Original Article

Identification of Candida Species and Antifungal Susceptibility in Cancer Patients with Oral Lesions in Ahvaz, Southern West of Iran

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Abstract

Background: Oral candidiasis is a common disease in cancer patients subject to chemotherapy. The aim of this study was to evaluate the risk factors of rising oral candidiasis incidence and to identify the Candida species isolated from oral lesions of cancer patients and their antifungal sensitivity. **Materials and Methods:** A total of 645 patients with cancer were examined. Several *Candida* species were isolated from specimens and identified by morphological and molecular methods. The susceptibility of isolates to amphotericin B, fluconazole, and nystatin was also investigated. **Results:** A total of 74 isolates of *Candida* were recovered from oral cavity of 61 cancer patients with oral candidiasis. The isolates included *Candida albicans* (*n* = 56; 75.5%), *Candida glabrata* (*n* = 4; 5.4%), *Candida krusei* (*n* = 5; 7%), *Candida tropical* (*n* = 7; 9.4%), and *Candida kefyr* (*n* = 2; 2.7%). A total (*n* = 72; 98.65%) of isolates were susceptible to nystatin, (*n* = 58; 78.4%) of them were susceptible to fluconazole, and (*n* = 8; 10.8%) of susceptible dose-dependent isolates were specified, (*n* = 46; 62.16%) of isolates were susceptible to amphotericin B. **Conclusion:** Finally, in addition to emphasis on topical nystatin application in the first stage of oral candidiasis in these patients, using alternative systemic drugs such as fluconazole and amphotericin B can be considered for the resistant candida isolates to nystatin.

Keywords: *Amphotericin B, Candida, chemotherapy, fluconazole, nystatin, oral candidiasis*

Introduction

Candida albicans, as the most important commensal opportunistic yeast, is usually present in mouth, digestive system, and urinary tract; however, it can cause infection when the host is vulnerable or immunocompromised. The infection can be superficial or on mucous membranes, which may infiltrate into blood and cause internal organ infection.[1-4] *Candida* species have been identified as human pathogens, including *C. albicans, Candida glabrata, Candida krusei, Candida parapsilosis, Candida tropicalis, Candida kefyr, Candida lusitaniae, Candida dubliniensis*, and *Candida guilliermondii*.[5,6] Oral candidiasis is common in patients undergoing chemotherapy. Over the years, the prevalence of oral candidiasis in chemotherapy patients has been reported to be 7.2%–52%, which is affected by various factors such as therapeutic intervention, type of cancer, and disease stage.[7-10] *Candida* species are opportunist pathogens and their pathogenicity is influenced by the inherent potential of the organism as well as host factors.[11,12] The main factors of aggressive candidiasis in a host are long-term hospitalization in intensive care unit and administration of broad-spectrum antibiotics or immunosuppressive drugs.[13,14]

Several studies around the world show that colonization rate and infection in mouth vary among different groups of patients under chemotherapy, and most studies have been conducted on a specific population of patients, for example, those with head and neck cancer.[7,15] There have been few studies on the incidence of oral candidiasis in different groups of cancer patients to compare its prevalence among these groups. Increasing resistance of *Candida* species (especially non-*Albicans* ones) to antifungal agents such as fluconazole and amphotericin B have been reported in some centers.[16,17] Widespread use of these drugs for prophylaxis of fungal diseases in cancer patients with neutropenia is the reason for such resistance.[18] In general, early diagnosis and identification

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of fungal pathogens for targeted antifungal therapy is essential among these patients.[19,20] On the other hand, nystatin in the form of pastille is used to treat or prevent oral candidiasis lesions. In one study, nystatin pastille proved more effective than fluconazole in the treatment of oral candidiasis.[21] Fluconazole is an antifungal drug administered orally or intravenously to treat various fungal diseases. Moreover, it is used to prevent infection in patients immunocompromised due to chemotherapy drugs (e.g., neutropenic patients), those with organ transplants, and premature neonates.[22] Amphotericin B is considered as the treatment of choice for systemic fungal infections; however, resistance to amphotericin B has been reported in some species such as *C. lusitaniae*, *C. guilliermondii*, and *C. kefyr*.[16,23] Resistance to antifungal drugs has a major impact on the development of fungal infections and is significantly associated with increasing dissatisfaction with treatment, mortality, and prolonged hospital stay. This research was conducted at Jundishapur Ahvaz University of Medical Sciences in Baqaee, Golestan, and Shafa hospitals with the aim of evaluating the frequency of oral candidiasis in a variety of patients undergoing chemotherapy to evaluate the risk factors associated with oral candidiasis among patients with various types of malignancies. Another goal of this research was to investigate antifungal susceptibility patterns of different *Candida* isolates against three antifungal agents of fluconazole, amphotericin B, and nystatin that are commonly used in these patients in Iran.

