Original Article

# "Determining The Antifungal Susceptibility Pattern Of *Candida Albicans* And The Molecular Characterization Of ERG11 Gene In Fluconazole Resistant *Candida Albicans* Isolates At A Tertiary Care Centre, Kanpur, Uttar Pradesh".

**Prema Nandini[1]*, R.Sujatha[2]**

[1]*Ph.D Scholar, Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: pushpa.choclate@gmail.com
[2]Professor and Head of Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: drsujatha152@gmail.com

***Corresponding Author:** Prema Nandini

*Ph.D Scholar, Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: pushpa.choclate@gmail.com
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## Abstract

Introduction: The Candida species are responsible for various clinical infections ranging from mucocutaneous infection to life threatening invasive diseases. The treatment of choice is the use of azoles, such as fluconazole (FLC). The mutations in ERG11 and amino acid substitutions in the target enzyme ERG11 leads to changes in the tertiary structure of enzyme and subsequently alter the abilities of azole antifungals.

Aim and Objective: To determine the Antifungal Susceptibility Pattern of *Candida albicans* and the Molecular Characterization of ERG11 gene of Fluconazole Resistant *Candida albicans* isolates at a Tertiary Care Centre.

Material and Methods: This was a cross sectional study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre, Mandhana ,Kanpur for a period of 1 year i.e, August 2021 to August 2022. A total of 70 consecutive Candida species were isolated from 823 clinical specimens.
Growth on Sabouraud dextrose agar were evaluated for colony appearance, microscopic examination, Gram staining, germ tube test and urea hydrolysis test. Further, they were processed for *Candida* speciation on CHROMagar. Antifungal susceptibility testing was performed as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines 2021. The DNA isolation was done using the Qiagen DNA extraction kit followed by the PCR for the molecular detection of ERG11 gene.

Results: Out of the total 70 *Candida* isolates, *Candida albicans* 29 (41.4%) was the most common species. Among the non-*albicans Candida* species, *Candida tropicalis* 20 (28.5%) was the predominant isolate followed by *Candida glabrata* 17 (24.2%) and least by *C. krusie* 4 (5.7%). The ratio of Males 18 (62%) was more as compared to that of the Females 11( 37.9%) with the maximum age of 31-40 years and least in the age group above 61 years of age. The number of isolates was maximum in the urine sample. The ERG11 gene was detected in all the fluconazole resistant 4 (13.7 %) strain of *candida albicans*.

Conclusion: In the current study nearly 4(13.7%) of the *Candida albicans* indicates a reduced sensitivity to the effects of azole drugs. Therefore, understanding how all Candida spp. display resistance to fluconazole is crucial if we are to maintain the efficacy of this essential antifungal treatment.

**Keywords:** *Candida albicans*, Antifungal, Molecular Characterization, ERG11, PCR

## INTRODUCTION

The genus Candida consists of about 200 species and around 20 distinct Candida species are known to cause human disease [1]. They are defined as a normal commensal flora of human body inhabiting skin, mucous membranes and gastrointestinal tract but may be associated with superficial and deep seated fungal infections [2].

The switch of Candida species from commensal to a potent pathogen is facilitated by various virulence factors such as adherence to host tissues, medical devices, biofilm formation, and secretion of extracellular hydrolytic enzymes [2]. Also, in recent years non-*albicans Candida* (NAC) species are considered as major pathogens causing severe infections in human [3]. More than 90-95% of invasive disease is caused by 5 most common pathogens, namely the *C.albicans, C.tropicalis, C.glabrata, C.parapsilosis* and *C.krusei* [4,5].

Among the available antifungal agents, the preferred and most frequently used drugs in the treatment of Candida infections are azoles [6] (e.g., cotrimazole, miconazole, fluconazole (FLC)), polyenes (e.g., nystatin and amphotericin B), echinocandins (e.g., caspofungin, micafungin), nucleoside analogues and allylamines [6,7].
Azoles inhibit ergosterol biosynthesis by interfering with the enzyme lanosterol 14-_-demethylase (CYP51A1, Erg11p) encoded by the ERG11 gene, which is involved in the transformation of lanosterol into ergosterol [6,8]. Ergosterol (ergosta-5,7,22-trien-3_-ol) is the primary sterol in the fungal cell membrane [8] and plays a major role in maintaining plasma membrane (PM) integrity and function [9,10]. Hence, its disruption has become a focus of antifungal therapies.

