Effects of Two Different Growth Media on the Postantifungal Effect Induced by Polyenes on Candida Species

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There are no data on the effects of different growth media on polyene-induced postantifungal effect (PAFE) in Candida species. Hence, the nystatin- and amphotericin B-induced PAFEs in six Candida species (26 isolates) grown in Sabouraud’s dextrose broth (SAB) and RPMI broth were evaluated, following limited exposure to the MICs of the two polyenes, using an automated turbidometric method. For nystatin, PAFE varied between 1.88 and 4.87 h in SAB and 0.66 and 6.89 h in RPMI, and for amphotericin B, the equivalent values were 3.13 to 10.98 h in SAB and 0.97 to 7.01 h in RPMI. These highly significant (P < 0.001) variations in the PAFE with both drugs, noted with most Candida strains grown in different media, call for standardization of intralaboratory methodology in measuring this parameter in order to obtain universally comparable data.

Opportunistic oral infections caused by both Candida albicans and non-C. albicans Candida species are becoming increasingly common in immunocompromised patients. For instance, the vast majority of human immunodeficiency virus-infected patients suffer from oral candidosis, the most common AIDS-associated oral infection, during the course of their disease (1). Nystatin and amphotericin B, which belong to the polyene group of antifycotics, are common therapeutic agents for oropharyngeal candidosis (5). Nystatin is widely used as a topical agent in the management of oral candidosis, and oral application of this antifungal agent can also be of considerable benefit in preventing the systemic spread of oral candidosis in immunocompromised persons (5). Though not popular, topical amphotericin B oral preparations are available and used by some clinicians for the management of oral candidosis (5). However, due to the diluent effect of saliva and the cleansing effect of the oral musculature, their availability tends to fall below the effective therapeutic concentrations and the organisms undergo only a brief exposure to antifungal agents (7). Hence, the term “postantifungal effect” (PAFE) has been used in recent years to describe the suppression of fungal growth that persists after brief exposure of organisms to the antifungal agent in question. PAFE could be used as a secondary benchmark in determining the antifungal activity of an antimycotic in addition to the antifungal activity of an antimycotic in addition to the conventional MIC measurement. It may also have therapeutic relevance in determining the antifungal dosing regimens in a clinical setting (2).

There are only a few reports on the PAFE of polyene antifungal agents, particularly with non-C. albicans Candida isolates. These studies to determine the PAFE have essentially investigated the interactions of a few isolates with mainly a single polyene agent (4, 10, 11). There is also no comprehensive information on amphotericin B-induced PAFE on different oral Candida species or the impact of growth media on the PAFE. Hence, the aim of this study was to compare the PAFE on oral isolates of Candida belonging to six different species following limited exposure to nystatin and amphotericin B in two commonly used growth media, namely, Sabouraud’s dextrose broth (SAB) and RPMI 1640 broth.

A total of 26 Candida isolates were studied: four isolates each of C. albicans, C. tropicalis, C. parapsilosis, C. glabrata, C. krusei, and C. guilliermondii, plus C. albicans ATCC 90028 and C. tropicalis ATCC 13803 used as the reference laboratory strains. All isolates were identified using API-20C AUX (BioMérieux, Basingstoke, United Kingdom). Stock cultures were maintained at −70°C. After recovery, these were maintained on Sabouraud’s dextrose agar and stored at 4 to 6°C during the experimental period.

Nystatin and amphotericin B (both from Sigma, St. Louis, Mo.) were dissolved in a mixture of dimethyl sulfoxide (DMSO) and absolute ethanol (3:2 ratio), respectively. They were prepared initially as 2,000 µg/ml solutions and stored at −70°C before use. Since the antifungal agents used were dissolved in DMSO and absolute ethanol, equivalent amounts of the latter chemicals were tested to ascertain whether they had an effect on the isolates tested. The minute volumes of the chemicals used did not have any effect on yeast growth compared with the controls.

SAB (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England) was prepared with double-distilled, sterile water. RPMI 1640 medium, buffered with 0.165 M MOPS [3-(N-morpholino)propanesulfonic acid] containing l-glutamine and lacking sodium bicarbonate (Gibco BRL Products, Life Technology, Gaithersburg, Md.), was dissolved in 1 liter of sterile distilled water, adjusted to pH 7.2, and filter sterilized.

