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Microplate alamarBlue Assay for *Paracoccidioides* Susceptibility Testing

A. C. A. de Paula e Silva, H. C. Oliveira, J. F. Silva, F. Sangalli-Leite, L. Scorzoni, A. M. Fusco-Almeida, M. J. S. Mendes-Giannini

Faculdade de Ciências Farmacêuticas, UNESP—Universidade Estadual Paulista, Araraquara, Departamento de Análises Clínicas, Laboratório de Micologia Clínica, São Paulo, Brazil

CLSI method M27-A3 is not available for use with dimorphic fungi, such as those of the *Paracoccidioides* genus. In this study, we developed a microdilution method and added the alamarBlue reagent to test the responses of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* against amphotericin B and itraconazole antifungals. The test proved to be sensitive, practical, and inexpensive and can be used to monitor the activity of low-growth microorganisms and their response to various drugs.

Paracoccidioides brasiliensis and *Paracoccidioides lutzii* are dimorphic fungi and the etiologic agents of paracoccidioidomycosis, a disease with multiple clinical presentations, prolonged evolution, and high rates of mortality and morbidity (1–4). Until now, a lack of routine tests has made it difficult to determine whether patients who did not respond to treatment were infected with resistant strains (5–8). Amphotericin B (AMB) is still the first-choice drug despite its high toxicity, and itraconazole (ITZ) is effective for the mild and moderate forms of the disease (9, 10). A long course of therapy and sometimes even lifelong secondary prophylaxis are required, especially in AIDS patients, who have a high incidence of relapse (11). A more effective drug with a better safety profile than the currently available drugs would greatly improve the treatment of dimorphic fungal infections (10, 12).

CLSI (Clinical and Laboratory Standards Institute) document M27-A3 has proposed a method for determining the *in vitro* susceptibility of yeasts to different drugs. However, no standardized method is available for dimorphic fungi because of the difficulty in culturing them. For these reasons, more reliable assays are needed for use with the genus *Paracoccidioides*. The ability to determine the MICs of different compounds would inform clinicians in the choice of an antifungal agent and the management of therapy, as well as facilitating screens of new molecules for their antifungal activity.

However, the microdilution method used today presents diffi-

culties in visually reading the MIC because there is a lack of significant fungal growth. Nevertheless, this test has been used in studies in the literature (12–15). In contrast, the microplate alamarBlue assay (MABA) has been evaluated by several authors in *Aspergillus fumigatus* (16), *Mycobacterium* spp. (17–19), and *Trypanosoma cruzi* (20). Resazurin, the active ingredient in the MABA (alamarBlue Biosource International, Invitrogen's BioSource division), is permeable, blue, and virtually nonfluorescent, but after entry into viable cells, resazurin is continuously reduced by the cells to resorufin, a highly fluorescent red compound.

The aim of our study was to compare the reference broth microdilution microplate assay (MMA) and microdilution microplate assay (MMI) methods with the MABA to measure the presence of fungi of the *Paracoccidioides* genus and the activity of two drugs, AMB and ITZ.

The MMI and MMA were performed according to docu-

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Address correspondence to M. J. S. Giannini, giannini@fcar.unesp.br.

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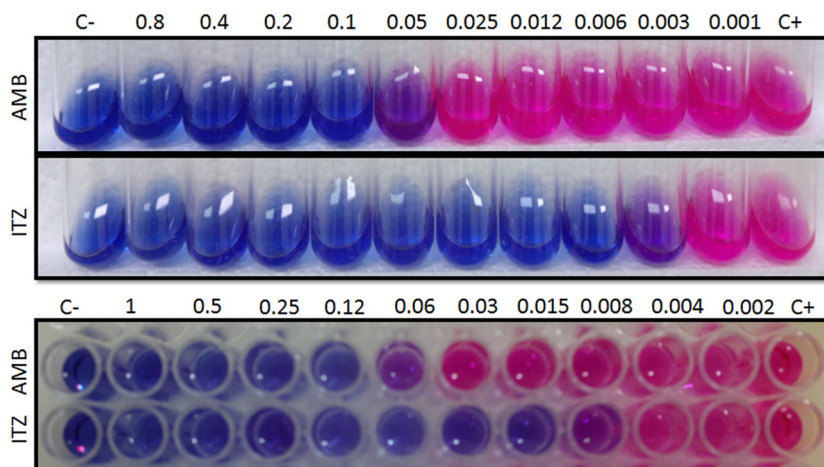


FIG 1 MMA and MMI tests developed with *P. brasiliensis* 18 against AMB and ITZ. The MIC values were determined after the addition of alamarBlue.

