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IN-VITRO ANTIFUNGAL SUSCEPTIBILITY REVEALS OCCURRENCE OF AZOLE AND ALLYLAMINE RESISTANCE AMONG CLINICAL ISOLATES OF *CANDIDA ALBICANS* AND *CANDIDA NON ALBICANS* FROM CENTRAL INDIA

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Keywords:

Drug Resistance, *Candida*, Antifungal, CLSI M-27-A method

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ABSTRACT: Background: Drug resistance among *Candida* species constitutes the most significant problem in the treatment of Candidiasis.Systematic studies on antifungal drug susceptibility which may be useful in deciding clinical strategies are not routinely done in India and other developing countries. **Objective**: Aim of this study was testing sensitivity of clinical isolates of *Candida albicans and* non *albicans* to frequently prescribe antifungal drugs, fluconazole, ketoconazole, Itraconazole, Amphotericin B and Terbinafine. **Material and methods:** 25 strains of *C. albicans* and non *albicans* were tested *in vitro* for susceptibility to five antifungal agents, by using standard broth macro dilution method (CLSI M 27-A). **Results:** The present study revealed the percentage and extent of emerging drug resistance and cross resistance among Central Indian clinical isolates of *C. albicans* and non *albicans* against azoles and allylamine. In the present study, the total percentage of resistance was found to be 84% (21/25). The drug for which maximum resistance were found was Ketoconazole (64%) followed by Itraconazole (44%) Terbinafine (24%) and lastly Fluconazole (20%). The total percentage of cross resistance was 62% (13/21) and the maximum seen in *C. albicans* followed by C. *glabrata, C. krusei* and *C. guilliermondi*. No resistance was found for polyene drug Amphotericin B. **Conclusion:** This short study from India has exhibited the increasing frequency of resistance and Cross resistance of *C.andida* species against azoles and allylamine. We suggest a comprehensive study to determine the extent and degree of antifungal drug resistance among *Candida* species in India.

INTRODUCTION: The genus *Candida* includes several species implicated in human pathology. *Candida albicans* is by far the most common species causing infections in humans. The emergence of non-albicans *Candida* species as a significant pathogens has however been well recognized during the past decade.[1]

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Candida spp. have been shown to cause a similar spectrum of disease ranging from oral thrush to invasive disease, yet differences in disease severity and susceptibility to different antifungal agents have been reported [2, 3] Immunocompromised population is increasing throughout the world as well as in developing countries including India.

It includes, HIV/AIDS patients, diabetics, cancer patients, people under long term antibiotic treatment and chemotherapy, increasing number of organ transplantation patients, patients undergoing surgeries and using prosthetic implants/ catheters inside the body. This will add to those susceptible to *C. albicans*, resulting in increase in the incidence

of Candidiasis infections in immunocompromised population.[4]

Options of the antifungal drugs available for treatment of systemic and invasive Candidiasis are restricted to polyenes, allylamines, azoles and recently developed echinocandin class of molecules [5, 6]. Amphotericin B, a polyene fungicidal agent, has been used for the treatment for invasive Candidal infections, but cost and dose related side effects limit its use [7]. Azole group of drugs, are commonly used in treating many forms of Candidal infections for a long time, however, their prolonged use has led to the development of drug resistance in C. albicans and other species. Azole resistance is more seen in non Candida albicans spp. compared to Candida albicans .Undesirable side effects, toxicity and emergence of drug resistance are the limitations for use of these drugs. Emergence of drug resistance in C. albicans is reported from all over the world [8]. Studies on prevalence of infections and antifungal susceptibility testing are useful in deciding clinical strategies [9, 10]. Although routinely done in developed countries, such systematic studies are lacking in countries like India, except few isolated reports [11]. Antifungal susceptibility test was done by broth macrodilution method, as per CLSI guideline in M27-A document. By doing early speciation of Candida and performing antifungal susceptibility tests, it will be helpful in guiding physicians to select the proper antifungal drug and thus therapeutic failures can be prevented. Aim of this study was to detect resistance and cross resistance if any among Central Indian clinical isolates of *C. albican* and *C.non albicans.*

MATERIALS AND METHODS:

Study design: - The study design followed the recommendations of new document (M 27-A) macro dilution method for determining '*in vitro*' susceptibility pattern of *Candida* spp. against antifungal drugs.

