Original Article

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# *In vitro* antifungal susceptibility of *Candida albicans* isolates from oral cavities of patients infected with human immunodeficiency virus in Ethiopia

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**Summary**

***Objective:*** Oral Candidiasis is the most common HIV related oral lesion. Most patients are infected with a strain originally present as a commensal of the oral cavity. The chronic use of antifungal agents, in the treatment of candidiasis mainly in HIV/AIDS patients leads to the selection of strain resistant to this therapy. The objective of this study was to evaluate the *in vitro* susceptibility of *Candida albicans* to commonly used antifungal agents in Ethiopia.

***Methods:*** *In vitro* susceptibility tests were performed using the broth microdilution method following the National Committee for Clinical Laboratory Standards (NCCLS) M27-A guidelines. Data were then analyzed using SPSS for windows version 16.0. Tests of proportions were done with Chi-Square, and a p value of <0.05 was considered as statistically significant.

***Results:*** A total of 42 oral *C.albicans* isolates from HIV-infected patients were included in this study. Forty one (97.7%) of all isolates were determined fully susceptible to amphotericin B, 40 (95.3%) to nystatin, and 39 (92.9%) to ketoconazole and miconazole. On the other hand, the isolates showed highest rates of resistance against fluconazole (11.9%) relatively. There was little difference in the antifungal susceptibilities of *C.albicans* isolated from patients who had a history of previous antifungal therapy compared with those who had not received antifungal treatment.

***Conclusion:*** The *in vitro* antifungal susceptibility testing of *C.albicans* in this study showed relatively high resistance to commonly used azoles. As with the prescribing of any antimicrobial agent, the use of a systemic antifungal drug must be justified. Efforts must be maintained to avoid inappropriate or unnecessary prescribing of these antifungal.

**Key words:**
Antifungal agents; *Candida albicans*; Ethiopia; Resistance

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## Introduction

*Candida albicans* is among the gut flora, the many organisms which live in the human mouth and gastrointestinal tract. Up to 75% of healthy individuals carry the yeast *Candida* as part of their normal commensal oral microflora. However, *Candida* is an opportunistic pathogen which can cause acute or chronic infection in certain individuals. Predisposing factors to oral candidiasis include the wearing of a denture (prosthesis), smoking, immunosuppression, xerostomia and the receipt of broad-spectrum antibiotic therapy [1]. *C.albicans* is generally considered the most pathogenic *Candida* species and has been identified as the most prevalent yeast encountered in oral candidosis. During the course of HIV infection, 36 to 88% of all patients suffer from oral candidiasis [2-4]. In one clinical trial, it was shown that the development of oral candidiasis in patients at an early stage of HIV infection might promote the onset of full-blown acquired immunodeficiency syndrome (AIDS) [5].

*Candida* species have also been implicated in other forms of oral disease such as epithelial dysplasia, squamous cell carcinoma, angular cheilitis, burning mouth syndrome, lichen planus, endodontic infections, and periodontitis [6-9].

Although the introduction of highly active antiretroviral therapy (HAART) has reduced the prevalence of most opportunistic infections [10], Oropharyngeal candidiasis (OPC) is the most common HIV related oral lesion. About 90% of patients were found to suffer from oropharyngeal or esophageal candidiasis in various stages of AIDS [11]. The presence of *Candida* in the oral cavities of HIV/AIDS patients predicts the subsequent development of oral candidiasis [12].

Antifungal agents are often prescribed to manage oral candidiasis [13]. The polyenes (amphotericin B or nystatin) and the azole, miconazole, all of which are applied topically, are most frequently used to treat superficial candidosis. However, systemic azole antifungal therapy (fluconazole or itraconazole) can also be used to treat superficial candidosis and chronic forms of the infection. Prophylactic use of antifungals is also frequently employed in the management of oral candidosis in immunocompromised individuals, such as those suffering with AIDS or leukaemia.

