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Microtiter Method for MIC Testing with Spherule-Endospore-Phase *Coccidioides immitis*

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A method was developed for susceptibility testing with spherule-endospore-phase *Coccidioides immitis* by using a microtiter format. Isolated endospores were used to inoculate wells containing modified Converse medium with various concentrations of azole or nikkomycin antifungal substances which then were sealed with an acetate film. The plate was incubated at 37°C with shaking for 96 h, after which the control wells had visible turbidity and endpoints were discernible. Microscopic examination revealed that both control and treatment wells maintained cells predominantly in the spherule-endospore phase of growth.

Although a reasonable level of standardization exists for antibacterial susceptibility testing, methods for antifungal testing are numerous and have resulted in divergent test results (16). Adding difficulty to this situation are the complex environmental requirements of some fungi, such as the dimorphic pathogenic fungi, and certain yeasts, such as the lipophilic *Malassezia furfur*. Among the former group, the parasitic phase of *Coccidioides immitis* has perhaps the most stringent requirements for maintaining the parasitic cycle. Such factors as pH, appropriate nutrients, temperature, presence of a detergent, and cell density must be rigidly controlled for liquid-based spherule-endospore (SE) cultures to be successfully propagated. Under the conditions established for in vitro growth (3–6, 13), liquid-phase MIC testing would not be practicable without nephelometric measurement because the culture is typically seeded to a density already detectable with the unaided eye. At least three previous reports made use of the SE phase in determinations of MICs (2, 11, 17). Other reports of susceptibility testing with *C. immitis* have been concerned either with the saprophytic phase (7, 10, 17) or with an endospore inoculum which was allowed to convert to the filamentous stage during the incubation period (12, 14). We report herein a practical microtiter method for susceptibility testing which uses and maintains the SE cycle for the duration of the test procedure and yields results consistent with previously reported data.

The compounds tested were the azoles miconazole, ketoconazole, and itraconazole from Janssen Pharmaceutica, Beese, Belgium; fluconazole from Pfizer Inc., Groton, Conn.; and R 3783, N 7133, U 7476, clotrimazole, and bifonazole from Bayer AG, Wuppertal, Federal Republic of Germany; and the chitin synthase inhibitors nikkomycin X and nikkomycin Z, also from Bayer. Ketoconazole was first dissolved in 0.2 N HCl, fluconazole and the nikkomycins were dissolved in modified Converse medium (4, 5, 13), and all other compounds were first dissolved in 95% ethanol. All drugs were subsequently diluted in the growth medium in 96-well microtiter plates (Costar Plastics) in 100-μl volumes.

A fresh endospore inoculum of *C. immitis* Silveira was prepared by differential centrifugation of a liquid culture of the parasitic phase inoculated 72 h earlier by following the conditions described by Levine et al. (13). Endospores were separated by spinning at 500 × *g* for 5 min, followed by a high-speed spin of the upper portion of the supernatant at 800 × *g* for 10 min. The pellet was suspended to an optical density of approximately 0.01 at 440 nm in modified Converse medium, and 100-μl portions were added to all drug and control wells. At this density, a 100-ml portion has the usual turbidity used for culture maintenance, but the 100-μl volumes added to test wells appear transparent. The final volume in the wells was 200 μl. The wells were sealed with an adhesive acetate film (Dynatech Laboratories, Inc., Alexandria, Va.), and the plates were incubated with shaking (80 rpm) at 37°C. Plates were read at 96 h, with the MIC determined as the lowest concentration with no macroscopically visible growth. At 7 days, minimum lethal concentrations were determined by transferring 2.5 μl from wells showing no visible growth to tubes containing 5 ml of glucose-yeast extract broth and incubating at 37°C for 7 days. The minimum lethal concentration was defined as the lowest concentration showing a complete absence of growth.

The MIC results (Table 1) for miconazole, fluconazole, and clotrimazole were consistent with previously reported values (7, 8, 10, 14, 17), although the present method resulted in values for ketoconazole considerably higher than those of other reports (7, 12). This latter finding cannot likely be blamed on a pH effect such as is found with fungi tested in yeast nitrogen base medium, since the present growth medium has a pH much closer to neutral (6.4). The results for the nikkomycins were consistent with the calculated K_i values (15) and were considerably lower than MICs reported for this class of agent versus *Candida albicans* (1). Microscopic examination of control and several test wells at 96 h revealed that the fungus was maintained in the SE phase.

Although the importance of testing the parasitic versus the saprophytic phase of dimorphic fungi has yet to be established, using the former has an inherent appeal. In tests of the susceptibility of *C. immitis* to amphotericin B, the MICs for the SE phase were approximately 8- to 10-fold lower than those for the mycelial phase (2, 11). Similarly, although the SE phase of this fungus was found to be very sensitive to the chitin synthase inhibitor polyoxin D, the mycelial phase appeared to be refractory (9).

The method described above proved to be simple to conduct; it conserved inocula, media, and antifungal agents; and it resulted in data deemed consistent with other published data. Additionally, determining MICs with the SE phase instead of an arthroconidial inoculum offers the obvi-

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TABLE 1. MICs for *C. immitis* Silveira

Agent	MIC[a]	MLC[b]
R 3783	0.25 μg/ml	32 μg/ml
Itraconazole	1.0 μg/ml	32 μg/ml
Miconazole	1.0 μg/ml	4 μg/ml
Clotrimazole	1.0 μg/ml	16 μg/ml
N 7133	2.0 μg/ml	>64 μg/ml
Bifonazole	4.0 μg/ml	Not done
Ketoconazole	32 μg/ml	8 μg/ml
U 7476	>64 μg/ml	>64 μg/ml
Fluconazole	>64 μg/ml	>64 μg/ml
Nikkomycin X	1.56 μM[c]	50 μM
Nikkomycin Z	1.56 μM	6.25 μM

[a] The MICs were read at 96 h.
[b] The MLCs (minimal lethal concentrations) were read at 7 days.
[c] 1.56 μM = 0.7 μg/ml.

ous advantage of safety for laboratories which may not have the containment facilities to handle the saprophytic phase of the fungus. However, since this requires the conversion of the isolate from the saprophytic to the SE phase, the method may be restricted to those reference centers with the capability and the experience to effect such a conversion.

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