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Molecular Characterization Of Mdr1 Gene Inassociation To Antifungal Resistance In Pathogenic Candida Species

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ABSTRACT

Introduction: A very common illness that poses serious health hazards, particularly to immunocompromised people, is candidiasis. More than a billion infections occur each year, making it the most prevalent opportunistic fungal infection affecting human health worldwide. Numerous antifungal drugs have caused pathogenic Candida species to develop both acquired and innate resistance.

Aim And Objective: To study the molecular characterization of MDR1 genein association to its antifungal resistance in pathogenic *candida* species

Material And Methods: This was a cross sectional study conducted in the Department of Microbiology at a tertiary care centre. A total of 962clinical samples were screened. The Culture identification, specification, Antifungal Susceptibility testing was performed according to the CLSI guidelines. The DNA was extracted using the Qiagen DNA extraction kit and the resistant gene MDR1 was detected using the PCR.

Results: In the present studyout of 962clinical samples, 51.1% (492) were culture positive, among them 28% (138) were Candida isolates. Out of which 53(38.4%) were *Candida albican*s while 85(61.6%) wereNon-albicans Candida. Among Non-albicans Candida,the frequency of *Candida tropicalis* was observed to be maximum with urine samples and least for ET secreation. It was observed that the maximum sensitivity was observed with Amphotericin-B (95%), followed by Voriconazole(85%) and itraconazole (49.2%).The prevalence of MDR1 expression was 5.7% among Candida spp.

Conclusion: Characterizing the resistance genes in Candida species isolated from a range of clinical specimens will help us better understand the pathophysiology and clinical outcomes of candidiasis.

Keywords: *Molecular Characterization,Virulence factors, Fluconazole, DNA, PCR, MDR1, Gene Expression*

1. INTRODUCTION

Candida albicans is an important opportunistic fungal pathogen of humans. The azole antifungal agent fluconazole is a widely used compound to treat candida infections [1,2]. It is effective against a wide range of Candida species, including C. *albicans*, C. *glabrata*, C. *tropicalis*, C. *krusei*, and C. *parapsilosis*.In recent years, however, the incidence of treatment failures has been rising [3]. Fluconazole antifungal works by inhibiting the growth of *Candida albicans* by targeting the fungal cell membrane. The drug targets an enzyme called 14-alpha-demethylase, which is responsible for converting

lanosterol to ergosterol which is a component of the fungal cell membrane [4]. Recently, resistance to common antifungals has been reported in different *Candida* species[5,6]&the prevalence of drug resistance to fluconazole among *Candida albicans* is an increasing concern in the medical community [7].

Candida spp can develop resistance to fluconazole by different molecular mechanisms, including alterations in the sterol biosynthetic pathway, overexpression of *ERG11gene*, which encodes the target enzyme of fluconazole (sterol 14α-demethylase, or Erg11p), mutations in *ERG11* that result in a reduced affinity of Erg11p for fluconazole, and overexpression of genes encoding membrane transport proteins (*CDR1, CDR2*, and *MDR1*) that actively transport fluconazole out of the cell.Though alteration in the ergosterol syntheses pathway is one of the known potential resistance mechanism of azoles , overexpression of efflux pumps is one of the important causes. [8,9,10].

The two types of azole transporters in *C. albicans* have been identified: the major superfamily transporter encoded by MDR1 and the ATP-binding cassette (ABC) transporters encoded by CDR1 and CDR2 [11].These pumps differ in the specificity of the azole molecule and in the source of energy used to translocate the compounds across the cell membrane. The Cdr proteins are primary transporters able to transport all azole compounds using the hydrolysis of ATP; on the contrary, Mdr1p pump is a secondary transporter which uses proton gradient for extrusion of fluconazole which is encoded by MDR 1gene. [12,13]. The *MDR1* gene is not detectably expressed in vitro in fluconazole-susceptible *C. albicans* isolates but is strongly activated in many strains after the development of fluconazole resistance[14-16].While *MDR1* is normally expressed only at low levels in standard laboratory media, many fluconazole-resistant clinical *C. albicans* isolates constitutively overexpress *MDR1*. Deletion of the *MDR1* gene from *MDR1*-overexpressing *C. albicans* isolates resulted in decreased fluconazole resistance of the mutants, confirming that *MDR1* overexpression contributed to the resistant phenotype of these isolates [11]. Fluconazole resistance is usually a stable phenotype that is maintained in the absence of selection pressure by the drug. This implies that genetic alterations occurrs in the resistant isolates that result in a constitutive overexpression of the drug efflux pumps [17]. The effect of overexpression of these efflux pumps is the decreased intracellular concentration of azole available for inhibition of the target enzyme (lanosterol 14α- demethylase). Mutations in the transcription factors TAC1 (transcriptional activator of CDR genes) and MRR1 (multidrug resistance regulator 1) are responsible for upregulation of CDR1/CDR2 and MDR1, respectively To date, nineteen point mutations in different domains of TAC1 have been identified and fifteen mutations for MRR1 [18].The upregulation of MDR1 is responsible for fluconazole resistance and upregulation of ABCtransporters results in multi azole resistance [19].

