ORIGINAL ARTICLE

GENOTYPE-BASED ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA ALBICANS IN ICU PATIENTS AT A TERTIARY CARE SETTING

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ABSTRACT

Objective: Oral Candidiasis is one of the most common opportunistic infections of Candida albicans. Treatment of C. albicans is becoming a challenge due to the acquired resistance to antifungal drugs. Resistance to antifungal drugs may vary among the C. albicans genotypes. The objective of the study was to find the genotype-based antifungal susceptibility of C. albicans isolated from oral candidiasis of ICU patients.

Material & Methods: This cross-sectional study was conducted at Ayub Medical Complex and Khyber Medical University Peshawar, Pakistan. Sampling was done from ICU patients, and identification of Candida species was carried out by inoculating samples on Sabouraud's and CHROM agar (Oxoid Germany), by incubation at 37oC for 48 hours. The pathogenicity of C. albicans was confirmed by the formation of a germ tube. The antifungal sensitivity of C. albicans was determined on Moller Hinton agar according to the CLSI 2022 guidelines. Sensitivity to Fluconazole, Voriconazole, Clotrimazole, Nystatin, Amphotericin, (Oxoid) was assessed by disk diffusion method. Furthermore, the genotype identification was done by PCR through amplification of 25SrDNA amplification using specific primers.

Results: Out of 260 samples, 145 (55.5%) samples were of candida species. Among the positive samples prevalence of C. albicans was predominant at 111(76.5 %) followed by C. tropicalis 15(10.34%), C. kruzei 11(7.58%), and C. glabrata 8(5.5%) high incidence was reported in females (59.4%) compared to males (49.55%). The most susceptible age groups were 61-70 years (27%) and 1- 10 years (20.70%), while the lowest incident was reported in the age group 20-30 years (2.70%). Among C. albicans genotype-A was more prevalent 65(58.5%) followed by genotype-B 33(28.82%), and genotype-C 14(12.6%). Overall, high resistance was reported against Voriconazole (70.2%) followed by Miconazole (61.2%), and fluconazole (58.5%) while comparatively lowest resistance was reported against clotrimazole (31.53%). Nystatin and Amphotericin were found effective against oral candidiasis in ICU patients with a resistance of 9.9% and 11.71%, respectively. Genotype-A of C. albicans was found to be highly resistant to the azole group of antifungal drugs among all three genotypes.

Conclusion: Candida infection is common among ICU patients, especially at extremes of ages due to immune-compromised status. Overall, resistance to the Azole group of drugs is very high, however, the polyene group of drugs was found to be effective. The prevalence of Genotype-A of C. albicans was predominant in ICU patients with high resistance to Azole antifungal drugs.

Keywords: Azole antifungal drugs. C. albicans. Genotype, Oral candidiasis, Polyenes

This article may be cited as: Akhtar M, Asghar S, Jehangir F, Khurshid S, Jadoon Z, Khwaj A, Ullah I. Genotype-Based Antifungal Susceptibility Of Candida Albicans In Icu Patients At A Tertiary Care Setting. J Med Sci 2025 January - March;33(1):31-36

INTRODUCTION

Oral mucosa is susceptible to infection by bacterial, viral, parasitic, and fungal microbes. [1] Fungi cover a small portion of the oral microbiome, of which the major contributor is C. albicans, which can become pathogenic in an immune-compromised state and underlying diseases. [2, 3] The prevalence of C. albicans is very high, ranging from 17- 80%. Other candida species include C. glabrata, C. krusei, C. tropicalis, and C. parapsilosis have also been reported in different studies (4). C. albicans takes advantage of the impaired immune system, which helps the fungi to proliferate in the oral mucosa and form a characteristic white plaque in the oral cavity (5). Oral candidiasis is the most frequent form of infection, with predisposing factors including prolonged hospitalization, use of broad-spectrum antibiotics, poor oral hygiene, Diabetes Mellitus, and other immune-compromised states. [6]

The pathogenicity of C. albicans is linked to the host predisposing factors, as well as microbial factors such as phenotypic switching, biofilm formation, secretion of hydrolyzing enzyme, and drug resistance(7). Phenotyp-

