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A study on phenotypic traits of *Candida* species isolated from blood stream infections and their *in vitro* susceptibility to fluconazole

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Abstract: *Background & objectives:* Fungal infections are increasing very rapidly. *Candida* is a major pathogen involved and constitutes a part of normal microflora of humans. This organism is a leading agent associated with blood stream infections. The present study involves phenotypic characterization of *Candida* isolates. The isolates were obtained from National Culture Collection of Pathogenic Fungi at Post Graduate Institute of Medical Education and Research, Chandigarh, India. Keeping in view the rising trend of resistance by the *Candida* species to various antifungal agents their susceptibility has also been studied. *Methods:* All the isolates were identified upto species level by studying their phenotypic and biochemical characteristics. Phenotypic traits of *Candida* isolates studied included: formation of pseudohyphae, chlamydospore production, germ tube production, fermentation and assimilation of various sugars by the different species of *Candida*. All the isolates were tested for their susceptibility to fluconazole by disc diffusion and micro broth dilution methods. ***Results:*** The isolates could be clearly differentiated from each other by their phenotypic traits studied. A total of 51 out of 67 isolates examined (76.11%) were sensitive to fluconazole, 6 were sensitive dose dependent and 10 exhibited resistance to this drug. Among the resistant species *C. albicans* figured on the top followed by *C. tropicalis*, *C. guillermondii* and *C. glabrata*. However, resistance to fluconazole was not observed by *C.parapsilosis* isolates. *Conclusions:* The present study would help in the diagnosis of *Candida* species from clinical samples and give clue about the susceptibility of different species of *Candida* to fluconazole which might helpin the management of candidal infections.

Keywords: *Candida,* Phenotypic characterization. Ergosterol pathway, Antifungal, Fluconazole

Introduction

Candidial infections constitute the major fungal infections of humans in our country which are caused by *Candida* species. This fungus is a part of normal microflora of human body; however it has the potential to cause disease in the immunocompromised host, thus acting as an opportunistic pathogen [1-2]. In India, candidal infections are on the rise, with *Candida albicans* being the most common cause.

There are several reports of increasing incidence of infection with non *Candida albicans* species like *C. glabrata, C. parapsilosis and C. tropicalis* which now have been listed among etiological agents. There have been reports which show the prevalence of *Candida* species as major fungal pathogen in the country [3-4]. Several antifungal agents are being used for treatment of candidiasis. The sterol pathway in *Candida* species is the target for many classes of antifungal drugs [5]. Many types of isoprenoids viz steroids, cholesterol, retinoids, carotenoids, ubiquinones, and prenyl groups bound to proteins are essential components of fungal cells because of their vital role in a number of biological processes [6]. Azoles are amongst the most useful antifungal drugs used for the treatment of candidiasis which inhibit ergosterol pathway by inhibiting 14-alpha-demethylation step. This results in the accumulation of methylated sterols which leads to disruption of fungal cell membrane integrity [5]. Drug resistance poses a major problem in the treatment and cure of

infections due to *Candida* species. Mechanisms for such resistance have been elucidated by various studies. White and coworkers in 1998, described the molecular basis of fluconazole resistance in *Candida albicans* which was associated with the increased expression of genes that encode multiple drug resistance, ATP binding casette transporters and target enzymes and nucleotide substitutions at various positions of 14-alpha- demethylase enzyme coding ERG11 gene in ergosterol pathway [7]. Due to the development of drug resistance in *Candida* species there is an alteration in physiological and biochemical activities which may influence virulence of these drug resistant species [8]. Many strategies can be adopted to overcome resistance against fluconazole in *Candida* species such as multidrug chemotherapy [9-10]. The present study has been designed for phenotypic characterization of various phenotypic traits of different *Candida* species and determining their susceptibility pattern against fluconazole.

Material and Methods

Collection of isolates of Candida species: A total of 67 *Candida* isolates used in study were collected from the National Culture Collection of Pathogenic Fungi at Post Graduate Institute of Medical Education and Research (P.G.I.M.E.R) Chandigarh, India. The origin of these isolates was blood stream infection. The isolates were collected during the period 2009-12 and included *Candida albicans* (19/67, 28.35%) and non albicans (48/67, 71.65%) Among non albicans group, the distribution of different species was as follows: *C. tropicalis*20 (58.33%), *C. guilliermondii*8 (16.66%), *C. glabrata*8 (16.66%) and *C. parapsilosis* 12 (25%).The isolates were preserved in 10% glycerol at -80ºC as per standard protocol. The study was cleared by the Institute Ethics Committee (IEC) of this University vide letter no SUBMS/IEC/10/57-60 dated 26.10.2010, project number SUIEC/10/03.

