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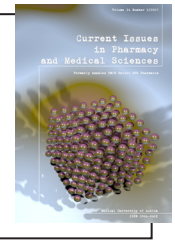

# Susceptibility *in vitro* of clinical *Candida albicans* isolates to the selected azoles

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**ABSTRACT**

*Candida* spp. is the most prevalent cause of fungal infection worldwide, and their increasing resistance to anti-fungal agents, especially to azoles, has become problematic. The aim of this work was to establish the susceptibility to fluconazole, itraconazole, voriconazole and posaconazole of 50 clinical *C. albicans* isolates from hematooncological patients. This has been evaluated using the following parameters: MIC (Minimum Inhibitory Concentration), $MIC_{50}$ (MIC required to inhibit the growth of 50% of organisms), as well as $MIC_{90}$ (MIC required to inhibit the growth of 90% of organisms). Susceptibility of the studied clinical isolates to all azoles was high, being 86% for itraconazole, 90% for fluconazole and posaconazole and 92% for voriconazole. The resistance rates ranged from 8% (voriconazole), to 12% (itraconazole). The emergence of azole-resistant yeast strains creates a necessity to determine and monitor the sensitivity of the isolated *Candida* spp., including *C. albicans*, especially in patients predisposed to life-threating fungal invasive disease.

## INTRODUCTION

*Candida*, a genus of yeasts that belongs to the Saccharomycetales, is considered to be the most common cause of fungal infections worldwide [1]. A few hundred *Candida* species have been identified. Dozens of them are opportunistic pathogens, causing fungal infections named as 'candidiases', especially in patients with impaired immune systems, e.g. oncological and hematooncological patients. These infections include superficial lesions, affecting the skin or mucous membrane, or invasive, life-threatening diseases [2]. They have mainly an endogenic character because human microbiota can be regarded as the reservoir of *Candida* spp. [3]. The most commonly isolated pathogenic species is *Candida albicans* [4]. Although the diagnostic and treatment options have been improved, fungal infections, including candidiases, remain a problem, especially those being invasive diseases with high mortality [5].

Polyenes, fluoropyrimidines, echinocandins and azoles are antifungal agents used in *Candida* infections treatment [4]. Azoles are antifungals used globally to treat *Candida* spp. infections, as well as for preventive purposes [2]. These agents belong to the family of imidazole derivatives (e.g. clotrimazole, ketoconazole, miconazole) and triazole derivatives such as itraconazole, fluconazole, posaconazole and voriconazole [4,6]. Antifungal activity of azoles results from inhibition of the enzyme encoded by gene *ERG11*, i.e. sterol 14alfa-demethylase, which takes part in the transformation of lanosterol into ergosterol. Therefore, these agents interrupt the biosynthesis of ergosterol – an important *Candida* spp. cell membrane compound, leading to inhibition of fungal growth. In addition, 14α-methyl-3,6-diol, being a fungistatic compound, is accumulated inside the yeast cell [4].

Increasing resistance and multiresistance to antifungal agents has become more and more serious. The most predominant among *Candida* spp. is resistance to azoles, caused by the wide use of these medicines in the last decades in clinical practice – both for prophylactic and therapeutic purposes [1]. *Candida* spp. has evolved several resistance mechanisms against azoles. Among these are: point mutations in the gene *ERG11* (interference in azoles binding), gene *ERG11* up regulation (influence on ergosterol production), reduced azoles affinity to cell membrane, overexpression or amplification of multiple classes of drug efflux pumps (rapid azole extrusion from fungal cells), regulation of cellular stress response pathways and biofilm creation [1,3].

The overall goal of this work was to determine the susceptibility to fluconazole, itraconazole, voriconazole and posaconazole of clinical *C. albicans* isolates from

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hematooncological patients in order to assess the prevalence of resistance to these azoles.

## MATERIALS AND METHODS

The yeast population used in these studies included 50 *C. albicans* isolates. These microorganisms were from the collection of the Department of Pharmaceutical Microbiology, Medical University of Lublin, and were primarily isolated from clinical specimens obtained from hematooncological patients such as swabs from oral cavity, upper respiratory tract, cervix, vagina, urethra and ears, as well as sputum, blood, stool and urine. The isolates were identified by standard diagnostic methods. The Ethical Committee of the Medical University of Lublin approved the study protocol (No. KE-0254/75/2011).

The assay of sensitivity of clinical *C. albicans* isolates to fluconazole, itraconazole, voriconazole and posaconazole was performed using the commercial Sensititre YeastOne YO9 AST antifungal panel (Thermo Fisher Scientific) based on the broth microdilution method, allowing for the determination of minimal inhibitory concentration (MIC). This method enables the qualitative and quantitative evaluation of the MIC (Minimum Inhibitory Concentration).