TABLE 1 Comparison of MMA and MMI for isolates of two *Paracoccidioides* species

Drug	MIC (mg/liter) in indicated assay for:			
	<i>P. brasiliensis</i> phylogenetic species isolate 18		<i>P. lutzii</i> strain 01	
	MMA	MMI	MMA	MMI
AMB	0.05	0.06	0.1	0.06
ITZ	0.003	0.008	0.001	0.004

ment M27-A3, and ITZ (Sigma) and AMB (Sigma) were prepared with RPMI 1640 medium (Gibco) supplemented with 2% glucose (RPMI 1640-2% Glc) (4). The final concentrations of ITZ and AMB varied from 1 to 0.002 mg/liter and 0.8 to 0.0015 mg/liter, respectively, after the addition of the inoculums. The antifungal activity was analyzed against the *P. brasiliensis* phylogenetic species S1 isolates 18 (São Paulo), D03, and 339 (São Paulo), S2 isolate 02 (Venezuela), and PS3 isolate Epm83 (Colombia) and *P. lutzii* strain 01 (ATCC MYA-826/Goiania) and isolates EE (Mato Grosso) and 8334MMT (Goiania). All the strains were isolated from human patients and were maintained in Fava-Netto medium at 37°C, and subcultures were performed every 4 days.

The cells were suspended in sterile saline and allowed to settle for several minutes to eliminate large aggregates, and then the supernatants were collected. The number of viable cells was estimated by staining with Trypan blue and counting with a hemocytometer, and the final concentration was 10^6 cells/ml. This suspension was diluted 1:50 in sterile saline and 1:20 in RPMI 1640-2% Glc for the MMI test. For the MMA test, the same dilutions were performed, but the first dilution was 1:100. The final inoculum used was 0.5×10^3 to 2.5×10^3 cells/ml after the addition of the antifungal. The plates were incubated at 35°C at 150 rpm for 48 h. After this period, the MABA was employed (according the manufacturer's instructions), and the plates were incubated for an additional 24 h, totaling 72 h for the MIC final reading. The lowest antifungal agent concentration that substantially inhibited the growth of the organism was visually determined at the point at which there was no change in the original blue color of the reagent.

Similar results were obtained when the MMA and MMI were compared for *P. brasiliensis* 18 (Fig. 1) and *P. lutzii* 01 (Table 1). The MICs ranged from 1 to 2 dilutions, which are acceptable values according to document M27-A3 (CLSI).

The visual readings of MICs are easily performed, and the MIC values obtained by both methods are equivalent. Additionally, these values are similar to those described in the literature (12–15, 21–26).

There are several studies using MMA with the *Paracoccidioides* genus, and these generally follow the method suggested by Shadomy et al. (21–23, 27) or that suggested by the NCCLS standardized method in document M27-A (24, 25). The problem with this method is the high cost due to the need for larger amounts of culture medium and other reagents and materials. Also, there are in the literature studies (12–15) of MMI with the *Paracoccidioides* genus; however, they used larger final inoculums than those recommended in document M27-A3. This approach improved the ability to identify the MIC in the slow-growing fungus but is a significant modification in relation to the methods recommended

TABLE 2 MICs of AMB and ITZ in *Paracoccidioides* MABA test

Drug	MIC (mg/liter) for indicated isolate or strain							
	<i>P. lutzii</i>			<i>P. brasiliensis</i> phylogenetic species				
	Strain 01-like isolate		Strain 01	S1 isolate		S2 isolate	PS3 isolate	
AMB	EE	8334MMT	01	18	D03	339	02	Epm83
AMB	0.25	0.25	0.06	0.12	0.008	0.06	0.12	0.06
ITZ	0.008	0.03	0.008	0.015	0.015	0.015	0.008	0.015

in document M27-A3. Therefore, we followed the CLSI standardized method in document M27-A3, and the use of MABA made the MMI more sensitive and reliable, allowing the detection of fewer than 50 viable cells per well.

The use of MABA can replace the MFC (minimum fungicide concentration) test that is inconclusive in most cases because the long incubation of the plate results in dehydration of the solid medium and the consequent death of cells which might have been viable, leading to false-negative results. Similarly, the MIC could be considered equivalent to the MFC because it is a sensitive method to assay the viability of fungal cells.

Thus, the MMI/MABA has been shown to be a reliable and reproducible test for this genus. The test was extended to different isolates of the *Paracoccidioides* genus, confirming that this methodology can be applied for a wide range of isolates (Table 2). Comparing the MIC values determined for the antifungals against different isolates shows that MIC values for ITZ and AMB were within the expected ranges found in the literature (12–15). The MIC values varied independently of the *Paracoccidioides* species from 0.008 to 0.25 mg/liter for AMB and 0.008 to 0.03 mg/liter for ITZ.

Thus, the *Paracoccidioides* MABA test is reliable, reproducible, quick, and an accurate tool for routine laboratory testing.

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