The introduction of systemic antifungal drugs which act upon different targets is the main issue of the in vivo antifungal resistance control. Different factors, such as growth curve phase, quality of the specimen, quantity of the inoculum, temperature, pH, culture medium composition, incubation duration and solvent, are believed important factors affecting minimum inhibitory concentration (MIC) value to most of the antifungal agents. We assayed an in vitro susceptibility test with 40 isolates of dermatophytes: Microsporum canis, Trichophyton rubrum, Trichophyton mentagrophytes and Epidermophyton floccosum against griseofulvin, fluconazole, itraconazole and terbinafine, using the guidelines of the M38-P document approved by the NCCLS. We determined the growth curves, to estimate the specific growth rate (μ max) and the generation time (G) of each dermatophyte, using dry weight and spectrophotometry methods. We demonstrate that, at 192 h, all fungi tested had a constant growth curve and we considered this as the optimal time for MIC determination. Terbinafine, griseofulvin and itraconazole possessed the highest antifungal activity against the four groups of dermatophytes studied. Fluconazole demonstrated no efficacy. Our MIC results differ from other authors and this difference is due to the timing of the MIC determination based on the growth curve of each fungi tested.

Keywords antifungal susceptibility, growth curve, minimum fungicidal concentration, minimum inhibitory concentration

Introduction
Dermatophytes are the result of invasion of queratinized tissues, skin, hair and nails by dermatophytes. They cause a high morbidity, with a recent report world-wide increase of 50% in patients older than 25 years [1–3]. Probably the widespread use of antibiotics and immunosuppressor therapies; the dissemination of human immunodeficiency virus; neutrophil T cells and cytotoxic lymphocyte abnormalities; large numbers of transplant and burn patients and other pathological conditions, as well as changes in personal habits and close contact with domestic animals have contributed to enhance the incidence [4].

In routine clinical practice, dermatophytoses respond satisfactorily to most antifungal drugs. Nevertheless, we have found recalcitrant infections to systemic and topical treatment, most of them caused by Trichophyton rubrum [2,4,5].

It has been difficult to create a standardized procedure to measure in vitro susceptibility test. There are some conditions to be concerned of the filamentous fungi in determining the minimum inhibitory concentration (MIC), such as quality of the specimen, quantity of the inoculum, composition of the medium, temperature, pH, incubation duration, solvent, antifungal drug [5–13] and growth curve.
The National Committee for Clinical Laboratory Standards (NCCLS) has created a guideline reference for studies with filamentous fungi, Document M38-P, 1998 [10,12,14]. This document does not include terbinafine in the antifungal drug testing.

In this study, we aim to investigate the in vitro susceptibility test with the broth micro-titration method by standardizing at the exponential phase of the growth curve, the inoculum size by colony-forming units (c.f.u./ml) of each of the dermatophytes studied.

Materials and methods

NCCLS method

Our study was performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, document M38-P) [10] with small modifications adopted to our protocol.

Fungal Identification

Forty isolates of dermatophytes were tested. Ten isolates each of Trichophyton rubrum, Trichophyton mentagrophytes, Epidermophyton floccosum and Microsporum canis were collected from skin lesions, hair and nails from patients of the outpatient clinic of the Mycology and Dermatology Departments of our research institute between December, 1999 and May 2000. All the strains belonged to patients who had not received topical or systemic treatment with antifungal drugs during a previous period of 12 weeks. All clinical samples were examined by 10% KOH microscopic morphology exam and positive samples were cultured in Sabouraud chloramphenicol agar at 25°C. The organisms were identified with lactophenol blue. Cultures were maintained by periodic passage on Sabouraud chloramphenicol agar. Prior to the assay, dermatophytes suspensions were obtained from fresh cultures on potato dextrose agar after 7 days of incubation at 25°C, in order to obtain greater sporulation.