Test Organism: - A panel of 25 well characterized pathogenic yeast isolate from the Medical Mycology Research laboratory, Department of Biosciences, Jabalpur (M.P.), India, was used in this study **(Table 1)** Before testing; all isolates were sub cultured on to SDA slants to ensure optimal growth characteristics.

Quality control strains: Quality control strains were used in every testing batch recommended by CLSI. *C. albicans* ATCC 90028 and C. *tropicalis* ATCC 750 and C. *glabrata* ATCC 90030. MICs for these reference strains were compared with published control limits and used to guide antifungal susceptibility testing and validation as per CLSI guidelines [12, 13, 14.]

Antifungal Drugs: The following five antifungal drugs were used. Amphotericin B (AMFOCAN, Dabur India Ltd.), Ketoconazole (NIZRAL; Johnson and Johnson) Janssen Pharmaceutical, Regd. trademark of Johnson and Johnson, USA), Itraconazole capsules (CANDISTAT; E Merk India Ltd; Licensed user of T.M.), Fluconazole (Flustan^{TM}; Dr. Reddy's Lab. Ltd.)^{TM} Trademark under registration and Terbinafine (DASKIL); Novartis India Ltd).

Drug Dilutions: In this study drug dilutions were prepared according to CLSI M27-(A) Protocol with slight minor modification [15.] Stock solution of drug (100 xs) was prepared in 100 % DMSO (dimethyl sulphoxide (Sigma chemicals. co. St. Louis. Mo.) for (itraconozole, ketoconazole and amphotericin B) or sterile distilled water (fluconazole) or PEG-400 (Polyethylene glycol (union carbide, Danbury, Conn) for terbinafine immediately prior to use. From these stock solutions intermediate test drug dilutions were prepared which is to be 10 x the strength of the final drug concentrations, with RPMI 1640 medium as diluent (by two fold drug dilution scheme);CLSI standards (Approved M27-A) of macrodilution broth reference method across the concentration range (0.03125µg/ml-64µg/ml).

Inoculum Preparation: All isolates were subcultured at least twice to ensure purity and viability. Yeasts were grown on SDA (Sabouraud's Dextrose Agar) plates for 24 hr at 35° C. Five colonies each at least 1mm in diameter were suspended in 5ml of sterile 0.85 % saline. The resulting yeast suspension was mixed for 15s with a vortex. The turbidity of the suspension was adjusted spectrophotometerically to match the transmittance of a 0.5 McFarland barium sulphate turbidity standard at 530 nm. This procedure yielded a yeast stock suspension of 1 x 10^6 to 5 x

10^6 cells/ml A working suspension was made by 1: 100 dilution followed by 1: 20 dilution of the stock suspension with RPMI 1640 broth medium, which results in 0.5 x 10^3 to 2.5 x 10^3 cells/ml [16]. Test inocula were made in sufficient volumes to directly inoculate each MIC tube with 0.9 ml.

Test Medium: Medium used was RPMI 1640 (10.3 g/L) with L-glutamine without bicarbonate (Himedia, India) buffered to pH 7.0 with MOPs buffer (3-N-morpholino Propane sulphonic Acid) to a final molarity 0.165 M.

