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In Vitro Antifungal Susceptibility of *Cryptococcus gattii*

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We have determined the in vitro susceptibilities of 57 strains of *Cryptococcus gattii* to nine antifungal agents and have compared the MICs for these strains with those for *C. neoformans*. MICs were determined by a microdilution reference method. Albaconazole and ravuconazole (MICs of 0.04 and 0.05 μg/ml, respectively) showed the best activities. Micafungin showed no activity (MIC of >128 μg/ml). In general, *C. gattii* was less susceptible than *C. neoformans* to all drugs tested, with the exception of amphotericin B and flucytosine.

Cryptococcosis is a relevant human infection generally associated with high mortality. *Cryptococcus neoformans*, the responsible agent, has been traditionally classified into two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. However, recent molecular studies have indicated that the two varieties should be recognized as separate species, *C. neoformans* and *C. gattii* (4, 8). Although both affect the lungs and central nervous system, the infections caused by the two species have important differences in epidemiologies, clinical presentations, and therapeutic outcomes (7, 18). *C. neoformans* causes infections worldwide, mainly in immunocompromised hosts. By contrast, *C. gattii* is geographically restricted to tropical and subtropical regions and affects mainly immunocompetent hosts (3, 19). In the north and northeast regions of Brazil, *C. gattii* is endemic, prevailing in 62.7% of the cryptococcosis cases (14). Also, infections caused by *C. gattii* seem to have poorer responses to antifungal therapy than those caused by *C. neoformans* (22). Despite these differences, only *C. neoformans* has been included in the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (13) for testing yeast. Some studies on the in vitro antifungal susceptibility of *C. neoformans* have been performed (11, 16, 21), but scarce data exist on the other species. Only a few strains of *C. gattii* have been tested up to now. Also, the results of these studies have been very contradictory (1, 12, 22). The aim of our study, therefore, was to determine the in vitro activities of nine agents against a large number of isolates of *C. gattii* from clinical and environmental origins and to compare the results with those for *C. neoformans*.

Eighty-seven strains of *Cryptococcus* spp. were selected for testing. Among these were 57 strains of *C. gattii* (52 of clinical origin and 5 of environmental origin) and 30 of *C. neoformans* (23 of clinical origin and 7 of environmental origin). The clinical strains were from patients with cryptococcal meningitis and were isolated in the north, northeast, southeast, and central regions of Brazil and maintained at FIOCRUZ (IPEC/INCQS) Culture Collection, Rio de Janeiro, Brazil. Species identification was performed by using standard methods (9). All isolates were maintained in 20% skim milk at −20°C until the study was performed. Prior to testing, each isolate was subcultured on Sabouraud dextrose agar (SDA) to ensure optimal growth. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains and included each time that a set of isolates was tested. MIC ranges were within the control limits recommended by the NCCLS (13). Ranges of MICs of albaconazole (ABC) and micafungin (MFG) were as follows: 0.1 and 16 to >16 μg/ml, respectively, for *C. parapsilosis* and 0.06 to 0.125 and 2 to 4 μg/ml, respectively, for *C. krusei*.

ABC (J. Uriach & Co, S.A., Barcelona, Spain), amphotericin B (AMB; E. R. Squibb & Sons, Barcelona, Spain), flucytosine (5FC; Hoffmann-La Roche, Basel, Switzerland), fluconazole (FLC; Pfizer, Madrid, Spain), itraconazole (ITC) and ketoconazole (KTC; Janssen Research Foundation, Beerse, Belgium), MFG (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), ravuconazole (RVC; Bristol-Myers Squibb Company, New Brunswick, N.J.), and voriconazole (VRC; Pfizer, Madrid, Spain) were obtained as assay powders. Stock solutions (all drugs) were prepared in dimethyl sulfoxide, with the exception of those of 5FC, FLC, and MFG, which were prepared in water. Serial twofold dilutions were performed as described by the NCCLS (13). Final dilutions were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (Sigma, Madrid, Spain). Aliquots (100 μl) of each antifungal agent at twice the final concentration were dispensed into the wells of microdilution trays. The microplates were stored at −70°C until used. The final concentrations of the drugs ranged from 0.25 to 128 μg/ml for MFG, from 0.125 to 64 μg/ml for 5FC and FLC, and from 0.03 to 16 μg/ml for all remaining agents.