Antifungal drugs: Fluonazole powder and discs (25mcg) used in the study were purchased from Hi-Media Laboratories, Mumbai (India).

Morphological examination on Corn meal agar: Pseudohyphae and chlamydospore production by *Candida* species was observed by streaking the isolates on to Corn meal agar (Hi-Media) plates supplemented with tween 80 (1%). Each plate was divided into 4 quadrants. Using a sterile needle or straight wire, the yeast colony was lightly touched in each quadrant and streaked. Cover glass slip was flame sterilized and placed over control part of streak after it had cooled. The plates were incubated at 25ºC for 3-5 days. The cultures were examined for various morphological features such as pseudohyphae, blastospores or chlamydospores.

Morphological examination on Hicrome Candida diffrential agar: Overnight growth of all the isolates was streaked on Hicrome *Candida* differential agar (Hi-Media). All the plates were incubated at 25ºC for 24-48hrs and observed for the appearance of different color by individual species of *Candida*.

Germ tube test: This test is used for presumptive identification of *Candida albicans*. It is a rapid screening test where the production of germ tube is observed within two hours. For this test, a fresh growth from a pure culture was obtained. A very light suspension of the test organisms in 0.5ml of sterile serum (pooled from human serum or calf serum) was prepared. The optimum inoculum being 10^5 to 10^6 cells per ml, incubated at 37ºC for exactly two hrs. One drop from incubated serum was transferred on a slide with coverslip. Observed under microscope for the production of germ tubes. Germ tube represents the initiation of hyphal growth arising from the yeast cell. *Candida albicans* strain ATCC 90028 was used as control for germ tube test. Each test was considered positive only if 30 % of the cells produced the germ tubes.

Sugar fermentation test: Liquid fermentation medium containing Peptone-1%, NaCl-0.5%, and Andrade's indicator-0.005% was sterilized by autoclaving at 120ºC for 15 min at 15 pound pressure. Filter sterilized sugar (2%) was added to the medium. This was poured in to the sterile test tubes in which sterile Durham tubes were placed. Inoculum preparation was done by suspending heavy inoculums of yeast grown on Yeast nitrogen base (YNB, Hi-Media),a sugar free medium. The carbohydrate broth was inoculated with approx. 0.1ml of inoculums, incubated at 25ºC for up to 1 week and examined after every 48-

72hrs interval for the production of acid (pink color) and gas in Durham tubes.

Sugar assimilation test: Yeast suspension was prepared by adding heavy inoculum from a 24-48hr old culture in 2ml of YNB (Hi-Media). Molten agar in a vol. of 18ml cooled to 45ºC was added to this suspension and mixed thoroughly. Entire volume was then poured into 90 mm Petri plate, allowed the agar to set in the plate at room temperature. Carbohydrate impregnated sterile discs (Hi-Media) were then placed on the surface of agar plate. A drop of 10% filter sterilized sugar solution was placed on each disc. The discs were dried at 37ºC and stored at 4ºC in air tight container. The plates were incubated at 37ºC for 3-4 days. The presence of growth around the disc was considered as assimilation for that particular carbohydrate.

Susceptibility testing of Candida species against antifungal agents: The susceptibility of *Candida* isolates was performed by disc diffusion and broth dilution methods by following CLSI M 44 A2 and M27A3 protocols.

Disc diffusion method: The susceptibility of *Candida* strains to antifungal was performed by disk diffusion method as per CLSI M44-A2 protocol. In this method, Muller-Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue dye (GMB) medium was used. The discs of fluconazole stored at 8 °C or below, or frozen at -14 °C or below, in a nonfrost-free freezer were used in the test. Inoculum was prepared by picking five distinct colonies of approximately 1 mm diameter from a 24-hour-old culture of *Candida* species, which were suspended in 5 ml of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).