Materials and Methods

Isolation of samples

This study, which was approved by Ethics Committee of Jundishapur University of Medical Sciences (code number IR.AJUMS.REC.1397.699), was conducted on patients with solid tumors and hematologic malignancies who needed daily care. Patients with immunodeficiency, mental retardation, and those receiving antifungal agents over the past 4 weeks were excluded from the study.

Of 645 patients with cancer, samples were taken from patients with signs and symptoms such as inflammation/mucositis and/or presence of white plaques and feeling changes in their taste, leading to the detection of 61 oral candidiasis cases.[24-26] Specifications of patients including age, sex, chronic diseases, type of cancer, surgery, chemotherapy, radiotherapy, and oral dryness were determined. Sampling was done using two sterile swabs placed in tubes containing 0.5 ml sterilized distilled water, which were used to prepare direct smears and culture on CHROMagar Candida medium (CHROMagar Company, France). The culture medium was incubated at 35°C for 48 h. The sample was considered positive if there were growth of ≥ 10 colony-forming units (CFUs).[26-28]

Identification of samples using morphological methods

Primary diagnosis was based on the color of colony on CHROMagar Candida medium. Chlamydoconidia formation on corn Meal Agar-Tween 80 medium (Merck, Germany) and incubation at 25°C for 3 days distinguished *Candida albicans* from non-*Albicans* species.

Identification of isolates using molecular methods

After isolation, the samples were cultured on Sabouraud Dextrose Agar (SDA) medium (Merck, Germany) and the species were detected by polymerase chain reaction (PCR)-restriction fragment length polymorphism using *MSP1* enzyme for all isolates.[28] Then, to isolate *C. albicans* from *Candida dublinensis*, Duplex PCR was run directly on DNA extracted using two pairs of primers targeting ITS-1 and ITS-2 regions. The primer sequences were as follows.[29]

- CAL F: 5′ TGGTAAGGCGGGATCGCTT 3′
- CAL R: 5′ GGTCAAAGTTTGAAGATATAC 3′
- CDU F: 5′ AAACTTGTCACGAGATTATTTTT 3′
- CDU R: 5′ AAAGTTTGAAGAATAAAATGGC 3′.

Drug susceptibility

Drug susceptibility test was performed using microdilution according to CLSI guidelines. Minimal inhibitory concentrations (MICs) for fluconazole and nystatin were determined as the lowest drug concentration significantly reducing the growth of organisms compared with positive control well, which was identified as MIC well.

Clinical breakpoints for fluconazole and amphotericin B were determined based on CLSI M27-S3 and M27-A4 guidelines.[30,31] To determine MIC values for fluconazole, the drug was diluted in 0.125–64 range, so that ≤8 values were susceptible, 16–32 susceptible dose-dependent (SDD), and ≥64 resistant. For amphotericin B, drug dilution ranged 0.003–2, and ≥2 values were considered as resistant.[30] MIC for topical nystatin was determined based on CLSI M27-A2 instructions.[32] For nystatin, 0.003–2 μg/ml dilution of the drug was prepared, with ≤2 and >2 values showing susceptibility and resistance, respectively.[33]

Briefly, all *Candida* isolates were cultured on SDA medium. After incubation of isolates for 24 h at 30°C, a suspension of 0.5 McFarland standard was prepared from colonies, which was diluted with RPMI-1640 medium buffered with (3-(N-morpholino) propanesulfonic acid) to pH 7.0–7.2 at a rate of 1:100 CFU/ml. One hundred microliter of the mentioned dilution range of each drug was added to the wells. One hundred microliter of diluted yeast suspension was then added to each well, and two wells were used as a positive and negative control. Microplates were incubated at 35°C for 24–48 h, and the opacity of solution was assessed with the naked eye.[34,35]

Statistical analysis

Mann–Whitney nonparametric test in SPSS V23 (IBM, Armonk, NY, USA) with error rate of $P < 0.05$ was used to analyze the effects of variables of age, radiotherapy in addition to chemotherapy, surgery, and dryness of oral mucosa.