Susceptibility testing procedure: The 10 X drug dilution were dispensed in 0.1 ml volumes into sterile glass bottles. Each bottle was then inoculated by 0.9 ml volumes of diluted yeast inoculum suspension. This step brought the drug dilutions to the final test drug concentration (64 µg/ml - 0.03125 µg/ml) for all five antifungal drugs. The growth control tube (s) contained a 0.9 ml volume of an inoculum suspension and a 0.1 ml volume of drug free medium. Quality control organism was tested in the same manner as the other isolates and was included each time with the tested batch. In addition, 1 ml of un-inoculated drug free medium was included as a sterility control. All bottles were incubated at 35^0 C and MICs were read after 24 and 48 hr of incubation according the CLSI M27-A protocol [12, 13, 14] and [17]. All the experiments were performed in triplicates. Results obtained are the outcome of the three different observations.

Interpretation of results: MICs for the antifungal drugs were read after 48 hours and the interpretive breakpoints were as suggested by CLSI. These were as follows. The tested strains were categorized into three groups: **Susceptible (S), Susceptible Dose Dependent (SDD) and Resistant (R).** Amphotericin B susceptibility breakpoint-≤ 0.25µg / ml (S), Breakpoints for fluconazole were ≤ 8µg / ml (S), 16-32µg / ml (SDD), ≥ 64 µg / ml (R), for itraconazole ≤ 0.125 µg / ml (S), 0.25-0.5µg / ml (SDD), ≥ 1µg / ml (R), for ketoconazole ≤ 0.0625µg / ml (S), > 0.125 µg / ml (R) (10,11,12). Terbinafine susceptibility breakpoints were ≤ 8µg / ml (S), 16-32µg / ml (SDD), ≥ 64 µg / ml (R) [12,13].

Stastical analysis: Student 't" test was employed to determine the significance of the difference between the geometrical means of MICs values. Statistically significant was set at $P<0.05$

RESULT:

Susceptibility testing procedure for twenty five clinical isolates was carried out and all the *C. albicans* and *non albicans* isolates showed susceptibility to Amphotericin B with MICs in the range of 0.0625-0.25µg/ml. For the drug Terbinafine, 76 % isolates were categorized as sensitive (0.03125- 4ug /ml) and resistance was exhibited by 24 % isolates (MIC > 64ug/ml).For Azoles, only Fluconazole showed all three pattern of susceptibility i.e 40% of isolates showed susceptibility (MICs 0.25-4ug/ml), 40% showed SDD (16 ug/ml) and rest 20% showed resistance(64 ug/ml). For Ketoconazole only 36% of isolates showed susceptibility (MICs 0.03125-0.125ug/ml) yet 64% showed resistance (1-64ug/ml). For Itraconazole 56% isolates showed susceptibility (0.03125-0.125ug/ml) and 44% showed resistance (1-8ug/ml) **(Table 1, 2)**. Therefore **Fig.1**.express the drug for which the maximum number of isolates from total number (25) with maximum frequency showed resistance were against ketoconazole (64%) followed by Itraconazole (44%), Terbinafine (20%) and fluconazole (20%). The total percentage of resistance was found to be 84% (21/25).

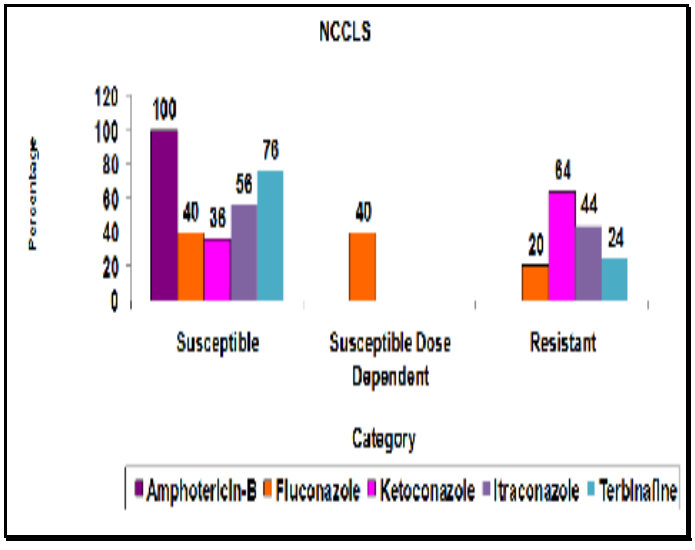

FIG 1. OVERALL PERCENTAGE OF ANTIFUGAL SUSCEPTIBILITY PATTERN AGAINST 25 CLINICAL ISOLATES OF *C. ALBICANS* AND NON *ALBICANS*