MICs of nystatin and amphotericin B for each isolate were determined in duplicate using the twofold broth microdilution technique as outlined by the National Committee for Clinical Laboratory Standards (9). Both RPMI 1640 and SAB were used to determine the MIC. In brief, 10 µl of the 1 x 107- to 5 x 106-CFU/ml inoculum of the cell suspension was inoculated into each well of a 96-well microplate containing 150 µl of medium with a twofold-diluted concentration of the drug. The MIC was read as the highest dilution of the drug that
inhibited growth after 24 h of incubation at 37°C in a shaking incubator (Lab-Line).

For the PAFE assay, yeast cells maintained on Sabouraud's dextrose agar were inoculated onto fresh plates and incubated overnight prior to use. The organisms were harvested, and a cell suspension was prepared in 0.15 M phosphate-buffered saline (pH 7.2; PBS) to an optical density at 520 nm of 1.5. From this cell suspension, 0.5 ml was added to tubes containing 2 ml of medium (control) and 2 ml of medium-drug solution (test): the drug concentration was the MIC of the drug. This gave a cell suspension of 10^6 to 10^7 cells/ml in each assay tube.

The control and test tubes were then incubated at 37°C for 1 h in a shaker incubator. Following this procedure, the drug that was carried over and contaminating the yeast cells was removed by two cycles of centrifugation for 10 min at 3,000 rpm. The supernatant was completely decanted, and the pellets were resuspended in 2.5 ml of sterile PBS. This procedure has given a cell suspension of 10^6 to 10^7 cells/ml in each assay tube.

An aliquot of 100 μl from each cell suspension was added to a microtiter well containing 150 μl of medium. Then the microtiter plate was placed in a computerized spectrophotometric incubator (Spectramax 340; Molecular Devices Corp., Sunnyvale, Calif.) and incubated at 37°C for 24 h. Plates were kept stationary on the automated reader but were automatically shaken at 30-min intervals immediately prior to the optical density readings. Growth of yeast cells was centrally monitored by the computerized instrument in terms of the change in the turbidity (absorbance at 595 nm) at 30-min intervals. The PAFE was determined as the difference in time (in hours) required for the growth of the drug-free control and drug-exposed test cultures to increase to the 0.05 absorbance level (3, 4, 6).

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The MICs (24 h) for all tested isolates in SAB and RPMI 1640 broth for nystatin ranged between 1.56 and 6.25 μg/ml, while the corresponding values for amphotericin B ranged between 0.049 and 0.78 μg/ml. These MICs were all within the reported in vitro susceptibility values for nystatin and amphotericin B (8), implying that all tested isolates were susceptible to the latter polyenes.

The PAFEs of nystatin on the 26 isolates ranged between 1.88 and 4.87 h in SAB broth and 0.66 and 6.89 h in RPMI broth, whereas equivalent figures for amphotericin B were 3.13 to 10.98 h in SAB and 0.97 to 7.01 h in RPMI broth (Table 1). These differences in the PAFE values when yeasts were cultured in the two different media were highly significant (P < 0.001) for both nystatin and amphotericin B, with the exception of the amphotericin B-induced PAFE observed with C. albicans and C. glabrata in these media (Fig. 1). A significant interspecies variation in polyene-induced PAFE was also observed (Table 1). For instance, the mean values of amphotericin B-induced PAFE in SAB ranged from 3.13 (for C. glabrata) to 10.98 h (for C. tropicalis).

As demonstrated previously (3, 4, 6), growth curves revealed a period of fungistasis after removal of the polyene antimycotics, illustrating a PAFE, in both SAB and RPMI 1640 medium. This was followed by a growth rate comparable to that of the unexposed controls. However, the growth rates in the two media were different, as shown by the different PAFE values (Table 1 and Fig. 1).

The incidence of oral candidosis continues to escalate, with increasing emergence of inherent or acquired resistance to antifungal agents (12), and it would be desirable if clinicians could rely on in vitro antifungal susceptibility tests to obtain critical information regarding dosage regimens. The MIC of an agent is the universally accepted criterion for evaluating dosages regimens, and the goal with respect to therapy with antifungals is to maintain the drug concentration above the MIC throughout the dosing period (10). However, in reality, constant drug levels in the infective focus are not easily achieved, especially in the oral environment, where the salivary flushing action is constant. Therefore, additional parameters to guide and determine the dosage regimens are needed. The recently introduced concept of PAFE may serve as a further benchmark in this context together with MIC determinations.

