ISSN: 2320 - 4230

Journal of Drug Discovery and Therapeutics 1 (9) 2013, 27-41

RESEARCH ARTICLE

STUDIES ON PHENOTYPIC TRAITS AND GENETIC VARIABILITY IN ERG 11 GENE AMONG DRUG RESISTANT *CANDIDA* SPECIES

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Received 20 August 2013; Revised 30 August 2013; Accepted 05 September 2013

ABSTRACT

Candida is an opportunistic pathogen of human beings and other mammals. It is the leading agent of various types of diseases in immunocompromised individuals. The most common infecting species is *C. albica*ns where as other *non albicans* species are *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* also causes various clinical diseases. Candidemia is the fourth leading cause of bloodstream infections and carries a 35–55% mortality. The widespread use of azoles has led to increasing azole resistance among *Candida albicans* strains. One mechanism of azole resistance involves point mutations in the *ERG11* gene, which encodes the target enzyme (cytochrome P450 lanosterol 14α-demethylase). Aim of study was to isolate azole-drug resistant *Candida* species and to observe genetic variability in ERG11 gene, also phenotypic characterization of different *Candida* species. In the present study, 24 strains were examined for phenotypic characters such as the pseudohyphae, chlamydospore formation and germ tube production and some colored colonies on chrome agar. Antifungal susceptibility testing was also done by placing fluconazole and voriconazole disk on Muller Hinton agar. Zone of inhibition of various diameters were formed around these disks by which we identified resistant and sensitive *Candida* sp. Two isolates of *Candida tropicalis* were resistant to both the antifungal agents. The ERG 11 gene of two azole drug resistant isolates was then amplified using forward and reverse primers. The two drug resistant amplified products were then sequenced and the sequence results were compared with homologous species.

INTRODUCTION:

Opportunistic pathogens such as *Candida albicans* which often reside in the host whose immune system is weakened as benign, commensal organisms. Opportunistic pathogens may gain access to vulnerable tissues in patients undergoing organ transplants or chemotherapy, or when indigenous competitors are eliminated upon antibiotic treatment, causing death in more than 50% of infected patients (Wilson *et al*., 2002). It is important to understand the genetic mechanisms underlying the survival and adaptation of opportunistic pathogens to growth in host environments (Margolis *et al*., 2007). The most commensal fungus of the human microbial flora, *C. albicans* provides a model for the study of opportunistic pathogens because it reproduces asexually and demonstrates a high degree of genetic and genomic variability among isolates (Cowen *et al*., 1999; Iwaguchi *et al*., 2000; Joly *et al*., 2002; Pujol *et al*., 2002). Many commensal and harmless symbionts may become invasive pathogens in immunocompromised hosts, the mechanisms underlying the maintenance of genetic variation and of the commensal state bear investigation (Levin *et al*., 2000). *Candida albicans* is pathogenic yeast that causes oral, vaginal, and systemic infections (Odds *et al*., 1988). These infections are commonly associated with immune dysfunction, as they are frequently found in AIDS patients and bone marrow transplant patients. Antifungal drugs that are available for the treatment of these infections include the polyene amphotericin B and the azoles, such as fluconazole. In the 1990s, many human immunodeficiency virus (HIV)-infected patients received long-term, low-level azole antifungal therapy, which resulted in azole-resistant isolates of *C. albicans* (White, T. C. *et al*., 1998). Many studies have identified azole-resistant strains of C. albicans from AIDS patients, whereas we focused on those isolates from non-AIDS Candidiasis patients, with the idea of identifying other mutations in ERG11 that contribute to azole resistance.

