparative evaluation of its performance has be made.

The ASTY method, another colorimetric microdilution method, has not been evaluated as often as the YeastOne Panel. In general, CLSI and colorimetric tests give similar MIC ranges for antifungal agents (7–9). Our results indicate that, for susceptibility of *T. asahii* isolates, the ASTY method shows good concordance with the reference microdilution method. The obtained MIC ranges for MCZ, ITCZ, and VRCZ were narrow and low; in contrast, the MIC ranges for FLCZ were narrow and high. For AMPH-B and 5-FC, the MIC were higher and broader than those for other antifungal agents.

To our knowledge, no other study has examined the antifungal susceptibility of *T. asahii* isolates using the ASTY colorimetric method. Therefore, our study provides important comparisons between the reference method and colorimetric panels.

A few studies have evaluated the ASTY panel. A multicenter evaluation study compared the susceptibility of 802 *Candida* species using ASTY and reference microdilution methods (10). In that study, the MIC of ITCZ, 5-FC, and AMPH-B were compared and the overall agreement was 93% at 24 hr and 96% at 48 hr. Agreement ranged from 90% with ITCZ and 5-FC to 96% with AMPH-B at 24 hr, and from 92% with ITCZ to 99% with AMPH-B and 5FC at 48 hr. Thus the ASTY colorimetric microdilution panel method appeared to be comparable to the CLSI reference method for testing the susceptibility of *Candida* spp. to some antifungal agents. Other studies have reported antifungal susceptibility results obtained using the ASTY method. These studies used ASTY to determine the MIC

© 2008 The Societies and Blackwell Publishing Asia Pty Ltd

of *Candida* spp. and *G. capitatum* clinical isolates without comparison to the reference method (11–13). Therefore, we could not compare our results with these studies. We believe that the ASTY panel has certain advantages in a clinical setting. Namely, standardized antifungal dilutions, media, and inoculum concentrations minimize the requirement for an expert in antifungal susceptibility testing and ASTY can be performed easily, even by inexperienced laboratory personnel.

Our antifungal susceptibility results should be compared to those with similar research methodology. For example, Metin *et al*. (18) compared the Etest and disk diffusion methods with the CLSI M27-A2 reference method for 27 clinical isolates of *Trichosporon* spp. (14 *T. mucoides*, 13 *T. asahii*). The observed agreement was 81–100% for AMPH-B, FLCZ, ITCZ, and VRCZ. With microdilution they found relatively high MIC for FLCZ and relatively low MIC for VRCZ. However, they found lower AMPH-B and ITCZ MIC than our MIC results (18). In another study, Arikan *et al*. (17) investigated the *in vitro* activity of AMPH-B, FLCZ, and ITCZ against 43 *T. asahii* isolates by comparing the Etest and CLSI method. They found microdilution ranges following 48-hr incubations of 1 to 8, 0.25 to 16, and 0.06 to 4 μg/ml for AMPH-B, FLCZ, and ITCZ, respectively, and reported relatively lower MIC than in our study (17).

In this study, some *T. asahii* isolates showed high MIC values for AMPH-B ($>16\,\mu$g/mL), FLCZ ($>64\,\mu$g/mL), and VRCZ ($>1\,\mu$g/mL). Thirty strains showed MIC values of $>32\,\mu$g/mL for 5-FC. ASTY is a valuable method for detecting resistant strains in a clinical setting, because both ASTY and CLSI methods show good agreement regarding the high MIC of antifungal agents.

Using a commercial product does not guarantee product performance in an individual laboratory under actual testing conditions. Therefore, it is still necessary to evaluate the colorimetric panels for relatively uncommon pathogens, such as *Trichosporon* spp., in individual laboratories.

ACKNOWLEDGMENTS

This study was supported in part by research grants from the Asian and Africa Science Platform Program of the Japan Society for the Promotion of Science, Clinical Hematology Oncology Treatment Study Group, and a Health Science Research Grant for "Research on Emerging and Re-emerging Infectious Diseases" from the Ministry of Health, Labor, and Welfare. We thank the physicians who provided the clinical isolates. Dr. Ayse Kalkanci, who is the first author, was funded as a visiting scientist by an Onda Scholarship provided by Meiji Pharmaceutical University.

