Microplate alamarBlue Assay for Paracoccidioides Susceptibility Testing


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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 difficulties 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-

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Paracoccidioides species isolate 18</th>
<th>P. lutzii strain 01</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMA</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>MMI</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

TABLE 2 MICs of AMB and ITZ in Paracoccidioides MABA test

<table>
<thead>
<tr>
<th>Drug</th>
<th>Strain 01-like isolate E834MMT</th>
<th>Strain S1 isolate 02</th>
<th>S2 isolate 03 Epm83</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMB</td>
<td>0.25</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>ITZ</td>
<td>0.008</td>
<td>0.015</td>
<td>0.008</td>
</tr>
</tbody>
</table>

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-A. 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 in document M27-A. Therefore, we followed the CLSI standardized method in document M27-A, 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 different isolates of the Paracoccidioides species from 0.008 to 0.25 mg/liter for AMB and 0.008 to 0.3 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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REFERENCES


