In vitro Activity of New Azole Luliconazole Compared to Fluconazole against Candida Strains Isolated from Oral Lesions of Cancer Patients

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ABSTRACT

Introduction: Oral candidiasis is the most common fungal infection in patient's undergone chemotherapy. The aim of this study was to investigate the incidence and causative agents of oral candidiasis along with in vitro activity of new azole luliconazole compared with agents of oral candidiasis in a population of cancer patients.

Materials and Methods: A total of 385 oral samples from patients with various types of cancer and undergone chemotherapy were subjected to fungal culture. The yeast isolates were then identified by using PCR-RFLP method. The MIC values for fluconazole and luliconazole were determined using broth microdilution according to the M27-S3 protocol of the CLSI. The MICs, MIC50, MIC90 and geometric mean (GM) values were evaluated for all the isolates.

Results: Totally, 36 yeast strains were isolated which were found to be as Candida albicans (n=26; 72.2%), C. glabrata (n=5; 13.8%), C. kefyr (n=3; 8.3%), Pichia kudriavzevii (C. krusei) (n=1; 2.8%) and C. stellatoidea 1 (n=1; 2.8%) species. The MICs for luliconazole against all Candida isolates ranged from 0.007 µg/mL to 2 µg/mL; compared to 0.25 μg/mL to 128 μg/mL for fluconazole. The lowest GM values were 0.85 for C. glabrata and 1.14 μg/mL for C. kefyr isolates. The GM values of both antifungal drugs showed no significant differences between the C. albicans isolates.

Conclusion: C. albicans remains the most common agent of oral candidiasis in patients with different forms of cancers. Compared to fluconazole, Luliconazole showed more activity against all Candida species and potentially can be considered as an effective antifungal agent alternative to fluconazole.

Key words: Candida, Luliconazole, Fluconazole, Chemotherapy

INTRODUCTION

Oral candidiasis (known as oral thrush) is the most frequent fungal infection of the oral cavity caused by the genus Candida [1]. Under normal conditions, members of the genus Candida, as the most frequent fungi resident in the oral cavity, coexist with the other flora and do not cause disease but, any alteration in the oral microenvironment and/or systemic environment may led to an overgrowth of mycotic flora of the mouth and subsequently to oral fungal infections. Such alterations can especially be seen in babies less than one month, pregnant women, HIV-infected patients, and individuals with diabetes mellitus, renal failure, and immune disorders [2]. Finally, cancer patients under chemotherapy have an increased risk for developing oral candidiasis up to 40% [3,4].

Despite some surveillance health cares and the advances in medical interventions and prolonged life expectancy, the incidence of invasive systemic mycoses has increased markedly. These patients should be regularly monitored in term of oral candidiasis [1,5]. However in patients, with systemic candidiasis mortality rate is from 71% to 79%
Among the healthier population, the carrier rates have been reported 20% to 75% with no sign of infection [7]. In patients with leukemia who, receiving immunosuppressive or broad-spectrum antibiotic therapy and those with AIDS the carrier rates increased to 90%, and 95%, respectively [8-11]. The diagnosis of oral candidiasis is fundamentally on the basis of clinical findings and recognition of the lesions; however it should be confirmed by mycological detection of yeast in the oral samples and/or isolation in culture. Oral candidiasis can be treated with either topical or systemic antifungal agents. Polyene antifungals drugs such as nystatin and amphotericin B were the most drugs used topically, in the treatment of oral candidiasis. Currently one of the most important medicines, particularly for patients with extreme immune weakness, is azole compounds. Among the azoles, Fluconazole was found the drug of choice in the previous established PCR based tests. Firstly, the genomic DNA was extracted from each isolate with boiling method [18]. The nuclear 5.8S rRNA gene and its flanking internal transcribed spacer regions (ITS1 and ITS2) were amplified through the PCR in a 25 µl reaction mixture volume using the fungal universal ITS1/ITS4 primer pair [19], and thereafter digested by restriction endonuclease MspI (Thermo Fisher Scientific, Waltham, MA, USA). The fragmented products were then separated through electrophoresis on 2% gel agarose. Final identification of each isolate was accomplished by comparison of the banding patterns with those specific banding profiles demonstrated in the previous report for Candida species [18]. Discrimination of C. dubliniensis which shares the same RFLP pattern with C. albicans was performed in a duplex-PCR approach using two species-specific PCR primer pairs, targeting sequences in ITS-1 and ITS-2 regions [20]. The sequences of the primers were as follows: CDF (5’-AA ACTTGTCACGAGATTATTTTT) and CDUR (5’-AAA GTTTGAAAGATAAAATGCCC-3’) for specific identification of C. dubliniensis and CALF (5’-TGGTAAGCCGGGATGCCTF-3’) and CALR (5’-GGTTTCAAGGAGATTTATTTT) for detection of C. albicans. The duplex-PCR was conducted in a reaction mixture and thermal condition as previously described [20]. Identification of isolates was achieved by comparing the size of amplified products in a 1% gel electrophoresis.