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Date Received: 21/08/2024
Date Revised: 21/01/2025
Date Accepted: 18/02/2025

ic switching from yeast to pseudohyphae helps Candida albicans evade the immune response, invade the tissue, cause systemic infection, and facilitate the formation of biofilm, which further enhances the survival of the candida. [8] The hydrolytic and proteolytic enzyme of C. albicans helps in tissue disintegration and facilitates its invasion into the oral mucosal layer. The pathogenic strain of C. albicans produces hemolysin enzymes; besides this, they also produce more than ten different types of proteinase enzymes with diverse pathological functions. [6] The drug resistance in C. albicans is mainly associated with biofilm formation and efflux pumps, which makes the treatment option more difficult. [9]

Azole antifungal agents, particularly fluconazole, have been used as a prophylaxis agent or treatment of oral candidiasis in patients with underlying disease. Continued exposure to azoles in the oral cavity leads to the development of resistance in the candida species due to the selective pressure of the drugs, which is a high point of concern for clinicians. [10] The resistance is achieved either in the modification of the enzyme the cytochrome P-450 lanosterol, 14-α-demethylase which is mediated by chromosomal gene ERG11, or the activated efflux pump that expels the drugs out of the cell cytoplasm makes these drugs less effective against the oral candidiasis infection. [11] Furthermore, Nystatin is considered highly effective against oral candidiasis. Its widespread use in oral candidiasis led to the emergence of nystatin resistance among the candida species. [12] Similarly, resistance to amphotericin B has also been on the rise. All of these conditions make the infection worse, which eventually leads to a high mortality rate due to candidiasis. [13]

Molecular typing for candida is important in epidemiological studies as different strains exhibit a diverse range of pathogenicity and drug resistance patterns. [14] So for PCR-based, various typing methods have been used for typing the Candida species, such as multi-locus sequence typing (MLST), pulse field gel electrophoresis (PFGE), and restriction fragment length polymorphism (RFLP), while several studies used the ABC genotyping method for C. albicans in clinical samples. [15, 16] The ABC genotyping is based on the presence, and absence of transposable intron in the 25Sr DNA gene, which is after PCR confirmed on the gel electrophoresis, thus classifying C. albicans to genotype A, B, C, D, and E. [17] Studies evidence that genotype A of C. albicans is more prevalent in oral, vaginal, and skin infections, furthermore, genotype A is comparatively more resistant to antifungal agents. [18]

The present study is designed to find a genotype-based antifungal susceptibility pattern of Candida albicans among ICU patients having oral candidiasis.

MATERIAL AND METHODS

This cross-sectional study was conducted at Ayub Medical Complex Abbottabad and Khyber Medical University Peshawar. A total of 261 samples were collected from ICU patients with suspected oral candidiasis at Ayub Teaching Hospital, Abbottabad. Ethical approval for the study was obtained from the ethical review board of Khyber Medical University and the concerned hospital. Sample swabs were then transported aseptically to the microbiology lab of the Institute of Pathology and Diagnostics Medicine, Khyber Medical University (IPDM-KMU) Peshawar for culture sensitivity and molecular identification.

ISOLATION AND CONFIRMATION OF THE C. ALBICANS

Samples were cultured on Sabouraud's Dextrose agar (Oxoid Germany) and incubated at 37°C for 48 hours. After incubation, pinpoint white colonies were picked from the SDA agar for further confirmation of C. albicans. The colonies were tested for germ tube formation and growth pattern on CHROM agar (Paris France company).

ANTIFUNGAL SENSITIVITY OF C. ALBICANS

Antifungal sensitivity of C. albicans was performed by the disc diffusion method according to the guideline of Clinical Laboratory Standard Institute (CLSI) documents M44-A2. Six standard antifungal drugs were included in this study that is Amphotericin B (20ug/disc), Nystatin (50ug/disc), Fluconazole (25ug/disc), Clotrimazole (10ug/disc), Voriconazole (15ug/disc), and Miconazole (10ug/disc) were obtained from Oxoid (Turkey). A suspension of C. albicans was prepared 10^6 colonies/ml and was spread on the surface of Muller Hinton Agar (Oxoid Germany). The antifungal discs were placed on the agar plate with equal distance and incubated at 37°C for 48 hours. After incubation, the zone of Inhibition was measured in millimeters.