Up to now, more than 160 distinct amino acid substitutions have been reported however only ten of them cause FLC resistance [11]. This information suggests that the enzyme encoded by this gene is highly susceptible to structural changes. Previous reports of mutations in the ERG11 gene have defined three hot-spot regions located within residues 105 to 165, 266 to 287, and 405 to 488, which are particularly permissive to amino acid substitutions [12].
Amino acid replacement in these hot-spot regions could correspond to conformational changes in the protein [13]. Frequently, clinical isolates of *C. albicans* reveal several amino acid substitutions as a result of long-term exposure to the antifungals. However, not all amino acid substitutions contribute equally to azole resistance [12].
Unfortunately, the emergence of antifungal-resistant isolates constitutes a significant problem for treatment strategies [8]. In particular, the broad usage of azoles, such as FLC, has given rise to concerns regarding the emergence of resistance to this class of antifungal agents [4]. It is possible to distinguish several drug resistance mechanisms to azoles in *C. albicans* mutations in ERG11 and amino acid substitutions in the target enzyme ERG11 lead to changes in the tertiary structure of the enzyme and subsequently alter the abilities of azole antifungals resulting in resistance in *C. albicans* [8,11]. The purpose of the present study was to determine the Antifungal Susceptibility Pattern of *Candida albicans* and the Molecular Characterization of ERG11 gene in Fluconazole Resistant *Candida albicans* isolates at a Tertiary Care Centre, Kanpur, Uttarpradesh.

## MATERIAL AND METHODS

This was a cross sectional study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre Mandhana Kanpur for a period of 1 year i.e, August 2021 to August 2022. The Ethical clearance was obtained from the Ethical Committee of RMCH&RC, Mandhana, Kanpur. The Demographic details and clinical history along with the relevant clinical investigations was recorded after the informed consent. Candida isolates from all clinical specimen in pure culture were included in the study whereas, repeat isolates from same clinical specimen of same patient and isolation of Candida species from mix culture were excluded from the study.
All the clinical samples were subjected to culture on 5% Blood agar, and MacConkey agar. Gram staining of all the positive cultures was performed, and those showing yeast like budding cells were sub-cultured on SDA and HiChrome agar for species identification. Germ tube test was performed to differentiate Candida albicans and NACA. Further identification was done by Chrom agar, sugar assimilation tests using commercially prepared sugar discs sucrose, maltose, dextrose, trehalose, lactose and dulcitol from HiMedia and studying micro morphology on corn meal agar.

A total of 70 isolates of Candida species from different clincal specimens like blood,BAL,Urine, Pus ,Et secreation and Vaginal secreation were included in our study.
Antifungal sensitivity of Candida isolates was done by Kirby-Bauer disc diffusion method. Mueller Hinton agar supplemented with 0.2% glucose and 0.5µg/ml methylene blue dye medium (MH-GMB) was used for this purpose against azole group Fluconazole 25ug from Hi-media Laboratories Pvt Ltd India. The broth micro dilution method was done to determine the minimum inhibitory concentrations (MICs) according to the CLSI guidelines 2021 [14].

### Molecular Identification of ERG11 gene of Fluconazole Resistant *Candida albicans*

The DNA was isolated using the Qiamp DNA Blood Mini Kit (QIAGEN, Germany) as per the manufactures guidelines. The DNA was eluted in 60 µl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 µL reaction mixture with 35 no. of cycles. The primers were purchased from "**Saha gene'** and was reconstituted with sterile double distilled water based on the manufacturer's instruction.


**Figure No.1:** The DNA Extraction kit

**Figure No.2:** The Reagents used for the DNA Extraction

| Fragment | Gene | Primer sequence | Length (bp) |
|---|---|---|---|
| A | ERG11-FA<br>ERG11-RA | 5'- ATGGCTATTGTTGAAACTGTC-3'<br>5'- CGTTCTCTTCTCAGTTTAATTTC-3' | 785 bp |
| B | ERG11-FB<br>ERG11-RB | 5'- GAAGAGAACGTGGTGATATTGATC-3'<br>5'- CACTGAATCGAAAGAAAGTTGCC-3' | 826bp |

**Table No. 1:** Primers used to amplify ERG11 gene fragments.

### Polymerase Chain Reaction (PCR)

The amplification of the ERG11 gene sequence was performed using PCR. Due to its length (1587 bp), the sequence was amplified in two fragments (A and B) using the primers shown in Table No. 1. The starters were designed using the SnapGene program and the Oligo Analyzer tool and were synthesised by Genomed (Warsaw, Poland). The primers were designed to include a sequence of 20 bp downstream and 12 bp upstream (at positions 20 bp ERG11 or +1599 bp ERG11, respectively) to ensure the amplification of the entire ERG11 gene sequence [15].

