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Research Article

Prevalence, Antifungal Susceptibility, and Biofilm-Forming Ability of Candida Species from Clinical Samples

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ABSTRACT

Introduction: Candida species, as opportunistic fungal pathogens, can form biofilms on biotic and abiotic surfaces, leading to resistance to current antifungals used in the treatment of candidiasis. This study aimed to identify Candida species from clinical samples and to assess antifungal susceptibility patterns and biofilm-forming ability.

Objectives: This study aimed to identify different Candida species from clinical samples and to assess the antifungal susceptibility patterns and biofilm formation ability.

Methods: In this study, 100 Candida isolates were obtained from various clinical samples (blood, urine, body fluids, etc.) by culturing. Antifungal susceptibility assays for Candida isolates were performed using the broth microdilution technique in accordance with the Clinical and Laboratory Standards Institute M27-A3/S4 guidelines. Biofilm formation was measured by the colorimetric microtiter method.

Results: Candida albicans (48%) was the most common Candida species identified, followed by C. glabrata (34%), C. dubliniensis (9%), C. tropicalis (5%), C. parapsilosis (1%), C. krusei (1%), and two mixed colonies were identified. The antifungal susceptibility pattern shows that Candida isolates exhibit varying susceptibilities. C. albicans was 100% susceptible to amphotericin B, whereas C. glabrata was 70.58% resistant to amphotericin B. The Candida parapsilopsis isolate was susceptible to all antifungals. According to the results, Candida isolates have a moderate ability to form biofilms.

Conclusions: Therefore, periodic monitoring of Candida species prevalence and antifungal susceptibility patterns necessitates selecting the appropriate therapeutic protocol, as well as the development of novel antifungal compounds and treatment strategies.

Keywords: Antifungal, Biofilm formation, Candidiasis, Drug resistance

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1. Introduction

Candidiasis is an opportunistic fungal infection caused by Candida species, which normally inhabit the mucosal membranes of the mouth, gastrointestinal tract, and genital tract. Among Candida spp., C. albicans was the most identified and prevalent, followed by C. glabrata, C. lusitaniae, C. parapsilosis, C. kefyr, C. famata, C. africana, and C. orthopsilosis, in descending order of frequency (1,2). On the other hand, Candida albicans is identified as the most prevalent causative agent of candidiasis. In recent years, the prevalence of non-albicans species has increased significantly. Risk factors for candidiasis include the use of broad-spectrum antibiotics or steroids, prolonged intensive care unit (ICU) stays, central venous catheter placement, organ transplantation, chemotherapy or radiotherapy, mechanical ventilation (invasive or noninvasive), and diabetes (3-5).

Nowadays, emerging resistant species to first-line antifungal drugs (such as echinocandins and azoles) are leading to therapeutic failure and poor patient outcomes (6). A limited number of effective antifungals, drug-drug interactions, and drug toxicity exacerbate this issue. On the other hand, approximately half of all nosocomial infections are linked to the use of indwelling medical devices. Candida spp can attach, adhere, and form biofilms on abiotic surfaces such as medical devices (1,7,8). The biofilm's structure, as an impermeable barrier against antifungals, reduces the sensitivity of Candida isolates to antifungals, especially azoles.

Given the increased prevalence of Candida infections and the varying risk factors across regions, assessing antifungal susceptibility patterns of Candida spp. and pathogenesis factors, such as biofilm formation, allows for predicting the prevalence of fungal infections and for providing appropriate treatment protocols (9,10).

2. Objectives

This study aimed to identify Candida species from clinical samples and to assess the antifungal susceptibility patterns and biofilm-forming ability.