To overcome this problem, combination therapy using different classes of antifungal medications may be effective in treating fluconazole-resistant *Candida albicans* infections[20].

Overuse of the medications, however, may cause resistance to develop, making the fungus more difficult to cure. As a result, it's critical to employ alternative treatments wherever feasible and to use fluconazole only when required [18]. Given these worries, it's critical to assess the scope of the issue, identify the species of Candida causing infections, and ascertain how susceptible they are to antifungal medications. Furthermore, identifying the MDR1 gene in fluconazole-resistant Candida species can contribute in the development of more potent treatments by shedding light on the mechanisms behind resistance.

Therefore, the present study was undertaken for the detection of the molecular characterization of MDR1 gene in association to its antifungal resistance in pathogenic *candida* species.

2. MATERIAL AND METHODS

This was a cross-sectional study carried out in the Department of Microbiology at a tertiary care centre, for a period of 1 year i.e, December 2023 to December 2024. The Demographic details and clinical history along with the relevant clinical investigations were recorded.

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

Exclusion Criteria: Isolates of Candida species from mix cultures and repeat isolates from the same clinical specimen of the same patient were excluded.

Isolation and Identification of *Candida species:*

Samples: Urine, sputum, blood, vaginal swabs,pus, ET secretions, pleural fluid, ascitic fluidwere collected using aseptic precautions as per the Standard guidelines.

Sample Processing: Direct gram staining were performed to see the presence of yeast and pseudohyphae of Candida species from the different samples. Urine samples were inoculated on CLED agar while others were inoculated on Blood agar for 24 hrs at 37ºC. Then the colonies from these plates were cultured on SDA and CHROMagar and incubated for 24-48hrs at room temperature.Growth of Candida was identified by colonial characteristics as white to creamy and pasty colonies.

Species Identification: Candida species were identified phenotypically by Gram staining, Germ tube test, Colonies on CHROMagar, Biochemical tests like urease test & Carbohydrate assimilation test.

Antifungal susceptibility testing: 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 2024 [21].

Molecular Identification of MDR1 gene of Fluconazole Resistant *Candida species*

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 instructions.

Primer sequence ATGTTGGCATTCACCCTTCGAAAACTTCTGGGAAAACTGG of 426bp length was used to detect the MDR 1 target gene. [22]. For the PCR amplification, 2 µl of template DNA was added to 18 µl reaction containing 10 µl of Qiagen master mix, 2 µl of primer mix (1 µl each of the respective forward and reverse primers) and 6 µl of molecular-grade water. The cyclic conditions for MDR1 gene, initial denaturation at 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 59 °C for 1 min 30 s and 72 °C for 1 min 30 s were followed by extension of 72 °C for 10 min.

The Agarose gel preparation and visualized by Gel Doc™ EZ Gel Documentation *System: 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.

Statistical analysis: Data was recorded in the Microsoft Excel. The values were represented in the Numbers, percentage and bar diagram...

3. RESULTS

In the present study,a total of 962 different samples were received in the laboratory. Out of the these samples, 51.1% (492) were culture positive, among them 28% (138) were Candida isolates. Among the 138 Candida isolates, 53(38.4%) were *Candida albican*s while 85(61.6%) were Non-albicansCandida.**Frequency of culture positive and culture negative is represented in** [Table no. 1]

Table No.1: Frequency of culture positive and culture negative.