### The PCR cycling conditions

The PCR Reactions were carried out in the following reaction mixture where 12.5 µLof the Master Mix (BioRad, Hercules, CA, USA), 0.5µ L of each of the 5 µ M primers, 2 µ L of isolated genomic DNA and 9.5 µ L of sterile water (total volume 25 µ L). Fragment "A" of the ERG11 gene sequence was amplified using a program called ZL-ERG11A and fragment "B" using the ZL-ERG11B program. The Thermal Cycler (BioRad, Hercules, CA, USA) was used to perform the PCR reaction. The PCR cycling conditions have been illustrated below shown in Table No. 2.

| Step | Program | | | | Cycles |
|---|---|---|---|---|---|
| | ZL-ERG11A | | ZL-ERG11B | | |
| | Time | Temperature | Time | Temperature | |
| **Initial denaturation** | 5 min | 98 ºC | 5 min | 98ºC - | |
| **Denaturation** | 30 s | 98 ºC | 28 s | 98º C | |
| **Annealing** | 30 s | 51 ºC | 29 s | 55 ºC | |
| **Extension** | 30 s | 72º C | 30 s | 72º C | 35 |
| **Final extension** | 5 min | 72º C | 5 min | 72º C | |
| | ∞ | 4ºC | ∞ | 4ºC | |

**Table No. 2:** The PCR cycling conditions to amplify ERG11 gene fragments.

The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 1 kb DNA Ladder (Thermo Fisher Scientific ™, Waltham, MA, USA) was used as the marker to evaluate the PCR product of the sample [15].

## RESULTS

A total of 70 consecutive Candida species were isolated from 823 clinical specimens out of which 29 (41.4%) isolates were confirmed to be *C.albicans.* Among the non *albicans candida* (NAC) the *C.tropicalis (*28.5%) was the most common isolate followed by *C.glabrata* (24.2%) and least for *C. krusie* with 5.7% [Table No. 3].

| Type of Fungal isolates | Number of Isolates | Percentage |
|---|---|---|
| *C. albicans* | 29 | 41.4 % |
| *C.tropicalis* | 20 | 28.5 % |
| *C.glabrata* | 17 | 24.2 % |
| *C. krusie* | 4 | 5.7 % |

**Table No. 3:** The Type of Candida species isolates

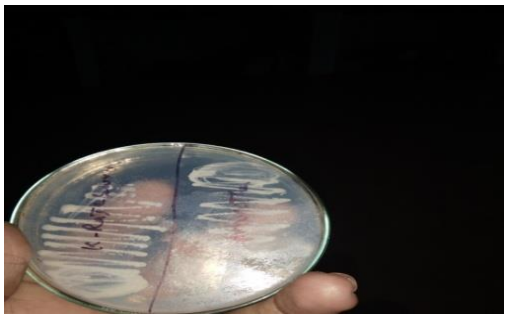

**Figure No.3:** *Candida albicans* growth on cornmeal agar

**Figure No.4:** *Candida albicans* growth on hichrom agar

| Gender | Total no. of Cases studies (N=29) | Percentage |
|---|---|---|
| **Male** | 18 | 62% |
| **Female** | 11 | 37.9% |

**Table No. 4:** Genderwise distribution of the *Candida albicans*

The ratio of Males 18 (62%) was more as compared to that of the Females 11( 37.9%) [Table No. 4] with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age [Table No. 5]. There was no *candida albicans* isolated in the age group of 0-10 years of age.

| S.No. | Age (in years) | No. of Cases | Percentage |
|---|---|---|---|
| 1. | 0- 10 | - | - |
| 2. | 11-20 | 3 | 10.3 % |
| 3. | 21-30 | 4 | 13.7 % |
| 4. | 31-40 | 10 | 34.4 % |
| 5. | 41-50 | 9 | 31 % |
| 6. | 51-60 | 2 | 6.8 % |
| 7. | ≥61 | 1 | 3.4 % |

**Table No.5:** Age wise distribution of *Candida albicans* patients from the study

| Type of Sample | Number of Isolates |
|---|---|
| **BAL** | 1 |
| **Urine** | 15 |
| **Pus** | 5 |
| **Et secretion** | 3 |
| **Vaginal secretion** | 4 |
| **blood** | 1 |

**Table No. 6:** Type of Sample Isolated from *Candida albicans*

The maximum number of isolates was found in the urine sample followed by the pus and least in the BAL and the blood sample [Table No. 6].