Results

Patients and organisms

Samples were taken from 286 patients (51.4% females and 48.9% males) with various malignancies, among whom 61 were diagnosed with oral candidiasis (57.4% females and 42.6% males) with a mean age of 26–87 years. The incidence of oral candidiasis was 14.8% higher in women than in men. Oral candidiasis was detected in types of cancers [Table 1].

Table 1: Characteristics of 61 cancer patients with oral candidiasis caused by *Candida* species

Parameter	Results
Median age, year (range)	59 (26-87)
Male sex, *n* (%)	26 (42.6)
Female sex, *n* (%)	35 (57.4)
Malignancy, *n* (%)	
Leukemia	13 (21.5)
Lung	10 (16.4)
Colon	10 (16.4)
Breast	9 (14.7)
Liver	4 (6.5)
Lymphoma	4 (6.5)
RCC	4 (6.5)
Uterine	3 (5)
Gastric	2 (3.3)
Throat	1 (1.6)
Bladder	1 (1.6)

RCC: Renal cell carcinoma

Out of these patients with 11 types of cancer, 74 different isolates of *Candida* were isolated, including 75.5% *Candida albicans* and 24.5% non-*albicans* species, namely *C. albicans* (*n* = 56). *glabrata* (*n* = 4), *C. krusei* (*n* = 5), *C. tropicalis* (*n* = 7) and *C. kefyr* (*n* = 2) [Table 2].

Risk factors

Oral candidiasis in patients undergoing chemotherapy and radiation therapy was 67.21% versus 32.7% in those with chemotherapy alone. Considering error rate of $P < 0.05$, the difference was statistically significant ($P = 0.045$). Furthermore, 68.85% of patients with oral candidiasis were diagnosed with dryness of mouth, which was significant in comparison with their patients having normal saliva ($P = 0.014$). In cancer patients with oral candidiasis, the effect of ≥60 years of age variable was 62.29%. On the other hand, more than half of the patients (63.93%) underwent surgery, for whom nonparametric analysis to determine the effect of age and surgery in the incidence of oral candidiasis was $P = 0.042$ and $P = 0.036$, respectively, indicating the significance of these values [Table 3].

Antifungal susceptibility

In this study, three antifungal agents of fluconazole, amphotericin B, and nystatin were used for 74 isolates of *Candida.*

After 24 h, 21.6% of isolates were resistant to fluconazole (MIC ≥64 μg/ml), including 20.3% and 1.4% of *C. albicans* and *C. kefyr* species, respectively. Ten and eighty percent of isolates were SDD (MIC = 16–23 μg/ml), 6.7%, 1.4%, and 2.7% of which were *C. albicans, C. tropicalis,* and *C. krusei*, respectively, and the remaining isolates (67.6%) were susceptible (MIC ≤8 μg/ml).

After incubation for 24–48 h, 37.8% of isolates were resistant to amphotericin B (≥2), including 36.4% and 1.4% of *C. alb*icans and *C. kefyr* species, respectively,

Table 2: *Candida* species isolated from different cancer patients

Cancer type	*Candida Species*					Total, *n* (%)
	Candida albicans, *n* (%)	*Candida glabrata*, *n* (%)	*Candida kefyr*, *n* (%)	*Candida krusei*, *n* (%)	*Candida tropicalis*, *n* (%)	
Leukemia	12 (16.2)	1 (1.4)	1 (1.4)	-	1 (1.4)	15 (20.4)
Lung	9 (12.1)	1 (1.4)	-	1 (1.4)	1 (1.4)	12 (16.2)
Colon	10 (13.5)	-	-	1 (1.4)	-	11 (14.9)
Breast	8 (10.8)	-	-	1 (1.4)	-	9 (12.1)
Liver	3 (4)	2 (2.7)	-	-	2 (2.7)	7 (9.4)
Lymphoma	3 (4)	-	-	1 (1.4)	2 (2.7)	6 (8.1)
RCC	4 (5.4)	-	-	-	1 (1.4)	5 (6.8)
Uterine	4 (5.4)	-	-	-	-	4 (5.4)
Gastric	2 (2.7)	-	-	1 (1.4)	-	3 (4)
Throat	1 (1.4)	-	-	-	-	1 (1.4)
Bladder	-	-	1 (1.4)	-	-	1 (1.4)
Total, *n* (%)	56 (75.5)	4 (5.4)	2 (2.7)	5 (7)	7 (9.4)	74 (100)

RCC: Renal cell carcinoma

and the remaining isolates (62.2%) were susceptible to fluconazole (<2 μg/ml).