DNA EXTRACTION

The Genomic DNA of confirmed C. albicans was obtained by using the Thermo Fisher Scientific, Yeast Genomic DNA extraction kit (Cat no.: 78870), following the manufacturer's guidelines. The integrity of the genomic DNA was checked on 1% agarose gel, while its concentration was checked on Spectrophotometric Nanodrop (Thermo-Fisher).

PCR AMPLIFICATION OF 25S RIBOSOMAL ENCODED GENE

For the C. albicans genotype, a 25S ribosome-encoded gene was selected and already published, and optimized primers were used(19). The primer sequence and condition mentioned as forward primer -ATAAGGGAAGTCGGCAAAATAGATCCGTAA, and Revers primer R-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT, the primer was further confirmed through the primer blast, after

fulling the criteria, 20ul reaction mixture volume was prepared that contain 10ul PCR master Mix (cyber green thermo scientific), 1ul forward and 1ul Reverse primer, 2ul genomic DNA, and 6ul PCR water (thermo scientific). PCR conditions were initial denaturation of 95°C for 5 minutes, second denaturation at 95 °C for 35second, with 35 cycles then annealing at 60 °C for 45 seconds followed by primer extension at 72 °C for 45 sec and then last extension at 72 °C for 3 minutes and a holding temperature of 4 °C for 10minutes. The PCR product was confirmed based on its product size on 1.5% agarose gel electrophoresis with a 100bp ladder (Thermo Fisher).

RESULTS

Out of a total of 261 suspected oral samples collected from ICU patients, 145(55) were cultured positive for candida species on Sabouraud's dextrose agar and Chrom agar. The most prevalent species was C. albicans 111 (76.5%) followed by C. tropicalis 15 (10.34%) C. Kruzi 11(7.58%), and C. glabrata 8(5.5%). Samples were taken from patients of both genders; the incidence of candida albicans was found higher in females 59.4% (66) compared to their counterpart males 49.5% (55).

Overall, the prevalence of C. albicans was found higher in the aged group 60-70 years, 17.1% (19), followed by 1-10 years 13.5% (15) while the lowest incident was reported in the aged group 20 to 30 years below Table 1 explains the age and gender-wise prevalence of C. albicans.

Table No 1 Antifungal susceptibility profile of candida albicans

Antifungal drug	Resistance n (%)	Sensitivity n (%)	SSD n (%)
Fluconazole	65 (85.5)	40 (36.1)	6 (5.4)
Clotrimazole	35 (31.53)	67 (60.36)	9 (8.1)
Miconazole	68 (61.2)	33 (29.7)	10 (9)
Voriconazole	78 (70.2)	25 (22.5)	8 (7.2)
Amphotericin	13 (11.71)	90 (81)	8 (7.2)
Nystatin	11 (9.9)	94 (84.6)	6 (5.4)

PREVALENCE OF C. ALBICANS GENOTYPES IN ORAL CANDIDIASIS

The prevalence of C. albicans different genotypes in oral candidiasis was identified by using a primer that targeted the 25SrDNA gene. In our studies, a high prevalence of C. albicans genotype A, 65 (58.55%), was found in the oral candidiasis, followed by genotype B, 33 (28.82%), and genotype C, 14 (12.6%). The below picture shows the result confirmation of all three genotypes on 1.5% agarose gel.

ANTIFUNGAL SENSITIVITY

Candida albicans susceptibility was checked. The antifungal drugs used are Fluconazole, Clotrimazole, Miconazole, and Voriconazole of the Azole group, while

Table No 3.2: Genotype, antifungal sensitivity profile of C. albicans; R stands for Resistance, S: Sensitive, SDD: Susceptible dose-dependent

Drugs	Genotyeps			
	Pattern	Genotype A	Genotype B	Genotype C
Fluconazole	R	47(34%)	16(14.41%)	2(1.8%)
	S	16(14.41%)	13(11.71%)	11(9.9%)
	SDD	2(1.8%)	3(2.7%)	1(0.9%)
Clotrimazole	R	25(22.52%)	7(6.3%)	3(2.7%)
	S	36(32.43%)	20(18.01%)	11(9.9%)
	SDD	4(3.6%)	5(4.5%)	0(0%)
Miconazole	R	43(38.73%)	20(10.01%)	5(4.5%)
	S	16(14.41%)	10(9%)	7(6.3%)
	SDD	6(5.4%)	2(1.8%)	1.8%(2)
Voriconazole	R	50(45%)	22(19.81)	6(5.4%)
	S	12(10.81%)	6(5.4%)	7(6.3%)
	SDD	3(2.7%)	4(3.6%)	1(0.9%)
Amphoteracine	R	13(11.71%)	0(0%)	0(0%)
	S	51(45.94%)	28(25.22%)	13(11.71%)
	SDD	1(0.9%)	4(3.6%)	3(2.7%)
Nilstaine	R	11(9.9%)	0(0%)	0(0%)
	S	52(46.84%)	32(28.82%)	12(10.8%)
	SDD	2(1.8%)	2(1.8%)	2(1.8%)