Although a reasonable database on the PAFE of the major antifungals against a number of Candida species is available (3, 4, 6), the impact of different growth media on the PAFE of these antimycotics has not been studied. The media used in our study, namely, SAB and RPMI 1640 broth, differ in their composition and their pH. SAB contains pancreatic digest of casein, peptic digest of fresh meat, and dextrose, and its pH is 5.7 (Oxoid manual). RPMI 1640 broth is a chemically defined

### TABLE 1. Mean PAFE of nystatin and amphotericin B on Candida species in SAB and RPMI 1640 broth

<table>
<thead>
<tr>
<th>Species (no. of isolates)</th>
<th>Nystatin (SEM)</th>
<th>Amphotericin B (SEM)</th>
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<tbody>
<tr>
<td></td>
<td>SAB</td>
<td>RPMI</td>
</tr>
<tr>
<td></td>
<td>SAB</td>
<td>RPMI</td>
</tr>
<tr>
<td>C. albicans (5)</td>
<td>1.88 (0.08)</td>
<td>3.09 (0.24)</td>
</tr>
<tr>
<td>C. glabrata (4)</td>
<td>2.63 (0.08)</td>
<td>3.39 (0.26)</td>
</tr>
<tr>
<td>C. guilliermondii (4)</td>
<td>3.70 (0.37)</td>
<td>1.71 (0.21)</td>
</tr>
<tr>
<td>C. krusei (4)</td>
<td>4.87 (0.18)</td>
<td>6.89 (0.33)</td>
</tr>
<tr>
<td>C. parapsilosis (4)</td>
<td>3.18 (0.20)</td>
<td>5.64 (0.94)</td>
</tr>
<tr>
<td>C. tropicalis (5)</td>
<td>3.14 (0.24)</td>
<td>0.66 (0.08)</td>
</tr>
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* In hours. Data are means for three experiments conducted on separate occasions in duplicate for each isolate (i.e., means of 30 raw values for C. albicans and C. tropicalis and 24 values for the other species).
medium which contains a range of amino acids, vitamins, salt, and glucose, and its pH is 7.2 (Gibco BRL product guide). SAB is the recommended supporting medium for laboratory culture of fungi and has been so used for decades (2). RPMI 1640, on the other hand, was originally formulated for suspension cultures or monolayer cultures of human leukemic cells. It was subsequently recommended as the medium of choice for antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards (9). Both these media are used extensively in mycological research, especially in studies of Candida.

Our results indicate clearly that the growth patterns of the isolates in SAB and RPMI 1640 broth were variable and elicited different PAFE values depending on the medium, even with the same Candida isolate. In general, Candida growth was extensive in SAB, whereas it was comparatively low in RPMI 1640. One reason for this may be the limited nutritional sources in RPMI 1640 broth compared with SAB. Despite the differences in the media, a PAFE was observed for all isolates following brief exposure to the polyenes, and this is likely to be due to the time taken by Candida to recover before active multiplication after such exposure. Similar polyeone-induced PAFE values have been observed, particularly with C. albicans, in previous studies (3, 4, 6). However, this study is the first to report the significant differences in the polyeone-induced PAFE on a battery of six different Candida species in different growth media.

The present results therefore clearly illustrate that standardization of laboratory regimens is essential in order to obtain globally comparable data on PAFE of antymycotic agents. As only a few reports from a handful of laboratories on the subject of PAFE are yet available, authorities such as the NCCLS should take early steps to design and issue guidelines (akin to that for MIC measurement of antifungals) that could be universally used in evaluation of the PAFE and to generate data that are globally acceptable.

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REFERENCES


FIG. 1. PAFE induced by nystatin and amphotericin B on different Candida species growing in SAB and RPMI 1640 media. The first bar for each species represents the PAFE induced in SAB and the second bar represents the PAFE induced in RPMI 1640. Highly significant (P < 0.001) variations in the PAFE values were noted, with both nystatin and amphotericin B, when the organisms were grown in SAB and RPMI 1640 (with the exception of amphotericin B-induced PAFE observed in C. albicans and C. glabrata [asterisks]).