The resulting suspension was vortexed for 15 seconds and its turbidity was adjusted visually with 0.5 McFarland standard, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into it. The dried surface of a sterile Mueller-Hinton + GMB agar plate was inoculated by evenly streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. Fluconazole discs (25 mcg) and one sterile disc (negative control) were dispensed onto the surface of the inoculated agar plate, Incubated in inverted position at 35^0C (± 2 °C). Each plate was examined after 20 to 24 hours of incubation for zone of inhibition. The diameter of each zone was measured to the nearest whole millimeter at the point at which there was a prominent reduction in growth. For susceptibility testing, *Candida albicans* (ATCC 90028) was used as reference strain whereas *Candida krusei* (ATCC 6258) was used as quality control strain [11].

Broth dilution method: The susceptibility testing of *Candida* isolates against fluconazole was also performed by the micro - broth dilution method as per CLSI M27-A3 protocol with some modifications. Dilutions of fluconazole powder was made and the susceptibility testing was carried out in RPMI 1640 medium with L glutamine and without sodium bicarbonate (Hi-Media). The pH was adjusted to 7.0±0.1 in MOPS buffer (Hi-Media) and I N NaOH.

The inoculum was prepared by picking five colonies, 1mm in diameter, and suspended in 5ml of sterile 0.145mol/L saline (8.5g/l NaCl) or sterile water. The suspension was vortexed for 15 sec. and cell density was adjusted with spectrophotometer by adding sterile saline or sterile water. A working solution was made by 1:20 dilution followed by 1:50 dilution of stock suspension with RPMI 1640 broth medium which resulted in 1 x 10^3 to 5.0x 10^3 cells per ml. The test strain and the drug dilutions were mixed in equal volumes in a microtitre plate, incubated for 24 to 48 hrs at 35^0C (± 2 °C). The amount of growth in tubes containing the agent was compared visually with the amount of growth in positive control tubes. For susceptibility testing, *Candida albicans* (ATCC 90028) was used as reference strain whereas *Candida krusei* (ATCC 6258) was used as quality control strain. By this method, the MIC values of fluconazole were determined [12].

Calculation of minimum inhibitory concentration values for fluconazole: MIC_{50} and MIC_{90} values for fluconazole were calculated by arranging the MIC data in

ascending manner and selecting cumulative % nearest to 50 as MIC_{50} and cumulative % nearest to 90 as MIC_{90} respectively.

Results

Phenotypic characterization of the isolates: Among phenotypic traits, colony morphology of each isolate was studied using Sabroud's dextrose agar and Hicrome *Candida* differential agar. On SDA medium, colonies of *Candida albicans* were white to cream colored smooth and soft, whereas *Candida tropicalis*,, *C. glabrata*, and *C. guilliermondii* produced white to cream colored colonies. The colonies of *C. parapsilosis* were cream to yellowish.

On Hicrome *Candida* differential agar, *Candida albicans* produced were apple green colonies (Fig 1a) , *Candida tropicalis* produced dull blue to purple colonies that diffused into the surrounding agar with pale pink edge (Fig 1b). *C. guilliermondii* produced small pink to purple colonies while *C. glabrata* had white large glossy pale pink to violet colonies (Fig 1c) and *C. parapsilosis* produced white to pale pink colonies (Fig 1d).

Production of pseudohyphae, chlamydospore and blastospores was observed on Corn meal agar supplemented with Tween 80. *Candida albicans* on CMA showed dimorphic morphology i.e. yeast as well as pseudohyphae with terminal chlamydospores, *Candida tropicalis* exhibited pine forest arrangement of pseudohyphae, , *C. glabrata* Yeast only (Fig-2a) ,*C. parapsilosis* shown Giant hyphae and blastospore at nodes (Fig 2b) and *C. guilliermondii* had scant pseudohyphae with chains of blastoconidia(Fig 2c).

Fig-1: Morphology of *Candida* species on Hicrome *Candida* differential agar (a) *Candida albicans* (b) *Candida tropicalis* (c) *Candida glabrata* (d) *Candida parapsilosis*

