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RESEARCH ARTICLE
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"DETERMINING THE ANTIFUNGAL SUSCEPTIBILITY PATTERN OF CANDIDA ALBICANS AND THE MOLECULAR PROFILING OF ERG11 GENE IN FLUCONAZOLE RESISTANT CANDIDA ALBICANS ISOLATES AT A TERTIARY CARE CENTRE, UTTAR PRADESH".

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ABSTRACT

Introduction: Candida is the most opportunistic fungal pathogen, which results in various manifestations ranging from mucocutaneous lesions 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 detect antifungal drug-resistant Antibiotic Susceptibility Pattern of *Candida albicans* and the Molecular Characterization of ERG11 gene of Fluconazole Resistant *Candida albicans* isolates.

Material and Methods: This was a cross sectional study carried out in the Department of Microbiology for a period of 1 year i.e, June 2022 to June 2023. A total of 72 consecutive Candida species were isolated from 764 clinical specimens. Growths 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 2022. The DNA isolation was done using the Qiagen DNA extraction kit followed by the conventional PCR for the molecular detection of ERG11 resistant gene.

Results: Out of the total 72 *Candida* isolates, *Candida albicans* 33 (45.83%) was the most common species. Among the non-*albicans Candida* species, *Candida tropicalis* 20 (27.7%) was the predominant isolate followed by *Candida glabrata* 12(16.6%) and least by *C. krusie* 7 (9.72%). The ratio of Males 21 (63.6%) was more as compared to that of the Females 12(36.3%) 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 5 (15.15%) strain of *candida albicans*.

Conclusion: In the current study nearly 5(15.15) 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, DNA, PCR

INTRODUCTION

The genus Candida comprises of about 150 yeast species, which is composed of a heterogeneous group of organisms & consists of more than 17 different Candida species that are responsible for different human infections [1]. Candida is the most opportunistic fungal pathogen, which results in various manifestations ranging from mucocutaneous lesions to life threatening invasive diseases. Although *Candida albicans* is the most common cause of candidiasis accounting for about 60-80% of infections, a shift towards non albicans Candida species is evident in recent years [2].

Candidiasis is an opportunistic infection occurring in presence of predisposing factors like extensive and prolonged administration of broad-spectrum antimicrobials, corticosteroids, immunosuppressive agents and cytotoxic drugs, diabetes mellitus, HIV, chronic renal failure, haemodialysis, renal transplantation or indwelling urinary catheter [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].

Azole resistance mechanisms are different and may contribute to mutations in encoding gene *ERG11*. It is based on the spatial configuration alterations of the target enzyme 14a-demethylase (ERG11p) [4]. Erg11p is a significant protein of the cytochrome P450 superfamily enzyme and has an important role in the ergosterol synthesis pathway of *C. albicans*. Ergosterol (ergosta-5,7,22-trien-3β-ol) is a kind of sterol found in the fungal cell membranes and play a major role in maintaining the integrity and function of *C. albicans* membrane [11].

The modification of the target protein represents one of the commonest mechanisms of MDR where the target protein of azoles, ERG11p, is modified by the chromosomal mutations leading to the replacement of native amino acids. This is evident from the fact that several point mutations in ERG11 gene which encodes ERG11p have been identified in clinical drug resistant isolates of

Candida. Interestingly, these mutations appear to be predominantly restricted to certain hot spot regions of ERG11p. The exact placement of all the identified mutations in a 3D model of the protein confirms that these mutations are not randomly distributed but rather are clustered in select hot spot regions [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 affluent nations, fungal infections and candidemia are becoming more common, which has led to a rise in morbidity and mortality. The concerning rise in infections involving bacteria resistant to drugs is a result of the abuse of wide spectrum antibiotics, which promotes the overgrowth of Candida spp. and increases the likelihood that the bacterium will cause illness. There has been a change in each Candida species' relative frequency. Only polyenes, allylamines, azoles, and the more modern echinocandin class of compounds are accessible as antifungal medicines for the treatment of systemic and invasive candidiasis. Healthcare practitioners are extremely concerned about the sharp rise in the incidence of Candida spp. resistance to antifungal treatment during the last few decades.

