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*Original Article*

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# *Phenotypic identification of Candida species and relative expression of CDR1 gene in fluconazole sensitive and resistant Candida albicans*

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## ABSTRACT

**BACKGROUND & OBJECTIVE:** Candida species belong to the genus Candida. It is a commensal yeast in healthy people and may cause life-threatening infections in people with impaired immune systems.The objective of the current study was to observe the frequency of Candida species, their resistance against fluconazole, and the upregulation of the candida drug-resistant gene in both fluconazole-sensitive and resistant isolates.

**METHODOLOGY:** A comparative study with 206 Candida species was collected on Sabouraud Dextrose agar from Jinnah Hospital from 2016-2018. The isolates were re-confirmed by using various microbiological techniques.APIC 20AUX made identification up to the species level. Antifungal susceptibility testing of Candida species against fluconazole was determined by the broth microdilution method. Real-time PCR was carried out on the sensitive and resistant isolates to observe the part of the CDR1 gene against fluconazole resistance.

**RESULTS:**With a frequency of 66 (32.0%), C. tropicals was the most frequent species, followed by C. albicans 41(19.9%), C. glabrata 24 (11.7%), C. lusitaniae 14 (6.8%) and several less frequent species. Testing for antifungal susceptibility showed that 80% of Candida species were sensitive to fluconazole. According to gene expression data, the CDR1 gene significantly contributes to fluconazole resistance in Candida isolates

**CONCLUSION:**The most prevalent isolate among all isolated species of Candida was C. tropicalis. A chi-square test was done, and a P-value of 0.418 showed that there was no association between gender and Candida species. All Candida species were susceptible to fluconazole as first-line treatment. Anova test was applied to compare the mean Attitude, Practice.

**KEYWORDS:** Candida Drug Resistant Gene 1, Polymerase Chain Reaction.

## INTRODUCTION

The genus Candida consists of a heterogeneous group of organisms, and it is known that more than 17 different Candida species are responsible for the etiology of human infections. Candida albicans stands out as the predominant pathogen linked to significant fungal infections, responsible for approximately 90% of all reported cases among the various candida species [1].

Epidemiological studies conducted in several regions, including the Middle East, Europe, and the United States, consistently demonstrate that fungal infections are predominantly caused by C. albicans [2].

The pathogenesis of candidiasis is influenced by several virulence factors, such as morphological transition between yeast and hyphal forms, expression of invasins and adhesions on their surface, thigmotropism, biofilm formation, and production of hydrolytic enzymes [3].

All pathogenic Candida species are creating protection against antifungals, specifically triazole compounds, by mechanisms, for example, modification of antifungal target protein's structure, articulation of efflux pumps that reduce drug accumulation, and a change in the membrane sterol's composition. Resistance to antifungal drugs leads to treatment failure in patients and also results in variation in the prevalence of Candida species [4].

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One of the mechanisms of resistance to azole drugs is the over-expression of CDR1, CDR2, and MDR1 genes. The products of these genes are active transport pumps that can efflux the drugs from the pathogen's cell [5].

As Pakistan is a developing country, a low spending sum is designated for medical services. Tertiary healthcare hospitals are either denied cutting-edge research facilities or simply have bacterial culturing labs. Low consideration has been set down on fungal isolation, characterization, determination of contagious infections, and risk factor evaluation underlying fungal infection epidemics. Resistance against antifungal drugs is becoming an emerging problem nowadays, especially against fluconazole; that is why this research was designed to find out the role of the CDR1 gene in fluconazole resistance.

## METHODOLOGY

This comparative study was conducted at the Department of Microbiology and Resource Lab. The duration of the study was 12 months, starting from the year 2016-2018, after the approval of the synopsis (UHS/Education/126-17/3464). The sample size was determined using the formula:

$$n = Z^2 1\text{-}\alpha/^2 P(1-P) \quad / \quad d^2$$

Z21-α/2 for 95% confidence level = 1.96

P= Anticipated proportion of C. albicans 16% [6].

d = Margin of error = 5%

n = Sample Size =206

The inclusion criteria encompassed all candida species.

A total number of two hundred and six Candida species were taken by using a convenient sampling technique from Jinnah Hospital Lahore. Using sabouraud dextrose agar, all obtained strains were sub-cultured to keep the organism fresh and confirmed by Gram stain, wet preparation, germ tube test, and API 20C up to the species level. Testing for drug susceptibility was performed using the broth microdilution technique in the Department of Microbiology, University of Health Sciences (UHS). The fluconazole-resistant and sensitive C. albicans were processed further for mRNA extraction and CDR1 gene expression by real-time PCR.

