COMPARISON BETWEEN E-TEST AND CLSI BROTH MICRODILUTION METHOD FOR ANTIFUNGAL SUSCEPTIBILITY TESTING OF Candida albicans ORAL ISOLATES

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SUMMARY

Thirty Candida albicans isolated from oral candidosis patients and 30 C. albicans isolated from control individuals were studied. In vitro susceptibility tests were performed for amphotericin B, fluconazole, 5-flucytosine and itraconazole through the Clinical and Laboratorial Standards Institute (CLSI) reference method and E test system. The results obtained were analyzed and compared. MIC values were similar for the strains isolated from oral candidosis patients and control individuals. The agreement rate for the two methods was 66.67% for amphotericin B, 53.33% for fluconazole, 65% for flucytosine and 45% for itraconazole. According to our data, E test method could be an alternative to trial routine susceptibility testing due to its simplicity. However, it can not be considered a substitute for the CLSI reference method.

KEYWORDS: Candida; NCCLS; E test; Antifungal drugs.

INTRODUCTION

Candida albicans is a commensal yeast of the normal oral microbiota. However, several local and systemic factors can predispose to the development of oral candidosis. Thus, conditions such as age extremes, immunodeficiency, endocrine disorders, radiotherapy, malignant diseases, xerostomia, denture wearing, poor oral hygiene and orthodontic treatment can be cited as predisposing factors. Nystatin and amphotericin B are common therapeutic agents for oropharyngeal candidosis. The increasing number of clinical isolates resistant to antifungal therapy, as well as the necessity of a guide to the selection and follow-up of the treatment led to a demand for susceptibility testing of fungi. For this purpose, the Clinical and Laboratorial Standards Institute (CLSI) approved a reference method for antifungal susceptibility testing of yeasts, the National Committee for Clinical and Laboratorial Standards (NCCLS) M-27 A2 document. The E test has been introduced as an easier testing procedure and an alternative for the NCCLS method. The great advantage of E test is the simplicity of the methodology. However, not all antifungal agents are available in E test and there is a difficulty associated with endpoint interpretation, due to the growth of micro-colonies in the inhibition zone, leading to lower reproducibility when the test is performed by several technicians. The aim of this study was to compare the results of fluconazole, itraconazole, flucytosine and amphotericin B susceptibility testing of C. albicans oral strains obtained by the CLSI reference method and the E test.

MATERIALS AND METHODS

Isolates: Thirty Candida albicans isolated from denture-associated oral candidosis patients and 30 from control individuals were studied. The patients with candidosis were randomly selected among the patients of the Prosthetics Department of São José dos Campos Faculty of Dentistry (University of the State of São Paulo-UNESP, São Paulo, Brazil). No patient was under treatment with antifungal drugs. The individuals from the control group were randomly selected among students from this University. Patients with basis diseases or smokers or under medication were excluded. The strains were isolated from saliva and identified by biochemical, physiological and morphological tests. Candida spp. strains were transferred onto Sabouraud dextrose agar (Difco Laboratories, Detroit, USA) and stored at 4 °C. Thirty C. albicans strains were transferred onto fresh Sabouraud dextrose agar (Difco Laboratories, Detroit, USA) slants 24 hours prior the realization of antifungal susceptibility testing.

Susceptibility testing:

1. NCCLS reference method: The susceptibility assays were determined by the microbroth dilution method performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, USA) as described previously in NCCLS guidelines, M-27 A document (NCCLS, 2002). Briefly, isolates were inoculated at 35 °C and observed at 24 and 48 hours. Five colonies greater than 1 mm in diameter were selected, suspended in saline solution and adjusted to a final concentration of 0.5 x 10^3 to 2.5 x 10^3 in RPMI 1640 medium (Sigma, St. Louis, USA) buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS; Sigma). The antifungal agents fluconazole (Pfizer, São Paulo, Brazil), itraconazole (Janssen Pharmaceutica, São Paulo, Brazil), amphotericin B (Sigma) and flucytosine (5-fluorocytosine; Hoffman La Roche, Basel, Switzerland) were added to the broth mixture and the plates were incubated at 35°C for 24 hours.