Out of 70 isolates a total of 29 isolates of *Candida albicans* were isolated. A total of 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method [Table No. 7].

| Antifungal-Fluconazole | Number of isolates N=29 | Percentage of isolates |
|---|---|---|
| Sensitive | 27 | 93 % |
| Resistant | 2 | 6.8 % |

**Table No. 7:** Antifungal Sensitivity pattern of *Candida albicans* against fluconazole by Kirby Bauer disc diffusion method according to the CLSI guidelines

| Antifungal-Fluconazole | Number of isolates N= 29 | Percentage of isolates |
|---|---|---|
| Sensitive | 25 | 86.2% |
| Resistant | 4 | 13.7% |

**Table No. 8:** Antifungal Sensitivity pattern of *Candida albicans* by CLSI broth Microdilution method

In the Table No. 8 it was illustrated that out of 29 isolates of *C.albicans* tested for susceptibility pattern by CLSI broth microdilution method 25 isolates (86.3%) were sensitive and 4 isolates(13.7%) were resistant to Fluconazole showing Mic>/=64ug/ml.

| Antifungal | Kirby bauer disc diffusion Method | | Broth microdilution method | |
|---|---|---|---|---|
| | Sensitive | Resistant | Sensitive | Resistant |
| Fluconazole | 27(93%) | 2(6.8%) | 25(86.2%) | 4(13.7%) |

**Table No. 9:** Antifungal Sensitivity pattern of *Candida albicans* against Amphotericin B by CLSI broth microdilution method

| Type of Sample | Fluconazole |
|---|---|
| **BAL** | 0 |
| **Urine** | 1 |
| **Pus** | 0 |
| **Et secretion** | 0 |
| **Vaginal secretion** | 1 |
| **blood** | 0 |

**Table No. 10:** Sample wise resistance pattern of *C.albicans* against Fluconazole by CLSI Kirby bauer disc diffusion method

| Type of Sample | Fluconazole |
|---|---|
| **BAL** | 0 |
| **Urine** | 3 |
| **Pus** | 0 |
| **Et secretion** | 0 |
| **Vaginal secretion** | 1 |
| **blood** | 0 |

**Table No. 11:** Sample wise resistance pattern of *C.albicans* against Fluconazole by CLSI broth microdilution method

In the present study it was observed that by Kirby bauer disc diffusion method 2(6.8%) were resistant to Fluconazole whereas, 4(13.7%) showed resistance to Fluconazole by Broth microdilution method..
In the present study it was also observed that the maximum number of sample observed resistant was found in the urine sample.

The Molecular characterization of the ERG11 gene of Fluconazole Resistant *Candida albicans* isolates was performed and the DNA was been extracted.

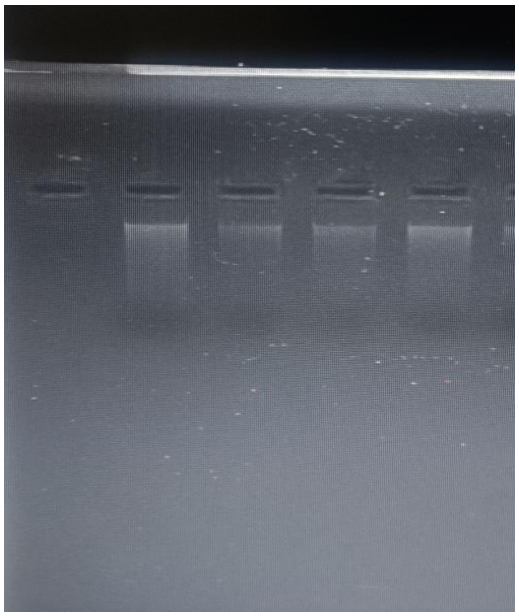

**Figure No.5:** The DNA Extraction of Fluconazole Resistant *Candida albicans*

The obtained genomic DNA was a matrix for the PCR reaction in which fragments of the ERG11 gene were amplified. The efficiency of the PCR reaction was checked using the gel electrophoresis technique. The illustration below (Figure No. 6) shows a pictorial result of this procedure. Expected product sizes were: 785 bp (fragment 5'to 3') and 826 bp (fragment 3'to 5').