**Materials and Methods**

** Patients, samples and isolates**

The ethics permission for the study was granted by Isfahan University of Medical Sciences/Ethics Committee (IR.MUI.REC.1394.3754). 385 patients undergoing chemotherapy who were hospitalized in some diagnostic and therapeutic centers in Isfahan and were suspected to oral candidiasis were included in the study. The patients were clinically investigated and those with pseudomembranous, erythematous, and hyperplastic lesions were selected for sampling. Two specimens were collected from each accessible and defined oral lesion by gently rubbing a sterile plain swab over the lesion. The specimens were examined by using direct microscopy and culture of the samples. A direct wet slide using 10% KOH was prepared from each sample and another swab was cultured on the chromogenic CHROMagar™ Candida medium (CHROMagar, Paris, France) and incubated at 35°C for 48h. The Diagnostic criteria for oral candidiasis were based on the clinical recognition of the lesions confirmed by mycological demonstration of yeasts, pseudohyphae or hyphae in direct smear and culture (≥ 10 CFU yeasts) [17].

**Identification of the isolates**

Identification of grown yeasts in the study was primarily made following the color of colonies grown on the CHROMagar™ Candida medium as per the protocol of the manufacturer. The depressive identification of the isolates to the species level performed by the previous establishment PCR based tests. Firstly, the genomic DNA was extracted from each isolate with boiling method [18]. The nuclear 5.8S rRNA gene and its flanking internal transcribed spacer regions (ITS1 and ITS2) were amplified through the PCR in a 25 µl reaction mixture volume using the fungal universal ITS1/ITS4 primer pair [19], and thereafter digested by restriction endonuclease MspI (Thermo Fisher Scientific, Waltham, MA, USA). The fragmented products were then separated through electrophoresis on 2% gel agarose. Final identification of each isolate was accomplished by comparison of the banding patterns with those specific banding profiles demonstrated in the previous report for Candida species [18]. Discrimination of C. dubliniensis which shares the same RFLP pattern with C. albicans was performed in a duplex-PCR approach using two species-specific PCR primer pairs, targeting sequences in ITS-1 and ITS-2 regions [20]. The sequences of the primers were as follows: CDF (5’-AA ACTTGTCACGAGATTATTTTT) and CDUR (5’-AAA GTTTGAAAGATAAAATGCCC-3’) for specific identification of C. dubliniensis and CALF (5’-TGGTAAGCCGGGATGCCTF-3’) and CALR (5’-GGTTTCAAGGAGATTTATTTT) for detection of C. albicans. The duplex-PCR was conducted in a reaction mixture and thermal condition as previously described [20]. Identification of isolates was achieved by comparing the size of amplified products in a 1% gel electrophoresis.

**Antifungal susceptibility testing**

Antifungal susceptibility testing was carried out following the microdilution method outlined in CLSI document M27-S3 [21]. Briefly, the stocks of fluconazole (Serva, USA) and luliconazole (Nihon Nohyaku Co., Osaka, Japan) were prepared in water and dimethyl sulfoxide (DMSO), respectively. Serial two-fold dilutions of the antifungals were prepared in RPMI 1640 medium buffered with MOPS buffer to pH 7.0-7.2, and 100 µl of each dilution was dispensed into 96 well round bottom microdilution plates (SPL, South Korea). The final concentrations of drugs ranged from 0.125 µg/mL to 5.00 µg/mL for fluconazole and 0.001 µg/mL to 1 µg/mL for luliconazole. To prepare the fungal inocula, all identified isolates were sub-cultured on Sabouraud dextrose agar (SDA) and after overnight growth, the yeasts suspensions were diluted in RPMI 1640 medium and adjusted spectrophotometrically at 530 nm wavelength to yield final inoculum of 1 × 10³ CFU/ml to 5 × 10³ CFU/ml. 100 µl of this suspension was distributed in each well of the
microplate for testing. The micro-plates were incubated at 35°C for 48 h. Turbidity was typically determined by subjective observation [17].