**API 20C AUX:**

To create the inoculum, three to four colonies from the sabourd agar were grown in 2 ml of ordinary saline for each species of Candida. We compared its turbidity to the 2.0 McFarland Standard. The API C medium was filled with about 100 µl of this suspension according to the kit's instructions. Incubation of the API strip with suspension-filled capsules was placed for 48 to 72 hours at 29ºC±2ºC; opacity higher than the negative control indicated a positive reaction (BioMerieux).

**Antifungal susceptibility testing of C. albicans against fluconazole by broth microdilution method:**

Using the equation M1V1 = M2V2, a fluconazole stock solution with a concentration of 1280 g/ml was created. 640µl of the stock solution and 340µl is a medium for culture of organisms in biological solution were combined in Tube 1 to make the working solution. Ten labelled tubes, each with a final volume of 1 ml, were set with concentrations ranging from 640 g/ml to 1.25 g/ml. Five isolated colonies were selected from SDA and overnight cultured in 5 ml of sterile saline to prepare the inoculum. The test organism's working suspension was made by combining 20µl of the aforementioned suspension with 1 ml of RPMI-1640 buffered with MOPS; this was further diluted 1:20.

Fluconazole concentrations were arranged in tubes (1–10) in descending order, and 0.1 ml of each concentration was applied to the corresponding wells on the microtitration plate. As positive and negative controls, the 11th well-received 0.1 ml of RPMI, while the 12th well received 0.2 ml. Wells 2 through 11 of the microtiter tray were filled with the working inoculum solution (0.1 ml). Before incubation, purity control agar plates with colony counts ranging from 5 to 30 CFUs on each plate were made using ten microliters of inoculum from the positive control for colony counts and streaking [7].

**RNA Extraction:**

The total RNA of C. albicans isolates was isolated using the RiboExTM kit with ready-to-use chemicals.

**RNA Quantification by Nanodrop Method:**

3 µl of dissolved RNA was dispensed into labelled PCR tubes and carried on ice for RNA quantification by Nanodrop. The Nanodrop values were used to check the concentration (ng/ µl) and purity of RNA and further in synthesizing complementary DNA (cDNA).

**cDNA Synthesis:**

The first strand of cDNA was created using a cDNA kit following RNA isolation (GeneDierX). One microgram of extracted RNA was used to synthesize cDNA by Reverse Transcriptase enzyme (GScript RTase) at 65°C for 3 to 5 minutes. Then, incubated at 50°C for 60 minutes and last, the de-activation of the enzyme was done at 70°C for 15 minutes.

**cDNA confirmation by Actin PCR:**

Conventional ACTIN PCR was used to verify that all RNA samples had been converted to cDNA. This was done by using CDR1 reverse and forward primers and Actin reverse and forward primers 0.5µl each, as mentioned in the below table. 10µl Green PCR Master Mix (2X) and 2µl cDNA as a template in each labelled PCR reaction tube. The following primers were used for gene expression analysis.

**Forward and Reverse primers of CDR1 and ACT**

CDR1[8]

Forward primer 5′-GAAAGAGAACCATTACCAGG-3′

Reverse primer 5′-AGGAATCGACGGATCAC-3′

ACT1 [5]

Forward primer: 5′-TTGAGAGTTGCTCCAGAAGAACATC-3′

Reverse primer: 5'- AGTCATCTTTTCTCTGTTGGATTTTGGA-3'

**Gel Electrophoresis:**

Beta Actin and CDR1 PCR products underwent gel electrophoresis. A 2% agarose gel was formed by mixing 2g agarose with 100ml 1X TAE buffer, heated for homogeneity, and mixed with 3.0 µl ethidium bromide. After loading 8µl PCR product into each well, the gel ran at 90V for 60min, observed under UV light using gel doc (XR+ Bio-Rad).

**Real-Time PCR for CDR1 gene expression analysis:**

To create cDNA, one microgram (1 g) of total RNA was utilized, and Syber Green Master Mix with fluorescent dye Syber was then employed in real-time polymerase chain reaction (RT-PCR). Using the CDR1 and ACT primers, the amplification process required a 95 °C starting cycle, followed by 40 cycles of 95 °C for 20 sec, 56 °C for 30 sec, and 72 °C for 40 sec. Actin was used as an internal control in the study of gene expression.The IBM SPSS software version 20 was used to conduct the statistical analysis. Mean±SD was determined for quantitative variables like MIC. Frequency and Percentages were determined for Qualitative variables. Raised CDR1 gene expression was detected in drug-resistant strains of Candida albicans.