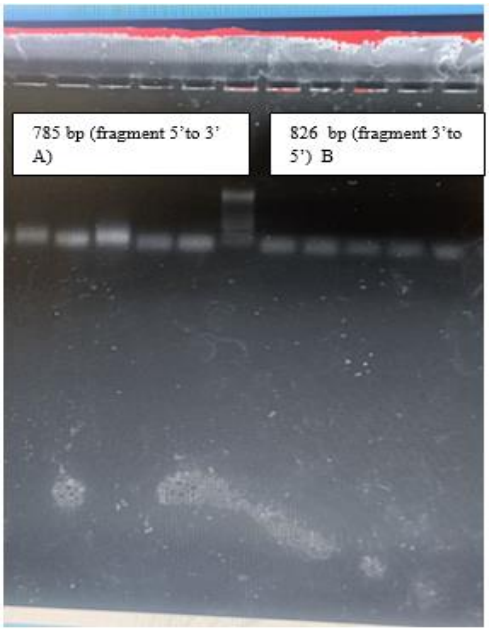


**Figure No.6:** The Photograph of amplified ERG11 gene in Fluconazole Resistant *Candida albicans* along with the positive control ATCC 10231.

The amplified band size of the PCR product was obtained of 785 bp (fragment 5'to 3') and 826 bp (fragment 3'to 5'). Lane No. 1,3, 4 and 5 were ERG11 gene positive for 785 bp. Lane 2 was the Positive Control for *Candida albicans* Fluconazole Resistant. Lane 6 was the 1 Kb DNA Ladder. The Lane No. 7 to 10 were the fragment B positive for 826 bp. Lane 11 been the positive Control. The size of obtained DNA fragments was compared to GeneRuler ™ 1 kb DNA Ladder.

## DISCUSSION

*Candida albicans* is the most prevalent fungal species of the human microbiota; this species asymptomatically colonizes many areas of the body, particularly the gastrointestinal and genitourinary tracts of healthy individuals [16].The studies indicate that Candida sp., specifically *C. albicans,* is responsible for most fungal infections [17]. The problem is the growing number of Candida strains resistant to conventional antifungal drugs such as azoles which are the drugs of choice for most Candida infections [18].

In the present study a total of 70 consecutive Candida species were isolated from 823 clinical specimens out of which 29 (41.4%) isolates were confirmed to be *C.albicans.* This study was similar to the study performed by other authors where *Candida albicans* was the most frequently isolated species reported by Sasso et al [19], Mnge et al [20], and Zeng et al [21].

Among the non *albicans candida* (NAC) the *C.tropicalis* (28.5%) was the most common isolate followed by *C.glabrata* (24.2%) and least for *C. krusie* with 5.7%. This study was similar to the study performed by the other author where the maximum isolates was found of *Candida albicans.* In case of the Non *Candida albicans* (NAC) the *C.tropicalis* was found to be maximum and least was *C. krusie.* This study was parallel to the study performed by the other authors where *C. glabrata* and *C. tropicalis* was reported as a 2nd predominant NAC species [22-26] but in contrast with the study by Elias Seyoum et al., where *C. krusie* was found to be the maximum [27].

It was found that the ratio of Males 18 (62%) was more as compared to that of the Females 11( 37.9%) with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age. There was no *candida albicans* isolated in the age group of 0-10 years of age. There were other studies which were parallel to our study where the male was more common. R A Kashid et al.,[28] reported the isolation of Candida species was higher in males (55.10%) with male to female ratio of 1:0.81. In another study by Amar CS et al., more Candida isolates from male and the male female ratio was reported as 0.66:1[29]. The study by B S G Sailaja et al.,[30] was similar to our study where the maximum age of 31-40 being affected the most but in contrast with the study by Arasi et al., which reported that more Candida strains in age group >60 years [31].

The maximum number of isolates was found in the urine sample followed by the pus and least in the BAL and the blood sample. These finding were similar to the study by CA Kauffmann et al., [32] Sankarankutty Jay and Vipparti Harita [33 ]where the maximum strains were isolated from Urine sample.

In the current study a total of 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method and 25 isolates (86.3%) were sensitive and 4 isolates(13.7%)were resistant to Fluconazole showing Mic>/=64ug/ml by broth microdilution method according to the CLSI guidelines 2021. This study was in accordance with many other studies conducted by Lulu Zhang.et.al., [34] which showed 10.6% resistance and 89.2% sensitivity to fluconazole and study conducted by shirshaklamsalet.al.,[35]which showed 80.9%susceptibility and 9.1% resistance to fluconazole.

