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Short Communication

Serotypes of Clinical Cerebrospinal Fluid *Cryptococcus neoformans* Isolates from Southern Taiwan and Their In Vitro Susceptibilities to Amphotericin B, Fluconazole, and Voriconazole

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SUMMARY: In this study, 34 clinical cerebrospinal fluid isolates of *Cryptococcus neoformans* were serotyped, and their in vitro susceptibilities to amphotericin B, fluconazole, and voriconazole were analyzed. Of these 34 isolates, serotype A was found in 29 isolates and serotype B in the other five. The voriconazole geometric mean MIC was significantly lower than the amphotericin B/antibiotic medium 3 geometric mean MIC ($P < 0.0001$ at both 48 and 72 h), as well as the fluconazole geometric mean MIC ($P < 0.0001$ at both 48 and 72 h). Of the three antifungal agents, only fluconazole, with geometric mean MICs at both 48 and 72 h, showed significant difference between the serotypes A and B of *C. neoformans*.

Amphotericin B alone or combined with flucytosine and fluconazole have been important antifungal agents used in the treatment of cryptococcal meningitis (1). However, the therapeutic result for cryptococcal central nervous system infection has not been satisfactory, prompting the need for more effective antifungal agents in the treatment of this serious infectious disease. Voriconazole, a triazole antifungal agent derived from fluconazole, has shown a range of activities against a wide variety of clinically important fungal pathogens (2); however, an understanding of its potential role in the clinical treatment of cryptococcal meningitis has been limited. In this study, 34 clinical isolates of *Cryptococcus neoformans* cultured from the cerebrospinal fluid (CSF) specimens of patients with crytpococcal meningitis were serotyped, and their in vitro susceptibilities to amphotericin B, fluconazole, and voriconazole were analyzed.

Thirty-four clinical isolates of *C. neoformans*, cultured from the CSF specimens of patients with cryptococcal meningitis collected over a 5-year period (January 1998 - December 2002) at Chang Gung Memorial Hospital (CGMH)-Kaohsiung, were included in this study. During the study period, brain-heart infusion agar or broth and Sabouraud dextrose agar slants and plates were used for routine cultures of suspected yeasts and fungi. *C. neoformans* was identified by standard mycological techniques based on the characteristics of growth appearance on Sabouraud dextrous agar at 37°C, assimilation of carbohydrates, production of urease, and the presence of a capsule on India ink preparation. Isolates were stored frozen at −20°C in 20% glycerol until the study was performed. Prior to testing, each isolate was subcultured at least twice on potato dextrose agar slants (Remel, Lenexa, Kans., USA) to ensure its viability, purity, and optimal growth.

In variety typing of *C. neoformans* isolates, L-canavanine-glycine-bromothymol blue (CGB) agar (3) and glycine-phenol red agar without cycloheximide (GOP) (4), respectively, were used. Interpretations of the color changes and designation of serogroups followed the standard criteria (5). Agglutination serotyping was performed with eight factor-specific sera (Iatron Co., Tokyo, Japan) and interpreted according to the description of Ikeda et al. (6). In vitro susceptibility testings for amphotericin B, fluconazole, and voriconazole were performed using the broth macrodilution method according to NCCLS (NCCLS M27-A2) (National Committee for Clinical Laboratory Standards, 2002). These three antifungal agents were provided as standard powders of known potency. Two standard strains, *C. neoformans* ATCC 90112 and 90113, were used as internal controls in each run of the test. Susceptibility testing was performed in a RPMI 1640 medium with L-glutamine and without sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo., USA) for all three antifungal agents. In addition, antibiotic medium 3 (Difco Laboratories, Detroit, Mich., USA) was also used to determine MICs of amphotericin B. MICs were read after 48 and 72 h. The geometric means of MICs at 48 and 72 h between amphotericin B/RPMI and amphotericin B/antibiotic medium 3 were compared using the Wilcoxon rank-sum test. We used the Wilcoxon rank-sum test to analyze the geometric means of MIC value at 48 and 72 h between the two antifungal agents (amphotericin B/antibiotic medium 3 versus fluconazole; amphotericin B/antibiotic medium 3 versus voriconazole; and fluconazole versus voriconazole). The 48- and 72-h geometric mean MICs of the three antifungal agents against the different varieties of *C. neoformans* were also analyzed using Wilcoxon rank-sum test. A P-value < 0.05 was considered statistically significant.

The serotypes of the tested 34 *C. neoformans* isolates were serotype A in 29 isolates and serotype B in the other five.

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Table 1. In vitro susceptibility of *Cryptococcus neoformans* (n = 34)

Antifungal agents	MIC (μg/ml) 48 h			MIC (μg/ml) 72 h		
	Range	MIC_{90}	Geometric mean	Range	MIC_{90}	Geometric mean
Amphotericin B/RPMI	0.03-1	0.5	0.35	0.125-1	0.5	0.42
Amphotericin B/ATM3	0.03-1	1	0.54	0.125-1	1	0.71
Fluconazole	0.03-4	2	1.30	0.03-8	2	1.50
Voriconazole	0.0075-0.5	0.03	0.03	0.0075-0.5	0.03	0.03

MIC: minimum inhibitory concentration, RPMI: RPMI 1640 medium, ATM 3: antibiotic medium 3.