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 present study was undertaken to determine the Antifungal Susceptibility Pattern of *Candida albicans* and the Molecular Characterization of ERG11 gene in Fluconazole Resistant *Candida albicans* isolates.

MATERIAL AND METHODS

This was a cross sectional study carried out in the Department of Microbiology for a period of 1 year i.e, June 2022 to June 2023. The Ethical clearance was duly obtained from the Ethical Committee. The Demographic details and clinical history along with the relevant clinical investigations was recorded after the informed consent.

Inclusion Criteria: Candida isolates from all clinical specimen in pure culture were included in the study.

Exclusion Criteria: 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 2022 [14].

GENOTYPIC METHOD:

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	5'- ATGGCTATTGTTGAAACTGTC-3'	
	ERG11-RA	5'- CGTTCTCTTCTCAGTTTAATTTC-3'	785 bp
B	ERG11-FB	5'- GAAGAGAACGTGGTGATATTGATC-3'	826bp
	ERG11-RB	5'- CACTGAATCGAAAGAAAGTTGCC-3'	

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

The PCR ASSAY:

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 Snap Gene 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 µL of 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 ZL-ERG11A Time	Temperature	ZL-ERG11B Time	Temperature	Cycles
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	35
Extension	30 s	72º C	30 s	72º C	
Final extension	5 min	72º C	5 min	72º C	
	∞	4ºC	∞	4ºC	

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

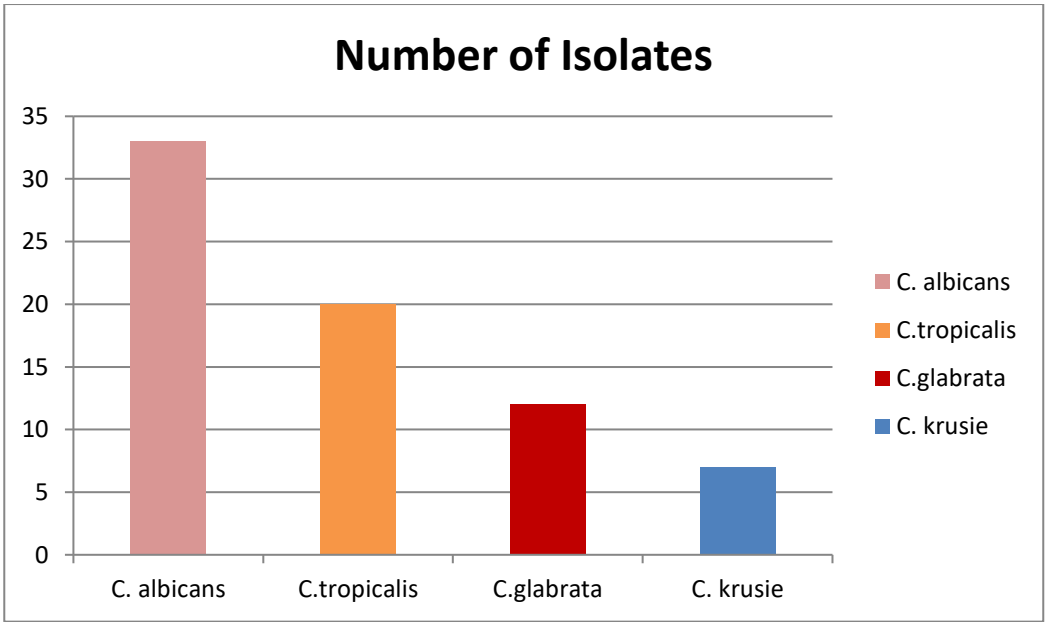
The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories 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

In the present study a total of 72 consecutive Candida species were isolated from 764 clinical specimens out of which 33(45.83%) isolates were confirmed to be *C.albicans.* Among the non *albicans candida* (NAC) the *C.tropicalis 20(*27.77%) was the most common isolate followed by *C.glabrata 12*(16.66%) and least for *C. krusie* with 7 (9.72%) [Table No. 3].