Amphotericin and Nystatin are from the polyene group. Among azoles, high resistance was reported against Voriconazole at 70.2% (78), followed by miconazole at 61.2% (68), and fluconazole at 58.5% (65), while the lowest was against clotrimazole at 31.53% (35). In Polyene, the lowest resistance was reported against both amphotericin 11.71 % (13) and nystatin 9.9 % (11). Comparatively, polyene is safe in the sense of resistance, as resistance to both of the drugs is within the WHO recommendation range. C. albicans collectively antifungal sensitivity resistance, and susceptible dose dependence is shown in table 2.

Out of 111 samples, 9% (10) samples were showing an MDR pattern of drug resistance they were resistant to all the tested antifungal drugs used in this research study.

R*- Resistance, S*- Sensitive, SDD*- Susceptible dose-dependent

GENOTYPE ANTIFUNGAL SENSITIVITY PROFILE OF C. ALBICANS.

The antifungal sensitivity of each genotype was determined. genotype A was found highly resistance to all of the azole antifungal agents, with lower resistance to the polyene agent, followed by genotypes B, and C, both show up to some extent resistance to the azole group while highly sensitive to the polyene antifungal agents. among azole group, clotrimazole was comparatively found effective against all genotype of the C. albicans in oral candidiasis patients. The below table explains % wise prevalence of each tested drug to all of the C. albicans genotypes.

ALBICANS GENOTYPES.

DISCUSSION

Recently, the incidence of fungal infection, particularly that of C. albicans, increased in patients with underlying diseases, which eventually raised the mortality rate due to fungal infection. Besides C. albicans' high morbidity, other fungal etiology have been in an uprising trend, which is an alarming situation as most of them are highly resistant to currently available antifungal agents. [20] For efficient treatment, rapid diagnosis and correct identification of fungal species are crucial to the timely management of the infection. In the case of fungal infection, commonly used phenotypic methods are time-consuming and cannot differentiate among genotypes, even among species. Therefore, the Rapid molecular method offers accurate results with high discriminate power. [21] The molecular methods used for the identification of Candida species are RFLP, PFGE, and MLST. The genotype of C. albicans is differentiated based on the presence or absence of transposable intron in the 25SrDNA gene. The PCR-based amplification of the 25SrDNA gene differentiates C. albicans into 5 different genotypes, A to E genotypes. The most commonly reported are A to C genotypes, while D and E are rare genotypes (19). In the current study, both Phenotypic and genotypic methods were used for the identification of C. albicans. In phenotypic confirmation, the growth pattern was checked on SDA, CHROMagar, and the formation of germ tube. CHROMagar, although efficient in the differentiation of Candida species, cannot differentiate all of its species. Therefore, molecular methods are required. Based on the characteristic growth color of Candida species on CHROMagar, a high prevalence of C. albicans was reported at 76.5% (111) followed by C. tropicalis at 10.35%, C. kruzi at 7.58%, and C glabrata 5.5%, a previous study from Pakistan also report likewise pattern of Candida species prevalence (22). Similar findings were reported in China, where the prevalence of C. albicans was in the top 75.35%, followed by C. tropicalis at 15%, then Kruzi 2.7%, while C. glabrata that has recently risen having a 2.4% prevalence, The data was from four years, in the last years the prevalence of glabrata increased in their study. Another study from India also reports a similar pattern of Candida species prevalence in patients with oral squamous cell carcinoma where C. albicans was leading, followed by C. tropicalis, Kruzi, and glabrata. [23]

In the current study, samples were taken from both genders aged 1-80 years old. Patients were categorized based on age group and gender. The prevalence of candidiasis was found to be higher in females (59.4%) in all age groups compared to males (49.5%), which is in concurrence with a previous study conducted in Karachi, Pakistan. [22] Similar findings were also reported from China and India, where oral candidiasis was found to be more common in females compared to their male counterparts (4, 22). The high prevalence of oral candidiasis in females may be due to hormonal changes, poor oral hygiene, the low immune status of females, and the excess use of oral antibiotics in developing countries like Pakistan and India.