All the used yeast isolates were stored at -75°C in Sabouraud broth (bioMerieux) with the addition of 50% glycerol. They were first subcultured on Sabouraud agar (bioMerieux) and incubated at 35°C for 24 h. The yeast suspensions were then prepared in sterile water according to the manufacturer's instruction to obtain optical density corresponding to 0.5 Mc Farland standard. The final inocula were prepared by adding 20 µL of a given suspension into 11 mL of OneYeast broth to obtain a density of approximately 1.5-8 × 103 CFU (Colony Forming Units)/mL. The 96-well plates with antifungal agents at serial two-fold concentrations (fluconazole: 0.12 to 256 mg/L, itraconazole: 0.015 to 16 mg/L, posaconazole: 0.008 to 8 mg/L and voriconazole: 0.008 to 8 mg/L) were filled up by 100 µL of the final inoculum and then incubated at 35°C for 24 h. After incubation, a visual reading was made on the basis of changing the yeast growth indicator color from blue (negative) to red (positive). MIC was defined as the lowest concentration of the compound showing complete fungal growth inhibition. The experiments were performed in triplicate. The representative data are presented.

In these studies, MIC interpretative criteria (mg/L) for *C. albicans*, according to CLSI M27 (Clinical and Laboratory Standards Institute standard M27) were as follows: fluconazole – susceptible (≤ 8), dose-dependent susceptible (16-32), resistant (≥ 64); itraconazole – susceptible (≤ 0.125), dose-dependent susceptible (0.25-0.5), resistant (≥ 1); posaconazole and voriconazole – susceptible (≤ 1), dose-dependent susceptible (2) and resistant (≥ 4) [7].

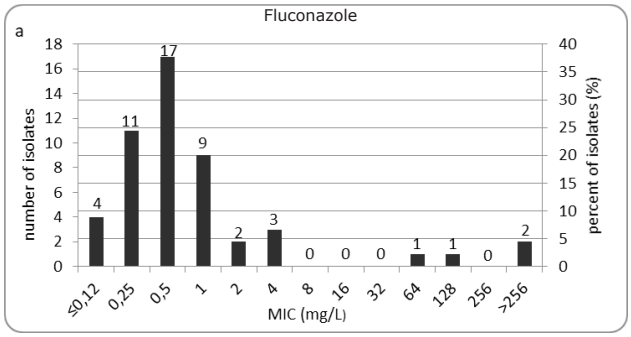
## RESULTS

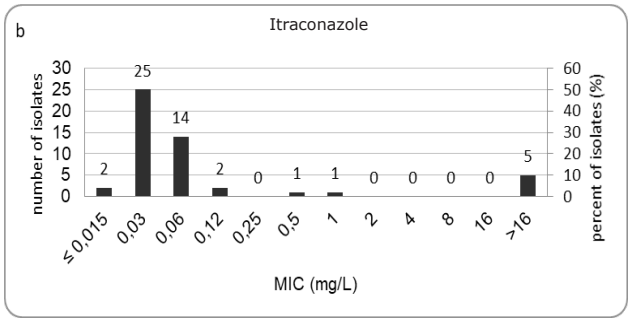
The susceptibility of clinical *C. albicans* isolates was first determined to fluconazole, itraconazole, voriconazole and posaconazole. Table 1 presents the obtained data. The majority of these isolates were sensitive to all azoles within the range 86-92%. The resistance rates ranged from 8 to 12%. We identified one isolate (2%) as susceptible to itraconazole, depending on dose.

***Table 1.*** The rate of susceptibility of clinical *C. albicans* isolates to the selected azoles assessed by MIC determination

| Category of sensitivity | Number (percentage) of isolates | | | |
|---|---|---|---|---|
| | Fluconazole | Itraconazole | Voriconazole | Posaconazole |
| Sensitivity | 46 (92%) | 43 (86%) | 46 (92%) | 45 (90%) |
| Sensitivity dependent on dose | 0 (0%) | 1 (2%) | 0 (0%) | 0 (0%) |
| Resistance | 4 (8%) | 6 (12%) | 4 (8%) | 5 (10%) |

Subsequently, we established the range of MIC of each azole for both sensitive and resistant *C. albicans* isolates (Fig. 1). We found that the MIC of fluconazole for susceptible strains ranged from ≤ 0.12 to 4 mg/L, and about one-third of isolates was characterized by MIC = 0.5 mg/L. MIC for fluconazole-resistant isolates was estimated at ≥ 64 mg/L (Fig. 1a). According to the obtained data (Fig. 1b), MIC of itraconazole ranged within ≤ 0.015-0.5 mg/L. In the case of this drug, we noted that for 50% of the isolates, MIC = 0.03 mg/L. In turn, MIC for strains with sensitivity depending on itraconazole dose amounted to be 1 mg/L, while for resistant isolates, MIC ≥ 16 mg/L. Furthermore, we saw that the MIC of voriconazole against sensitive isolates was within the range ≤ 0.008-0.12 mg/L (Fig. 1c). Moreover, the most frequent MIC value was MIC ≤ 0.015 mg/L found for 72% isolates. Voriconazole-resistant strains were characterized by MIC = 16 mg/L. In addition, our work revealed that the MIC of posaconazole for susceptible isolates were in the range of ≤ 0.008-0.06 mg/L, and most isolates (56%) were characterized by MIC = 0.015 mg/L. We also established that posaconazole-resistant strains possessed MIC ≥8 mg/L (Fig. 1d).