Inoculum size preparation

All the experiments were carried out under sterile conditions in a laminar flow hood. Small samples of each dermatophyte were removed from the colony surface by adding sterile saline solution to the slant and gently rubbing the colony surface with a sterile applicator stick to suspend the conidia and hyphae, until the turbidity of all the samples were similar. Then they were diluted 1:100 in saline solution. Samples of 10 μl were inoculated on Petri dishes containing Sabouraud chloramphenicol agar. Quantification of CFU/ml during 7 days at 25°C were carried out using Quantity One® software (Bio-Rad Laboratories, Hercules, CA) and image analysis in a Bio-Rad Fluor-S Multi-Imager. The total number of colonies of each strain were multiplied by the dilution factor in order to obtain the original concentration of each isolate expressed in c.f.u./ml. The resulting sample of each suspension, equivalent to 3 x 10⁵ cells/ml, were diluted in RPMI 1640 with 10 g/l glucose (Sigma®, Chemical, St Louis, MO) without sodium bicarbonate at a final concentration of 0.165 mol/l HEPES buffer (Gibco®, Grand Island, NY) and adjusted to pH 7.0. The sterility control of the RPMI 1640 medium was performed by inoculation on a blood agar plate, which permits the detection of any contaminants.

Growth curve by dry weight determination

From each isolate, 3 x 10⁵ cells/ml were diluted in 60 ml RPMI 1640 medium. A volume of 6 ml was placed in each of the Erlenmeyer flasks. They were incubated at 25°C under continuous shaking at 72 cycles/min. From each flask, duplicate samples of 1 ml, previously homogenized, were placed in previously weighted Eppendorf tubes. After centrifugation at 14 000 g for 20 min, the sediments were dried in an oven at 60°C, and differential weight was determined using an analytic balance. The readings were made at 0, 3, 5, 7, 9 and 10 days.

Spectrophotometric growth curve method

The optical density was measured from each of the control wells which contained the dermatophytes and RPMI 1640 medium every 48 h until 10 readings had been made.

The generation time (G) and the specific growth rate (μmax), were determined, according to the equation $G = 0.693/\mu_{\text{max}}$; $\mu_{\text{max}} = \frac{\ln X_0 - \ln X_t}{t_{1/2} - t_1}$. $X = $ biomass (gr), $t =$time (h) [15]. The purpose was to determine the growth rate of each dermatophyte and the biomass reached during the incubation. In this case, 192 h corresponded to the exponential phase of the growth curve as the time of constant growth and the optimal moment for MIC determination.

Antifungal drug dilutions

The in vitro susceptibility test of the isolates of dermatophytes were performed against griseofulvin (Calox Pharmaceuticals, Caracas, Venezuela), fluconazole (Techno Químicos Pharmaceuticals, Caracas,
Venezuela), itraconazole (Janssen Research Foundation, Beerse, Belgium) and terbinafine (Novartis Research Institute, Vienna, Austria).

The antifungal drugs were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma). Each antifungal drug was freshly prepared in a stock solution of 1 mg/ml and then diluted in RPMI 1640 medium to the following concentrations per ml: 0.001, 0.005, 0.01, 0.05, 0.1, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 60.0 μg.

**Determination of in vitro susceptibility by microtiter reading method**

From the inoculum size of 3 × 10³ c.f.u./ml, 100 μl were inoculated in each well of a 96-well flat-bottom microtiter plate (Nunclon™ Brand Products, Denmark) with 100 μl of the specific antifungal drug concentration. The positive control contained 100 μl RPMI 1640 medium plus 100 μl of the respective isolate and the negative control contained 200 μl RPMI 1640 medium as the optical density blank. Each assay was carried out in triplicate.

Optical density was determined at 570 nm (BT 2000 Microkinetics Reader; Bio-Tek Instruments, Highland Park, VT) at 24, 72, 144, 192 and 240 h.

The MIC was defined as the lowest drug concentration that reduces growth by 80% relative to the growth control. Optimally evaluated of the different grades of turbidity was done. No turbidity score was defined.

The MFC was determined as the lowest concentration of the antifungal drug that substantially inhibits the growth in 100% relative to the growth control. The MFC was performed from each well of the microtiter tray. 20 μl (3 × 10³ cells/ml) of the content of each specific antifungal drug concentration were inoculated on Petri dishes with Sabouraud chloramphenicol agar, to evaluate the growth until 192 h. Each concentration was studied in duplicate.

**Statistical analysis**

The analysis was done by the optimal endpoint of the growth curve, which corresponded to the exponential phase reached at 192 h. Means and standard deviations of each of the optical densities of the antifungal drugs with their corresponding dermatophyte were determined.

