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Evaluation of Susceptibility to Fluconazole and Voriconazole in Oral *Candida glabrata* Isolates from Drug Addicts

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Abstract

Background: Candidiasis is a spectrum of opportunistic fungal diseases that can manifest in drug addicts in various forms, such as stomatitis.

Objectives: This study aimed to determine the prevalence of oral candidiasis caused by *Candida glabrata* in addicts and examine the susceptibility of isolates to two azoles.

Patients and Methods: After taking oral samples from 131 drug addicts suspected of having oral candidiasis, *Candida* species were identified by culture on chromogenic *Candida* agar, carbohydrate-assimilation (API test), and polymerase chain reaction. The minimum inhibitory concentrations (MICs) of fluconazole and voriconazole against the isolates were determined using the broth microdilution method. Data were analyzed by SPSS (version 23) using the *t*-test and one-way analysis of variance. A P value of less than 0.05 was considered statistically significant.

Results: Overall, 22.2% of the isolates were *C. glabrata*, which was more abundant among opiate addicts. Among these isolates, 61.1% were resistant to fluconazole and 44.4% were resistant to voriconazole. The MIC_{90} of voriconazole was 8 μg/mL, which was 16 times less than that of fluconazole (128 μg/mL). There was no statistically significant difference between the frequency of fluconazole- and voriconazole-resistant isolates ($P > 0.05$).

Conclusions: Oral candidiasis was a common problem among drug addicts. In addition, the prevalence of azole-resistant *C. glabrata* isolates was high among these individuals.

Keywords: *Candida glabrata*, Fluconazole, Voriconazole, Substance-Related Disorders

1. Background

Increased incidence of candidiasis, its epidemiological changes, and drug resistance highly justify the need to conduct studies on fungal infections. Pathogenic *Candida* species are found in both humans and animals. According to epidemiological studies, non-albicans *Candida* species are gradually replacing *Candida albicans* (1). *Candida glabrata*, formerly known as *Torulopsis glabrata*, is an opportunistic pathogenic yeast with a rising prevalence in immunocompromised individuals, such as HIV and cancer patients, transplant recipients, consumers of immunosuppressive drugs, and drug addicts (2).

Drug addiction is a global problem with serious health and financial consequences. Drugs can stimulate the brain's reward system, causing it to produce excessive dopamine as a pleasure neurotransmitter. Either way, the brain reinforces the behavior of drug use with dopamine-induced pleasure. Addiction is not only an individual aspect but also a social threat. In addition to threats to the physical and mental health of individuals, it has irreparable harm to the social and economic aspects of society. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), drug-related disorders are the problems associated with the use and abuse of drugs such as cocaine, heroin, and other substances that people have to change their thinking, feeling, and behavior (3). Patients with drug addiction have shown to have increased susceptibility to fungal infections. Also, drug abusers with a history of travel may serve as carriers of infection; thus, the fungal infection should be considered in narcotic users (4, 5). In addicts, *Candida* species can infect the oral cavity or bloodstream, thereby leading to severe infections (6).

Despite the increasing diversity of antifungal drugs, the efficacy of these agents for the treatment of fungal infections is not satisfactory (7). Azoles including fluconazole and voriconazole are among the most important antifungal agents used for the treatment of fungal infections. Voriconazole is commonly used for the treatment of fluconazole-resistant candidiasis (8). The drug affects the activity of cytochrome P450 and inhibits the demethylation of 14-alpha-lanosterol and ergosterol synthesis. This results in ergosterol depletion and accumu-

lation of methylated sterols, which disrupt the cell membrane structure and function (9).

2. Objectives

In this study, considering the drug resistance in fungal infections, we aimed to determine the prevalence of oral candidiasis in addicts and compare the antifungal activity of fluconazole and voriconazole against *C. glabrata* strains isolated from drug addicts with oral candidiasis in vitro.

3. Patients and Methods

In a cross-sectional study, 131 samples were studied from Oct 2017 to Dec 2018. The target population was drug addicts that used opium, opium sap, crack, naswar or methamphetamine (not alcohol) suspected of having oral candidiasis and recently admitted to three camps in Golestan Province, north-east of Iran. We selected those higher than 18 years of age. Addicts were identified and diagnosed based on the symptoms of bloodshot eyes, bad breathing, shakes or tremors, frequent bloody noses, or dizziness and headaches according to camps' psychiatrists and urine/blood tests. Moreover, 131 samples were prepared from non-drug users with oral candidiasis as a control group. The mean age of the subjects was 21 ± 18 years. Moreover, all personal data such as the name and address of them were confidential and samples were identified merely with a special code.