Amphotericin B is the main therapy for serious fungal infections for more than 40 years. Infusion-related side effects and dose-limiting nephrotoxicity associated with its use prompted continuous search for equally effective but less toxic alternatives. Azoles are safe and effective agents for the treatment of oropharyngeal candidiasis and have gradually replaced amphotericin B. However, resistance to azoles is now becoming common. Several reports suggest that susceptibility rates of *Candida* spp. to triazole antifungal amongst cancer patients have remained high with fluconazole resistance restricted to *C.krusei* [14, 15] and *C.glabrata* [14-16]. Other investigators have notified that *C.albicans* isolates from HIV positive and cancer patients are resistant to fluconazole and itraconazole [17, 18].

Information concerning the antifungal susceptibility of *Candida* is important in the prediction of the likely efficacy of subsequent treatment. *C.albicans* is generally assumed to be susceptible to most antifungal agents. The recent increased use of antifungal therapy has raised concerns over the potential for the emergence of resistance of *Candida* to antifungals. Indeed, the continued exposure of *Candida* to antifungals in certain patient groups has already been shown to alter the susceptibility of strains [19, 20].

The aim of the present study was to assess the *in vitro* susceptibility of oral *Candida* isolates from Ethiopian HIV/AIDS patients to five frequently used antifungal agents.

## Materials and methods

### *Patients*

Clinical samples were collected from Antiretroviral Therapy (ART) Clinic of Jimma University Specialized Hospital and isolation and experimentation were conducted in the Laboratory of Medical Microbiology of Jimma University. The samples were collected from all WHO defined stage III HIV/AIDS patients, visiting the hospital during the study period, who did not begin ART. Informed consent was obtained from participants and procedures were performed according to Jimma University board of ethical committee.

### *Candida albicans isolates*

Specimens were obtained by oral swab or the concentrated oral rinse method [21]. Isolates were cultured on Sabouraud's dextrose agar (Oxoid, Hampshire, UK) under aerobic conditions at 37°C for 48 h. These strains were identified by phenotypic methods such as germ tube formation in sheep serum, colored colony morphology on CHROMagar *Candida* medium (Paris, France), and API 2OC Aux (bioMerieux, Craponne, France).

### *Antifungal agents*

The five antifungal agents used for susceptibility testing of *C.albicans* isolates were amphotericin B, nystatin (Bristol-Myers Squibb, Middlesex, UK), miconazole, ketoconazole (Janssen, Beerse, Belgium), and fluconazole (Pfizer, Surrey, UK). The entire chemicals were supplied by Drug Admistration and Control Authority as working standards. Fluconazole was dissolved in sterile distilled water. Amphotericin B, nystatin, miconazole and ketoconazole were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to make stock solutions.

### *Assay media*

RPMI broth was prepared from RPMI 1640 broth medium (Sigma-Aldrich, Dorset, UK) containing 0.165 M morpholine-propanesulfonic acid (MOPS) and 2% glucose, which was incubated aerobically at 37°C for 48 h.

### *Inoculum*

Prior to antifungal drug testing, isolates were grown on Sabouraud's dextrose agar (Oxoid, Hampshire, UK) under aerobic conditions at 37°C for 48 h. Then suspensions of *C.albicans* were prepared from colonies by suspending in 0.85% saline and adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard. Dilutions, of that inoculum suspension, in RPMI 1640 were prepared and quantitatively subcultured to confirm the actual number of CFU/ml. The tests were repeated if the inoculum was not within the range of 0.5-2.5 x 103 CFU/ml [22].

### *Antifungal susceptibility testing*

The minimum inhibitory concentration (MIC) for each drug to the test isolates of *C.albicans* was determined using a broth microdilution method recommended by National Committee for Clinical Laboratory Standards (NCCLS) M27-A guidelines


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[22]. The range of ketoconazole, miconazole, amphotericin B and nystatin was 0.015-16 μg/ml and for fluconazole concentrations tested was 0.06-64 μg/ml.

The broth microdilution test was performed by using sterile, disposable multiwell microdilution plates. Aliquots of 100 μl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the well. In the case of the spectrophotometer readings, the azole cut-off value was 50% of the reading of growth control wells while for polyene antifungals, a cutoff value of 100% was used. Standard strains of *C.albicans* ATCC 10231 were used for quality control.