The statistical differences between the response–concentration antifungal drug curves were determined by the chi-squared test. MIC was estimated by the difference between the values of initial (24 h) and final (192 h) optical densities. The minimum dose was calculated for those of more than 80% and less than 60%. More than half of the MIC distributions were higher than 50% of the differential value between the initial and final readings. Values of P < 0.05 were considered significant and highly significant if P < 0.01. All analysis were performed by JMP and SAS 6.3 for Windows 95.

**Results**

The growth curves determined by spectrophotometry method in microtiter plates showed to be suitable for our study. The determination of the growth curve by the dry weight method in Eppendord tubes of each strain of dermatophyte demonstrated strong dispersion (data not shown). Figure 1 shows the growth curve measured by optic density method and demonstrates a short latency phase curve with *M. canis* at 48 h. The other dermatophytes had a latency phase curve at 72 h, after this time, there was an increase of the absorbance. *M. canis* had the faster growth curve. At 192 h, all the dermatophytes studied showed an active exponential phase curve. Based on this observation, this was the best time to carry out the *in vitro* assay of antifungal drug MIC susceptibility test.

Also the determination of the growth specific rate (μmax) and generation time (G) [15] at 192 h showed a constant μmax. The biomass of the specimens of dermatophytes grew constantly and progressively. This indicates that the dermatophytes had optimal viability in absence of the antifungal drugs. We observed that *T. rubrum* grew very slowly; requiring 105.96 h to reach a biomass of 0.00654 g in contrast to *M. canis*, reaching a biomass of 0.01 g in 69.30 h (Table 1). These results demonstrate differences in the growth rate curve of the strains studied.

**Minimum fungicidal concentration**

Visual readings of microculture fungal growth were made every 2 days to determine drug inhibition. In the determination of minimum fungicidal concentration (MFC), we observed greater activity with terbinafine against *T. mentagrophytes*; itraconazole for *M. canis*;

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cinetic parameters of the control group of dermatophyte fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatophyte Fungus</td>
<td>μ (per h)</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td>0.00654</td>
</tr>
<tr>
<td><em>Epidermophyton floccosum</em></td>
<td>0.007</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>0.00849</td>
</tr>
<tr>
<td><em>Microsporum canis</em></td>
<td>0.01</td>
</tr>
</tbody>
</table>

μ (per h): growth specific rate; G (h): generation time.
We determined the percentage of inhibition growth for each strain of dermatophyte against the four antifungal drugs. Our results for T. mentagrophytes, E. floccosum and M. canis showed: 90% of inhibition growth with terbinafine and griseofulvin. Meanwhile T. rubrum demonstrated 80% of inhibition growth with griseofulvin and itraconazole. Fluconazole showed less than 70% of inhibition growth for the four strains of dermatophytes studied (Fig. 2). The optical density measured gradually decreased, parallel to the increase of the drug concentration. All of these results were lectured at 192 h.

**Minimum inhibitory concentration**

Table 3 summarizes the range of MIC values for antifungal drugs against the different strains of dermatophytes in comparison with previously published data.

**Discussion**

This paper introduces the correlation of the in vitro antifungal susceptibility testing with the exponential phase of their growth curves as the best moment to assay this test.

It is well known the difficulty to standardize filamentous fungi. Most authors employ the spectrophotometry technique to standardize the inoculum size of them [16]. In our study, we observed significant variability readings to standardize the inoculum size using 570 nm wavelength absorbance. Fukuda et al. [17] have reported variability of sporulation of these dermatophytes. We evaluated the correlation of the results obtained by optical density with those of c.f.u./ml but we did not observe concordance. Pujol et al. [11] have reported similar results. It might be useful to standardize the size of the inoculum with the same proportions of sporulation (conidia and hyphae filter) [17] by employing different culture agar. The use of potato dextrose agar, rice agar and oat cereal agar, sequentially and alternatively, to obtain more conidial

---

**Fig. 1** Growth curves as measured by optical density (OD at 570 nm) of the dermatophyte species study. *Measured by growth in the control wells.
sporulation has been described [18]. The use of a convenient culture medium for the production of conidia is of critical importance, since the production of spores could limit the ability to prepare sufficient inoculum for the study as well as for the quantification [18].

We consider that quantification by the c.f.u./ml method is more precise to standardize the inoculum size. This has been confirmed by other authors [4,10,19].