A broth microdilution method for MIC determination was carried out as described in NCCLS document M27-A2 (13). Stock inoculum suspensions were prepared from 48-h-old cultures grown on SDA at 35°C. The suspensions were adjusted to a cell density that ranged from 1.0×10^6 to 5.0×10^6 cells/ml. Each suspension was diluted 1:50 and further diluted 1:20 in RPMI 1640 medium. An aliquot of 0.1 ml was added to each

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TABLE 1. MICs of nine antifungal agents for 87 isolates of *Cryptococcus* spp.

Species (no. of isolates)	MIC parameter[a]	MIC (μg/ml) of:								
		ABC	AMB	ITC	5FC	FLC	MFG	KTC	RVC	VRC
C. gattii (57)	Range	<0.03–0.5	0.25–2	0.03–0.5	0.5–>64	1–64	>128	<0.03–0.5	<0.03–0.5	<0.03–1
	GM	0.06	0.59	0.28	6.16	9.54	>128	0.10	0.10	0.15
	90%	0.125	1	0.5	16	32	>128	0.25	0.25	0.25
C. neoformans (30)	Range	<0.03–0.06	0.25–2	<0.03–0.5	2–16	1–32	>128	<0.03–0.25	<0.03–0.125	<0.03–0.25
	GM	0.02	0.51	0.11	5.12	3.89	>128	0.04	0.02	0.06
	90%	0.06	1	0.25	8	8	>128	0.125	0.06	0.125
Total (87)	Range	<0.03–0.5	0.25–2	<0.03–0.5	0.5–>64	1–64	>128	<0.03–0.5	<0.03–0.5	<0.03–1
	GM	0.04	0.55	0.20	5.75	7.07	>128	0.07	0.05	0.11
	90%	0.125	1	0.5	16	32	>128	0.25	0.25	0.25

[a] GM, geometric mean; 90%, MICs at which 90% of isolates tested were inhibited.

well of the microdilution tray to obtain a final inoculum concentration of 0.5×10^3 to 2.0×10^3 CFU/ml, as demonstrated by quantitative colony counts on SDA. Growth and sterility control wells were included for each isolate tested. The microplates were incubated at 35°C. The MIC endpoints were read after 48 and 72 h of incubation. The MIC of AMB was defined as the lowest concentration that produced 100% inhibition of growth, and the MICs of the other antifungal drugs were defined as the lowest concentrations that produced an 80% reduction in growth. After the MIC was measured, the minimum fungicidal concentration (MFC) was determined as described by other authors (5, 15). The microplates were shaken, and 10 μl from each well that showed complete inhibition (100% inhibition or an optically clear well) relative to the last positive well and the growth control (drug-free medium) was cultured on SDA plates at 35°C. The MFC was defined as the lowest drug concentration at which there was either no growth or fewer than three colonies. This parameter represents killing of approximately 99% of the original inoculum. Both on- and off-scale MICs and MFCs were included in the analysis. The high off-scale results were converted to the next highest concentration, and the low off-scale results were left unchanged. To facilitate the comparison of the activities of the drugs, geometric mean MICs and geometric mean MFCs were determined for each drug-isolate combination. Also, to determine the difference between in vitro fungistatic and fungicidal activities, each MFC was compared to the corresponding MIC for each isolate-drug combination. Comparisons of proportions were performed by using the Wilcoxon test or the Mann-Whitney test as appropriate and the statistical SPSS package (version 10.0). P values of <0.05 were considered statistically significant.

All isolates produced clearly visible growth only after 72 h of incubation. We could not read the MIC endpoint clearly at 48 h because the growth in all isolates was insufficient. Table 1 shows the MICs of the drugs tested. When all the strains of the two species were considered together, the widest ranges of MICs were those of 5FC (0.5 to >64 μg/ml) and FLC (1 to 64 μg/ml) and the narrowest range was that of the MICs of AMB (0.25 to 2 μg/ml). Although AMB, ITC, KTC, and VRC showed good activity, the two new triazoles ABC and RVC (MICs of 0.04 and 0.05 μg/ml, respectively) were more active than the other drugs tested ($P < 0.05$). On the other hand, MFG was the least active (MIC of >128 μg/ml). In general, *C. gattii* was less susceptible than *C. neoformans* to all the drugs tested ($P < 0.05$). The only exceptions were for AMB and 5FC, because the results between the two species were not significantly different. Table 2 shows the MFCs of the eight antifungal agents for 87 strains tested. For all isolates, the MFCs of AMB were either the same as or less than two dilutions higher than the MICs. In contrast, the MFCs of the other drugs for all isolates were much higher than the MICs, which may indicate that the other antifungal agents have fungistatic activity.