One of the most commonly prescribed antifungal drugs for *Candida* infections is fluconazole, a triazole antifungal [4]. The development of resistance against azole antifungals can be due to alteration of the lanosterol 14 alpha demethylase target enzyme because of either overexpression or mutation in ERG11 gene encoding the enzyme henry et.al 2000 [36].

Azoles work by inhibiting the biosynthesis of ergosterol, an indispensable component for maintaining the fluidity in the membranes of eukaryotic cells, which leads to the toxic accumulation of its precursor, lanosterol [37]. One of the most commonly prescribed azole antifungals used for the treatment of *C. albicans* infections is fluconazole [38-40] .Fluconazole inhibits the enzyme sterol 14–α demethylase (CYP51), which is essential for the biosynthesis of the fungal–specific membrane ergosterol [36][41,42]. *C. albicans* CYP51 (CaCYP51) whose normal substrate is lanosterol, catalyzes the demethylation reaction of lanosterol in a three-step process towards producing ergosterol. Azole antifungal drugs inhibit this enzyme by binding their nucleophilic N–4 atom to the enzyme's heme Fe (iron) at the sixth coordinate position, hence occupying the binding pocket competitively [8] [43,44]. CaCYP51 is encoded by the ERG11 gene and altering the nature of the enzyme at the sequence level is a common strategy for evading the inhibitory action of the azoles, leading to resistance [45].

In the present study there were four isolates of *Candida albicans* that were found resistant to Fluconazole, carrying the ERG11 gene. Many *C. albicans* clinical isolates overexpress ERG11, the gene encoding the target of the azoles. The significance of increased ERG11 expression is one of the important reason for fluconazole-resistant. However, other resistance mechanisms (not investigated in this present study) apart from mutations in ERG11 may also be responsible [46,47].

Various virulence mechanisms developed by Candida play a role in this increasing drug resistance . Current data show that nearly 13.7 % of the *Candida albicans* indicates a reduced sensitivity to the effects of azole drugs, which was similar to the study by other author where 7% indicates reduced sensitivity [48] .Understanding the mechanisms underlying fluconazole resistance is a crucial part of managing our limited antifungal repertoire.
As a result, the need to develop new therapeutic methods is becoming stronger and stronger.

## CONCLUSION

The development of drug resistance can be seen as an unavoidable result of the antifungal drug's selective pressures. Many genes and mutations that promote fluconazole resistance in clinical isolates, especially in *C. albicans*, have been revealed over the past 20 years. Understanding how all Candida spp. display resistance to fluconazole is crucial if we are to maintain the efficacy of this essential antifungal treatment.

## REFERENCES


1. Sowmya GS, Kulkarni M, Gopalakrishnan R. Speciation and antifungal susceptibility pattern of Candida isolates in a tertiary care hospital. Int J Adv Sci Technol. 2014; 2(3):12– 18.
2. Achkar JM, Fries BC. Candidal infections of the genitourinary tract. Clin Microbiol Rev. 2010; 23:253–273.
3. Gullo A. Invasive fungal infections: the challenge continues. Drugs. 2009; 69(Suppl 1):65–73.
4. PfallerMA,DiekemaDJ,Epidemiologyofinvasivecandidiasis:apersistentpublichealthproblem.Clin Microbiol Rev. 2007; 20(1):133–163.
5. Centers for Disease Control and Prevention. Candidiasis. USA: 2015 June. Available from: https://www.cdc.gov/ fungal/diseases/candidiasis/
6. Lopes, J.P, Lionakis, M.S. Pathogenesis and virulence of Candida albicans. Virulence 2022, 13, 89–121.
7. de Oliveira Santos, G.C., Vasconcelos, C.C., Lopes, A.J.O., de Sousa Cartágenes, M.D.S., Filho, A.K.D.B., do Nascimento, F.R.F., Ramos, R.M.; Pires, E.R.R.B., de Andrade, M.S., Rocha, F.M.G. et al. Candida infections and therapeutic strategies: Mechanisms of action for traditional and alternative agents. Front. Microbiol. 2018; 3, 1351.
8. Flowers, S.A., Colón, B., Whaley, S.G., Schuler, M.A., Rogers, P.D. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob. Agents Chemother. 2015; 59: 450–460.
9. Fumiyoshi, A.; Toshiki, H. Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in Saccharomyces cerevisiae. Biochim. Biophys. Acta (BBA)-Biomembr. 2009; 1788, 743–752.
10. Dupont, S., Lemetais, G., Ferreira, T, Cayot, P. Gervais, P. Beney, L. Ergosterol biosynthesis: A fungal pathway for life on land? Evolution .2012; 66, 2961–2968.
11. Xiang, M.J. Liu, J.Y. Ni, P.H. Wang, S. Shi, C. Wei, B. Ni, Y.X. Ge, H.L. Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS Yeast Res. 2013; 13, 386–393.
12. Marichal, P. Koymans, L. Willemsens, S. Bellens, D. Verhasselt, P. Luyten, W. Borgers, M. Ramaekers, F.C.S. Odds, F.C. Vanden Bossche, H. Contribution of mutations in the cytochrome P450 14 alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. Microbiology 1999; 145, 2701–2713.
13. Hargrove, T.Y. Friggeri, L. Wawrzak, Z. Qi, A.; Hoekstra, W.J. Schotzinger, R.J. York, J.D. Guengerich, F.P. Lepesheva, G.I.Structural analyses of Candida albicans sterol 14_-demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. J. Biol. Chem. 2017; 292, 6728–6743.
14. Clinical and Laboratory Standards Institute (CLSI).Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard, 3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA. 2021.
15. Aneta K. Urbanek 1 , Zofia Łapi ´ nska , Daria Derkacz 1 and Anna Krasowska . The Role of ERG11 Point Mutations in the Resistance of Candida albicans to Fluconazole in the Presence of Lactate. Pathogens 2022; 11, 1289.