Also the selection of the optimal period of growth in order to assay the antifungal drug in vitro susceptibility has been demonstrated by Guidry et al. [19] for Blastomyces dermatitidis and Bahmer et al. [20] for Trichophyton rubrum.

Not all dermatophytes strains have the same growth curve [19,21,22].

In our study, we did two growth curves for each strain of dermatophyte, where we could see parallel discrepancies at the latency and deceleration phase of the curve as well as retardation at the beginning of the exponential phase between dermatophytes of the same strain. We consider that our study is not comparable with other authors, who have estimated MIC at 72 h or when visible growth was seen in the drug-free control well [14,23–25].

In this study, we could observe that our dermatophytes, at 72 h, were still at the latency phase, which corresponds to the nutrients absorption and DNA replication [15].

Table 2 Minimum fungicidal concentrations (g/ml) of each of the antifungals to ten isolates of each of the indicated dermatophyte species studied.

<table>
<thead>
<tr>
<th>Dermatophyte fungi</th>
<th>Itraconazole</th>
<th>Terbinafine</th>
<th>Griseofulvin</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. rubrum</td>
<td>1–10</td>
<td>0.1–20</td>
<td>5–60</td>
<td>40–60</td>
</tr>
<tr>
<td>T. mentagrophytes</td>
<td>0.01–5</td>
<td>0.05–5</td>
<td>10–60</td>
<td>60–&gt;60</td>
</tr>
<tr>
<td>E. floccosum</td>
<td>0.01–1</td>
<td>1–10</td>
<td>0.1–5</td>
<td>5–30</td>
</tr>
<tr>
<td>M. canis</td>
<td>0.005–20</td>
<td>1–5</td>
<td>5–&gt;60</td>
<td>5–60</td>
</tr>
</tbody>
</table>

Fig. 2 Percent inhibition of the growth of dermatophyte species studied caused by antifungals. *Results lectured at 192 hours.
In our study, 192 h corresponded to the exponential phase of the growth curve for all of our specimens, and not at 144 h, described by other authors [7,10,12,26]. Concerning the two methods that we employed to measure the growth curve, by dry weight or by optical density, we observed a disadvantage for the former. The method of optical density offered the advantage of measuring the growth curve, by dry weight or by optical density, we observed a disadvantage for the former. The method of optical density offered the advantage of measuring the growth curve more precisely and concisely. According to our MIC results, measured by optical density, we found: terbinafine showed more efficacy in MIC to the four dermatophytes and lower MFC to *T. mentagrophytes*. These results indicate that terbinafine was the best *in vitro* antifungal drug according to its pharmacology properties. Nevertheless, high concentrations of terbinafine were required for *M. canis* and *T. rubrum*. Perhaps we are confronted with more resistant specimens, since this result is not comparable to other studies reviewed [25].

Fluconazole was not efficient in MIC determinations for none of the dermatophytes. MIC determination is difficult to develop and to interpret. Especially,azole derivatives must be evaluated with caution. The gradual inhibition growth of the dermatophytes should be carried out to high antifungal concentrations [4,26], as showed in this study.

Interpretative antifungal drug endpoints for dermatophytes have not yet been established and the clinical relevance of MIC results remains uncertain. Because of the difficulty to correlate the *in vitro* with the *in vivo* process [27].

Pujol *et al.* [11] and Goh *et al.* [26] have reported that MIC have a higher predictive value to clinical proximity and MFC is helpful in cases of severe fungal infections in immunocompetent patients. On the other hand, Buty *et al.* [28] suggest that MFC is more important than MIC in predicting *in vivo* resistance. We believe that in long-term treatment it is preferable to select antifungal drugs with low MFC and MIC.

In our study, *T. rubrum* showed slow drug free growth and required high antifungal drug concentrations to reach 80% of inhibition. Perhaps, the long latency phase observed or of a multidrug resistance phenomena, could explain us, the cause of recalcitrance to antifungal drugs [19,29,30]. We may infer that a combination of drugs that could produce a synergistic activity is a possible advance towards achieving higher cure rates [31].

**Acknowledgements**

This study was supported by CONICIT S1-07002420. We wish to thank Julman Cermeño (Mycology Department, Universidad de Oriente, Estado Bolivar, Venezuela) and Verónica Castillo and Douglas Angulo for their assistance.

**References**


© 2005 ISHAM, Medical Mycology, 43, 319–325