3. Methods

3.1 Study Design

This study was cross-sectional and descriptive, conducted on 100 Candida isolates obtained from culturing various clinical samples (blood, urine, body fluids, etc.) that were identified as Candida spp. in the microbiology laboratory at Imam Reza Hospital, Birjand, Iran, from December 2020 to March 2022. The following formula was used to calculate the sample size based on the prevalence of Candida species, according to a previous study:

$N = (Z_{1-\alpha/2})^2 P(1-P)/ (\delta)^2$

$Z = 1.96, P=0.93, \alpha=0.05, \delta= 0.2$

The standard strains of Candida albicans (ATCC 10231), Candida glabrata (ATCC 2001), Candida Parapsilosis (ATCC 22019), and Candida tropicalis (ATCC 20962) were also used to identify Candida isolates.

3.2 Participants

Our study population consisted of Candida isolates from the mycology archive of the Faculty of Medicine, collected during a comprehensive screening program for the elderly in Birjand in the summer of 2022, with ethics code (IR.bums. REC.1398.257). The Candida isolates used in the present study were collected from oral swab cultures of elderly participants in the screening program and are kept in the mycology archive of the Faculty of Medicine.

These isolates were collected from individuals over 60 years of age during oral and dental examinations using a swab. The isolates that were considered positive in terms of colony count on culture medium in the microbiology laboratory (cfu/ml>10^5) were considered (regardless of clinical conditions).

Individual's refusal to participate in the research project.

3.3 Scales

3.3.1 Culture and Phenotypic Identification of Candida Isolates: The isolates were cultured on CHROM agar (Becton Dickinson, Germany) and incubated at 37°C for 24-48 h to identify phenotypic and morphological characteristics (11,12).

3.3.2 Molecular Identification: The previously described DNA extraction method was used with some modifications (13). In brief, fresh colonies were suspended in distilled water. Phenol and chloroform were added to the suspension, and the mixture was then centrifuged. The supernatant was transferred to a clean tube, ethanol was added, and the mixture was chilled. The sample was centrifuged to precipitate the DNA. Finally, the precipitated DNA was dissolved in distilled water, washed with ethanol, and stored at low temperature (14,15). The extracted DNA was used as a template in a polymerase chain reaction (PCR) reaction at an appropriate dilution

3.3.3 Polymerase Chain Reaction (PCR) Condition: In this study, PCR was performed using primers specific to Candida species, previously designed by Aratefar (2019) (13). The primers were obtained from the National Center for Biotechnology Information (NCBI) nucleotide database

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(https://www.ncbi.nlm.nih.gov/nucleotide/). Their sequences underwent Basic Local Alignment Search Tool analysis and confirmation on NCBI. Sinacolon Company (Tehran, Iran) was responsible for synthesizing and shipping the primers. The sequences of the primers used in this study were shown in Table 1. The Act1 gene is the internal control. The PCR was optimized in a final volume of 25µL as follows: the mixture was prepared with 10µL of master mix (DNA biotech, Iran), 0.25µL of each primer, 2.5µL of each DNA template (14,15). Using diethyl pyrocarbonate water, the final volume of the mixture was adjusted to 25 µL. The PCR thermal cycling condition was as follows: initial denaturation at 95°C for 5min, followed by 35 cycles of incubation at 95°C for 45sec, 58°C for 45sec, and 72°C for 45sec. and finally 72°C for 7min as final extension. The PCR was carried out on a thermal cycler (PEQLAB, Germany). Candida albicans (ATCC 10231), Candida glabrata (ATCC 2001), Candida tropicalis (ATCC 20962), and Candida Parapsilosis (ATCC 22019) were used as the quality control strains.

Using agarose gel (1.2%) and gel documentation system (Bio Rad, USA), amplicons were performed to identify the species by discrimination of amplicon sizes (Table 1).