16. Achkar JM, Fries BC. Candida infections of the genitourinary tract. Clin Microbiol Rev. 2010; 23:253–73.
17. Dadar, M.; Tiwari, R.; Karthik, K.; Chakraborty, S.; Shahali, Y.; Dhama, K. Candida albicans—Biology, molecular characterization,pathogenicity, and advances in diagnosis and control—An update. Microb. Pathog. 2018; 117, 128–138.
18. Patil, S. Rao, R.S.Majumdar, B. Anil, S. Clinical appearance of oral Candida infection and therapeutic strategies. Front. Microbiol.2015; 6, 1391.
19. Sasso M, Roger C, Sasso M, Poujol H, Saber Barbar S, Lefrant J. Changes in the distribution of colonising and infecting Candida spp. isolates, antifungal drug consumption and susceptibility in a French intensive care unit: A 10-year study. Mycoses. 2017; 60:770–780.
20. Mnge P, OkeleyeBI, Vasaikar SD, Apalata T. Species distribution and antifungal susceptibility patterns of Candida isolates from a public tertiary teaching hospital in the Eastern Cape Province, South Africa. Braz J Med Biol Res. 2016. 10.1590/1414-431X20175797.
21. Zeng Z, Tian G, Ding Y, Yang K, Liu J, Deng J. Surveillance study of the prevalence, species distribution, antifungal susceptibility, risk factors and mortality of invasive candidiasis in a tertiary teaching hospital in Southwest China. BMC Infect Dis. 2019; 19:939.
22. Mohandas V, Ballal M. Distribution of Candida Species in different clinical samples and their virulence: Biofilm formation, proteinase and phospholipase production: A study on hospitalized patients in Southern India. J Glob Infec Dis. 2011; 3:4–8.
23. Bitew A, Abebaw Y. Vulvovaginal candidiasis: Species distribution of Candida and their antifungal susceptibility pattern. BMC Womens Health. 2018; 18:94.
24. Schmalrec AF, Willinger B, Haase BG, Lass-Flo C, Feeler K, et al. Species and susceptibility distribution of 1062 clinical yeast isolates to azoles, echinocandins, flucytosine and amphotericin B from a multi-centre study. Mycoses. 2012; 55:e124–e137.
25. Richter S. S., Galask R. P., Messer S. A., Hollis R. J., Diekema D. J., Pfaller M. A. Antifungal Susceptibilities of Candida Species Causing Vulvovaginitis and Epidemiology of Recurrent Cases. Journal of Clinical Microbiology. 2005; 43(5):2155–2162.
26. Badiee P, Alborzi A. Susceptibility of clinical Candida species isolates to antifungal agents by E-test, Southern Iran: A five year study. Iran J MicrobioL. 2011; 3:183–188.
27. Elias Seyoum, Adane Bitew, and Amete Mihret. Distribution of Candida albicans and non-albicans Candida species isolated in different clinical samples and their in vitro antifungal suscetibity profile in Ethiopia. BMC Infect Disease. 2020; 20:231.
28. R A Kashid S Belawadi G Devi D Dadich Characterization and antifungal susceptibility testing for Candida species in a tertiary care hospital J Health Sci Res 20112217
29. C S Amar Ashish J V Hajare Y Belagali Study of prevalence and antifungal susceptibility of candidaInt J Pharm Bio Sci. 2013. 4236181
30. Sailaja B, Prasad P**.** A study on isolation of Candida species in various clinical samples in a tertiary health care unit. Indian Journal of Microbiology Research. 2019; 6: 258-260.