Most of the earlier studies on oral candidiasis use only phenotypic way for the identification of Candida species, but here we used both phenotypic and genotypic methods even to explore different genotypes of C. albicans while targeting the 25SrDNA gene of the C. albicans. Based on 25SrDNA gene polymorphism, C. albicans are classified into different genotypes A to E (17). In the existing study, the prevalence of C. albicans genotype A (58.55%) was found, followed by genotype B (28.82%) and genotype C (12.6%). Although a study from Iran reports a high prevalence of genotype A in oral candidiasis, it was followed by genotype C rather than B. Additionally, they also report genotype D in their study. [24] In contrast to our finding, a study in Thailand reported a high prevalence of genotype B in oral candidiasis followed by genotypes A, C, and D. [25]

In this study, six antifungal agents of two classes (azole and polyene) were used. Collectively, C. albicans

was found to have high resistance to the azole group, particularly to the Voriconazole, miconazole, and fluconazole, while sensitive to the polyene antifungal agent, the nystatin, followed by amphotericin B (Table 2). This finding coincides with a study from Iran where the resistance ratio of C. albicans was found to be higher in the azole group, while the polyene group showed 100% sensitivity. [26] The current findings in China, the rate of resistance in C. albicans to azole in oral candidiasis is comparatively lower although they mentioned upraised resistance to azole, while the first-line treatment in oral candidiasis the topical nystatin was found highly effective (27). To the best of our knowledge, no such data is available that mentions the genotype-based antifungal sensitivity profile of C. albicans in oral candidiasis. In our study, genotype A was found to be highly resistant to azole, even though some strains of it show resistance to nystatin and amphotericin, while genotypes B and C were comparatively found less resistant to azole while highly sensitive to nystatin and amphotericin (Table 3). The rise of resistance to azoles, particularly to fluconazole and Voriconazole, is an alarming stage for clinicians. Numerous studies have been conducted exploring various aspects and mechanisms of azole resistance in C. albicans. Our result revealed an increased rate of resistance to azoles. Our study population was of ICU patients with already weakened immune status, and already exposed to a variety of antibiotics and antifungal agents, It may be possible that the organism acquired resistance via horizontal gene transfer, or due to selective antifungal pressure on the candida species in the hospital environments that leads to the development of drugs resistance among them.

This study has certain limitations. Firstly, the small size of the samples and short duration, including one center, we used only PCR-based identification of the C. albicans genotype, as clearly sequencing will further explore the new strain and will possibly help in rapid diagnosis.

CONCLUSION

Oral candidiasis is an opportunistic infection. Its occurrence is an alarming sign of immune-deficient status, as it often causes systemic infection in critically ill patients. Treating such patients is difficult if caused by drug resistance strain. In this study, the overall prevalence of C. albicans was found to be 55.5%. Females were found more susceptible to candidiasis, particularly in the age group 60-70 years, genotype A of C. albicans was mostly reported one with higher resistivity to both azole and polyene antifungal agents. Further studies are needed in this contest to design rapid diagnostic tests and empirical treatment strategies, as well as to manage oral candidiasis in critically ill patients.

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CONFLICT OF INTEREST: Authors declare no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE: NIL

Authors Contribution:

Following authors have made substantial contributions to the manuscript as under

Authors	Conceived & designed the analysis	Collected the data	Contributed data or analysis tools	Performed the analysis	Wrote the paper	Other contribution
Akhtar M	✓	✓	✓	✓	✓	✓
Asghar S	✓	✓	×	✓	✓	×
Jehangir F	×	✓	×	×	✓	×
Khurshid S	✓	✓	✓	×	✓	✓
Jadoon Z	✓	✓	×	✓	✓	×
Khwaj A	×	✓	×	×	✓	×
Ullah I	✓	✓	✓	✓	✓	✓

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethical Approval:

This Manuscript was approved by the Ethical Review Board of Khyber Medical University. Vide No. KMU/IPDM/IEC/2023 19. Dated: 31 07 2023