TABLE 1: CHARACTERISTICS OF THE PATIENTS AND MIC VALUES FOR ALL TWENTY FIVE *C. ALBICANS AND NON ALBICANS* CLINICAL ISOLATES AGAINST FIVE ANTIFUNGAL DRUGS BY ANTIFUNGAL SUSCEPTIBILITY TESTING.
(Abbreviation: AMB: Amphotericin–B, FLU: Fluconazole, ITRA: Itraconazole, KETO: Ketoconazole, TER: Terbinafine)

Isolate No.	Underlying Diseases	Source of isolates studied	Species of *Candida*	Drugs MIC (µg/ml)				
				AMB	FLU	ITRA	KETO	TER
1	Tuberculosis Vulvo Vaginal	Sputum	*C. albicans*	0.0625	0.5	1	0.03125	4
2	Candidiasis (VVC)	Vaginal wash	"	0.125	0.5	2	8	2
3	Septicaemia	Blood	"	0.25	1	2	0.03125	≥64
4	Diabetes mellitus	Blood	"	0.25	16	8	16	≥64
5	Cellulitis	Urine	"	0.0625	0.25	1	2	4
6	Oropharyngeal infection	Oral Wash	*C. parapsilosis*	0.0625	16	0.03125	0.03125	4
7	Post-operative case	Urine	"	0.0625	4	0.03125	8	4
8	Vulvo Vaginal Candidiasis (VVC)	Vaginal Wash	"	0.125	16	0.03125	1	4
9	Vulvo Vaginal Candidiasis (VVC)	Vaginal Wash	"	0.25	16	0.03125	8	0.03125
10	Post-operative case	Blood	"	0.25	4	0.0625	0.0625	4
11	Altered sensorium	Blood	*C. tropicalis*	0.125	16	0.03125	64	≥64
12	Cancer	Blood	"	0.0625	16	1	0.03125	1
13	Cancer	Blood	"	0.125	16	0.0625	0.03125	1
14	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	"	0.3125	16	0.03125	4	≥64
15	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	"	0.03125	16	0.03125	0.03125	2
16	Diabetes mellitus	Urine	*C. glabrata*	0.0625	16	2	8	4
17	Post operative case	Blood	"	0.0625	64	4	0.03125	0.03125
18	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	"	0.125	64	0.0625	2	1
19	Acute renal failure	Urine	"	0.25	16	0.0625	8	4
20	Cancer	Blood	*C.guilliermondii*	0.03125	64	2	8	0.03125
21	Pulmonary tuberculosis	Blood	"	0.0625	0.5	0.03125	8	≥64
22	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	"	0.0625	16	4	0.125	4
23	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	*C. krusei*	0.25	64	2	0.125	1
24	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	*C. krusei*	0.25	64	0.0625	0.125	4
25	Vulvo Vaginal Candidiasis (VVC)	Vaginal wash	*C. viswanathii*	0.0625	0.5	0.125	0.125	>64

TABLE 2. SUSCEPTIBILITY STATUS OF 25 CLINICAL ISOLATES OF CANDIDA SPECIES AGAINST FIVE ANTIFUNGAL DRUGS.
(Abbreviation: S, Sensitive; DD, Dose dependent; R, Resistant)