Table 1: Primer of Genes Used in This Study

Primers Name*	Sequences (5'→3')	Amplicons	References
C.alb	F-5'AGATTATTGCCATGCCCTGAG3' R5'CCATGTCGAACGTAGCGTATGC3'	606bp	22
C.gal	F5'ACCGTGCTTGCCTCTACA3' R5'GACATCTGAGCCTCGTCTGA3'	212bp	22
C.tr	F5'AGAACAAGAAAACAGTGAAGCAA3' R5'CCATGTCGAACGTAGCGTATGC3	126bp	22
C.par	F5'TACACCAAGCGACTCAGC3' R5'ACCAGCTGCTTTGACTTG3'	490bp	22
C.kr	F5'GGCGTTGTCCATCCAATG3' R5'CAGGAGAATTGCTGTTCCC3'	1159bp	22
C.dub	F5'GTCGGACATATACCTCCAACTC3' R5'CCATGTCGAACGTAGCGTAT3'	718bp	22

*C.alb: C. albicans; C.gal: C. glabrata;C.tr:C. tropicalis;C.par:C. parapsilosis; C.kr: C. krusei;C.dub:C. dubliniensis.

3.3.4 Antifungal Susceptibility Assays: Antifungal susceptibility assays for Candida isolates were performed using the broth microdilution technique according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 recommendations. Identified isolates were tested against a suite of antifungals, including fluconazole, voriconazole, clotrimazole, itraconazole, and amphotericin B (provided by Sigma-Aldrich, Canada). The procedure was executed in 96-well microtiter plates. Antifungal dilutions were serially prepared, followed by the addition of 100 µL of RPMI 1640 medium containing incremental drug concentrations to the wells. Thereafter, wells were inoculated with 0.5 - 2.5 × 10^3 CFU/mL of yeast suspension. Sealed plates underwent incubation at 35 °C for 24-48 hours. The evaluated concentration spectra ranged from 64–0.125 µg/mL for fluconazole to 16–0.03 µg/mL for amphotericin B, itraconazole, voriconazole, and clotrimazole. Each Candida isolate was assayed in duplicate for each antifungal. Lastly, Candida albicans (ATCC 10231), Candida glabrata (ATCC 2001), Candida tropicalis (ATCC 20962), and Candida Parapsilosis (ATCC 22019) were used for quality control purposes. Finally, susceptibility outcomes were interpreted according to the CLSI M-27 (S4) standard (Table 2) (16).

Table 2: The Antifungal Susceptibility Interpretations (µg/mL) Described in Clinical and Laboratory Standards Institute (CLSI) Document M27-A3

Antifungal	Susceptibility Interpretations (µg/ml)		
	Susceptible	Dose dependent	Resistant
Fluconazole	2≥	4	8≤
Clotrimazole	0.12≥	-	1≤
Voriconazole	0.12≥	-	1≤
Itraconazole	0.12≥	-	1≤
Amphotericin B	2≥	-	2≤

3.3.5 Quantitative Evaluation of Biofilm Production: The colorimetric microtiter plate technique (17) was utilized to assess biofilm production. Specifically, yeast suspensions standardized to 0.5 McFarland units were dispensed into triplicate wells. The microplate was incubated at 32°C for 24 hours. Following this phase, wells were decanted and rinsed three times with 300 µL of isotonic saline. After ambient air-drying, biofilms were immobilized by adding 300 µL of methanol for 30

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minutes. Wells were then decanted and air-dried. Subsequently, 150 µL of 0.1% (w/v) crystal violet dye was applied, followed by a 5-minute incubation, then rinsed with distilled water at the faucet. Finally, 150 µL of 33% (v/v) glacial acetic acid was added per well, and absorbance readings were obtained using an ELISA spectrophotometer (Stat Fax, 2000; Germany) at 360 nm. Biofilm density was interpreted according to:

$OD \leq OD_{CN}$:without biofilm formation
$OD_C < OD \leq 2 \times OD_C$:week biofilm formation
$2 \times OD_C < OD \leq 4 \times OD_C$:moderate biofilm formation
$4 \times OD_C < OD$:strong biofilm formation

3.4 Data Collection

In this study, Candida species were determined and genotyped by PCR, and the sensitivity of Candida isolates to nystatin, amphotericin B, and fluconazole was assessed using the broth microdilution method according to the CLSI protocol. Demographic information, including age, gender, antibiotic use, diabetes, and occurrence of oral lesions, was collected through a checklist for each patient.