Type of Fungal isolates	Number of Isolates	Percentage
C. albicans	33	45.83%
C.tropicalis	20	27.77%
C.glabrata	12	16.66%
C. krusie	7	9.72%

Table No. 3 : The Type of Candida species isolates

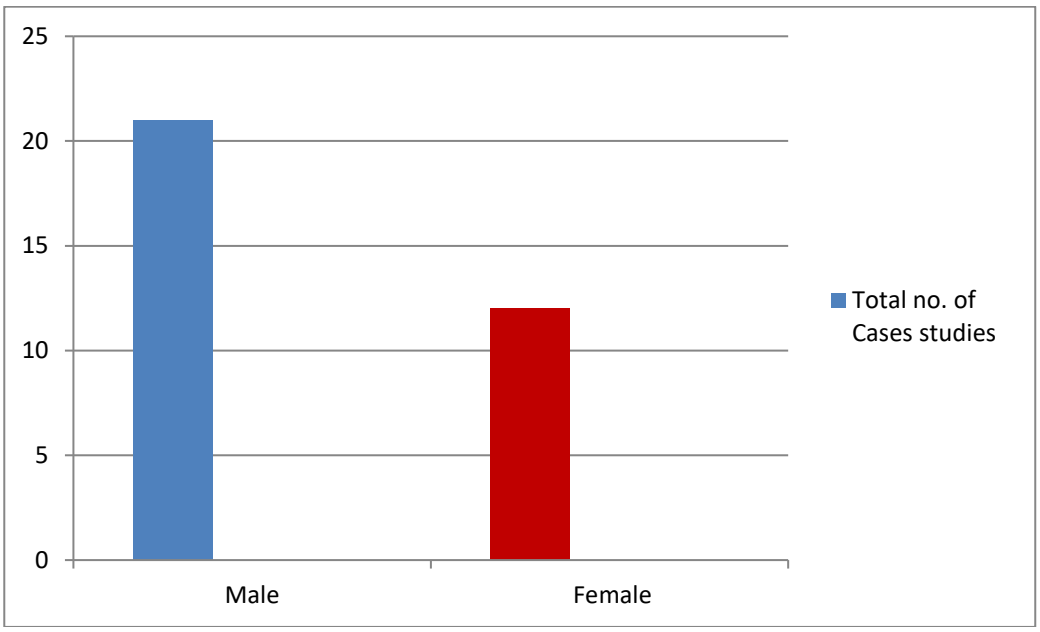


Graph No.1: Graphical Representation of the Type of Candida species isolates

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

Gender	Total no. of Cases studies (N=33)	Percentage
Male	21	63.63%
Female	12	36.36%

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



Graph No.2: Graphical Representation of the Genderwise distribution of the *Candida albicans*

In the current study it was observed that the ratio of Males 21 (63.63%) was more as compared to that of the Females 12 (36.36%) [Table No. 4] with the maximum age of 31-40 being affected the most followed by 21-30 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	4	12.12%
3.	21-30	9	27.27%
4.	31-40	11	33.33%
5.	41-50	4	12.12%
6.	51-60	3	9.09%
7.	≥61	2	6.06%

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

Type of Sample	Number of Isolates
BAL	2
Urine	16
Pus	6
Et secretion	5
Vaginal secretion	3
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 72 isolates a total of 33 isolates of *Candida albicans* were isolated. A total of 31(93.93%) samples of Candida were sensitive and 2(6.06%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method [Table No. 7].

Antifungal-Fluconazole	Number of isolates N=33	Percentage of isolates
Sensitive	31	93.93 %
Resistant	2	6.06%