All Candida species were then identified up to the species level by using API 20C AUX. C. tropicals was the most common species with a frequency of 66 (32.0%) followed by C. albicans 41(19.9), C. glabrata 24 (11.7%), C. lusitaniae 14 (6.8%), C. rugosa 13 (6.3%), C. famata 12 (5.8%), C. parapsilosis 11 (5.3%) and other less common species (Table-I).

**Table-I: Frequency of candida species (n=206).**

| Candida Species | n (%) |
|---|---|
| C. tropical | 66 (32.0) |
| C. albicans | 41(19.9) |
| C. glabrata | 24(11.7) |
| C. lusitaniae | 14(6.8) |
| C. rugosa | 13(6.3) |
| C. famata | 12(5.8%) |
| C. parapsilosis | 11(5.3%) |
| C .dubliniensis | 6(2.9%) |
| C. magnolia | 6(2.9%) |
| C. krusei | 5(2.4) |
| C. sphaerica | 4(1.9) |
| C. zeylanobles | 1(.5%) |
| **Total** | 206(100%) |

**Table- II: Distribution of candida species in various clinical specimens (n=206).**

| Clinical Samples | Total | C.albicans n (%) | Non-albicans n(%) |
|---|---|---|---|
| Urine | 133 | 28 (21.0%) | 105 (79%) |
| Sputum | 23 | 1 (4.3%) | 22 (95.6%) |
| Blood | 11 | 4 (36.3%) | 7 (63.6%) |
| Tracheal Secretion | 11 | 2 (18.1%) | 9 (82%) |
| CVP Tip | 9 | 0 (0.0%) | 9 (100%) |
| Pus | 9 | 3 (33.3%) | 6 (66.6%) |
| Foley's | 7 | 2 (28.5%) | 5 (71.4%) |
| HVS | 3 | 1 (33.3%) | 2 (66.6%) |
| Total | 206 | 41 (19.9%) | 165 (80.0%) |

According to the gender-specific distribution of Candida species, more females (60%) than men (40%) were infected. It was shown that females had higher concentrations of C. albicans and all NAC species than males.

Non-Candida albicans infected 100 (48.5%) more females than males 65 (31.5%). C. albicans infection rates were lower than non-albicans. To investigate the relationship between gender and Candida species, a Chi-square test was used. No significant correlation between the organism and gender was identified since the chi-square value was 0.656 and the P-value was 0.418, which is larger than 0.05 (Table-III).

**Table-III:Distribution of candida species in various clinical specimens (n=206).**

| Organism | Categories | Gender | | Total (n%) |
|---|---|---|---|---|
| | | Female(n%) | Male (n%) | |
| | C.albicans | 22 (10.67) | 19 (9.2) | 41 (19.9) |
| | Non-albicans | 100 (48.5) | 65 (31.5) | 165(80.1) |
| | **Total** | 122 (59.2) | 84 (40.7) | 206 (100) |

The susceptibility testing of fluconazole against Candida albicans, Candida tropicalis, and Candida glabrata was determined by broth microdilution method in RPMI medium after 48 hours of incubation. The MIC result was categorized into three groups according to CLSI document M27-A2. The result which was ≤8 was sensitive, equal to 16-32, S-DD, and ≥64 was counted resistant. The C. albicans isolates showed 80% susceptibility to fluconazole (Table-IV).

**Table-IV: Susceptibility pattern of C.albicans, C. tropicalis and C. glabrata to fluconazole.**

| Sensitivity | C. tropicalis | C. albicans | C. glabrata |
|---|---|---|---|
| Sensitive | 51 | 33 | 12 |
| Resistant | 7 | 4 | 7 |
| SDD | 8 | 4 | 5 |
| **Total** | 66 | 41 | 24 |

Anova testing was done to compare the mean of three fluconazole-resistant Candida species. The P-value of this test is .411, which is more significant than 0.05 and shows that all species have the same mean, which is why all three species show the same resistance pattern to fluconazole (Table-V and VI).

**Table-V: Comparison of the mean of three resistant Candida species.**

| Categories | N | Mean±SD | Standard Error |
|---|---|---|---|
| C. albicans | 4 | 105±30 | 15 |
| C. glabrata | 7 | 84±24.2 | 9 |
| C. tropicalis | 7 | 101.7±31.9 | 12 |
| **Total** | 18 | 95.5±28.6 | 6.7 |

**Table-VI: Anova Testing.**

| Resistance | Sum squares | df | Mean square | F | Sig |
|---|---|---|---|---|---|
| Between Groups | 1557.0 | 2 | 778.5 | 0.944 | 0.411 |
| Within Groups | 12367.4 | 15 | 824.4 | - | - |
| **Total** | 13924.4 | 17 | - | - | - |

**Upregulation of CDR1 in resistant and susceptible C. albicans**

Using gene-specific oligonucleotides, the mRNA levels of the ATP binding cassette transporter gene CDR1 were assessed in the susceptible and resistant isolates. In comparison to the four susceptible isolates, the four resistant isolates had higher levels of CDR1 mRNA overexpression, ranging from 1 to 2.5. ACT1 has been employed as a housekeeping gene as shown in (Figure-II).