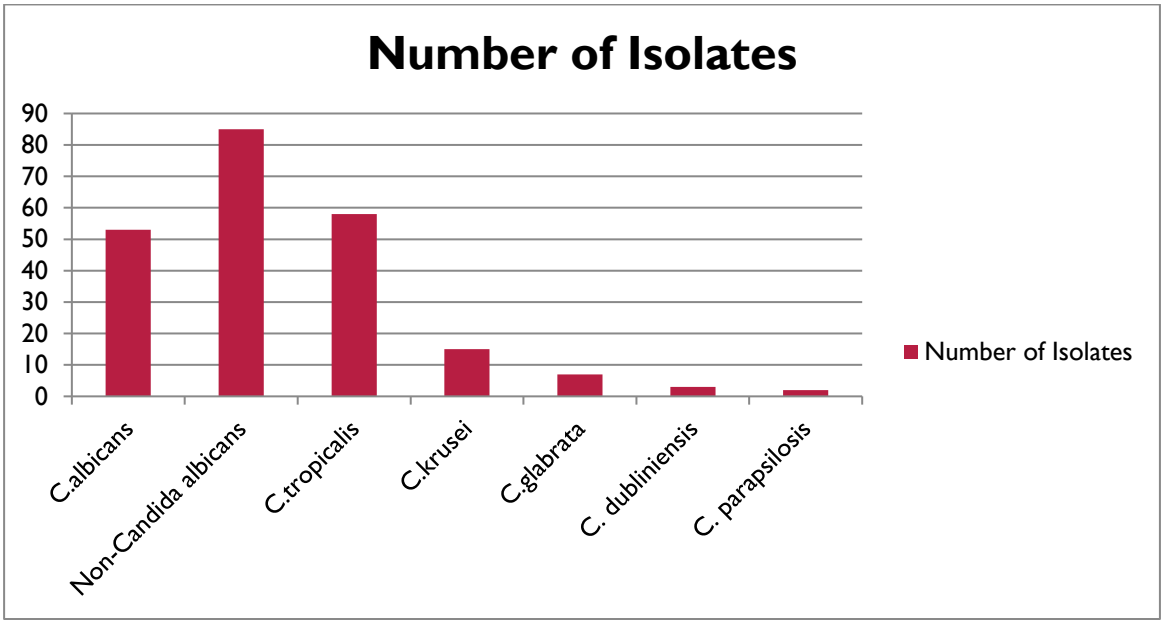
TOTAL SAMPLE	FREQUENCY	PERCENTAGE
Culture positive	492	51.1%
Candida sp	138	28%
other	354	71.9%
Culture negative	478	49.6%
Total	962	100%

Among Non-albicans Candida, 58 (68.2%) isolates were identified as *Candida tropicalis*.followed by *Candida krusei*15 (17.6%),*Candida glabrata* 7 (8.23%) while 5 (5.8%) belongs to other group [Table no. 2].

Table No. 2: Frequency of Candida isolates

CANDIDA ISOLATES	NO. OF ISOLATES	PERCENTAGE
C.albicans	53	38.4%
Non-Candida albicans	85	61.6%

C.tropicalis	58	68.2%
C.krusei	15	17.6%
C.glabrata	7	8.23%
C. dubliniensis *C. parapsilosis*	3 2	3.5% 2.3%
TOTAL	138	100%



Graph No. 1: Graphical representation of species distribution

Maximum number of Non-albicans candidawere isolated from Urine samples (44.7%) followed by Vaginal swab (22.3%), sputum(20%),ET secretions (7.05%), pus(7.07%), & Blood (1.17%). While pleural fluid and ascitic fluid showed no growth. While frequency of *C.albicans* was found more in Sputum sample(39.6%), followed by urine(24.5%),Vaginal swab (22.6%), pus (5.6%), blood (3.7%), and ET secretions(3.7%) as depicted in Table no3.

Table No.3: Distribution of *Candida species* among different samples

SAMPLE	*C.albicans*(N=53)	PERCENTAGE	Non-albicans Candida(N=85)	PERCENTAGE
Urine	13	24.5%	38	44.7%
Pus	3	5.6%	4	7.07%
Vaginal swab	12	22.6%	19	22.3%
Blood	2	3.7%	1	1.17%
Sputum	21	39.6%	17	20%
ET secretions	2	3.7%	6	7.05%
Pleural fluid	0	0%	0	0%
Ascitic fluid	0	0%	0	0%

Antifungal susceptibility test shows maximum sensitivity towards Amphotericin-B (94.7%), Voriconazole(85.1%) & itraconazole (49.7%) while fluconazole (33%), cotrimoxazole (15.3%), nystatin (10.4%), shows least sensitivity against Candida isolates as illustrated in table 5.

Table No. 4: Antifungal Drug Resistance Patterns .