After 24 h, 2.8% of isolates were resistant to nystatin (MIC >2 μg/ml), which was observed in only two isolates: One *C. albicans* and one *C. krusei*. The rest of the isolates (97.2%) were susceptible (MIC ≤2 μg/ml). MIC ranges, MIC50, MIC90, and geometric mean MIC were calculated for the three drugs [Table 4].

In this study, *C. albicans* isolates showed the highest resistance to amphotericin B (48.22%), followed by fluconazole and nystatin (26.79% and 1.35%, respectively), while the resistance of isolated non-*Albicans* species to fluconazole and amphotericin B was 5.55% and 5.56%, respectively, followed by nystatin (1.35%).

Dose-dependent species susceptible to fluconazole among *C. albicans* and non-*Albicans* isolates were 8.92% and 16.67%, respectively. The latter included *C. tropicalis* and *C. krusei* [Figure 1].

Table 3: Analysis of risk factors for oral candidiasis caused by *Candida species*

Risk factors	*n* (%)	*P*
Chemotherapy and radiotherapy	41 (67.21)	0.045
Dry mouth	42 (68.85)	0.014
Age ≥60 years old	38 (62.29)	0.042
Surgery	39 (63.93)	0.036

Discussion

Oral candidiasis is a common fungal infection in cancer patients and is currently recognized as the most prevalent fungal disease in humans.[36] Chemotherapy and radiotherapy in cancer patients can impair immune system cells and lead to neutropenia, resulting in colonization of *Candida* species in mucosal tissue, including oral cavity, which can pass into the blood and cause invasive candidiasis. Therefore, these patients are at high risk of invasive candidiasis.[27,37] The aim of this study was to investigate the frequency of various *Candida* species in different types of cancers and to evaluate the risk factors of increasing incidence of oral candidiasis in these patients. Antifungal sensitivity of the isolated species was investigated to achieve this goal. Our results showed oral candidiasis in 61 (21.3%) patients (57.4% women and 42.6% men) which was consistent with other studies. Schelenz *et al*. reported 18.9% rate of oral candidiasis in patients with cancer.[27] Bashir *et al*. reported 30% incidence of this disease among cancer patients.[19] In addition, Zollner-Schwetz *et al*. believed that the colonization of *Candida* species in the digestive

Table 4: *In vitro* antifungal susceptibilities of 74 clinical isolates against fluconazole, amphotericin B, and nystatin

Antifungal agents	Minimum inhibitory concentration (μg/mL)			
	MIC ranges	MIC 50%	MIC 90%	Geometric mean
Candida albicans (*n*=56)				
Fluconazole	64-0.06	0.5	64	19.73345
Amphotericin B	2-0.015	1	2	1.19563
Nystatin	4-1	1	2	1.26786
Candida glabrata (*n*=4)				
Fluconazole	4-0.12	4	4	3.03000
Amphotericin B	1-0.07	0.5	2	0.50175
Nystatin	2-1	2	2	1.75000
Candida tropicalis (*n*=7)				
Fluconazole	16-0.06	0.5	4	3.53429
Amphotericin B	1-0.015	0.12	0.5	0.31571
Nystatin	2-1	1	1	1.14286
Candida krusei (*n*=5)				
Fluconazole	64-0.25	8	32	12.0500
Amphotericin B	1-0.03	1	1	0.80600
Nystatin	4-1	2	4	2.20000
Candida kefyr (*n*=2)				
Fluconazole	64-2	.	.	.
Amphotericin B	2-0.25	.	.	.
Nystatin	1	.	.	.
All isolated yeasts (*n*=74)				
Fluconazole	64-0.06	1	64	16.43153
Amphotericin B	2-0.015	1	2	1.04750
Nystatin	4-1	1	2	1.3378

aMIC which inhibits 50% of *Candida* species isolates in test, bMIC which inhibits 90% of *Candida* species isolates in test, cGeometric mean MIC. MIC: Minimal inhibitory concentration