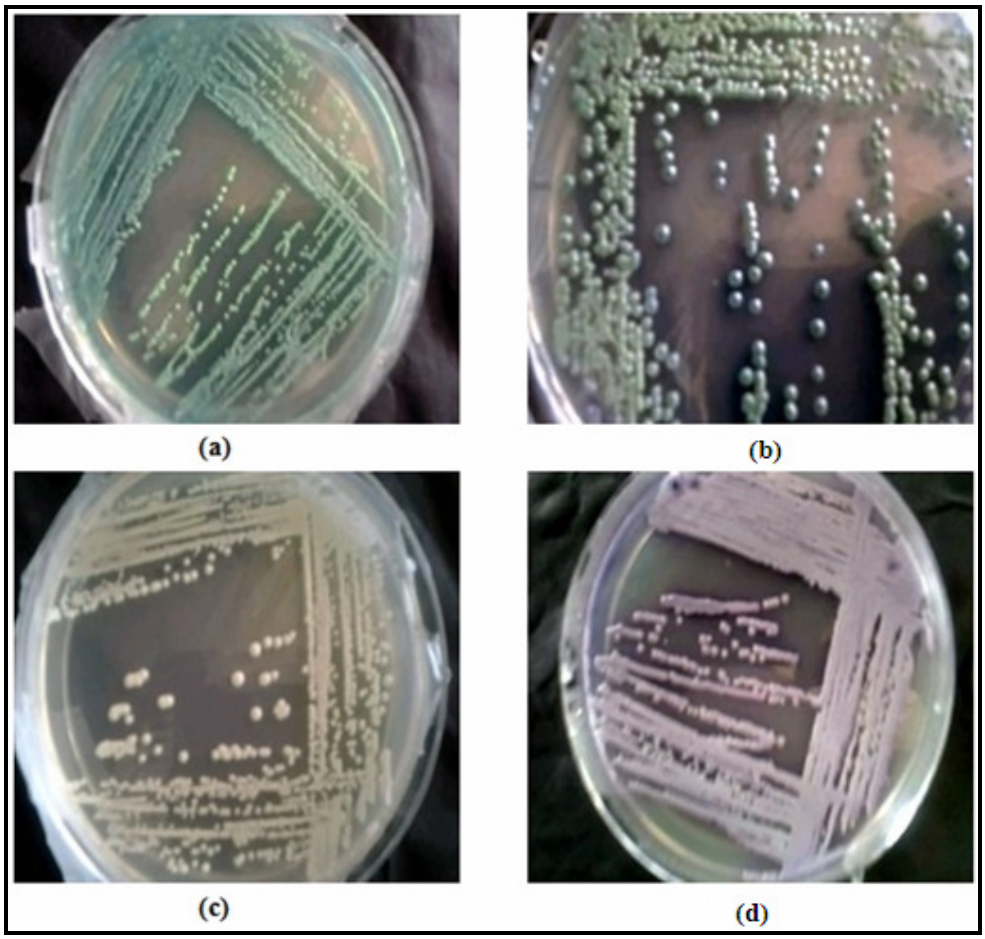
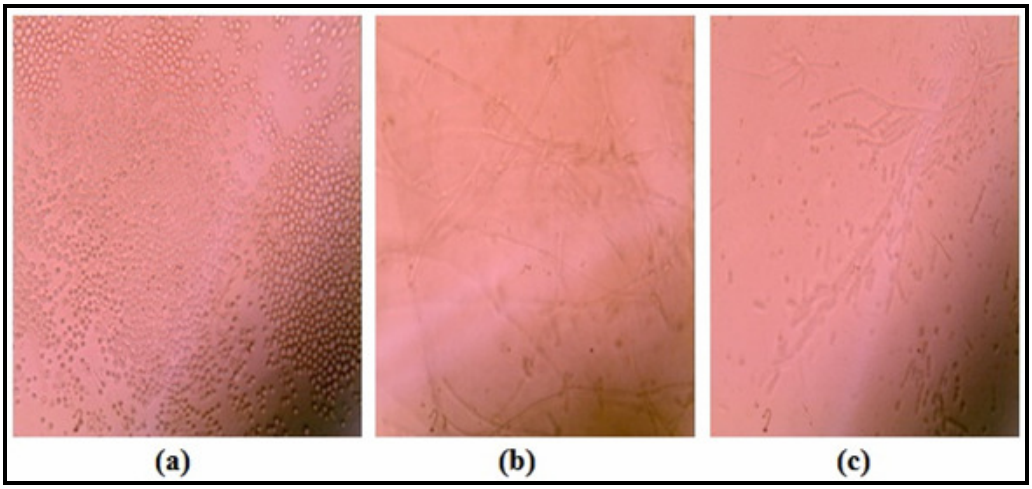


Fig-2: Morphology of *Candida* species on Corn meal agar (a) *Candida glabrata* (Yeast only) (b) *Candida parapsilosis* (giant hyphe and blastospores at nodes) (c) *Candida guillermondii* (Scant pseudohyphae with chains of blastoconidia)



Germ tube production: Germ tube production was observed only in case of *Candida albicans* isolates.