Antifungal activity was expressed as the MIC of each *C.albicans* isolate to the drug. The following resistance breakpoints were used according to NCCLS guidelines [22]:

-Fluconazole: resistant, ≥64 μg/ml; susceptible dose dependent, 16-32 μg/ml; susceptible, ≤8 μg/ml

-Ketoconazole: resistant, ≥4 μg/ml

-Miconazole: resistant, ≥8 μg/ml

-Amphotericin B: resistant, ≥2 μg/ml

-Nystatin: resistant, ≥16 μg/ml

***Quality controls***

Standard laboratory procedures were strictly followed. Control was taken so that mixture of different types of microorganism might not be tested on the same plate. A well identification of *C.albicans* was undertaken so that the result was not being grossly misleading. Standard strains of *C.albicans* ATCC 10231 was used for standardization and quality control purpose.

***Data analysis***

All data collected were analyzed using the Statistical Package for the Social Sciences (SPSS), version 16.0 software. Tests of proportions were done with Chi-Square, and a p value of <0.05 was considered as statistically significant.

***Ethics***

The proposal was reviewed and approved by the ethical clearance committee of Research and Publication office of Jimma University. Informed consent was obtained from the study subjects and those who volunteered participated in the study.

## Results

***Patient characteristics***

*C.albicans* was isolated from the oral cavities of 42 of 68 patients (61.8%). The rest non-*Candida albicans* strain is excluded from the study. The median age of the study participants was 31 and the majorities (58.4%) were females. 75.3% were from urban. Of the 42 candidapositive patients, 29 had typical clinical symptoms for oral candidiasis (69%), whereas 13 of them were only microbiologically positive, without evidence of *Candida* infection (31%).

***In vitro susceptibility results of the Candida albicans***

The isolates of *C.albicans* showed different susceptibilities to the tested antifungal drugs. The comparative *in vitro* susceptibilities of the yeast isolate to the antifungal agents were shown in Table 1. MICs for *C.albicans* ATCC 10231 was within the expected ranges. The determined MIC ranges were 1-66, 1-6, 0.5-9, 0.5-9, 0.12-3, and 0.25-17 μg/ml for fluconazole, ketoconazole, miconazole, amphotericin B and nystatin, respectively.

Overall, 41 (97.7%) of all isolates were determined susceptible to amphotericin B (MIC ≤ 2 μg/ml), 40 (95.3%) to nystatin, 39 (92.9%) to miconazole and ketoconazole. Two of all *C.albicans* isolates were found susceptible dose dependent (SDD) to fluconazole (MIC of 16-32 μg/ml) while 35 (83.4%) were fully susceptible. Five *C.albicans* isolates were found to be resistant to fluconazole (MIC ≥ 64 μg/ml). One (2.4%) of all *C.albicans* isolates were found resistant to amphotericin B, 3 (7.2%) to nystatin, 5 (11.9%) to miconazole and 6 (16.3%) to ketoconazole.

**Table 1.** *In vitro* antifungal susceptibilities of oral *C.albicans* isolates

| Antifungals (breakpoint, μg/ml) | MIC (μg/ml) | | | SDD (%) | R (%) |
|---|---|---|---|---|---|
| | Range | MIC50 | MIC90 | | |
| **Fluconazole (64)** | 1 – 66 | 2 | 8 | 2 (4.7) | 5 (11.9) |
| **Ketoconazole (4)** | 1 – 6 | 1 | 6 | - | 3 (7.1) |
| **Miconazole (8)** | 0.5 – 9 | 0.5 | 4 | - | 3 (7.1) |
| **Amphotericin B (2)** | 0.12 – 3 | 0.5 | 1 | - | 1 (2.3) |
| **Nystatin (16)** | 0.25 – 17 | 0.5 | 2 | - | 2 (4.7) |

SDD: susceptible dose dependent (for fluconazole only); R: resistant.
**Fluconazole**: resistant, ≥64 μg/ml; susceptible dose dependent, 16-32 μg/ml; susceptible, ≤8 μg/ml; **ketoconazole**: resistant, ≥4 μg/ml; **miconazole**: resistant, ≥8 μg/ml; **amphotericin B**: resistant, ≥2 μg/ml; **nystatin**: resistant, ≥16 μg/ml.