3.5 Data Analysis

The data were analyzed by descriptive statistics, chi-square test, and Spearman's correlation using SPSS software (version 16.0). In all statistical analyses, $P < 0.05$ was considered statistically significant.

3.6 Ethical consideration

In the present study, after obtaining approval from the Ethics Committee of Birjand University of Medical Sciences, Birjand, Iran (IR.BUMS.REC.1401.251), the patient demographic characteristics (age, history of infection, symptoms, diabetes, etc.) were collected. It was recorded on a checklist by the researcher using information from the patients' medical records.

4. Results

In this present study, the average age (±SD) of patients was 48.13±17.23 years. Table 3 presents the demographic characterization.

The results demonstrated that Candida isolates predominated, with C. albicans (48/100), followed by C. glabrata (34/100). Additionally, two cases were identified as mixed infections (C. albicans with C. glabrata) by multiplex PCR. Other species identified include C. krusei (1/100), C. tropicalis (5/100), C. parapsilosis (1/100), and C. dubliniensis (9/100) (Figure 1).

The pattern of antifungal susceptibility is as follows: C. albicans was 81.25% resistant to fluconazole and 100% susceptible to amphotericin B. C. glabrata had equal sensitivity to the two azole drugs, fluconazole and clotrimazole (26.47% and 73.52%, respectively), and, on the other hand, 70.58% were resistant to amphotericin B. The Candida parapsilopsis isolate was susceptible to all antifungals. C. krusei was resistant to all azoles but susceptible to amphotericin B. Both C. tropicalis and C. dubliniensis exhibited susceptibility patterns to the antifungals studied. Table 4 presents the antifungal pattern of Candida isolates. The statistical analysis showed significant indifference in antifungal susceptibility patterns between C. albicans and C. glabrata isolates ($P < 0.001$).

Figure 1: Frequency (%) of Candida spp. Isolates

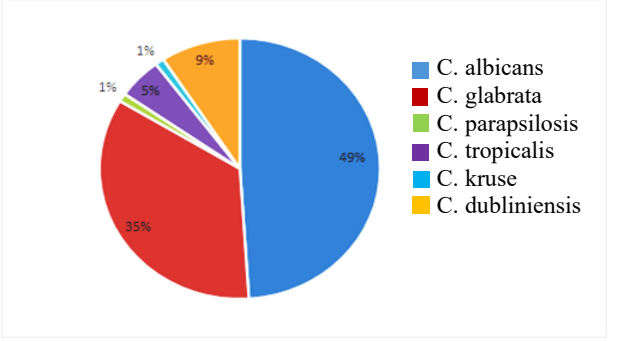


Table 3: Characterization and Distribution of Candida spp. Isolates

Demographic Factors		Frequency
Gender	Male	36
	Female	64
Inpatient department	Outpatient	48
	Surgery	9
	Infectious	1
	Obstetrics and Gynecology	5
	Internal Medicine (Male)	2
	Pediatrics	-
	ICU*	35
Sample	Urine	53
	Sputum	3
	BAL*	28
	Wound	12
	Vaginal secretion	1
	Body fluid	2
	Discharge from the hospital	1

*ICU: intensive care unit; ** BAL: Bronchoalveolar lavage

The results from examining biofilm formation in Candida isolates showed that the majority of isolates have a moderate ability to form biofilms (Table 5). According to the results, among Candida species, isolates of C. albicans, C. glabrata, and C. dubliniensis have shown the greatest ability to form biofilms at a moderate level. However, Strong biofilm formation

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was observed in isolates of C. albicans, C. glabrata, and Candida tropicalis. The chi-square test was used to compare biofilm-forming ability between C. albicans and C. glabrata isolates. Analysis of the results showed no significant difference in biofilm formation ability between C. albicans and C. glabrata isolates (P = 0.91).

Table 4: Antifungal Susceptibility Testing of Candida spp.