Biochemical characterization by sugar fermentation and assimilation: Further characterization of all the isolates was done on the basis of biochemical tests such as sugar fermentation and assimilation tests. All the *Candida* species exhibited different pattern of sugar fermentation and assimilation.*C. tropicalis* produced acid and gas in glucose, sucrose and maltose whereas the *Candida albicans*produced acid and gas from glucose and maltose only, *C.guilliermondii* produced acid and gasin glucose and sucrose only. Rest of the *Candida* spp. produced the acid and gas in glucose only.Regarding the pattern of sugar assimilation, most species assimilated glucose,galactose, maltose, trehalose, xylose and sucrose. The lactose was not assimilated by none of *Candida* species similarly dulcitol and mellibiose were not assimilated by *Candida* species except *C.guilliermondii.*

Confirmation of the isolates: On the basis of the above mentioned phenotypic and biochemical tests, all the 67 isolates of different *Candida* species were confirmed and no disconcordance between our results and identification by NCCPF (PGIMER, Chandigarh) was observed.

In vitro susceptibility of Candida species to fluconazole: The results of susceptibility ranges in terms of zone size and MIC values of fluconazole against different *Candida* species are presented in Table-1. Only 10 (14.92%) out of 67 isolates examined were resistant to fluconazole, 6(8.95%) were sensitive dose dependent and 51(76.11%) were sensitive However, no resistance was observed against fluconazole by *C. parapsilosis* isolates. Of these 50% isolates belonged to *Candida alibicans* group alone. The MIC values for sensitive strains ranged from 8 µg/ml to 0.25 µg/ml. For *Candida albicans* MIC range was from 0.25- ≥64 µg/ml. MIC range was worked out as 0.125-≥64 µg/ml for *C. tropicalis*, 0.125-≥64 µg/ml for *Candida guillermondii*, 0.125-2 µg/ml for *Candida parapsilosis.* The MIC range for *Candida glabrata* was determined as 0.50-≥64 µg/ml.

The MIC_{50} and MIC $_{90}$ values for *Candida albicans* were recorded as 2 µg/ml and 64 µg/ml respectively. For *Candida tropicalis,* these values were 1 µg/ml and 16 µg/ml respectively. These values for *Candida parapsilosis* were 0.25 µg/ml and 2 µg/ml respectively. However, keeping in view the small number of isolates of *Candida glabrata* and *C.guillermondi*, it was not feasible to determine these values. In this study the MIC values were highest for *Candida albicans* isolates followed by *Candida tropicalis* and *C. parapsilosis* isolates.

Table-1: Susceptibility range of *Candida* species against Fluconazole (Disc diffusion and broth dilution methods)

Species	Agent	Disc diffusion method	Broth dilution method		
		Zones of inhibition (in mm) 25 mcg disc Range	MICµg/ml Range	MIC_{50}	MIC_{90}
C. albicans(n=19)	Fluconazole	0-40	0.25-≥64 µg/ml	2µg/ml	64 µg/ml
C.tropilcalis(n=20)	Fluconazole	0-40	0.125-≥64 µg/ml	1 µg/ml	16 µg/ml
C.parapsilosis (n=12)	Fluconazole	21-40	0.125-2 µg/ml	0.25µg/ml	2 µg/ml
C. glabrata(n=8)	Fluconazole	0-34	0.50-≥64 µg/ml	ND	ND
C. guilliermondii(n=8)	Fluconazole	0-32	0.125-≥64 µg/ml	ND	ND
ATCC 6258 *C. krusei* (n=2)	Fluconazole	0	32-64 µg/ml	ND	ND
ATCC 90028 *C. albicans* (n=2)	Fluconazole	32-35	0.25-1 µg/ml	ND	ND

ND- Not determined as the number of isolates examined was less than 10. , isolates of ATCC 6258 *C. krusei* and ATCC 90028 *C. albicans* considered resistant and sensitive to Fluconazole used as quality control and reference strains.