ANTIFUNGAL DRUG	SENSITIVITY (%)	RESISTANCE (%)
Fluconazole	33% (45)	67.3% (93)
Cotrimoxazole	15.2% (21)	84.7% (117)
Nystatin	10.1% (14)	89.8% (124)
Itraconazole	49.2% (68)	50.7% (70)
Voriconazole	85% (117)	15.2% (21)
Micafungin	94.2% (130)	5.7% (8)
Amphotericin-B	95.6% (132)	4.3% (6)

The prevalence of MDR1 gene in *Candida* in the present study is 5.7%. Distribution of MDR1 gene is depicted in Table no 6

Table No. 5 Distribution of Drug resistance MDR1 gene among Candida isolates.

SPECIES	TOTAL SAMPLES	MDR1
Candida albicans	**53**	**3**
Candida tropicalis	**58**	**2**
Candida krusei	**15**	**2**
Candida glabrata	**7**	**1**
Other non-*Candida* spp.	**5**	**0**
Total	**138**	**8**

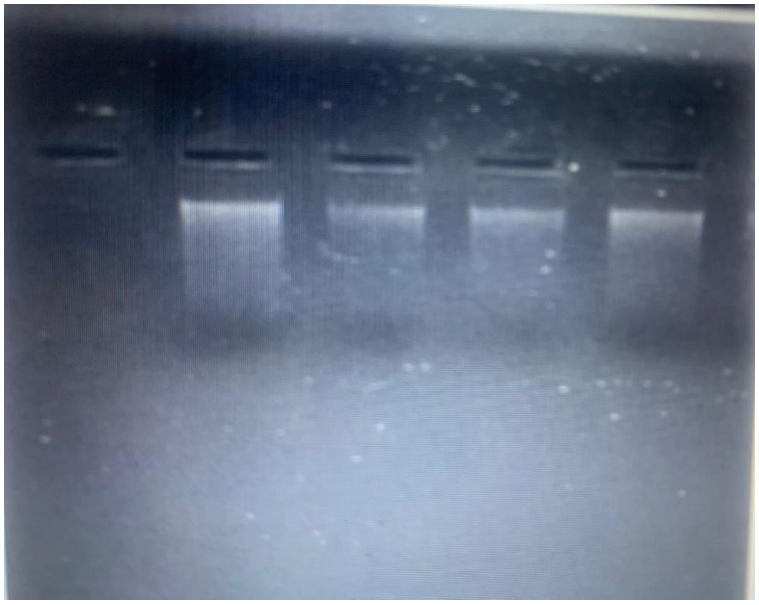


Figure No. 1: The DNA Extraction in Agarose gel

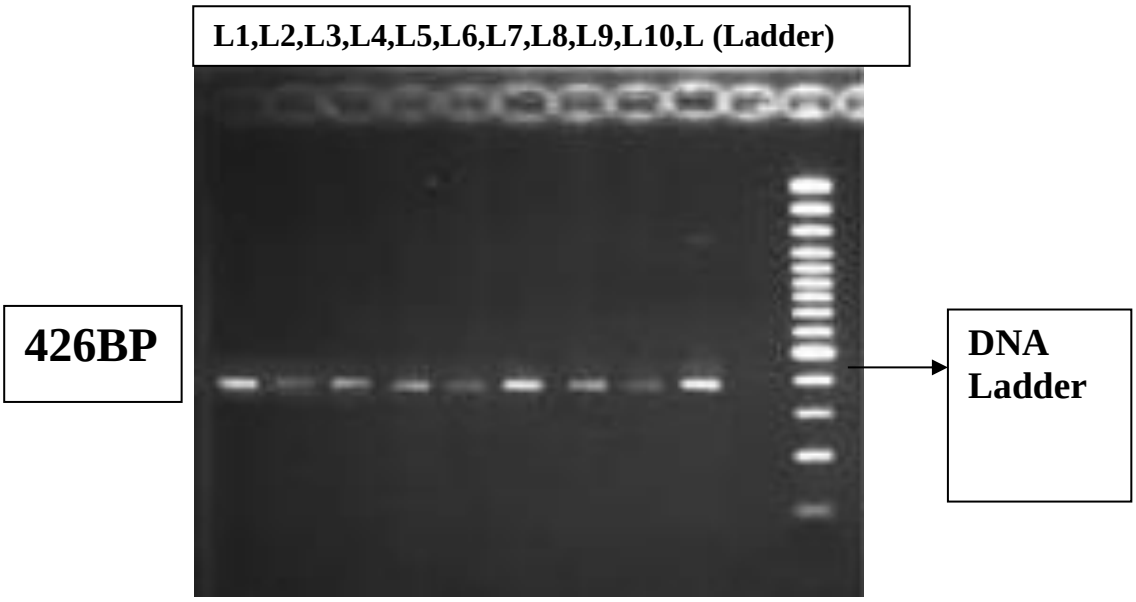


Figure No. 2: Gene *MDR1* geneL1 corresponds to the positive Control; L2-L9 Corresponds to the sample positive for MDR1 gene; L10 is the Negative Control to MDR1 ; L corresponds to the DNA Ladder