31. Chitralekha Saikumar A. Arasi Samyuktha Isolation, Identification and Speciation of Candida Species from Various Clinical Specimens in a Tertiary Care Hospital in ChennaiSch J App Med Sci201758F34608 .0
32. C A Kauffman Candiduria Clin Infect Dis 2005413717Supplement 6
33. Sankarankutty Jay Vipparti Harita Candida Species Isolated from Various Clinical Samples and their Susceptibility Patterns to Antifungals. J Med Microbiol Infec Dis. 201311226
34. Lulu Zhang,XiaodongShe,Daniel Merenstein et.al.Fluconazole Resistance patterns in Candida species that colonize women with HIV infection.Current Therapeutic research. 2014; 76(C):84-89.
35. Lamsal,S.,Adhikari,S.,Raghubhansi,B.R.,Sapkota,S.,et.al Antifungal Susceptibility and Biofilm formation of Casndida albicans isolated from different clinical specimens.Tribhuvan University Journal of Microbiology. 2021; 8(1),53-62.https://doi.org/10.3126/tujm.v8.41195.
36. Henry, K.W., Nickels, J.T., Edlind, T.D.(2000).Upregulation of Erg Genes in Candida Species by Azoles and other sterol Biosynthesis Inhibitors. Antimicrob.Agents chemother.44,2693-2700.doi:10.1128/AAC.44.10.2693-2700.2000 .
37. Ahmad A, Khan A, Manzoor N, Khan LA. Evolution of ergosterol biosynthesis inhibitors as fungicidal against Candida. Microb Pathog. 2010; 48(1):35–41.
38. Autmizguine J, Smith PB, Prather K, et al. Effect of fluconazole prophylaxis on Candida fluconazole susceptibility in premature infants. J Antimicrob Chemother. 2018;73(12):3482–3487.
39. Gong Y, Liu W, Huang X, Hao L, Li Y, Sun S. Antifungal activity and potential mechanism of n-butylphthalide alone and in combination with fluconazole against Candida albicans. Front Microbiol. 2019; 10. doi:10.3389/fmicb.2019.01461
40. Rybak JM, Muñoz JF, Barker KS, et al. Mutations in TAC1B: a novel genetic determinant of clinical fluconazole resistance in Candida auris. mBio. 2020; 11(3):1–16.
41. Warrilow AGS, Melo N, Martel CM, et al. Expression, purification, and characterization of Aspergillus fumigatus sterol 14-α demethylase (CYP51) isoenzymes A and B. Antimicrob Agents Chemother. 2010; 54(10):4225–4234.
42. Warrilow AGS, Mullins JGL, Hull CM, et al. S279 point mutations in Candida albicans sterol 14-α demethylase (CYP51) reduce in vitro inhibition by fluconazole. Antimicrob Agents Chemother. 2012; 56(4):2099–2107.
43. Bhattacharya, S. Sae-Tia, S. Fries, B.C. Candidiasis and mechanisms of antifungal resistance. Antibiotics . 2020; 9, 312.
44. De Petris A, Crestoni ME, Pirolli A, et al. Binding of azole drugs to heme: a combined MS/MS and computational approach. Polyhedron. 2015; 90:245–251.
45. Xiang MJ, Liu JY, Ni PH, et al. Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS Yeast Res. 2013; 13(4):386–393.
46. Teo JQM, Lee SJY, Tan AL, et al. Molecular mechanisms of azole resistance in Candida bloodstream isolates. BMC Infect Dis. 2019; 19(1):1–4.
47. Yao D, Chen J, Chen W, Li Z, Hu X. Mechanisms of azole resistance in clinical isolates of Candida glabrata from two hospitals in China. Infect Drug Resist. 2019; 12:771–781.
48. Howard, K.C.; Dennis, E.K.; Watt, D.S.; Garneau-Tsodikova, S. A comprehensive overview of the medicinal chemistry of antifungal drugs: Perspectives and promise. Chem. Soc. Rev. 2020; 49, 2426–2480.