Table-2: Comparative analysis of susceptibility patterns of *Candida* species against fluconazole by Disc diffusion and broth dilution methods

Species	Disc diffusion method (25 mcg disc)			Broth dilution method		
	S%	SDD %	R%	S%	SDD %	R%
C. albicans (n=19)	11 (57.85)	2(10.53)	6(31.57)	12 (63.15)	2 (10.53)	5 (26.32)
C.tropilcalis(n=20)	17(85)	1(5)	2(10)	16(80)	2(10)	2(10)
C.parapsilosis (n=12)	12(100)	0(0)	0(0)	12(100)	0(0)	0(0)
C. glabrata(n=8)	7(87.5)	0(0)	1(12.5)	7(87.5)	0(0)	1(12.5)
C. guilliermondii (n=8)	5(62.5)	1(12.5)	2(25)	4(50)	2(25)	2(25)

S, SDD, R by using interpretive breakpoint criteria of CLSI.

Comparative analysis of susceptibility patterns of Candida species against fluconazole by Disc diffusion and broth dilution methods: The comparative analysis of the susceptibility of different *Candida* species against fluconazole are presented in Table-2. It is evident from the table that the results of susceptibility by both the methods were similar and correlated fully in case of *Candida glabrata* and *Candida parapsilosis.* However, very minor differences were observed in case of *C. albicans, C. tropicalis and C. guillermondii.* Out of the total 67 isolate examined, 11 samples did not respond to disc of 25 mcg concentration of fluconazole. A total of 10 samples were confirmed as resistant by both disc diffusion (25mcg conc) and broth dilution method and thus, correlated well.

Discussion

Candidal infections have been on the progressive increase since past decades. *Candida* is the leading agent associated with blood stream infections. A marked increase in the incidence of patients of candidaemia has been reported in India during past five years [13]. The incidence of invasive mycosis associated with *Candida* species has increased rapidly in immuno-compromised patients [14]. In India, candidal infections are on the rise, with *Candida albicans* being the most common isolate. However, other non *Candida albicans* species like *C. glabrata, C. parapsilosis and C. tropicalis* are also frequently being isolated [3- 4, 15-16]. There

appears to be change in the trend of occurrence of candidal infections towards non albicans species. In our study, 67 isolates obtained from NCCPF PGIMER Chandigarh recovered from blood stream infections were phenotypically characterized by us and no disconcordance in the results was observed. Of these, a considerable proportion constituted non *Candida albicans* i.e. 48/67 (71.65%) while only 19/67 (28.35%) isolates represented *Candida albicans*. This shows the increasing trend of association of non *Candida albicans* in blood stream infections from patients at PGIMER Chandigarh (Personal communication).

However, it is difficult to clearly point out the demographic distribution for want of adequate information in this regard, it does certainly pinpoint the prevalence at this center covering northern part of India. The prevalence of candidal infections in our country as major fungal pathogen have been reported by other workers. Among non albicans group, we observed C. *tropicalis* as a prominent non *Candida albicans* species as also reported by Kothavade et al., 2010 in respect of other countries [17]. Novel species of non *Candida albicans* are also emerging as blood stream infections [15]. The *Candida albicans* is the prototype of *Candida* species in respect of various characteristics of the organism. It possesses certain virulence factors such as adherence to endothelial and epithelial cells, pseudohyphae formation, phenotypic switching, proteinases production and antigenic modification. *Candida albicans*and *Candida tropicalis*are known to produce aspartylproteinase which is also one of the contributing factor in their pathogenicity [4-18].

Hyphae formation, surface recognition molecules phenotypic switching and extracellular hydrophobic enzyme production have been considered to be some of the virulence traits for *Candida* species studied in details in recent years [19]. Shaheen and coworkers (2006) conducted the comparative study on *Candida* isolates from hospitalized and non hospitalized patients. These workers identified them on the basis of colony morphology on SDA, CHROM agar, pseudohyphae production on Corn meal agar and biochemical tests. The consistency in phenotypic traits was observed in different species of *Candida* by these workers [20]. Resende and co-workers (2004) studied phenotypic traits of 242 yeasts isolated from 200 patients from different clinics and identified them as *C. albicans* (105), *C. tropicalis*(62), *C. parapsilosis*(28), *C. glabrata*(19), isolates of *C. krusei* (8) and *C. guilliermondii* (5) [21]. The isolates exhibited the variation in the color of colonies produced on CHROM agar. Our findings were similar to those reported by Odds *et al.,* 1994 [22]. The sterol pathway in *Candida* species serves as a target for many types of antifungal drugs [5]. Many types of isoprenoids (*e.g.* steroids, cholesterol, retinoids, carotenoids, ubiquinones, and prenyl groups bound to proteins) are essential components of the cells of all organisms. These isoprenoids has a vital role in a number of biological processes [6].