Drugs	Amphotericin B			Fluconazole			Itraconazole			Ketoconazole			Terbinafine		
Category	S*	SDD**	R#	S*	SDD**	R#	S*	SDD**	R#	S*	SDD**	R#	S*	SDD**	R#
***C. albicans* (5)**															
CLSI	5	-	-	4	1	-	-	-	5	2	-	3	3	-	2
Percentage%	100%	-	-	80%	20%	-	-	-	100%	40%	-	60%	60%	-	40%
***C. parapsilosis* (5)**															
CLSI	5	-	-	2	3	-	5	-	-	2	-	3	5	-	-
Percentage%	100%	-	-	40%	60%	-	100 %	-	-	40%	-	60%	100%	-	-
***C. tropicalis* (5)**															
CLSI	5	-	-	-	5	-	4	-	1	3	-	2	3	-	2
Percentage%	100%	-	-	-	100%	-	80%	-	20%	60%	-	40%	60%	-	40%
***C. glabrata* (5)**															
CLSI	4	-	-	2	-	2	2	-	2	1	-	3	4	-	-
Percentage%	100%	-	-	50%	-	50%	50%	-	50%	25%	-	75%	100%	-	-
***C. guilliermondii* (3)**															
CLSI	3	-	-	1	1	1	1	-	2	-	-	3	2	-	1
Percentage%	100%	-	-	33%	33%	33%	33%	-	66%	-	-	10%	66%	-	33%
***C. krusei* (2)**															
CLSI	2	-	-	-	-	2	1	-	1	-	-	2	2	-	-
Percentage%	100%	-	-	-	-	100%	50%	-	50%	-	-	100%	100%	-	-
***C. viswanathii* (1)**															
CLSI	1	-	-	1	-	-	1	-	-	1	-	-	-	-	1
Percentage%	100%	-	-	100%	-	-	100%	-	-	100%	-	-	-	-	100%

Emerging resistance in *C.albicans* and non *albicans* isolates.

C. albicans showed highest and complete (100%) resistance for Itraconazole, then 60% for Ketoconazole and 40% for Terbinafine. *C. parapsilosis* showed 60%. Resistance only against Ketoconazole. *C. tropicalis* showed 40% resistance against both Ketoconazole and Terbinafine and only 20% resistance against Itraconazole. *C. glabrata* showed highest resistance 75% against Ketoconazole and then next resistance was of 50% for both Itraconazole and Fluconazole. *C. guilliermondi* showed highest and full resistance 100% against Ketoconazole then for Itraconazole 66% and then 33% for both Fluconazole and Terbinafine. *C. krusei* showed full and highest 100% resistance for both Ketoconazole and Fluconazole and 50% for Itraconazole ie it showed resistance to all the three azoles. *C. vishwanathi* showed 100% resistance for Terbinafine only. For all the three azoles highest resistance was showed by *C. krusei* followed by *C. gulliermondi* and *C. glabrata. C. albicans* and *C. tropicalis* showed resistance for both Itraconazole and Ketoconazole and lastly *C. parapsilosis* showed resistance only for Ketoconazole. For Terbinafine only *C. albicans*, *C.tropicalis*, *C. guilliermondi* and C. *vishwanathii* showed resistance **(Fig 2)**.

Cross resistance pattern among Candida species- *C. albicans* (Isolate no.2, 4, 5) showed cross resistance against Itraconazole and Ketoconazole and (Isolate no.3, 4) against Itraconazole and Terbinafine. *C. tropicalis* (Isolate no. 11, 14) showed cross resistance for Ketoconazole and Terbinafine. *C. glabrata* and *C. krusei* showed cross resistance for all azoles (16, 17, 18) and (23, 24) respectively.*C. guilliermondii*

showed cross resistance between Azoles (20) and Terbinafine too (21). The total percentage of cross resistance was 62% (13/21) and the maximum was seen in *C. albicans*, *C. glabrata*, *C.krusei* and *C. guilliermondii* **(Table 3)**.