The isolates demonstrated low amphotericin B MICs, in which 83.4% (35/42) presented values of 0.12 μg/ml, followed by nystatin, in which 66.6% (28/42) presented values of 0.25 μg/ml. We found the widest range and the highest MICs for fluconazole (range 1-66). Amphotericin B demonstrated better *in vitro* activity compared to nystatin and azoles with MIC90 1 μg/ml.

***Multi drug resistance (Cross-resistance)***

Resistance to two or more antifungals had been identified in many isolates. In the present study, a total of three *C.albicans* isolates were found to be resistant to ketoconazole. Two of the ketoconazole-resistant isolates and 3 out of 39 ketoconazole-susceptible isolates were found to be resistant to fluconazole. There was a significant difference in incidences of resistance to fluconazole between the ketoconazole-resistant and -susceptible *C.albicans* isolates ($p < 0.05$).

In addition, the prevalence of resistance to miconazole in ketoconazole-resistant *C.albicans* isolates (two of three) was also significantly higher than that recorded for ketoconazole-susceptible isolates ($p < 0.05$). Only one ketoconazole-resistant strain was resistant to nystatin and none was resistant to Amphotericin B. Amphotericin B resistant strain was resistant to all antifungal drugs used.

***History of antifungal therapy and in vitro antifungal susceptibility***

Information regarding previous antifungal therapy was obtained from 17 patients. A total of 13 individuals had been treated with antifungal systemic therapy prior to collection of the specimen; fluconazole (50 mg daily) had been taken by 9 patients and ketoconazole by 4 patients. The rest 4 patients were treated with topical nystatin. The mean duration of fluconazole therapy was 22 days (range, 10-42 days), for ketoconazole therapy 16 days (range, 10-22 days), and for nystatin 14 days (range, 10-24 days).

The MICs for all the antifungal agents tested against *C.albicans* isolates from patients who had received previous antifungal drugs were not notably different from those values for isolates obtained from individuals who had not previously taken antifungal therapy (Table 2).

## Discussion

*C.albicans* is one of the most frequently isolated yeasts in clinical laboratories from HIV/AIDS patients. Studies have shown that this organism can account for up to 75% of the yeasts recovered from sites of infection [23]. The current isolation rate of *C.albicans* was 61.8% which is closer to the reports from Mexico (58.3%) [24] and Sao Paulo, Brazil (66.4%) [25].

It has been suggested that due to the trailing growth phenomenon in the test medium, visual determination of MIC endpoints for some azoles can be complicated and unreliable. Some studies reported that spectrophotometric readings of broth microdilution tests provide a more objective assessment of MIC endpoints [26]. In the present study we used spectrophotometric readings assess MIC endpoints.

A variety of antifungal agents are now available for the treatment of *Candida* infections. However, worldwide reports indicated that pathogenic isolates of *C.albicans* have relatively high potentials for developing resistance [23]. Relatively, high resistance of *C.albicans* to common antifungal agents tested was observed in the present study.

Although triazole agents appear to be highly effective initially, the increase of resistance to them has been reported [13, 27]. The finding of this study demonstrated a relatively higher level of resistance to fluconazole and ketoconazole. Fluconazole is a triazole agent that is established as a first-line antifungal for the treatment of oral candidacies in Ethiopia. Several recent studies have also reported fluconazole resistance in *C.albicans* strains isolated from HIV-infected patients with oral candidacies [7, 28-30].

**Table 2.** *In vitro* susceptibility of 17 *C.albicans* isolates from patients who previously received antifungal therapy

| Antifungals | Previous nystatin (n=4) | | | Previous fluconazole (n=9) | | | Previous ketoconazole (n=4) | | |
|---|---|---|---|---|---|---|---|---|---|
| | MIC50 | MIC90 | R (%) | MIC50 | MIC90 | R (%) | MIC50 | MIC90 | R (%) |
| **Fluconazole** | 2 | 7 | 2.3 | 3 | 8 | 2.3 | 2 | 4 | 0 |
| **Ketoconazole** | 1.5 | 4 | 0 | 1 | 5 | 2.3 | 2 | 5 | 2.3 |
| **Miconazole** | 1 | 3 | 0 | 0.5 | 1 | 0 | 1 | 4 | 2.3 |
| **Amphotericin B** | 0.5 | 1 | 0 | 0.5 | 1 | 2.3 | 0.5 | 1 | 0 |
| **Nystatin** | 1 | 2 | 0 | 0.5 | 2 | 2.3 | 1 | 2 | 0 |