Azoles are amongst the most useful antifungal drugs for the treatment of candidiasis. They inhibit ergosterol pathway by inhibiting 14-alpha- demethylation step. This results in the accumulation of methylated sterols which leads to disruption of fungal cell membrane structure [7]. The resistance against azole class of antifungal agents has been increasing very rapidly due to frequent use of the drug [14]. There has been sequential increase in fluconazole resistance among *Candida* species. *In vitro* susceptibility testing revealed the development of resistance to fluconaozle in *C. albicans*. However, the resistance has also been observed in case of non albicans species due to continuous exposure to the drug [23].

In the present study, only 10 of 67 isolates tested (14.92%) were found resistant to fluconazole by both the disc diffusion and broth dilution methods. Only one sample proved additionally sensitive to fluconazole by disc diffusion method. The distribution of the resistant samples is as follows: *Candida albicans* 5 and non *Candida alibicans* group 5 (*Candida tropicalis* 2, *C. guillermondii* 2 and *C. glabrata* 1). However, no resistance was not observed against fluconazole in *C. parapsilosis* isolates. Six isolates were found to be sensitive dose dependent (SDD) in which *Candida albicans* (2), *C. tropicalis* (2), and *C. guilliermondii* (2). This reflects that the non *Candida albicans* can also develop

resistance to fluconazole and present difficulty in the control of human fungal infections, thus, gaining clinical significance. In a similar study conducted in Spain by Flo´rez*et* et al., 2009*, C. albicans* was found the most commonly isolated species (49.2%). In non albicans group *C. parapsilosis* (17.3%), *C. tropicalis* (15.2%), *C. glabrata* (13.7%) and *C. krusei* (3.6%) were implicated. A total of 8 isolates (4.1%) were found to be resistant to fluconazole and 7 (3.6%) resistant to itraconazole. However, all the isolates were found susceptible to other fungal agents. *C. krusei* and *C. glabrata* were found in over 18% of cases of candidemia [24]. In the present study, we examined the susceptibility of these isolates only to flucoanzole, however, susceptibility to other antifungal agents is under progress.

In another study conducted by Wang *et al.,* 2004, low resistance (3.7%) against fluconaozle was observed in 230 blood isolates of *Candida* as compared to isolates of other origins. The proportion of fluconazole resistance observed was high in non albicans species than *Candida albicans* [25].

We have further studied selective virulence traits such as protease and phospholipase activites, agar invasion, hemolysin and hyphae production on solid media and adherence, biofilm production of fluconazole resistant isolates of different *Candida* species. The study reflects that the fluconazole resistant strains appear to be more virulent as compared to sensitive ones [26]. The drug resistant strains tend to be more pathogenic for systemic infection due to a number of biochemical and physiological changes occurring during the development of drug resistance. These may include: composition of cell wall polysaccharides, cellular alterations affiliated with different expression more rapid and extensive hypha formation, increase in adherence to plastic materials and increased biofilm formation by the drug resistant species [8]. Thus due to development of drug resistance against many antifungal agents, treatment of these infections is becoming difficult [27]. The most difficult challenge for medical personnel is the early initiation of accurate antifungal therapy and diagnostic procedures [28].

In a similar study antifungal agents like terbinafine and fenpropimorph which targets other enzymes of the ergosterol pathway also exhibited synergistic antifungal activity against wild-type *C. albicans.* However, many other antifungal agents are also known to act as ergosterol biosynthetic pathway inhibitors [29]. The inhibitory action of these inhibitors can be increased by using these in combination [30]. Such studies can be undertaken to know the antifungal efficacy of other drugs which affect the pathway at other level i.e upstream or downstream of the 14-alpha- demethylation step. Also the combined effects in different combination of different antifungal agents can also be further studied in order to get an insight into the therapeutic potentials.

The isolates used in the study have thus been characterized for their phenotypic traits and susceptibility to fluconazole. Study on virulence traits of fluconazole resistant strains has been conducted as mentioned under reference no. 26. Further studies on susceptibility of different *Candida* species to the different inhibitors of ergosterol pathway and characterization of their ERG11 gene are underway.

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