Saliva or oral fluid samples were collected without stimulation. The samples were stored in sterile disposable containers and kept on ice for up to three hours. After a direct microscopic examination, the samples were cultured on Sabouraud dextrose agar containing chloramphenicol (Quelab, Canada) and incubated at 35°C for 48 hours. The isolates were identified by methylene blue staining, culture on chromogenic *Candida* agar, and carbohydrate-assimilation test using the API120C AUX kit. The identification of *Candida* species was confirmed by molecular evaluation via PCR. The PCR was performed according to the instructions of *Candida* spp. detection kit (Iranian Gene Fanavar Institute, Iran) using 18S rRNA universal primers (Table 1).

Table 1. Details of Primers Used for Molecular Detection of *Candida* spp.

	Sequence, 5'->3'	Length, nt	Tm, °C	GC, %
Forward primer	GCCAGCGAGTATTAACCTTG	20	58.4	50.00
Reverse primer	ATTTCAGCCAATGAGGA	20	56.4	45.00

The DNA was isolated using glass beads and the phenol-chloroform method. The PCR amplification was carried out in a final reaction volume of 25 μL containing 1 $\times$ PCR mix (19.7 μL), 0.3 μL Taq-DNA polymerase, and 5 μL of DNA sample. We also used PCR-negative and positive controls in the experiment. The mixture was vortexed for 3 - 5 seconds. The cycling conditions were as follows: 40 cycles of initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for one minute, and a final extension at 72°C for 5 minutes. The results were interpreted based on the kit instruction.

The minimum inhibitory concentrations (MICs) of fluconazole and voriconazole against *Candida* isolates were determined using the Clinical Laboratory Standards reference method for broth microdilution (NCCLS-M27-A3) (10). To prepare the drug stock solutions at the density of 256 - 0.5 μg/mL and 32 - 0.06 μg/mL for fluconazole and voriconazole, respectively, the powder of each antibiotic (Basel, Switzerland) was dissolved in distilled water and DMSO, in sequence, followed by a 1:100 dilution with RPMI medium (with glutamine, without sodium bicarbonate, pH indicator) (Sigma-Aldrich, USA). After preparing a yeast suspension (1×10^3 CFU/mL) from 48-hour cultures, the drug stoke solution and yeast suspension were equally inoculated into the wells of a 96-well plate containing the equal amount of RPMI medium. The lowest concentration of the drugs that inhibited the growth of isolates after 48 hours of incubation at 35°C was determined as the MIC. The growth rate was assessed in comparison with the positive control (no drug) and negative control (no microorganism) wells.

The MIC results were interpreted according to standard tables (10). For fluconazole, *Candida* species were considered susceptible, susceptible dose-dependent, and fluconazole-resistant if the MIC values were $\leq$ 8, 16 - 32, and $\geq$ 64 μg/mL, respectively. For voriconazole, *Candida* species were classified as susceptible, susceptible dose-dependent, and resistant if the MIC values were $\leq$ 1, 2, and $\geq$ 4 μg/mL, respectively. Data were analyzed by SPSS (version 23) using the *t*-test and one-way analysis of variance.

4. Results

Of 131 oral samples taken from addicts, 61.8% were identified as positive for *Candida* species. According to the phenotypic and genotypic assays, 65.4% of the isolates were *C. albicans* and the rest were non-albicans *Candida* species.

For analyzing the results after DNA replication, 10 μL of the multiplied product was loaded on the 1.5% agarose gel and TBE $\times$ 1 buffer without adding the loading buffer. One DNA marker was associated with samples for determining the size of bands that were then stained with the ethidium bromide gel (0.5 μg/mL). The images were generated by a

UV DOC device. The presence of 620 bp fragments compared to the DNA size marker indicated that the test was positive, implying that the identification of non-albicans *Candida* species was confirmed (Figure 1).