4. DISCUSSION

Candida is an opportunistic fungal pathogen with the potential to cause both serious systemic infections and superficial mucosal infections, particularly in patients with weakened immune systems. The antimycotic drug fluconazole, which prevents the manufacture of ergosterol, the primary sterol in the fungal cell membrane, is commonly used to treat C. albicans infections. Through a variety of molecular mechanisms, C. albicans can become resistant to fluconazole including overexpression of the genes encoding membrane transport proteins (CDR1, CDR2, and MDR1) that actively transport fluconazole out of the cell[23].

In the current studyout of 138 Candida isolates obtained from different clinical specimens over a period of one year. Maximum *C. albicans* were isolated from sputum followed by urine, vaginal swab, pus, and blood representing 39.6%%, 24.5%, 22.6%%, 5.6%, and 3.7% respectively. Non-albicans species was detected in urine (44.7%) followed by vaginal swab (22.3%), sputum (20%), ET secretions (7.05%), and pus (7.07%). The study conducted by the Vignesh Kanna B. et al [24] noted majority of isolates from high vaginal swab (34%) followed by sputum (28%), urine (18%), pus from surgical sites and others constituted to 20%.There was another study by Sharma et.al in 2023 where Candida were isolated from Urine 59.4%, Respiratory specimen (ET secretions and Broncho-alveolar lavage) 13.7%, Pus 5.2% and Blood 9.8% [25].

Potential clinical importance of species level identification has been recognized as Candida species differ in the expression of virulence factors and antifungal susceptibility [26]. Candida species also have a direct impact on the choice of empirical antifungal therapy and clinical outcome. Non-albicans candida species are on the rise due to increasing immunocompromised conditions. Candida albicans was the predominant species and *C. tropicalis* is reported to be the most predominant species among the non-albicans candida in the present study. Predominance of *C. albicans* was also seen in a study by Manjunath et al [27]. However,higher incidence of non-albicans candida ranging from 54-74% have been seen in numerous studies [28-30]. The study conducted by the Vignesh Kanna B. et alalso noted *Candida albicans* (51%) as the most common *candida* species, followed by *C. tropicalis* (25%), *C. krusei* (16%), *C. glabrata* (6%) and *C. dubliniensis* (1%) [24].

Overexpression of MDR1, which encodes a membrane transport protein of the major facilitator superfamily, is one mechanism by which the human fungal pathogen Candida albicans can develop increased resistance to the antifungal drug fluconazole and other toxic compounds. In the present study the prevalence of MDR1 drug resistance genes in *Candida is* 5.7%. This study was parallel to the study conducted by Ben-Ami et al. and Papon et al. where the MDR1 gene expression was 18% and 15 % respectively[31-32]. A study by Mehrnoush Maheronnaghsh et al stated that 12% MDR1 gene were expressed [33].It is crucial to consider the interplay of genetic modifications, phenotypic characteristics, and patient-related factors, allowing for a more comprehensive assessment of azole resistance [34,35].

5. CONCLUSION

In order to better treat Candida infections, additional investigation into the processes that connect virulence factors with drug resistance is needed, whichnecessitates tailored antifungal medication.After fluconazole resistance develops, the MDR1 gene

is substantially activated in many strains of Candida albicans, although it is not expressed in vitro in isolates that are susceptible to the drug. The molecular alterations that cause the MDR1 gene to constitutively activate in fluconazole-resistant needs to be better understood.

Further research is required to improve patient outcomes in clinical practice and address the evolving issues brought on by Candida infections. The combination of genetic changes, clinical characteristics, and patient-related factors allows for a more comprehensive assessment of azole resistance.

Declarations:

Conflicts of interest: There is not any conflict of interest associated with this study.

Consent for publication: There is consent for the publication of this paper.

Authors' contributions: Author equally contributed the work.

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