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

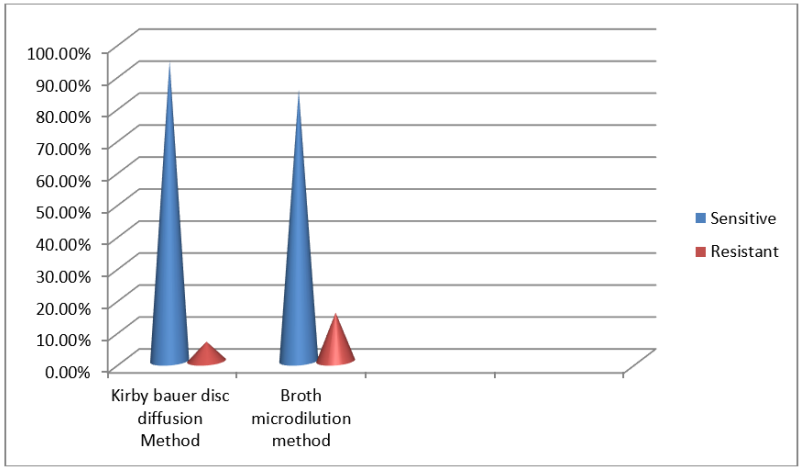
Antifungal-Fluconazole	Number of isolates N= 33	Percentage of isolates
Sensitive	28	84.85%
Resistant	5	15.15%

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 33 isolates of *C.albicans* tested for susceptibility pattern by CLSI broth microdilution method 28 isolates (84.85%) were sensitive and 5 isolates(15.15%) were resistant to Fluconazole showing Mic>/=64ug/ml.

Antifungal	Kirby bauer disc diffusion Method		Broth microdilution method	
	Sensitive	Resistant	Sensitive	Resistant
Fluconazole	31(93.93%)	2(6.06%)	28(84.85%)	5(15.15%)

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



Graph No. 3: Graphical Representation of the 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(6.06%) were resistant to Fluconazole whereas, 5(15.15%) showed resistance to Fluconazole by Broth microdilution method.

In the present study it was also observed that the maximum number of samples 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.

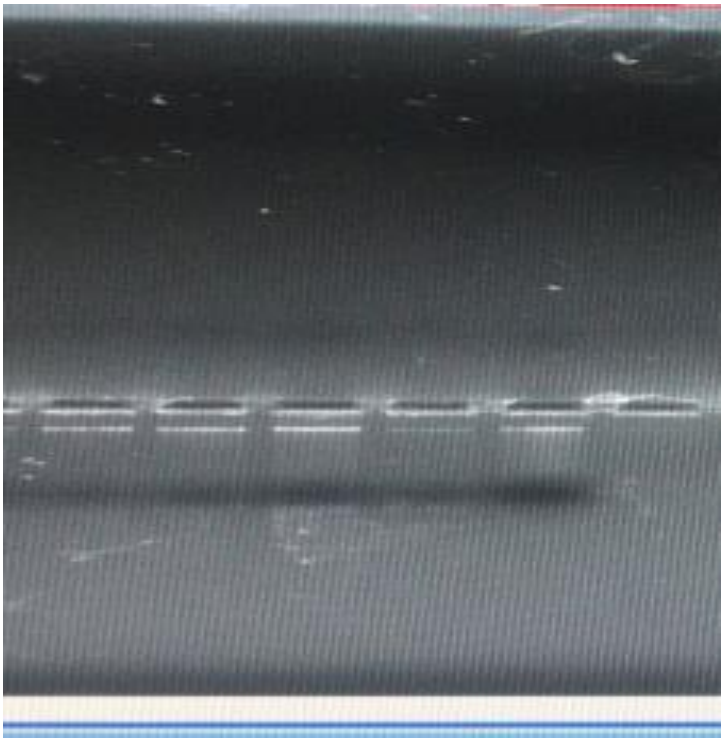


Figure No.4: The DNA Extraction of Fluconazole Resistant *Candida albicans*

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.5: L1-L5 are the sample positive for ERG11 gene, L corresponds to the DNA Ladder, L6-L10 are the sample positive for ERG11 gene, L11 is the positive control for ERG11 gene 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

Candidiasis is referred to both superficial and deep-tissue fungal infections often caused by *C. albicans.* A major increase in the incidence and prevalence of opportunistic fungal infections, specially caused by endogenous human commensal flora Candida species, has gained importance in the medical field worldwide. 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 [16]. The sequences of the *ERG11* gene of *C. albicans* species in the present study showed that they are highly mutated and possess all types of mutations; therefore, the mutation may be responsible for the development of fluconazole resistance. Many similar investigations addressing *ERG11* gene mutation reported different types of point mutations [17, 18].