Candida glabrata isolates were the most frequent species (22.22%) among non-albicans species and more frequent among males, those aged 20 - 25 years, and opium addicts (Figure 2).

In the control group, 46.6% were identified as *Candida* species and 12% of the isolates were *C. glabrata*. There was a significant difference in the frequency of isolates between drug users and non-users (P = 0.01).

The results of antibiotic susceptibility testing and MIC assay showed that 61.1% and 44.4% of *C. glabrata* isolates were resistant to fluconazole and voriconazole, respectively (Figure 3). However, 58% and 43% of *C. glabrata* isolates in the control group were resistant to fluconazole and voriconazole, respectively. There was no significant difference in the azole-resistance rate between drug users and non-users (P > 0.05).

The results of susceptibility testing showed that both fluconazole and voriconazole could inhibit the growth of *C. glabrata* isolates in a dose-dependent manner. The MIC_{90} of voriconazole was 8 μg/mL, which was 16 times lower than that of fluconazole (128 μg/mL). However, there was no significant difference between the rates of resistance to fluconazole and voriconazole among isolates from drug-users (Table 2).

Table 2. Comparison of Mean MICs of Fluconazole and Voriconazole Against *Candida glabrata* Isolates (P Value > 0.05)

Antibiotic	MIC value (Mean ± SD)
Fluconazole, μg/mL	
Resistant	64 ± 12.1
Susceptible	4 ± 5.7
Voriconazole, μg/mL	
Resistant	4 ± 12.1
Susceptible	0.5 ± 6.5

5. Discussion

Candidiasis is the most common fungal infection in the world. *Candida* is isolated almost invariably from cutaneous lesions, but rarely cultured from the blood or vitreous fluid. The sources of the fungus in these cases remain uncertain. Although some authors have suggested that *Candida* may contaminate heroin, attempts to culture *Candida* from heroin have been unsuccessful (11). The recreational use of illicit drugs has aroused concerns that drug abuse-mediated immune dysfunction increases host susceptibility to microbial pathogens. Opiates can also affect the immune system directly through opioid receptors on immune cells leading to reduced phagocytosis and chemotaxis. Endogenous *Candida albicans* endophthalmitis in injecting drug users has been extensively documented in the literature and is one of the most common ocular complications of drug use (12).

In the present study, we evaluated the prevalence of *C. glabrata* isolates in oral samples taken from drug addicts. The results showed that about 22% of the isolates were *C. glabrata*. Disruption of the mucosal defense or immune system can provide suitable conditions for opportunistic pathogens (13). In some studies, non-albicans species such as *C. glabrata* were the most frequently isolated *Candida* strains (14).

Previous studies reported *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* as the main causes of mucosal fungal infections (15). Similar to our findings, a study of the oral mucosa of immunocompromised patients in 1998 reported the high prevalence of non-albicans *Candida* species, including *C. glabrata* (16).

The importance of non-albicans *Candida* species has become more profound due to the emergence and development of resistance to antifungal drugs in some of these species, such as *C. glabrata* and *C. krusei*, which has led to a dramatic increase in the prevalence of candidiasis. Hence, there is an urgent need for its detection and determining the frequency of resistant *Candida* species to help develop and implement effective control measures (17).

In our study, 61% and 44.4% of *C. glabrata* isolates were resistant to fluconazole and voriconazole, respectively. In 2006, researchers stated that 43% of the strains isolated from blood samples of hospitalized patients were *C. glabrata*. Of these isolates, 23% were voriconazole-resistant and 41% were fluconazole-resistant (18), which are less than the rates observed in our study. This difference can be attributed to the difference in the characteristics of the studied populations, the type of samples taken, the type of infections, and the presence of underlying diseases. This highlights the necessity of determining susceptibility to azoles and other antifungal agents. In some studies, *C. glabrata* was the least susceptible species to voriconazole. Likewise 60% of *C. glabrata* isolates were susceptible to fluconazole, and 92%, were inhibited by $\leq 1\,\mu$g of voriconazole per mL (19).

Similar to our findings, in a study in Spain on 21 male and female drug addicts, the prevalence of *C. albicans* and *C. glabrata* was 67% and 28.9%, respectively (6). However, the mentioned study examined only heroin addicts, while our subjects were addicted to various substances.