Table 2. Antifungal susceptibilities of variety of *C. neoformans* isolates

Antifungal agents	MIC_{90} (μg/ml) 48 h						MIC_{90} (μg/ml) 72 h					
	Serotype A			Serotype B			Serotype A			Serotype B		
	Range	MIC_{90}	Geometric mean	Range	MIC_{90}	Geometric mean	Range	MIC_{90}	Geometric mean	Range	MIC_{90}	Geometric mean
Amphotericin B/RPMI	0.125-1	0.5	0.37	0.03-0.5	0.5	0.25	0.125-1	1	0.44	0.125-0.5	0.5	0.33
Amphotericin B/ATM3	0.25-1	1	0.58	0.03-1	1	0.38	0.5-1	1	0.73	0.125-1	1	0.57
Fluconazole	0.03-4	4	1.12	2-4	4	3.03	0.03-8	4	1.33	2-4	4	3.03
Voriconazole	0.0075-0.5	0.125	0.03	0.015-0.03	0.03	0.03	0.0075-0.5	0.125	0.03	0.015-0.125	0.125	0.04

Abbreviations are in Table 1.

Antifungal susceptibilities of the 34 *C. neoformans* isolates tested are presented in Table 1. When RPMI medium was used, the majority of MICs were clustered at concentrations of 0.25 μg/ml and 0.5 μg/ml. When antibiotic medium 3 was used, the majority of MICs were clustered at concentrations of 0.5 μg/ml and 1.0 μg/ml. The majority of fluconazole MICs were clustered at concentration ranges of 1.0 μg/ml to 4.0 μg/ml. The majority of voriconazole MICs were clustered at concentrations of 0.015 μg/ml and 0.03 μg/ml. The voriconazole geometric mean MIC was significantly lower than the amphotericin B/antibiotic medium 3 geometric mean MIC ($P < 0.0001$ at both 48 and 72 h), or the fluconazole geometric mean MIC ($P < 0.0001$ at both 48 and 72 h). Further, the amphotericin B/antibiotic medium 3 geometric mean MIC was significantly lower than the fluconazole geometric mean MIC ($P < 0.0001$ at both 48 and 72 h). The geometric mean MIC was also significantly different between amphotericin B/RPMI and amphotericin B/antibiotic medium 3 ($P = 0.003$ at 48 h, $P < 0.0001$ at 72 h) with a higher geometric mean MIC of amphotericin B/antibiotic medium 3. The results of in vitro susceptibility of the three antifungal agents against the two different varieties of *C. neoformans* (serotype A and serotype B) are shown in Table 2. The comparative results were as follows: amphotericin B/antibiotic medium 3 ($P = 0.953$ at 48 h, $P = 0.890$ at 72 h), fluconazole ($P = 0.0290$ at 48 h, $P = 0.035$ at 72 h), and voriconazole ($P = 0.411$ at 48 h, $P = 0.389$ at 72 h).

In this study, *C. neoformans* serotype A was the most common subtype of our *C. neoformans* isolates cultured from CSF specimens, accounting for 85% (29/34). The high incidence of *C. neoformans* serotype A infection in this group of patients is similar to that found in a report from northern Taiwan (7), as well as those in reports from other countries (8). The MICs of these three antifungal agents against the 34 *C. neoformans* isolates tested were all within the acceptable limits (9,10), with amphotericin B MICs $\leqq$ 1.0 μg/ml, fluconazole MICs $\leqq$ 8.0 μg/ml, and voriconazole MICs $\leqq$ 0.5 μg/ml. The geometric mean MIC of amphotericin B/antibiotic medium 3 was significantly higher than the value of amphotericin B/RPMI; this finding is consistent with the reported observations of Lozano-Chiu et al. (11); they found that only antibiotic medium 3 permitted a consistent and reliable detection of resistant isolates of *C. neoformans* when testing was performed in broth by the M27-A method. Generally, there has been no significant difference in drug susceptibility among the various serotypes of *C. neoformans* (8). However, var. *gattii* has been reported to be less susceptible than var. *neoformans* to amphotericin B and flucytosine (12,13), and this lower susceptibility of var. *gattii* to antifungal agents has been speculated as a contributor to a higher rate of complications, slower response, and longer duration of therapy for patients with var. *gattii* infection (12). In this study, only the geometric mean MIC of fluconazole at both 48 and 72 h showed a significant difference between the two different serotypes.

This study demonstrated that serotype A was the most common serotype of *C. neoformans* isolates cultured from the CSF specimens of meningitis patients at the CGMH-Kaohsiung, Kaohsiung, Taiwan, between 1998 and 2002. None of the CSF isolates tested during this time period showed in vitro resistance to amphotericin B, fluconazole, or voriconazole. Among these three antifungal agents, voriconazole was the most potent and may have great potential in future management of *C. neoformans* CNS infections.

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