In the present study a total of 72 consecutive Candida species were isolated from 764 clinical specimens out of which 33 (45.83%) 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 U.Chongtham et al [19], R.sharma et al [20], and H. bilal et al [21].

Among the non *albicans candida* (NAC) the *C.tropicalis* (27.7%) was the most common isolate followed by *C.glabrata* (16.6%) and least for *C. krusie* with 9.72%. This study was parallel 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 and C.glabrata.* This study was in support to the study performed by other research investigator Paul et al.,[22] where *C. tropicalis(30%)* was found to be the maximum followed by

C.Krusie (15%) and *C.glabrata* (8.3%) and similar study was performed by Kaur et al., [23]where *C.tropicalis* (35.2%) was the most common isolate followed by *C.glabrata* (16.7%) and least for *C. krusie* with 9.3% .

It was found that the ratio of Males 21 (63.6%) was more as compared to that of the Females 12(36.3%) with the maximum age of 31-40 being affected the most followed by 21-30 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. The finding was in accordance with Kaur et al. [23] were the ratio of males (64.8%) was more as compared to the females with 35.2%. and These finding were similar to the study by U.Chongtham et al [19] and R.sharma et al [20] .
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 was in accordance with R.Sharma et al[20] where the most was Urine sample (59.4%) was the common clinical sample followed by respiratory specimens (13.7%) and HVS (high vaginal swab) with positivity for candida infection. And similar with Umamaheshwari S.et al[24]. .
In the present study a total of 31(93.93%) samples of Candida were sensitive and 2(6.06%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method and 28 isolates (84.84%) were sensitive and 5 isolates(15.15%) were resistant to Fluconazole showing Mic>/=64ug/ml by broth microdilution method according to the CLSI guidelines 2022. This study was similar to the study performed by Lulu Zhang.et.al., [25] which showed 10.6% resistance and 89.2% sensitivity to fluconazole and study conducted by shirshaklamsalet.al.,[26]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 [27].
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 [28].
Overexpression of *ERG11* is common in azole-resistant clinical isolates of *C. albicans* and directly contributes to increased target abundance, ultimately lowering drug susceptibility· 29,30] .For *C. albicans*, the transcriptional activator, Upc2, is a crucial regulator of many ergosterol biosynthesis genes, including *ERG11*. Indeed, gain-of-function (GOF) mutations in *UPC2* cause the constitutive overexpression of ergosterol biosynthesis genes, a higher ergosterol content, and a reduction in fluconazole susceptibility [31-36]. Furthermore, disruption of *UPC2* in an azole-resistant clinical isolate abrogated *ERG11* overexpression and increased fluconazole susceptibility, highlighting the importance of this transcriptional activator in mediating *C. albicans* azole resistance.

Various virulence mechanisms developed by Candida play a role in this increasing drug resistance . Current data show that nearly 15.15 % 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 [37]. Understanding the mechanisms underlying fluconazole resistance is a crucial part of managing our limited antifungal repertoire.
The study purpose was to identify molecular mechanisms of resistance in a collection of arbitrarily selected susceptible and resistant isolates. Molecular tests based on our understanding of resistance mechanisms are unlikely to be sufficient to define clinical resistance in *C. albicans*. It may be important to consider alternate molecular tests that would monitor the general health or status of the fungal cell in the presence of drug in a shorter time than the present MIC determinations allow. Such tests might be able to accurately identify a resistant fungal isolate quickly, without the need for detailed knowledge about the exact molecular mechanisms of resistance.

Drug-resistant fungal infections have been found to be more prevalent, and azole cross resistance as well as decreased azole sensitivity, particularly to fluconazole, have been noted 3. These results lead us to conclude that AST is desperately needed, particularly in situations where doctors plan to use fluconazole—the least effective agent—or in situations where non-Candida albicans species are suspected due to their comparatively high rates of resistance.

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.

Declarations:
Conflicts of interest: There is no any conflict of interest associated with this study
Consent to participate: We have consent to participate.
Consent for publication: We have consent for the publication of this paper.
Authors' contributions: All the authors equally contributed the work.

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