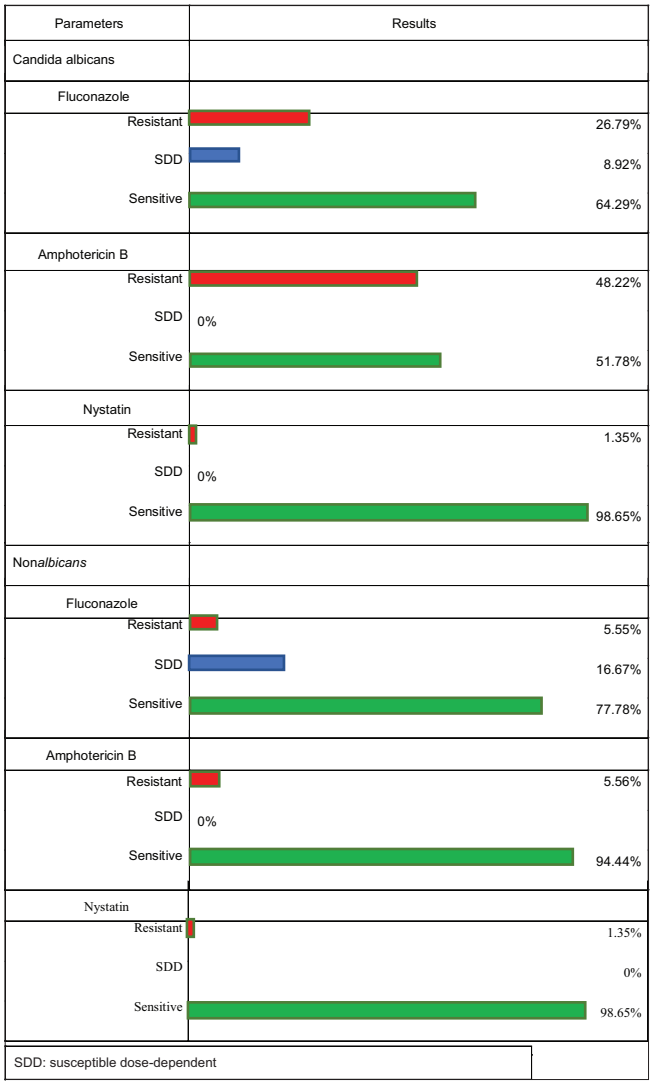

Figure 1: Antifungal susceptibility profiles of *Candida* isolates recovered from cases