2. E test method: The E test method was performed according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The E test strips were inoculated with logarithmic cultures of the strains and incubated at 35°C for 24 hours. MIC values were determined by the broth microdilution method performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, USA) slants 24 hours prior the realization of antifungal susceptibility testing.
Switzerland) were used in the susceptibility tests. Amphotericin B and itraconazole were dissolved in dimethylsulfoxide. Fluconazole and flucytosine were dissolved in sterile distilled water. The drugs were prepared at the following concentrations: 320 µg/mL for amphotericin B, 1000 µg/mL for fluconazole, 1250 µg/mL for flucytosine and 640 µg/mL for itraconazole. The solutions were diluted in RPMI medium and final drugs concentrations ranged from 32 to 0.04 µg/mL for amphotericin B, 64 to 0.04 µg/mL for itraconazole, 25 to 0.02 µg/mL for fluconazole and 128 to 0.06 µg/mL for flucytosine. After 48 hours of incubation at 35 °C, MIC (minimum inhibitory concentration) was determined visually by comparing its turbidity with the drug-free growth control well. For the azoles and flucytosine the MIC values were defined as the lower drug concentration which resulted in reduction of 80% in the turbidity in comparison with the drug-free growth control well and for amphotericin B the MIC value was defined as the lowest drug concentration for which the well was optically clear.

2. E test (AB Biodisk, Solna, Sweden): The E test was performed according to the manufacturer’s instructions (AB Biodisk, 1993). In brief, the inoculum concentration was adjusted to 0.5 in McFarland standard for *Candida* species. Then, 0.5 mL of this suspension was inoculated onto plates containing RPMI 1640 agar (1.5%) with 2% glucose using a cotton swab. After a period of 15 minutes, the E test strips were applied. The antifungal drugs amphotericin B, itraconazole, fluconazole, and flucytosine were tested. The plates were incubated at 35 °C and read after 24 and 48 hours.

*Analysis of the results:* The MICs (minimum inhibitory concentration) at which 50% (MIC$_{50}$) and 90% (MIC$_{90}$) of the isolates were inhibited were determined for each drug. A comparison between the results obtained by the NCCLS reference method and E test was performed. The rate of agreement was represented as proposed by SEWELL *et al.* (1994) in terms of percentage of agreement. It was considered agreement when MIC results of E test and NCCLS method were in exact agreement or were within ± 2 two-fold dilutions. The results were descriptively analyzed.

## RESULTS

The MIC ranges and values of MIC$_{50}$ and MIC$_{90}$ obtained for amphotericin B, fluconazole, itraconazole and flucytosine susceptibility testing determined by NCCLS method and E test are summarized in Table 1 and 2, respectively. MIC values were similar for the strains isolated from oral candidosis patients and control individuals.

### Table 1

**In vitro susceptibility testing of amphotericin B, fluconazole, itraconazole and flucytosine determined by NCCLS reference method**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MIC ranges (µg/mL)</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.04  - 1</td>
<td>0.08  - 2</td>
<td>0.125</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>B</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>0.012 - 0.19</td>
<td>0.012 - 0.78</td>
<td>0.049</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.04  - 2</td>
<td>0.04  - 2</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Candidosis group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.08  - 2</td>
<td>0.08  - 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.5   - 16</td>
<td>0.5   - 16</td>
<td>2</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>0.012 - 0.39</td>
<td>0.012 - 0.39</td>
<td>0.049</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.125 - 4</td>
<td>0.125 - 4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