R = resistance rate; R is calculated as 'number of resistant isolates to a drug' / 'number of total isolates'


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In the present study, overall resistance of *C.albicans* to fluconazole was 11.9%. These higher rates of resistance are not in agreement with those observed in Mexico, Brazil and United Kingdom [24, 25, 31] and other studies [7, 32] which reported lower rates of resistance to the studied antifungal. The reason for the high fluconazole resistance could be explained by the fact that fluconazole was prescribed to the most patients with oral candidacies as a standard care in Ethiopia.

Ketoconazole and miconazole are prescribed as an alternative to fluconazole for treating oral candidacies in Ethiopia. Ketoconazole is usually preferred as a topical antifungal agent because of high hepatotoxicity in systemic use. In this study, 7% of *C.albicans* isolates were found to be resistant to these azoles. This finding is supported by some other studies results [7, 33, 34]. Miconazole is employed topically to treat oral candidal infections. The results of this study would support continued clinical use of these agents.

Amphotericin B is both used topically in the treatment of superficial and systemic infections of hospitalized individuals. Our findings showed that oral *Candida* spp. isolated from HIV infected patients was highly sensitive to amphotericin B and only one (2.3%) of the isolates was resistant. The MIC distribution was concentrated in a narrow range and there was no strain that exhibited MICs greater than 3 μg/ml for this agent. The results of this study would indicate that amphotericin B remains an effective agent for the treatment of oral candidacies with a low incidence of *in vitro* resistance.

Some studies have also shown low [34] resistance or no resistance [12, 35] for amphotericin B, which is in accordance with our findings. In present study, the resistant strain was recovered from patients who had previously received fluconazole therapy which is in agreement with the previous study [7, 29, 34]. Nystatin is also a polyene antifungal. MICs for nystatin were higher than amphotericin B, and two strains were found to be resistant. These results would support the effectiveness of topical amphotericin B and nystatin therapy for superficial candidacies. Studies [36, 37] suggested that *C.albicans* demonstrate azole cross-resistance. In present study, the incidence of resistance to fluconazole and miconazole in ketoconazole-resistant isolates was significantly higher than recorded for ketoconazole- susceptible isolates.

The results would appear to confirm the presence of cross-resistance among oral *C.albicans* to these azole antifungal. Fluconazole and miconazole would not therefore be recommendable agents for treatment of oral candidacies that involve ketoconazole-resistant *C.albicans*. In the other case, only one ketoconazole-resistant strain was resistant to nystatin and none was resistant to amphotericin B. Therefore, these two agents may be used to treat candidal infections where ketoconazole resistance is evident. This finding supports the belief that there is no cross-resistance between the polyenes and azoles.

In this study, there is no difference in susceptibility of *C.albicans* to tested drugs, when patients who had previous antifungal exposure are compared to those who had not previously taken antifungal therapy. This finding does not replicate previous studies [19, 38]. Those studies that have reported a correlation between administration of antifungal and increased incidence of resistance have obtained samples from patients who had taken repeated and/or long-term antifungal therapy. In the present study patients had received antifungal therapy for a short period. These could also in part be due to the difference in population studied.

In conclusion, resistances to azoles among immunocompetent outpatient populations were relatively high. This study also highlights the presence of emerging cross resistance among azoles. The data presented in this study indicate that amphotericin B and nystatin are more effective than azoles for managing oral candidiasis. These local surveillance studies can help clinicians make treatment decision. As with the prescribing of any antimicrobial agent, the use of systemic antifungal drugs must be justified on a case-by-case basis. Efforts must be maintained to avoid inappropriate or unnecessary prescribing of these antifungals. Furthermore, careful periodical surveillance is needed in order to identify potential changes in the susceptibility pattern of fungi to these antifungal agents, with the increased use of these agents in Ethiopia.

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