Species	Antifungal Agent	MIC (µg/mL)			Susceptibility No. (%)		P. value*
		Rang	50%	90%	Susceptible	Resistance	
C. albicans	Fluconazole	≤8-≥32	16	32	9(18.75)	34(81.25)	>0.001
N= 48	Clotrimazole	≤2-≥8	8	16	10(20.83)	38(79.16)	
	Voriconazole	≤0.5-≥8	8	16	15(31.25)	33(68.75)	
	Itraconazole	≤0.2-≥4	2	4	22(45.83)	26(45.16)	
	Amphotericin B	≤0.125-≥1	0.5	1	48(100)	-	
C. glabrata	Fluconazole	≤2-≥32	16	32	9(26.47)	25(73.52)	>0.001
N=34	Clotrimazole	≤2-≥32	8	16	9(26.47)	25(73.52)	
	Voriconazole	≤0.5-≥8	4	4	11(32.35)	23(67.64)	
	Itraconazole	≤0.2-≥4	2	4	16(47.05)	18(52.94)	
	Amphotericin B	2-≥8≤	4	2	10(29.41)	24(70.58)	
C. parapsilosis	Fluconazole	0.12	0.2	0.12	1(100)	-	-
N=1	Clotrimazole	0.06	0.12	0.06	1(100)	-	
	Voriconazole	0.12	0.5	0.06	1(100)	-	
	Itraconazole	0.06	0.2	0.06	1(100)	-	
	Amphotericin B	0.06	0.2	0.06	1(100)	-	
C. tropicalis	Fluconazole	≤2-≥32	2	8	2(40)	3(60)	1.00
N=5	Clotrimazole	≤2-≥32	2	4	2(40)	3(60)	
	Voriconazole	≤0.5-≥4	1	2	2(40)	3(60)	
	Itraconazole	≤0.2-≥4	1	2	2(40)	3(60)	
	Amphotericin B	2-≥4≤	0.5	0.12	2(40)	3(60)	
C. krusei	Fluconazole	4	4	8	-	1(100)	
N=1	Clotrimazole	2	2	4	-	1(100)	
	Voriconazole	1	1	2	-	1(100)	-
	Itraconazole	0.5	0.5	1	-	1(100)	
	Amphotericin B	0.5	0.5	0.25	1(100)	-	
C. dubliansis	Fluconazole	≤2-≥32	16	32	3(33.33)	6(66.66)	1.00
N=9	Clotrimazole	≤2-≥32	8	32	3(33.33)	6(66.66)	
	Voriconazole	≤2-≥8	2	4	3(33.33)	6(66.66)	
	Itraconazole	≤2-≥8	2	4	3(33.33)	6(66.66)	
	Amphotericin B	2-≥8≤	2	4	3(33.33)	6(66.66)	

* Using Chi-square and t-test.

5. Discussion

To establish the frequency and species distribution of invasive infections in Birjand (South Khorasan, Iran), the results showed that the main Candida species identified from clinical samples was C. albicans, followed by C. glabrata, which had a significantly higher prevalence among these isolates. The antifungal susceptibility pattern shows that Candida isolates exhibit varying susceptibilities. Therefore, C. albicans isolates are susceptible to amphotericin B (about 100%), whereas C. glabrata and C. tropicalis showed

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significant resistance to amphotericin B (Table 4). In the study conducted by Khadka et al. (2020), a total of 100 Candida species were identified, with C. albicans as the predominant species, followed by C. tropicalis (18). Their findings showed reduced antifungal susceptibility to clotrimazole, fluconazole, and miconazole. However, in a study by Seyoum et al. (2020), the predominant species reported was C. albicans, and the highest antifungal susceptibility was observed with voriconazole (19). In another study by Hosseinpour et al. (2019), conducted on 156 urine samples, the most common Candida spp. identified was C. albicans, followed by C. tropicalis, and all isolates were susceptible to amphotericin B (20).