MIC - minimum inhibitory concentration

### Table 2

**In vitro susceptibility testing of amphotericin B, fluconazole, itraconazole and flucytosine determined by E test.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MIC ranges (µg/mL)</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>&lt;0.03 - 0.75</td>
<td>&lt;0.03 - 0.75</td>
<td>0.125</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.125 - &gt;256</td>
<td>0.023 - &gt;256</td>
<td>0.5</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>0.003 - &gt;32</td>
<td>0.006 - &gt;32</td>
<td>0.023</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.006 - 24</td>
<td>0.006 - &gt;32</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Candidosis group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.094 - 0.5</td>
<td>0.094 - 0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.064 - 16</td>
<td>0.064 - 16</td>
<td>0.25</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>0.003 - 32</td>
<td>0.003 - 32</td>
<td>0.023</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.016 - &gt;32</td>
<td>0.016 - &gt;32</td>
<td>0.064</td>
</tr>
</tbody>
</table>

MIC - minimum inhibitory concentration
The comparison of the MICs values obtained by NCCLS reference method and E test at 24 h and 48 h were compared and the percentages of agreement are presented in Table 3.

### DISCUSSION

The comparison between E test and CLSI methodology has been studied by several authors. Earlier studies related a good agreement between the NCCLS reference microdilution method and the E test for the azoles susceptibility testing. In these researches, the agreement percentages varied from 71 to 85.2% for ketoconazole\(^6,9\), from 80 to 96% to fluconazole\(^7,20,23\) and from 80 to 84% for itraconazole. However, in more recent publications, authors related poorer agreement rates that varied from 90 to 96%.

For amphotericin B susceptibility testing, good correlation was observed between the tested methods at 24h (66.67%) and 48 hours (71.67%). VAN ELDERE \(\text{et al.}\)\(^{25}\) and KOC \(\text{et al.}\)\(^{2}\) also related good agreement rates that varied from 90 to 96%.

For fluconosine, a good agreement between the NCCLS method and E test was observed at 24 hours (65%). These results are in accordance to those obtained by CHANG \(\text{et al.}\)\(^{5}\) and ISHIGAKI \(\text{et al.}\)\(^8\). At 48 hours, a moderated correlation could be observed (43.33%).

The present study demonstrated a lower percentage of agreement between the methodologies tested than other studies found in literature. The discrepancy might be due to interlaboratory differences or to the use of different media for E test methodology. This fact reinforces the importance of tests standardization. On the other hand, it is relevant to notice that the evaluator was not blind regarding the test or for the antifungal agent evaluated, which might be considered a limitation of the present work.

Minimum inhibitory concentrations obtained in the present study were similar for control samples and samples obtained from candidosis patients. A higher MIC could possibly be expected for candidosis patients, since virulence factors such as exoenzyme production have been correlated with antifungal resistance\(^{10}\).

In conclusion, E test method could be considered an alternative to trial routine susceptibility testing due to its simplicity. However, it can not be considered, at this moment, a substitute for NCCLS reference method, since a complete agreement between both methodologies has not been reached, as demonstrated by the present study and corroborated by others presented in literature. Due to the numerous variables associated to the E test method, further studies must be performed to standardize the medium and incubation conditions. Moreover, studies on the correlation of in vitro antifungal susceptibility testing and clinical response to these drugs are essentially important.

### RESUMO

Comparação entre E-test e o método da microdiluição do CLSI para teste de susceptibilidade a antifúngicos de isolados orais de *Candida albicans*

Trinta *Candida albicans* isoladas de pacientes portadores de candidose oral e 30 *Candida albicans* isoladas de indivíduos controle foram estudadas. Testes de susceptibilidade in vitro foram realizados com anfotericina B, fluconazol, 5-flucitosina e itraconazol pelo método do Clinical and Laboratorial Standards Institute (CLSI) e por E-test. Os resultados obtidos foram analisados e comparados. Os valores de CIM foram semelhantes para amostras isoladas de pacientes portadores de candidose oral e indivíduos controle. A concordância entre os dois métodos foi de 66,7% para a anfotericina B, 53,33% para o fluconazol, 65% para a flucitosina e 45% para o itraconazol. De acordo com estes
resultados, o método do E-test poderia ser uma alternativa para a triagem de casos de rotina pela sua simplicidade. Entretanto, este método não pode ser considerado como um substituto para o método de referência do CLSI.

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REFERENCES


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