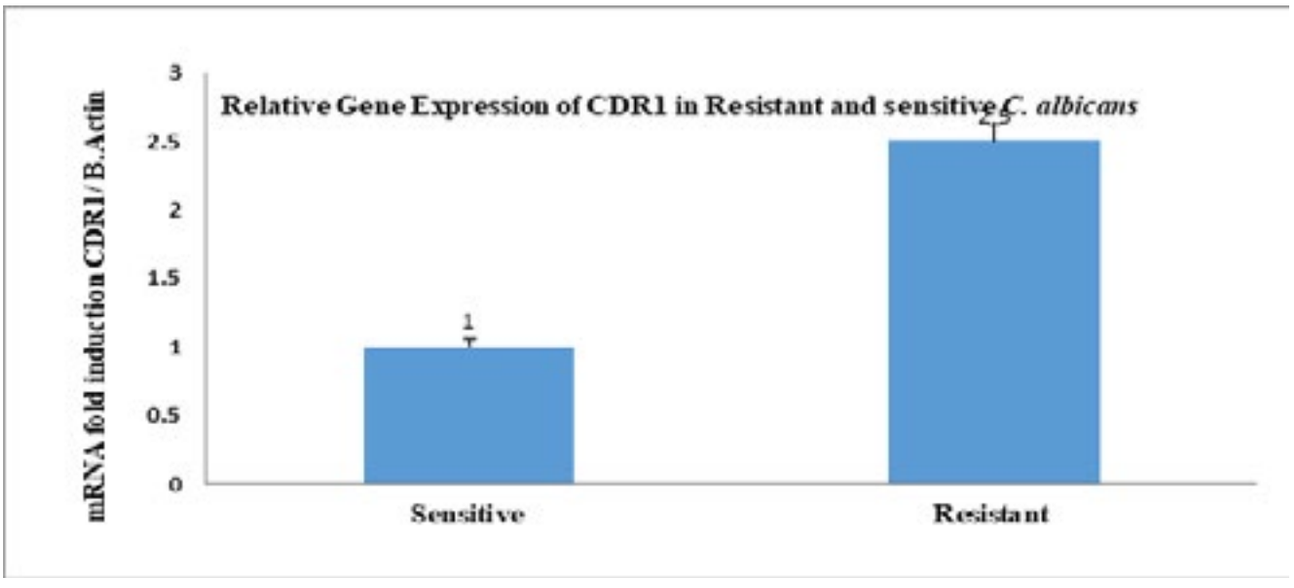


**Figure-I: CDR1 expression in resistant strains was ≥2.5 fold compared to sensitive C. albicans.**

## DISCUSSION

In the present study, Candida tropicalis was the most common species, representing 32.0% of the isolates. It was pursued by Candida albicans (19.9%), Candida glabrata (11.7%), and other less common species. Similar frequencies were observed in a study conducted in Karachi, Pakistan. Out of a total of 46,200 urine samples, 289 were positive for fungi. Out of the positive samples analyzed, it was discovered that five species were commonly present. Candida tropicalis was the most dominant, accounting for 60.9% of the cases, followed by Candida albicans at 30.1%, Candida parapsilosis at 4.8%, Candida glabrata at 2.8%, and Candida lusitaniae at 1.4[4].

According to the study, it was observed that C. tropicalis was the predominant species, representing 45% of the isolates. C. albicans ranked second in prevalence, accounting for 32% of the isolates. C. krusei and C. glabrata were comparatively less frequent, comprising 15% and 8% of the isolates, respectively. These results offer valuable insights into the occurrence and distribution of Candida species in cases of candiuria in the Bathinda region [9].

C. albicans accounted for 56% of the isolates, making it the most frequently identified species. The most prevalent was C. tropicalis comprising 20% of the total among non-albicans, followed by C. glabrata at 14% and C. krusei at 10%, as reported in Nepal[10].

The findings of our current study diverged from a previous study conducted in India, where the most prevalent Candida species among all isolates was reported as C. albicans, accounting for 31.42%, followed by C. tropicalis at 26.66% [11].