Because infections caused by *C. gattii* often have a worse prognosis than those caused by *C. neoformans* (18), and because in general these infections are not well studied, there is a critical need to determinate the in vitro susceptibility of *C. gattii* mainly to

TABLE 2. MFCs of eight antifungal agents for 87 isolates of *Cryptococcus* spp.

Species (no. of isolates tested)	MFC parameter[a]	MFC (μg/ml) of:							
		ABC	AMB	ITC	5FC	FLC	KTC	RVC	VRC
C. gattii (57)	Range	0.03–>16	0.25–8	0.25–>16	8–>64	0.25–>64	<0.03–>16	0.03–>16	0.06–>16
	GM	1.22	1.04	2.46	>64	46.65	1.90	2.17	2.91
	90%	>16	2	>16	>64	>64	>16	16	>16
C. neoformans (30)	Range	0.03–>16	0.5–8	0.06–>16	4–>64	2–>64	0.03–>16	0.03–>16	0.06–>16
	GM	2.28	1.02	2.46	>64	18.81	1.58	2.64	3.07
	90%	>16	4	16	>64	>64	>16	>16	>16
Total (87)	Range	0.03–>16	0.25–8	0.06–>16	4–>64	2–>64	0.03–>16	0.03–>16	0.06–>16
	GM	>16	2	>16	>64	>64	>16	>16	>16
	90%	>16	2	>16	>64	>64	>16	>16	>16

[a] The MFC of MFG was not determined (MIC, >128 μg/ml). GM, geometric mean; 90%, MFCs at which 90% of isolates tested were killed.

the new antifungal agents. To our knowledge, this is the first time that more than 21 strains of *C. gattii* have been tested.

Currently, AMB alone or in combination with 5FC remains the drug of choice for the treatment of cryptococcal meningitis, although FLC and ITC are also frequently used (17). In our study, we did not observe strains that were apparently resistant to AMB; in general, MICs for all isolates tested were ≤ 1 μg/ml (MICs for only two strains were 2 μg/ml). Similar results have been obtained by other authors (6, 11, 24) also using RPMI 1640 medium as the culture medium. However, strains resistant to AMB have been detected using antibiotic medium 3 (10). Although it is well known that FLC is more effective in vivo than ITC, several in vitro studies have reported opposite results (6, 16, 20). In our case, FLC also showed higher mean MICs than ITC (7.07 and 0.20 μg/ml, respectively) for all isolates. In general, ABC and RVC were the most active drugs tested. VRC also showed good in vitro activity, as has been reported by other authors (21, 23). In this study, we have compared the antifungal susceptibilities of clinical and environmental isolates. When all the strains of the two species were considered together, we did not find any statistically significant differences associated with origins ($P < 0.05$). Our results are in agreement with those obtained by other authors (6, 12), who also demonstrated that antifungal susceptibility is not dependent on the origin of the isolates tested. Comparative studies to determine the differences between the in vitro susceptibilities of the two species are scarce, and the results obtained are contradictory. For example, using a microdilution method, Calvo et al. (1) compared the activities of AMB, FLC, ITC, and 5FC against 89 isolates of *C. neoformans* and only 11 isolates of *C. gattii*. They obtained very similar MICs for both species. Similar results were obtained by Moraes et al. (12), who tested the same drugs by a macrodilution method, and by Chen et al. (2), who tested by a microdilution method. However, the results of these two studies disagree with those of the study of Yee-Chun et al. (22), who demonstrated that *C. gattii* is less susceptible than *C. neoformans* to 5FC and AMB. By contrast, our results indicated that *C. gattii* was as susceptible as *C. neoformans* to 5FC and AMB. However, *C. gattii* was more resistant than *C. neoformans* to the other antifungal agents tested.

New drugs such as posaconazole have been reported to possess fungicidal activity against *C. neoformans* (16). In this study, we have determined the MFCs of the nine antifungal agents but only AMB showed fungicidal activity.

In summary, we have demonstrated that there are some differences in the antifungal susceptibilities of the two species of *Cryptococcus*. However, further in vivo studies are needed in order to ascertain the predictive value of these in vitro data.

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