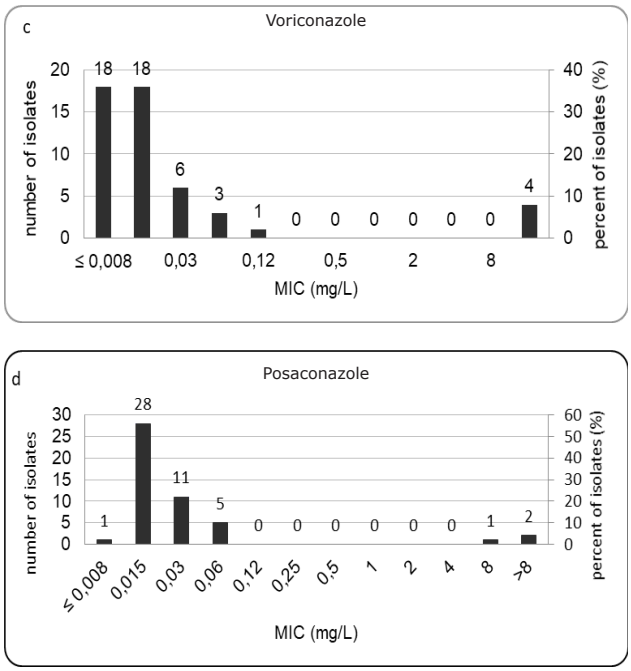



***Figure 1.*** MIC (mg/L) range of fluconazole (a), itraconazole (b), voriconazole (c) and posaconazole (d) for clinical *C. albicans* isolates

From Table 2 it can be noted that the population parameters, i.e. $MIC_{50}$ and $MIC_{90}$ of the studied azoles, inhibiting 50% or 90% of *C. albicans* isolates, respectively, were within the range of values for sensitive strains.

***Table 2.*** $MIC_{50}$ and $MIC_{90}$ (mg/L) values for the selected azoles of clinical *C. albicans* isolates

| Azole | $MIC_{50}$ | $MIC_{90}$ |
|---|---|---|
| Fluconazole | 0.5 | 4 |
| Voriconazole | 0.015 | 0.06 |
| Itraconaozle | 0.03 | 1 |
| Posaconazole | 0.015 | 0.06 |

## DISCUSSION

In recent years, the number of fungal infections, both superficial and life-threatening invasive diseases, has increased. *Candida* spp., mainly *C. albicans*, is the major etiological agent of fungal infections [8]. However, it should be not forgotten that excessive use of antifungals may lead to the growing resistance of *Candida* species, including *C. albicans*. The consequence of resistance may be therapeutic failure. Therefore, the susceptibility of clinical isolates to antifungal agents should be identified, allowing more appropriate drugs to be introduced to the treatment course. Literature data shows that sensitivity and affirming the "60-90 rule" may help in the results of therapy prediction. This rule suggests that infections caused by susceptible pathogens respond to suitable therapy approximately to 90%, while infections caused by resistant pathogens respond to suitable therapy only to 60% [9].

We found that the sensitivity of clinical *C. albicans* isolates from hematooncological patients to fluconazole, itraconazole, voriconazole and posaconazole was assessed at the high level, i.e. 86-92% with $MIC_{90}$ being 4 mg/L, 0.06 mg/L, 1 mg/L and 0.06 mg/L, respectively. The high sensitivity of clinical *C. albicans* isolates to azoles reported in this paper is in agreement with data presented by Lei *et al*. [10]. They found that fluconazole, itraconazole and voriconazole were highly active against clinical isolates of *C. albicans*, with $MIC_{90}$ of 1 mg/L, 0.125 mg/L and 0.0625 mg/L, while, about 90% isolates were susceptible to these azoles.

However, *C. albicans* can become resistant to azoles, especially to fluconazole, as this is frequently used to treat candidiases, mainly systemic infections [11]. The main mechanism of azole resistance in *Candida* spp. is related with increases in the number of efflux pumps in the cell due to gene overexpression [2]. We found that the resistance rate of clinical *C. albicans* isolates from hematooncological patients to fluconazole, itraconazole, voriconazole and posaconazole ranged from 8-12%. According to literature data, *C. albicans* isolates from candidemic patients have the lowest incidence of azole resistance, including fluconazole (0-5%) [11-14]. The incidence of fluconazole resistance in *C. albicans* isolates from oropharyngeal candidiasis was found to be higher, being above 10% [15]. However, studies performed by Yenisehirli *et al*. [16] showed that the overall resistance rates of *C. albicans* isolates to fluconazole, itraconazole, voriconazole and posaconazole were higher – 34%, 21%, 14% and 14%, respectively.

## CONCLUSIONS

The present study shows that susceptibility of *C. albicans* isolates from hematooncological patients to posaconazole, voriconazole, itraconazole and fluconazole was high (86-92%). However, emergence of azole-resistant strains creates a necessity to determine and monitor sensitivity to the isolated *Candida* spp., especially in patients with life-threating invasive disease.

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