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ORIGINAL ARTICLE

Susceptibility to 5-Fluorocytosine, miconazole and amphotericin B of *Candida albicans* strains isolated from the throat of non-AIDS patients

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ABSTRACT. Eighty *Candida albicans* strains, isolated from throat of patients at the Universitary Clinic of the Faculty of Superior Studies Iztacala of the National Autonomous University of Mexico, were analyzed. They were identified by microscopic and colony morphologies, germ tube test, and by auxanogram and zimogram. Minimal inhibitory concentrations (MIC) of 5-fluorocytosine, miconazole and amphotericin B were determined by microtiter broth dilution. MIC frequency distribution of 5-fluorocytosine showed a single peak (0.25-8.0 µg/ml), with 65% susceptible strains (MIC ≤ 1.0 µg/ml) and 35% intermediate susceptible strains (MIC = 1.1-8 µg/ml). MIC frequency distribution of miconazole was threemodal with 6.25% susceptible (MIC = 1.562 µg/ml), 48.75% intermediate susceptible (MIC = 3.125-12.5 µg/ml), and 45% resistant (MIC = 25-50 µg/ml) strains. All strains were susceptible to amphotericin B (MIC= 0.0156-0.125 µg/m). These results shows that amphotericin B was the more active antimycotic, followed by 5-fluorocytosine, against the strains analyzed, and that miconazole was the less effective one.

Key words: *Candida*, oral candidiasis, antimycotic susceptibility

RESUMEN. Se analizaron 80 cepas de *Candida albicans*, aisladas de las gargantas de pacientes que acudieron a la Clínica Universitaria de la Facultad de Estudios Superiores Iztacala de la Universidad Nacional Autónoma de México. Las cepas se identificaron por morfología colonial y microscópica, por formación de tubo germinativo y mediante auxanograma y zimograma. Las concentraciones mínimas inhibitorias (CMI) de 5-fluorocitosina, miconazol y anfotericina B se determinaron por microdilución en placa. La distribución de frecuencia de la CMI de 5-fluorocitosina mostró un solo pico (0.25-8.0 µg/ml), con 65% de cepas sensibles (CMI ≤ 1.0 µg/ml) y 35% de cepas con sensibilidad intermedia (CMI = 1.1-8 µg/ml). La distribución de frecuencia de la CMI de miconazol fue trimodal, con 6.25% cepas sensibles (CMI = 1.562 µg/ml), 48.75% con sensibilidad intermedia (CMI = 3.125-12.5 mg/ml) y 45% resistentes (CMI = 25-50 µg/ml). Todas las cepas fueron sensibles a anfotericina B (CMI = 0.0156-0.125 µg/ml). Estos resultados muestran que la anfotericina B fue el antimicótico más activo, seguido por la 5-fluorocitosina, contra las cepas de *C. albicans* estudiadas, y que el miconazol fue el menos efectivo.

Palabras clave: *Candida*, candidiasis oral, sensibilidad a antimicóticos

INTRODUCTION

Oropharingeal and esophageal candidiasis is a common opportunistic infection in immunocompromised patients. *Candida albicans* infections may be facilitated by debilitating illness (diabetes, tuberculosis, amoebic hepatic abscess), immunodeficiency (leukemia, AIDS, cancer, immunosuppressive drug administration after organ transplantation), catheterism, and by the use of broad spectrum antibiotics.[1,2,13,15] In order to treat infections caused by this microorganisms, two mainly categories of antimycotic agents have been developed: i) Polyenes (amphotericin B and nistatin), whose union to ergosterol of the cellular membrane causes pore formation with subsequent release of intracellular components and cell death. ii) Azoles (fluconazole, itraconazole, ketoconazole, etc.) which inhibits cellular membrane formation by interfering with ergosterol synthesis.[18] Another chemical employed against *C. albicans* is 5-fluorocytosine whose entry to the cell is mediated by the cytosine permease. This compound is transformed to 5-fluorouracil by cytosine deaminase. Incorporation of 5-fluorouracil into RNA interrupts protein synthesis leading to cell death.[7] Development of these antimycotic drugs has increased the therapeutic options, but its use has conducted to the selection of resistant *C. albicans*.[5,6,8,9,11,12] For instance, 50/100 *C. albicans* strains, isolated from the oral cavity of AIDS patients, were resistant to fluconazole.[3] In another study, resistance of 348 *C. albicans* strains was as follows: 17.5% to fluconazole, 3.4% to flucitosine, and 4% to amphotericin B.[13]

C. albicans molecular resistance mechanisms to azoles include: i)target enzyme alteration (lanosterol 14-a-demethylase), coded by ERG11 gene; ii)Expression of transporter proteins which function as efflux pumps (ABC), coded by CDR1 and CDR2 genes; and major facilitators genes (MDR1).18 It has been shown that CDR1 expression can be induced by azoles and, in less extent, by canavanina, calcofluor, and 5-fluorocytosine. Resistance of *Candida* sp. strains to amphotericin B can be expressed after azole exposition.[2]

The purpose of this work was to determine the MIC of 5-fluorocytosine, miconazole and amphotericin B for *C. albicans* strains isolated from throat of non-AIDS patients with hope that this piece of information may contribute to

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the proper treatment of infections caused by this opportunistic pathogen.