**RESULTS**

Out of 385 oral samples, in 36 cases a yeast strains arose from the culture, which were identified by PCR method. *C. albicans* was the most frequently isolated species (72.2%) in the oral cavities of patients using both CHROM agar culture medium and molecular method. The other detected *Candida*, were *C. glabrata* (n=5; 13.8%), *C. kefyr* (n=3; 8.3%), and each of *Pichia kudriavzevii (C. krusei)* and *C. stellatoidea* (n=1; 2.8%).

The band patterns obtained after treating MspI restriction digestion of the PCR products of *Candida* isolates are shown in (Figures 1 and 2). Duplex PCR with specific primers on 26 samples with RFLP profile shared for *C. albicans* and *C. dubliniensis* yielded an around 100 bp product indicative for *C. albicans* (Figure 3).

The ranges of luliconazole and Fluconazole minimum inhibitory concentrations (MICs) were ≥ 2-0.007 μg/ml and ≥ 128-0.5 μg/ml, respectively. The data obtained after doing broth microdilution method and also the geometric mean (GM), MIC50, MIC90 and MIC ranges of antifungal drugs against 36 clinical isolates are shown in Tables 1 and 2.

<table>
<thead>
<tr>
<th>Isolates no.</th>
<th>Isolate designation</th>
<th>Site of isolation</th>
<th>FLC(µg/ml)</th>
<th>LuL(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C. alb2</td>
<td>Lukemia</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>C. alb3</td>
<td>Bladder</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>C. alb4</td>
<td>Gastrointestinal</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>C. alb5</td>
<td>Lymphoma</td>
<td>&gt;128</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>C. alb9</td>
<td>Lukemia</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>C. alb11</td>
<td>Gastrointestinal</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Isolates</td>
<td>Antifungal</td>
<td>MICRange</td>
<td>MIC50</td>
<td>MIC90</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
<td>----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>C. albicans</td>
<td>FLU</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>LUL</td>
<td>2-0.007</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>FLU</td>
<td>&gt;128</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>C. krusei</td>
<td>FLU</td>
<td>&gt;128</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*a=Fluconazole; b=Luliconazole*

Table 2: MIC data for all clinical isolates
Considering the limited number detection of *Pichia kudriavzevii* (*C. krusei*) and *C. stellatoidea* isolates *C. stellatoidea* isolate was confirmed by sucrose sugar absorption test; MIC50, MIC90, and GM MIC were not calculated for these species. In addition *C. stellatoidea* possesses the same size band pattern of *C. albicans* by this method and showed creamy color on CHOROMagar. So identification of *C. stellatoidea* isolate was confirmed by sucrose sugar absorption test.

**DISCUSSION**

The main cause of candidiasis is still *C. albicans* although the other non-*albicans* species such as *C. glabrata* and *Pichia kudriavzevii* (*C. krusei*) which are less susceptible to azole antifungal drugs are increasing [22,23]. Nowadays the number of immunosuppressive patients has significantly increased due to malignant disease and despite some surveillance health cares and the advances in medical interventions and prolonged life expectancy, the various opportunistic fungal infections such as candidiasis, are also increased. From more than 150 known species of *Candida* only 15 species detected from the patients with candidiasis. It has been documented that in 95% of infections, the pathogens involved are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *Pichia kudriavzevii* (*C. krusei*) [24]. At the present study from the total of 36 detected isolates, 26 (72.2%) cases were confirmed to be *C. albicans* and 10 (13.8%) of the isolates were identified as non-*albicans* species using both phenotypic and molecular methods. There may be some factors such as previous azole therapy, underlying disease, geographical location, nutrition and age of the patients that cause variation in the kind of species and etiologic agents of candidiasis [25,26].

In a study done by Mohammadi et al. on diabetic patient in Iran, the most frequent *Candida* species in the oral cavities of diabetic patients were *C. albicans* (36.2%) followed by *Pichia kudriavzevii* (*C. krusei*) (10.4%) and *C. glabrata* (5.1%) [27]. The later study also has done in the same country so the different frequency of isolates should be due to the different underlying diseases in patients, however at the present study we found more *C. albicans* and less *C. glabrata*.