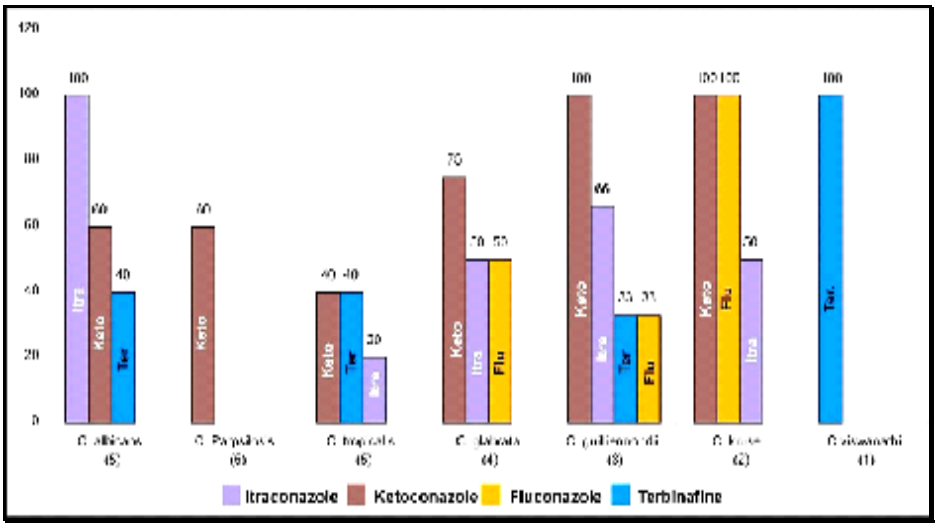

FIG 2. PERCENTAGE OF RESISTANCE SHOWED BY DIFFERENT SPECIES OF CANDIDA AGAINST ANTIFUNGAL DRUGS

TABLE 3: *CANDIDA ALBICANS* AND NON *ALBICANS* ISOLATES (WITH ASSIGNED ISOLATE NUMBERS) SHOWING CROSS RESISTANCE AGAINST AZOLES AND ALLYLAMINE

Isolates	Fluconazole	Itraconazole	Ketoconazole	Terbinafine
C.albicans	-	1,2,3,4,5	2,4,5	3,4
C.parapsilosis	-	-	7,8,9	-
C.tropicalis	-	12	11,14	11,14
C.glabrata	17,18	16,17	16,18,19	-
C.guilliermondii	20	20,22	20,21	21
C.krusei	23,24	23	23,24	-
C.vishwanathi	-	-	--	25

DISCUSSION: This is a systematic study on antifungal susceptibility testing of *C. albicans and non albicans* clinical isolates, from Central state of India causing superficial and systemic Mycosis. All the isolates included in this study were sensitive to the non-azole drug, Amphotericin B. In a study from southern India, Srinivasan and Kenneth [18] have reported that all the isolates were susceptible towards Amphotericin B. Mendiratta et al. [19] showed occurrence of *Candida* infections in neonatal ICU and reported all the *C. albicans* isolates susceptible to Amphotericin B . In a study done on immunocompromised patients, around 4 % of *C. albicans* isolates were found resistant to Amphotericin B. Therefore despite the widespread use of Amp B, resistance to polyenes antifungal agents remains an uncommon event among *Candida* isolate and has been the gold standard for the treatment of serious fungal infections [20].

Results obtained in our study have shown no resistance to Fluconazole in clinical isolates of *C. albicans*. Dose dependent susceptibility was observed in 20 % of the isolates, exhibiting MIC of 16 ug/ml drug concentration. In an extensive study from Latin America around 2 % of 10,000 *C. albicans* isolates from hospitalized patients were found Fluconazole resistant. Available studies on fluconazole susceptibility of *C. albicans* isolates from India have showed either no resistance or very low percentage of fluconazole resistance [21]. Mohanty et al. [22] found no Fluconazole resistance in *C. albicans* isolates from vulvo-vaginal candidiasis patients in Haryana, India. In Delhi, India, around 5 % of the *C. albicans* isolates from HIV positive OPC patients were shown to be Fluconazole resistant. Similar to the finding of other workers[23].Our strains of *C. albicans, C.*