tract of neutropenic patients was an important risk factor for invasive candidiasis and reported 48% prevalence of several *candida* species in oral and gut specimens of these patients.[38] In our study, oral candidiasis was significantly increased in patients undergoing chemotherapy and radiotherapy relative to those with chemotherapy alone. Dry mouth was more frequent among patients treated with both chemotherapy and radiation therapy. According to studies, radiation therapy leads to mucositis, dry mouth, and mucosal lesions that predispose to yeast infections. Moreover, the incidence of neutropenia due to long-term chemotherapy leads to the loss of mucosal layers and causes serious damage to immune system cells, which is associated with increasing risk of infection.[27] A similar trend has been observed in studies conducted around the world. Ramirez-Amador *et al*. found that radiotherapy caused hyposalivation, leading to colonization of *candida* yeasts on oral mucosa resulting in oral candidiasis.[39] Dahiya *et al*. reported increasing incidence of oral candidiasis in patients receiving radiotherapy along with chemotherapy.[28] Other risk factors investigated in this research were ≥60-year-old patients as well as those who underwent surgery, both of which significantly contributed to the incidence of oral candidiasis. In a research conducted on cancer patients and healthy subjects with oral candidiasis to evaluate the risk factors for candidiasis, it was found that ≥60-year-old cancer patients accounted for a significant share of patients with oral candidiasis. They also identified surgery as one of the risk factors for colonization of *Candida* species.[19] In this study, the highest frequency of oral candidiasis was observed in patients with leukemia as well as lung and colon cancers, and *C. albicans* was the most frequent causative agent of candidiasis in the mouth of patients with different types of cancers (75.5%). Many investigations have shown that *C. albicans* is the most typical cause of colonization as well as oral candidiasis among cancer patients.[27,28,40] Although *C. albicans* is recognized as the most frequent cause of colonization and candidiasis in patients, the increase in non-*Albicans* species such as *C. glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis* has been reported by several researchers in recent decades.[41,42] In Betts *et al*. study, the most common species of non-*Albicans candida* were *C. tropicalis* (37%) and *C. krusei* (11%). In the present study, the most prevalent non-*Albicans* species were *C. tropicalis* (9.4%), followed by *C. krusei* (7%), *C. glabrata* (5.4%), and *C. kefyr* (2.7%). Fluconazole is a triazole antifungal agent that is used as the first line of systemic therapy in patients undergoing chemotherapy. On the other hand, fluconazole and amphotericin B are widely used for prophylaxis of fungal infections in neutropenic patients with malignancies.[43,44] Infectious Diseases Society of America also recommends the use of nystatin in the form of suspension or pastille for the treatment of primary oral candidiasis and mentions easy access and convenience of this drug.[45] Reports have shown that the susceptibility of *C. albicans* and non-*Albicans* species such as *C. tropicalis, C. krusei*, and *C. glabrata* to fluconazole and amphotericin B has gradually decreased in the past decades.[46,47] In the present study, general resistance of *C. albicans* species to amphotericin B and fluconazole was detected, and only one *C. kefyr* isolate was found to be resistant to both drugs. In a research on patients undergoing chemotherapy, the resistance of *albicans* species to fluconazole was reported to be 47.2%, and the highest level of resistance was observed in *C. albicans*.[35] Increasing resistance of *C. albicans* species to fluconazole has been reported in several researches.[35,40] On the other hand, in an investigation conducted by Haddadi and colleagues, resistance to amphotericin B was reported in *C. albicans, C. krusei*, and *C. glabrata* species.[48] Therefore, Our results on *C. albicans* isolates were consistent with these studies, while *C. tropicalis, C. krusei*, and *C. glabrata* species isolated from patients were 94.4% and 77.78% susceptible to amphotericin B and

fluconazole, respectively. About 16.67% of dose-dependent species susceptible to fluconazole were *C. albicans, C. krusei*, and *C. kefyr*.

Overall, topical nystatin used in the form of suspension by patients had the best effect (98.65%) on *C. albicans* and non-*Albicans* species of *Candida in vitro*. Fluconazole and amphotericin B respectively had a stronger effect on *C. albicans* isolates while non-*Albicans* species were susceptible to amphotericin B and fluconazole, respectively. Consequently, the colonization of *Candida* species, which leads to fungal infections in hospitalized patients with various malignancies, is of high importance. In addition, multiple risk factors contribute to this problem in this vulnerable group. On the other hand, unchecked use of fluconazole as the first line of treatment in *C. albicans* species has led to the resistance of these species to amphotericin B. Given that cell membrane ergosterol in *C. albicans* isolates is the target of treatment by azole and polyene drugs, long-term treatment with fluconazole induces mutation in one or more alleles and eventual mutation in a number of genes, resulting in impaired synthesis and increasing the resistance of daughter cells. The change in ergosterol structure among refractory *C. albicans* species can also account for the resistance of these species to amphotericin B.[49] Based on the results of this study, it seems that among the three antifungal drugs used for the treatment of candidiasis over decades, application of topical nystatin is advisable in the first stage of treatment and even for the prevention of high-risk patients because most *Candida* isolates were susceptible to this drug, while the increasing resistance to fluconazole and amphotericin B demands novel antifungal drugs with a different function. Moreover, monitoring the epidemiological trend and assessment of drug susceptibility in various *Candida* species is suggested to achieve optimal drug response.

Conclusion

Data were shown that *C. albicans* is the most commonly identified species in oral candidiasis.

Chemotherapy, radiotherapy, surgery and ≥60-year-old patients significantly contributed to the incidence of oral candidiasis. Based on the results of this study, among the three antifungal drugs for the treatment of oral candidiasis, application of topical nystatin is advisable in the first stage of treatment and even for the prevention of high-risk patients because most *Candida* isolates were susceptible to this drug. In cases *Candida* species resistant to nystatin, using of systemic drugs fluconazole and amphotericin B is recommended.

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Conflicts of interest

There are no conflicts of interest.

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