REFERENCES

1. Erer B., Galimberti M., Lucarelli G., Giardini C., Polchi P., Baronciani D., Gaziev D., Angelucci E., Izzi G. (2000) *Trichosporon beigelii*: a life-threatening pathogen in immunocompromised hosts. *Bone Marrow Transplant* **25:** 745–9.
2. Tashiro T., Nagai H., Kamberi P., Goto Y., Kikuchi H., Nasu M., Akizuki S. (1994) Disseminated *Trichosporon beigelii* infection in patients with malignant diseases: immunohistochemical study and review. *Eur J Clin Microbiol Infect Dis* **13:** 218–24.
3. Goodman D., Pamer E., Jakubowski A., Morris C., Sepkowitz K. (2002) Breakthrough trichosporonosis in a bone marrow transplant recipient receiving caspofungin acetate. *Clin Infect Dis* **35:** E35–36.
4. Matsue K., Uryu H., Koseki M., Asada N., Takeuchi M. (2006) Breakthrough trichosporonosis in patients with hematologic malignancies receiving micafungin. *Clin Infect Dis* **42:** 753–7.
5. Falk R., Wolf D.G., Shapiro M., Polacheck I. (2003) Multidrug-resistant *Trichosporon asahii* isolates are susceptible to voriconazole. *J Clin Microbiol* **41:** 911.
6. Wolf D.G., Falk R., Hacham M., Theelen B., Boekhout T., Scorzetti G., Shapiro M., Block C., Salkin I.F., Polacheck I. (2001) Multidrug-resistant *Trichosporon asahii* infection of nongranulocytopenic patients in three intensive care units. *J Clin Microbiol* **39:** 4420–5.
7. Linares M. J, Charriel G., Solís F., Casal M. (2004) Comparison of two microdilution methods for testing susceptibility of *Candida* spp. to voriconazole. *J Clin Microbiol* **42:** 899–902.
8. Linares M.J., Charriel G., Solís F., Rodriguez F., Ibarra A., Casal M. (2005) Susceptibility of filamentous fungi to voriconazole tested by two microdilution methods. *J Clin Microbiol* **43:** 250–3.
9. Pfaller M.A., Espinel-Ingroff A., Jones R.N. (2004) Clinical evaluation of the Sensititre YeastOne colorimetric antifungal plate for antifungal susceptibility testing of the new triazoles voriconazole, posaconazole, and ravuconazole. *J Clin Microbiol* **42:** 4577–80.
10. Pfaller M.A., Arikan S., Lozano-Chiu M., Chen, Y-S., Coffman S., Messer S.A., Rennie R., Sand C., Heffner T., Rex J.H., Wang J., Yamane N. (1998) Clinical evaluation of the ASTY colorimetric microdilution panel for antifungal susceptibility testing. *J Clin Microbiol* **36:** 2609–12.
11. Kobayashi D., Kondo K., Uehara N., Otokozawa S., Tsuji N., Yagihashi A., Watanabe N. (2002) Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Antimicrob Agents Chemother* **46:** 3113–7.
12. Hattori H., Inoue C., Tomita Y., Kanbe T. (2007) A case of oral geotrichosis caused by *Geotrichum capitatum* in an old patient. *Jpn J Infect Dis* **60:** 300–1.
13. Ohta C., Yamane N., Nakasone I., Onaga S., Nakamura K. (2005) Antifungal susceptibility among the isolates of yeast from the University Hospital of the Ryukyus. *Rinsho Biseibutsu Jinsoku Shindan Kenkyukai Shi* **16:** 127–33.
14. Sugita T., Nakajima M., Ikeda R., Matsushima T., Shinoda T. (2002) Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. *J Clin Microbiol* **40:** 1826–30.
15. National Committee for Clinical Laboratory Standards. (2002) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved Standard. Second edition, document M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
16. Paphitou N.I., Ostrosky-Zeichner L., Paetznick V.L., Rodriguez J.R., Chen E., Rex J.H. (2002) *In vitro* antifungal susceptibilities of *Trichosporon* species. *Antimicrob Agents Chemother* **46:** 1144–6.

© 2008 The Societies and Blackwell Publishing Asia Pty Ltd

17. Arikan S., Hascelik G. (2002) Comparison of NCCLS microdilution method and E test in antifungal susceptibility testing of clinical *Trichosporon asahii* isolates. *Diag Microbiol Infect Dis* **43:** 107–11.
18. Metin D.Y., Hilmioglu-Polat S., Hakim F., Inci R., Tumbay E. (2005) Evaluation of the microdilution, Etest and disk diffusion methods for antifungal susceptibility testing of clinical strains of *Trichosporon* spp. *J Chemother* **17:** 404–8.
19. Makimura K., Suzuki T., Tamura T., Ikedo M., Hanazawa R., Takabashi Y., Yamada Y., Uchida K., Yamaguchi H. (2004) Comparative evaluation of standard dilution method and commercial kit for frozen plate antifungal susceptibility testing of yeasts using 200 clinical isolates. *Microbiol Immunol* **48:** 747–53.

© 2008 The Societies and Blackwell Publishing Asia Pty Ltd