parapsilosis, C. tropicalis and C. *viswanathii* showed no resistance for Fluconazole. However in the present study, *C. glabrata* exhibited 50% and *C. guilliermondi* exhibit 33% resistance for Fluconazole as reported by other workers [24].Similarly in India decreased susceptibility of *C. glabrata* and *C. guillermondi* for Fluconazole has been reported [22]. Our present study and review of Literature has revealed that *C. krusei* is intrinsically resistant to Fluconazole [25]. In the present study higher rate of Ketoconazole resistance was seen in *Candida guillermondii* (100%), followed by *Candida krusei* (100%), Candida glabrata (75%), *Candida albicans* (60%), *Candida parapsilosis* (60%), and *Candida tropicalis* (40%) i.e the highest percentage of resistance against all drugs used (64%) This finding is similar to the report from Central India by other workers who have reported ketoconazole as a least effective drug against all *Candida* spp.[26, 27.] Higher rate of ketoconazole resistance in *Candida glabrata* (16.6%) was also seen in several other studies. We have found that 44 % of the tested isolates were Itraconazole resistant where maximum resistance was shown by *C. albicans* (100%) followed by *C. guillermondii* (66%), *C. Parapsilosis* (60%) and *C. krusei* (50%) and 56 % isolates are susceptible. Earlier study from India reported resistance to Itraconazole, Ketoconazole and Fluconazole among 46% the *C. albicans* and 67% among non *albicans* isolates. [27]

Terbinafine is a synthetic antifungal drug with fungicidal activity against dermatophytes, moulds and fungistatic activity against *Candida* species [28.] Several researches have shown that Terbinafine is the first choice for the treatment of dermatophytosis [29]; however, few details are available about its effects on vaginal isolates of *Candida*. Despite the fact our results show that Terbinafine is effective against 76% of *Candida isolates* similar to the finding by Ryder [30, 31] .No Indian report is available on Terbinafine resistance in *C. albicans* [32]. Therefore, Terbinafine therapy can be considered as a good therapeutic option in the management of Candidiasis. Only 24% of tested isolates showed resistance.The total percentage of resistance was amazingly found to be (84%) with maximum for Ketoconazole followed by Itraconazole, Terbinafine and Fluconazole which suggested the emergence of resistance for *C. albicans* and non *albicans* against azoles and allylamine [33] .

There is possibility of cross resistance among antifungal azoles, due to previous exposure to one of the drugs mentioned above. This warns against occurrence of multiple drug resistance among the clinical isolates of *C. albicans and non albicans*. The present investigation showed (62%) of cross resistance among all *Candida* species of which maximum was shown by *C. glabrata, C. guilliermondi* and *C. krusei* against all azoles (Fluconazole, Itraconazole, Ketoconazole) and *C. albicans, C. tropicalis* between Itraconazole and Ketoconazole but also between Ketoconazole and Terbinafine. This finding is similar to earlier workers [33,34] .Pfaller et al. [35] reported multiple drug resistant *C. albicans* isolates showing resistance to four azoles- Fluconazole, Ketoconazole, Clotrimazole and Itraconazole .

Our study suggested that although Fluconazole can remain a drug of choice, care must be taken while prescribing it. Because, S-DD strains can acquire resistance to Fluconazole upon repeated exposure, as well as may show cross resistance to imidazoles. Cross resistance between azoles and allylamine deserves further exploration. Amphotericin B and Terbinafine could be used to treat infections by azole resistant *C. albicans and non albicans.*[36].We suggest a comprehensive epidemiological survey to establish the extent and degree of anti-fungal drug resistance among *Candida* species and need of antifungal susceptibility testing for better prophylaxis and treatment provisions against the infections caused by *Candida species.*

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