In our study, it was found that 80% of C. albicans were sensitive to fluconazole, while 10% were resistant, and the remaining 10% showed susceptibility to fluconazole in a dose-dependent manner. In a study conducted in Pakistan at Quaid-i-Azam University Islamabad, Candida species were examined for their prevalence and antifungal susceptibility. The results of that study revealed that 81.73% of C. albicans isolates were sensitive to the tested antifungal agent, while 14.6% were found to be resistant. The disk diffusion method was performed according to the CLSI M44-A guidelines [12].

A study conducted in Iran showed similar results. Testing for susceptibility was performed using the agar-based E-test method. Among the 285 C. albicans isolates tested, 10.5% were found to be resistant to fluconazole, while 89.5% were determined to be sensitive to the antifungal agent [13].

The susceptibility of the Candida strains to amphotericin B, fluconazole, voriconazole, and caspofungin was assessed using the broth microdilution technique. There were 111 positive samples in all, and there were 53 C. albicans. Fluconazole susceptibility and resistance were found in 3 (5.66%) and 6 (11.3%) of the C. albicans isolates, respectively [14].

A study in 2014 reported fluconazole resistance in Candida species in HIV patients. Fluconazole susceptibility testing was done on 227 C. albicans isolates, (81.9%) were sensitive, (7.5%) were SDD, and (10.6%) were resistant [15].

In an Egyptian study focusing on azole-resistant isolates of C. albicans, the findings revealed that among the tested isolates, 86.8% were sensitive to fluconazole, 3.8% showed susceptibility dose-dependent (SDD) response, and 9.4% were resistant [16].

In a recent study expression of the CDR1 gene was examined in four fluconazole-resistant C. albicans as compared to sensitive strains. The gene expression of CDR1 in resistant isolates was 2.5-fold more than in sensitive strains. Similar results were observed in a study in China. Eighteen fluconazole-resistant and sensitive strains were selected randomly for CDR1 gene expression and showed a statistically significant 3.16-fold relative increase as compared to sensitive strains [15].

In a 2014 study, the researchers explored the molecular process underlying C. albican's azole resistance. They specifically focused on investigating the mRNA levels of ABC transporter genes CDR1 to CDR4 in susceptible and resistant isolates. However, in the resistant strains, CDR1 was significantly overexpressed compared to the sensitive strains. The extent of up-regulation varied, ranging from 2.5-fold to 7.6-fold [17].

A study in 2001 to investigate the process of fluconazole resistance in C. albicans isolated from AIDS patients. The researchers compared four fluconazole-resistant strains with the fluconazole-sensitive ATCC 10231 strain. The findings indicated that the appearance of CDR genes in three of the resistant strains ranged from 2.1-fold to 4.4-fold in comparison to the susceptible strain. However, in one of the resistant isolates, the expression of CDR genes was only 1.3-fold as compared to the sensitive one [18].

The recent study's findings are consistent with the research project regarding fluconazole resistance. In this study, they examined fourteen fluconazole-resistant strains obtained from non-HIV patients, along with a susceptible control strain. Through real-time PCR analysis, they discovered increased expression of the CDR1 gene in unsusceptible species, with fold increases ranging from 1.6 to 8.0. These findings highlight the involvement of CDR1 and CDR2 genes in fluconazole resistance in Candida albicans [19].

In an Iranian study, researchers examined the up-regulation of CDR1, CDR2, MDR1, and ERG11 genes in two groups of C. albican isolates. One group included isolates that were sensitive to treatment, while the other group consisted of resistant isolates. The findings of the study demonstrated that among these genes, MDR1 exhibited the most frequent occurrence of overexpression, followed by CDR1[20].

## CONCLUSION

The most common isolate of culture was C. tropical 32%, C. albicans 19.9%, and C. glabrata 11.7% in 206 samples of human body process. The susceptibility pattern of C. albicans, C. tropicalis, and C. glabrata to fluconazole was 80% sensitive, and resistant isolates had higher levels of CDR1 mRNA overexpression. ACT1 has been employed as a housekeeping gene.

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***Author's Contribution:***

Muneeza Anwar : Data collection, translation, manuscript writing, and analysis.
Dr. Sidrah Saleem:Conceived, designed, and did statistical analysis and manuscript writing.
Dr. Shah Jahan : Substantial contributions to the conception.
Muhammad Imran: Design of the work.
Muhammad Roman: Drafting the work
Ayesha Ghazal: Reviewing it critically for important intellectual content.
Muhammad Usman Arshad: Final results writing.

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