Thus far, there are at least four explanations of azole resistance in *C. albicans*. The first is based on spatial configuration changes of the target enzyme 14α-demethylase (Erg11p) due to mutations in the encoding

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gene ERG11. Erg11p is a key enzyme in ergosterol synthesis pathway of *C. albicans*. Ergosterol is essential for maintaining the28 integrity and function of *C. albicans* membrane. Erg11p is a member of the cytochrome P450 superfamily. The protein is composed of 528 amino acids that form

13 α-helices of A to M, several β-pleated sheets and some other helix configurations such as A', J', K' and K''. The active centre of Erg11p is located deep inside the protein, near the haemachrome between helices I and L. The substrate interacts with a long access channel and is then demethylated (Ji H *et al*., 2000; Podust LM *et al*., 2001; Sanglard D *et al*., 1998; Marichal P *et al*.,1999). Azoles block this process and inhibit ergosterol synthesis. ERG11 contains 1851 bp. The transcription start codon is located at 148–150 bp and the stop codon at 1732–1734 bp (referring to a published ERG11 sequence in GenBank, accession no. X13296). If one or more mutations in ERG11 result in changes in the Erg11p spatial configuration, a decrease in the affinity between the azole and protein occurs. This altered phenotype often makes isolates resistant to azole. In addition, overexpression of ERG11 has been thought to increase resistance, although recent data indicate that overexpression is unrelated to azole resistance in *C. albicans* (White TC *et al*., 1997; White TC *et al*., 2002).

The present study is designed to study the phenotypic traits, antifungal susceptibility pattern and genetic variability in ERG11 in drug resistant *Candida* species. The study will help us in characterization, identification and to understand mechanism of drug resistance in *Candida* species.

METHODS:

Sample collection:

Urine sample of UTI suspected patients were collected from zonal hospital of Solan and throat sample were collected from students of SILB, Solan. The samples were transported to SILB laboratory in an air tight sample container and processed further immediately.

Isolation of *Candida* species:

Candida species were isolated from various clinical samples using SDA. The SDA media was prepared according to manufacturer's instructions. The sub-cultures on SDA were incubated at 37°C for 24 - 72 hours before observation and subsequent test were carried out. The isolated samples were then maintained at 4°C by inoculating in glycerol.

Characterization of *Candida* species:

Candida species were identified up to species level through examination of morphological, characteristics and biochemical tests. The phenotypic traits like germ tube production, pseudohyphae and chlamydospore formation were performed as per methodology described by (Arunaloke C *et al*., 2002). The biochemical like sugar fermentation and sugar assimilation were also performed.

Germ tube test:

Germ tube test was done for the presumptive identification of *Candida* species. It is a rapid screening test where the germ tube will be produced within two hours in contact with the serum in corn meal agar (Arunaloke C *et al*., 2002).

CHROM agar:

CHROM agar was used for presumptive identification of different *Candida* species and detects any mixed colonies. The method is based on the differential release of chromogenic breakdown products from products from various substrates following differential exoenzyme activity. CHROM agar was purchased as powdered media and the plates were prepared according to the manufacturers' instructions. Using an inoculating needle, a single colony from a pure culture was seeded into CHROM agar media and incubated at 35°C for 48 hours after which color changes were noted (Kangogo MC *et al*., 2011).

Pseudohyphae production:

The ability to produce pseudohyphae was determined by observing their morphology on corn meal agar. Corn meal agar was prepared containing tween 80. With the help of sterile needle yeast colonies were streaked on CMA plate, sterile cover slip will placed over streaked colonies. The plate was incubated for 3-5 days at 25°C. The plates were observed after 3-5 days under microscope to see the presence of pseudohyphae (Arunaloke C *et al*., 2002).

Chlamydospore production test:

Chlamydospore production test was performed by inoculating *Candida* isolates on corn meal agar supplemented by 8ml of tween 80. The samples which were previously grown in SDA were seeded as 4 parallel streaks in rectangular piece of agar placed in between two slides and the plates were incubated in wet chamber (Fisher F *et al.*, 1998) at 30°C for 72 hour. The plates were visualized under an optical microscope (Gatica JLM *et al.*, 2002). The double walled rounded spore will be observed as chlamydospore.