In recent decades, Candida species, particularly C. albicans, have emerged as formidable nosocomial pathogens. Differentiation of Candida spp. predominantly hinges upon biochemical profiling, germ tube induction assays, and macroscopic colony characteristics across varied culture substrates. Employing CHROMagar for Candida speciation, predicated on chromatic disparity, offers an expeditious, pragmatic, and robust modality for discerning clinically pertinent isolates compared with arduous traditional methodologies (21). In resource-limited developing economies, CHROMagar is a pragmatic phenotypic diagnostic modality superseding molecular methods. Notwithstanding, extensive empirical studies demonstrate an elevated prevalence of isolation of non-albicans Candida (NAC) taxa relative to C. albicans, thereby heralding the ascendance of NAC as consequential etiological agents. On the other hand, an increase in resistance to the azole group, as the first-line antifungal therapy, among Candida spp. has, in this regard, rendered the therapeutic strategy with azoles defective and recurrent (22,23). Due to the widespread use of azole for fluconazole, resistance to it has increased and is of serious concern. Azoles are cornerstone therapeutics for mycotic infections, exerting their efficacy by suppressing ergosterol biosynthetic pathways in the phospholipid constituents of the fungal plasmalemma. Alternatively, amphotericin B exerts antifungal activity by depleting ergosterol from membrane lipid raft domains, precipitating cytolysis, concomitant with the accumulation of reactive oxygen intermediates, thereby fostering oxidative injury, apoptotic cascades, and lethal cytotoxicity (24,25). Given the rising resistance to commonly used antifungals for treating fungal infections, especially candidiasis, we are concerned about the obsolescence of this class of antifungals. Therefore, the advance of a new generation of antifungal compounds is a research priority.

In this study, the assessment of biofilm formation showed that most Candida species can moderate it. However, no significant relationship was observed among the various species. In a study by Marak et al. (2020), C. albicans was reported as the most main Candida species; they acknowledged that the majority of isolates could moderate biofilm formation (25). According to the study results, the capability of Candida isolates to form biofilms is a clear reason for the emergence of resistance to each of the drugs studied. Understanding the mechanisms of resistance and the biological drivers of its emergence is essential for advancing therapeutics, enhancing diagnostics, and implementing interventions to overcome and prevent resistance.

Table 5: Evaluation of Biofilm Formation by Various Candida spp.

Candida spp.	Biofilm Formation Type (%)		
	Week No. (%)	Moderate No. (%)	Strong No. (%)
C. albicans (n=48)	11(18.75)	28(54.32)	9(18.75)
C. glabrata (n=34)	7(20.58)	23(67.64)	6(17.64)
C. parapsilosis (n=1)	0	1(100)	0
C. tropicalis (n=5)	0	2(40.00)	3(60.00)
C. kruse (n=1)	0	1(100)	0
C. dubliniensis (n= 9)	0	9(100)	0

Therefore, the challenges of treating Candida infections are not only related to antifungal treatment protocols but also to eliminating pathogenic factors of the microorganism, including biofilm formation, and to developing rapid and accurate diagnostic methods. Consequently, the limited treatment options and the increasing prevalence of antifungal-resistant strains necessitate the development of novel antifungal compounds and novel treatment strategies. Therefore, it is suggested that, while periodically monitoring the prevalence of nosocomial fungal infections, future studies should focus on the design and synthesis of naturally derived antifungal agents with fewer side effects.

5.1 Conclusion

According to the present study, periodic and regional surveys of the prevalence of fungal infections, especially Candida infections, and monitoring the antifungal susceptibility patterns of identified isolates can be beneficial for treatment, selecting appropriate therapeutic protocols, and preventing the emergence of strains resistant to common antifungals. Hence, this requires the use of accurate laboratory techniques to detect the fungal agent causing the infection early.

Statements

Authors' Contribution: All authors contributed to the study design, FN and MHN. FN was involved in

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the initial idea of the study. FN, MM, and MHN participated in the data collection process. FN was involved in the data analysis. MM, MHN, and FN contributed to the drafting of the manuscript. All authors have read and approved the content of the manuscript.

Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication. The data are not publicly available.

Ethical Approval: The Ethics Committee of Birjand University of Medical Sciences approved the research protocol (IR.BUMS.REC.1401.251)

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Conflict of Interest: The authors declared no conflicts of interest.

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