In a prospective study done in cancer center of New York, to investigate *Candida* colonization and infection in cancer patients, *C. albicans* was the predominant species (67.3%) followed by *C. glabrata* (45.6%). They found the overall resistance among all isolated *Candida* was 9.4% to fluconazole [28]. In the present study, out of the 26 *C. albicans* isolates, 12 species were resistant to Fluconazole using microdilution method (more than 128 µg/ml). It should be noted that only 5 species of *C. glabrata* detected from the mouth of the patients and 4 (80%) isolates were sensitive to fluconazole. In this case, it can be concluded that 20% of the *C. glabrata* species and about half of the isolates of *C. albicans* (50%) were resistant to fluconazole that this result is opposed to the previous reports, therefore correct identification and alternative fungal sensitivity drugs should be considered. In agreement with findings of DiNubile et al. results *C. albicans* and *C. glabrata* were predominant yeasts isolated from the oral cavity of patients with periodontitis. They reported *C. albicans* and *C. glabrata* 75% and 12.5% respectively the same as the results of the present study *C. albicans* 72.2% and *C. glabrata* 13.8%. This finding may guide our empiric treatment to shift for old azole in high-risk patients with known predisposing factors from developing serious candida infection particular with *C. albicans*. The MIC of luliconazole against *Candida* species has been reported to be higher than that against filamentous fungi that the increasing and extensive use of fluconazole prophylaxis and triazole treatment are very much related [29]. At the present study PCR-RFLP showed fast and easy recognition alongside using CHOROMagar-*Candida* pheno-typing method and for the early detection of yeasts. In between, these PCR-based techniques for many species it is reliable [30,31]. PCR-RFLP method is able to detect very small amounts of DNA [32]. In this study, identification of *C. albicans* and *Pichia kudriavzevii* (*C. krusei*) by CHROMMagar Candida and PCR-RFLP method showed the same results but *C. glabrata* and *C. kefyr* were identified only by molecular methods. In addition *C. stellatoidea* possess the same size band pattern of *C. albicans* by this method and showed creamy color on CHOROMagar. So identification of *C. stellatoidea* isolate was confirmed by sucrose sugar absorption test.

Luliconazole is an imidazole antifungal agent with a unique structure, as the imidazole moiety is incorporated into the ketene dithioacetate structure. Luliconazole, a new imidazole agent exhibited *in vitro* antifungal activity against several molds and yeasts. That is used in the form of a topical cream of 1%. The purpose this part of the study was to compare the *in vitro* antifungal activities of luliconazole with fluconazole for therapy of candidiasis against clinical *Candida* isolates of patients undergoing chemotherapy, since the majority resistant clinical *Candida* strains to fluconazole have been isolated from gastric cancer and lymphoma. Luliconazole with MIC

<table>
<thead>
<tr>
<th>C. stellatoidea</th>
<th>FLU</th>
<th>&gt;128</th>
<th>-</th>
<th>1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUL</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

R=Resistance; S=Susceptible

a=MIC which inhibits 50% of Candida species isolates in test

b=MIC which inhibits 90% of Candida species isolates in test

\[ \text{MIC}=\text{MIC} \]

\[ \text{MIC} \]
range: 0.007 μg/ml-1 μg/ml demonstrated greater potency against detected isolates than the Fluconazole antifungal drug. In a study conducted by Clarkson et al. to determine the susceptibility of Candida species isolated from chemotherapy patients, it was found that the sensitivity of them to ketoconazole and clotrimazole is more than fluconazole [3]. Previous studies have shown that luliconazole had low MICs against black fungi, Aspergillus fumigatus and dermatophyte species. Uchida et al. also showed that the GM MICs of luliconazole for Malassezia furfur, Malassezia sympodialis, and Malassezia slooffiae were approximately 1.4 μg/ml, 0.1 μg/ml, and 1 μg/ml, respectively [33]. Luliconazole showed the best activity with the lowest geometric mean 0.85 for C. glabrata isolates in comparison with 1.14 μg/ml against C. kefyr isolates. The geometric mean of luliconazole and fluconazole for C. albicans was not significantly different. In a luliconazole drug sensitivity research conducted in 2017 on candida isolates detected from different patients, the MIC range and MIC90 of vaginal isolates (HIV-) were 1-0.063 and 1μg/mL [34].

CONCLUSION

In conclusion, there are no clinical trials evaluating the efficacy of luliconazole in candida infections, preclinical studies have supported such a role. In this study, given that in vitro activities of luliconazole against azole-resistant candida isolates were superior to fluconazole. The evaluation of antifungal susceptibility patterns can provide useful information on the resistance patterns of the isolates. It should be considered that luliconazole may emerge as an effective and broad-spectrum antifungal agent in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

REFERENCES