In the present study, the MIC90 value of voriconazole

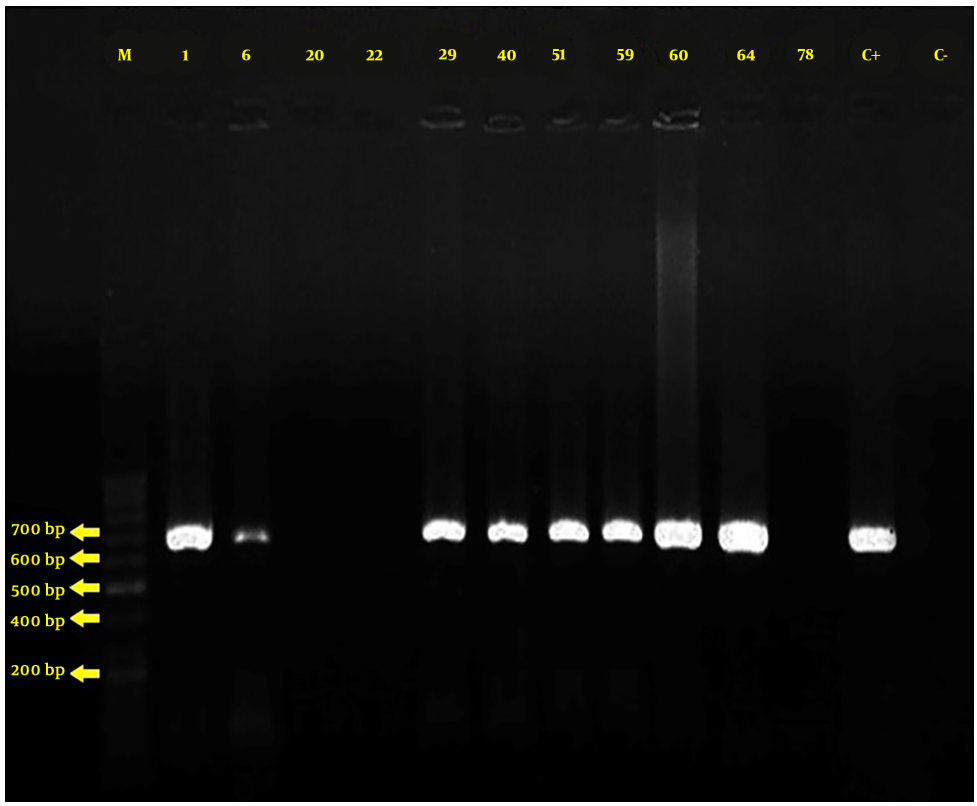

Figure 1. Results of electrophoresis of PCR products of *Candida glabrata* isolates on 1.5% agarose gel. Columns 1, 6, 29, 40, 51, 59, 60, and 64 contain the 620 bp fragment. C+, Positive control; C-, negative control.

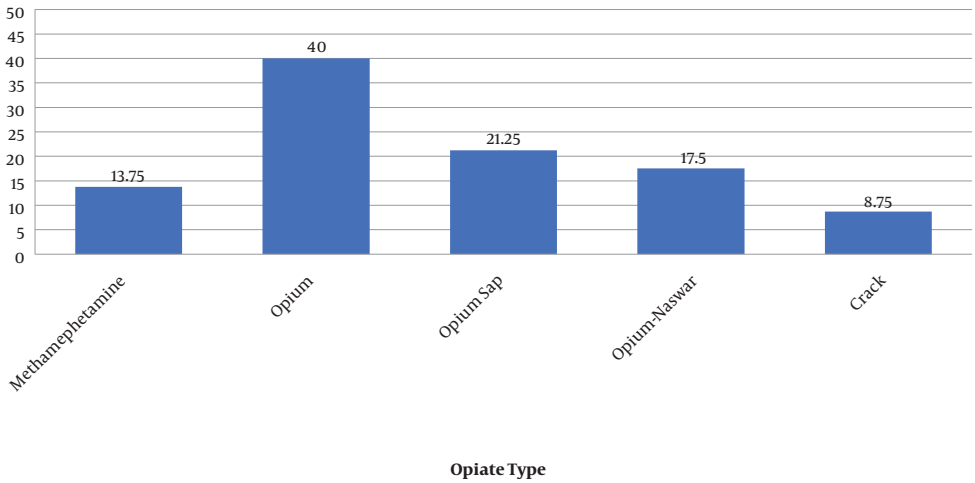

Figure 2. Relative frequency of *Candida glabrata* strains isolated from oral samples of addicts based on the type of addiction

was 16 times less than that of fluconazole against these isolates. Nevertheless, there was no statistically significant difference between voriconazole- and fluconazole-resistant isolates.