MATERIAL AND METHODS

Source of the strains. Eighty strains of *C. albicans*, isolated from the throat of non-AIDS patients at the Clinical Laboratory of the Universitary Clinic at FES-Iztacala, UNAM, in the period of one year, were analyzed. Patients do not have received antifungal or antibiotic treatment in the last two months. Strains were identified by microscopic and colony morphologies, germ tube production and by biochemical tests (zimogram and auxanogram).

Chemicals. 5-Fluorocytosine (Sigma Chemical Co. St. Louis Mo.) was diluted in bidistilled water, whereas Miconazole (Sigma Chemical Co. St. Louis Mo.) and Amphotericin B (Sigma Chemical Co. St. Louis Mo.) were diluted in Dimethyl sulfoxide (DMSO, Sigma Chemical Co. St. Louis Mo.). All working solutions were stored at 4°C protected from light.

*Media and growth conditions***.** *C. albicans* strains were grown in Sabouraud Agar (BIOXON, Mexico) at 35°C for 24 h. Afterwards, a single colony was used to inoculate 1 ml of RPMI-1640 media (Sigma Chemical Co. St. Louis Mo.) and incubated at 35°C for 24 h in CO_2 atmosphere. Broth was diluted 1:100 in sterile saline and again 1:100 in RPMI-1640. This final dilution contains about 10^4 cfu/ml, which were routinely verified by plate count in Sabouraud Agar.

Antimycotic susceptibility testing. MIC of the antimycotics was done by microtiter broth dilution in RPMI-1640.[9] Each microtiter well was inoculated with 100 ml of the diluted *C. albicans* culture (about 10^3 cfu) in a final volume of 200 ml. Positive and negative growth controls were included in each MIC determination: strain plus RPMI-1640 or strain + RPMI-1640 + DMSO, and RPMI-1640 alone. Microtiter plates were incubated at 35°C for 24 h in CO_2 atmosphere. Each well was microscopically observed for the presence of *C. albicans* growth and the first well showing no growth was recorded as the MIC for the antimycotic employed.

Table 1. Age of patients from which Candida strains were isolated.

Age (years)	Number of strains (%)
0 - 10	22 (27.5)
11 - 20	7 (8.75)
21 - 30	14 (17.5)
31 - 40	10 (12.5)
41 - 50	11 (13.75)
51 - 60	10 (12.5)
61 - 70	5 (6.25)
71 - 80	1 (1.25)

RESULTS

Eighty Candida strains isolated from the throat of HIV-negative patients with oral yeast carriage and clinical complaint, were obtained. All of them were classified as *C. albicans* by the characteristics described above. Fifty-four strains (67.5%) were isolated from female and twenty-six (32.5%) from male. Twenty-two (27.5%) of the strains were obtained from patients younger than 10 years (Table 1).

In Figure 1, it can be seen that MIC distribution of 5-Fluorocytosine for the *C. albicans* strains tested was unimodal, in the range 0.25-8.0 mg/ml, with 65% susceptible strains (MIC ≤ 1.0 µg/ml), and 35% with intermediate susceptibility (MIC =1.1-8 µg/ml). MIC_{50} and MIC_{90} (concentrations that inhibit 50% and 90% of the strains, respectively) of 5-Fluorocytosine were 0.736 µg/ml and 2.836 µg/ml, respectively.

MIC of miconazole showed a three modal distribution (Fig. 2), with a susceptible subpopulation of strains (6.25%) inhibited at ≤ 1.562 mg/ml; another group (48.75%) with intermediate susceptibility (MIC = 3.125-12.5 µg/ml) and 45% of resistant strains (MIC =25-50 µg/ml). MIC_{50} and MIC_{90} of miconazole were 10.15 µg/ml and 25 µg/ml, respectively.

Amphotericin B MIC distribution was unimodal in the range 0.0156-0.125 µg/ml, with 48.75% of strains being inhibited at 0.0625 µg/ml (Fig. 3). MIC_{50} and MIC_{90} of amphotericin B were 0.0380 µg/ml y 0.0771 µg/ml, respectively.

DISCUSSION

Candida albicans is the most frequently isolated fungal pathogen in humans with yeast infections, and the infection ranges from superficial to systemic. During the last two decades, human yeast infections have increased dramatically due to a variety of factors, e.g., the widespread use of broad-spectrum antibiotics, the expanded use of immunosuppressive drugs, the use of intravascular devices, and a longer survival of neonates and immunocompromised individuals. Progress in the management of severity ill patients has been accompanied by an increase in the number of *Candida* infections.[3]

In this study, we found that among 80 *C. albicans* strains isolated from throat of patients, 27.5% of them were obtained of infants ten or less years old, suggesting that this age group is one of the most susceptible to *C. albicans* infection due to its anatomic and immunologic immaturity. The second age group was 21-30 years old (17.5% of the isolates) and the minor ones were 61-70 years old (6.25% isolates) and 71-80 years old (1.25%) (Table 1). The ma-

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jority of the isolated *C. albicans* strains (67.5%) were obtained from women . We do not know the reason of this high percent because strains were isolated from an open population that assisted to this Universitary Clinic. However, in a previous work,[16] it was observed that the frequency of *Candida* carriage was highest in the mouth (56%), followed by the vulvovaginal (40%) and anorectal (24%) regions of healthy women. *C. albicans* was also reported as the most common species, among the *Candida* species identified from the oral yeast isolates obtained from Europe and North America.[4]

Here it is reported that 5-Fluorocytosine MIC distribution for the strains tested was unimodal (Fig. 1). In a previously reported study,[15] a similar distribution for 84 *Candida* sp. strains (63 *C. albicans*) was observed, with 53.3% of 5-Fluorocytosine susceptible strains (MIC ≤ 1 mg/ml), 32.4% intermediate susceptible (MIC =1.1-16 mg/ml) and 14.3% resistant ones (MIC > 16 mg/ml). Most strains reported in our study were susceptible to 5-Fluorocytosine (65%), requiring 2.83 mg/ml of this drug to inhibit 90% of them (Fig. 2). Despite we do not use reference strains to establish cut points between susceptible and resistant strains, and even though there were methodological differences, our results are in good agreement whit the cut points reported for 5-Fluorocytosine susceptible or resistant reference strains of *C. albicans* which showed MICs > 1 mg/ml (ATCC 90028) and > 64 mg/ml (ATCC90029), respectively (19). Also, in a previous work[16] 82% of the *Candida* strains, isolated from patients with various diseases of the respiratory tract, were identified as a *C. albicans.* Strains susceptibility tests showed that 5-fluorocytosine was the best antimycotic *in vitro*, in a similar manner as was observed in our study.

By the other hand, miconazole MIC distribution showed three peaks (Fig. 2). We suggest that they may be interpreted as three C. albicans strains subpopulations: a susceptible one (MIC = 1.56 mg/ml), an intermediate susceptible (MIC = 3.125-12.5 mg/ml) and a resistant one (45% of the strains; MIC =25-50 mg/ml). These results are similar to that reported by others, who found a threemodal miconazole MIC distribution for *Candida* sp. strains.[15] MICs values for resistant, intermediate susceptible, and susceptible subpopulations were > 32 mg/ml, 8.1-32 mg/ml, and ≤ 8 mg/ml, respectively.[15] Therapeutic options often are limited in patients with azole resistance oropharingeal and esophageal candidiasis, particularly when there is cross-resistance to other antifungal triazoles. Intravenous amphotericin B may be the only alternative antifungal compound available to treat esophageal candidiasis and advanced oropharingeal candidiasis, although, amphotericin B resistance has been described.[5,17]

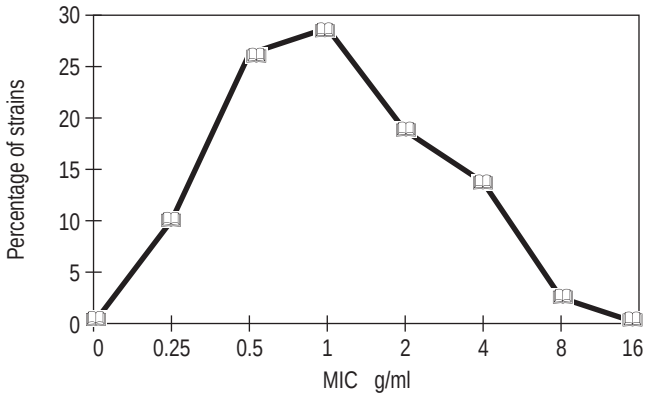

Figure 1. *5-Fluorocytosine susceptibility testing was done by microtiter broth dilution. Strains were classified as susceptible or intermediate susceptible according to the cut points reported by Radetsky.[15] MIC determinations were done by threeplicate.*