It has been suggested that voriconazole breakpoints are dependent on the pharmacodynamic analysis (19), but

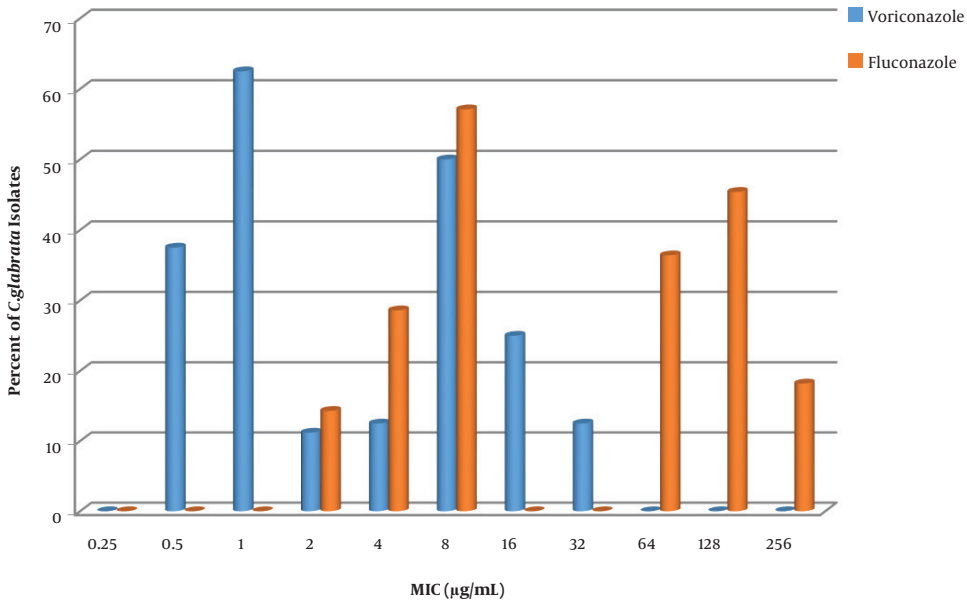

Figure 3. Relative frequency of fluconazole and voriconazole MICs of *Candida glabrata* isolates

the pharmacodynamic properties of the antifungal agents are not fully understood. In a study on the pharmacokinetic/pharmacodynamic parameters of voriconazole, an AUC_{24}/MIC ratio of 20 at a dose of 200 mg was effective for the treatment of infections caused by *C. albicans* in a mouse model with MIC of 0.25 μg/mL (20).

A new formulation of antifungal drugs, combination therapy, and the development of new bioactive compounds may improve therapeutic outcomes. Regarding the use of combination therapy for the management of candidiasis, researchers demonstrated that a combination of fluconazole and *Silybum marianum* extract was four-fold more effective than fluconazole or the extract alone (7).

In general, determining the susceptibility of pathogenic fungi before treatment may be beneficial for improving treatment efficacy and preventing drug overuse and the subsequent drug resistance.

5.1. Conclusions

Our results demonstrated the high prevalence and high rate of drug resistance among non-albicans *Candida* isolates, particularly *C. glabrata*, among the studied population of addicts. Given the high prevalence of resistance to fluconazole and voriconazole in these subjects, there might be the need to use higher doses of the drugs for favorable therapeutic outcomes. Therefore, it is proposed to accurately diagnose infections in drug users and exploit combination therapy at low doses or seek novel antifungal agents for the treatment of drug-resistant candidiasis.

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Footnotes

Authors' Contribution: Leila Fozouni contributed to the study concept and editing of the final manuscript. Narges Golirad performed laboratory examinations and interpreted the data. All authors discussed the results and implications and provided their comments on all stages.

Conflicts of Interests: The authors declare that there is no conflict of interest.

Ethical considerations: The ethical code is IR.IAU.AK.REC.1398.009.

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