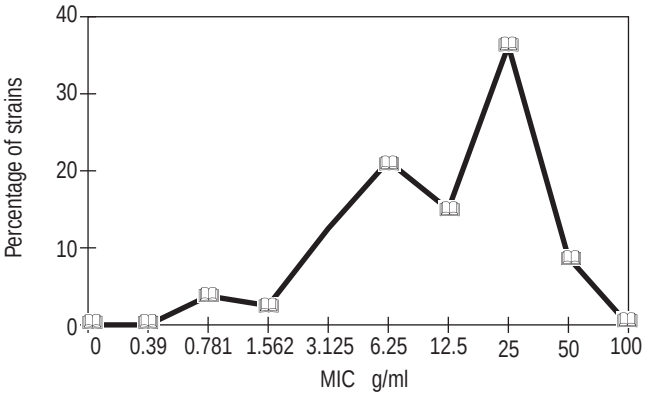

Figure 2. *Miconazole susceptibility testing was done by microtiter broth dilution. Strains were classified as susceptible, intermediate susceptible or resistant according to the cut points reported by Radetsky.[15] MIC determinations were done by threeplicate.*

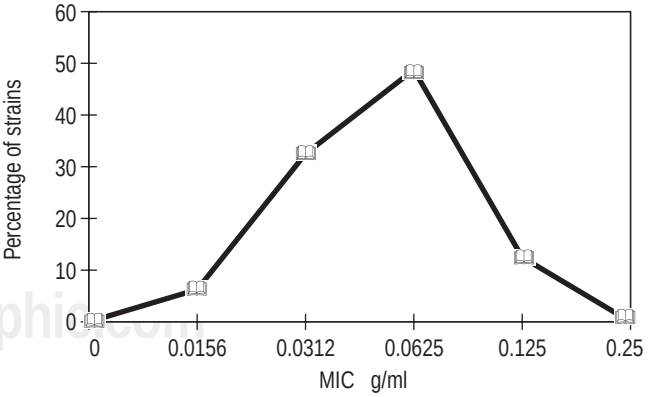

Figure 3. *Amphotericin B. susceptibility testing was done by microtiter broth dilution. Strains were classified as susceptible according to the cut points reported by Radetsky.[15] MIC determinations were done by threeplicate.*

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It has been reported that *C. albicans* miconazole resistance may be due to mutations in ERG11 gene, leading to altered target-enzyme (lanosterol 14 a-demethylase), or to expression of transporter proteins that function as efflux pumps (ABC) coded by CDR1 and CDR2 genes, or to major facilitators genes (MDR1).[11] Due to the fact that miconazole is one of the most employed antimycotics used against *C. albicans* infections, it is tempting to suppose that the high fraction of resistant strains (45%) reported in this work may possess any of these mechanisms that confers them the resistance phenotype.

It has been previously suggested that amphotericin B MIC for susceptible strains is in the range 0-0.8 mg/ml; whereas moderately susceptible strains are inhibited at concentrations of 1.6-3.2 mg/ml, and resistant ones at > 6.4 mg/ml.[17] However, in spite of the great information available about *Candida* species susceptibility to amphotericin B, there is not a firmly established cut point; although isolates with MIC > 1.0 mg/ml are considered as resistant. By both criteria mentioned, we can conclude that all the *C. albicans* isolates tested in this work were susceptible to amphotericin B (MIC =0.0156-0.125 mg/ml; see figure 3). Amphotericin B is one of the few compounds that has fungicidal activity in vitro and is still considered as a kind of paradigm for antifungal therapy in vivo. Moreover, the conventional form of amphotericin B is toxic, and its utilization is often limited by side effects. The lipid formulations of amphotericin B are better tolerated but are much more expensive.[10]

In summary, most of the *C. albicans* strains reported here were isolated from the throat of ten or less aged children. Amphotericin B susceptible or 5-Fluorocytosine intermediate susceptible, but miconazole resistant strains were detected. Susceptibility testing of *Candida* isolates is not standardized and not routinely available, and information related to this problem is scarce in non-AIDS patients,[14] mainly in developing countries. We fell that the results presented in this work are helpful for knowing the conservation or modification of antifungal susceptibility of *Candida* isolates prevalent in Mexico. These data prompt us to recommend the routinely antimycotic MIC determinations for *C. albicans* strains at clinical laboratories in order to prescribe the correct drug.

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