Sugar assimilation:

Sugar assimilation was done by preparing YNB and nutrient agar separately. Yeast suspension was prepared from old culture in 2ml of YNB by adding heavy inoculums. This suspension was added to 18ml of molten

agar and mixed well and poured to petri plate. Placed carbohydrate-impregnated discs onto the surface of the agar plate and incubated at 37°C for 3-4 days (Arunaloke *et al*., 2002).

Sugar fermentation:

Liquid fermentation medium was prepared by adding peptone (1%), sodium chloride (0.5%), Andrade's indicator (0.005%). The media was autoclaved at 120°C for 15 min at 15 psi and sterilized sugar was added at concentration of 2% to media. It was then poured to test tubes and placed durham's tube in each test tube. Heavy inoculum of yeast was inoculated and incubated at 25°C up to 1 week (Arunaloke *et al*., 2002).

Antifungal susceptibility testing:

The antifungal susceptibility of *Candida* strains was performed by disk diffusion method as per CLSI M44-A protocols.

Preparation of Inoculum:

Four-five colonies from pure growth of each organism were transferred to 5 ml of Mueller- Hinton broth. The broth was incubated at 37°C for 18-24 hours. The turbidity of the culture was compared with 0.5 McFarland Nephelometer standards to get 150 × 106 CFU/ml. The standardized inoculum suspension was inoculated within 15-20 minutes (Arunaloke *et al*., 2002).

Disk diffusion method:

The antifungal susceptibility of *Candida* strains was performed by disk diffusion method as per CLSI M44-A protocol. In this method Muller-Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue dye (GMB) medium were used. The disks of antifungal were used and stored at 8^0 C or below, or freeze at -14 °C or below, in a non frost-free freezer until needed. Inoculum was prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture of *Candida* species. Colonies was suspended in 5 ml of sterile 0.145 mol/L saline (8.5 g/100mL NaCl; 0.85% saline).The resulting suspension was vortexed for 15 seconds and its turbidity were adjusted either visually or with a spectrophotometer. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension. The dried surface of a sterile Mueller-Hinton + GMBagar 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°C each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. Antimicrobial disks were dispensed onto the surface of the inoculated agar plate. The plates were incubated inverted position at 35^0 C (± 2 °C) within 15 minutes after the disks were applied. Each plate was examined after 20 to 24 hours of incubation for zone of inhibition. Zone diameter was measured to the nearest whole millimeter at the point at which there was a prominent reduction in growth (Arunaloke *et al*., 2002).

Genetic variability in ERG11 gene among drug resistant *Candida* isolates:

The DNA of drug resistant species was extracted by method described ahead. The ERG11 gene is then amplified by polymerase chain reaction.

DNA extraction:

The different azole drug resistant *Candida* isolates were taken for DNA isolation. Fungi were grown on Sabouraud Dextrose Agar for 48 h at 30°C and suspended in sterile 0.9% NaCl solution at a concentration of 106 CFU/ml (McFarland 0.5 corresponds to 106 cells). DNA was extracted as described previously with some modifications (Pearce *et al*., 1991; Robert *et al*., 1995). For the extraction of whole-cell DNA, yeast suspensions were incubated with lysis buffer containing 0.1 mg/ml proteinase K, 150 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.5% SDS for 3 h at 55°C. After the phenol chloroform extraction, pellet was kept overnight at -20°C in 95% ethanol with sodium acetate. The supernatant was discarded, and the pellet was allowed to dry. DNA was then dissolved in 50 µl of sterile water and 5 µl of DNA solution was used for amplification procedure**.**

Polymerase Chain Reaction (PCR) amplification of ERG 11 gene:

Extracted DNA was used as template for the amplification of coding region of ERG11 genes by Polymerase Chain Reaction with the following primers: Forward 5'-GTTTCTACTGGATCCCATGG-3' and Reverse 5'-TACATCTGTGTGTCTACCACC-3'. PCR was carried out in 50µl volume containing 10 x PCR buffer 5µl, genomic DNA 20µl, 2.5mmol/L of each dNTP 5µl, 25 mmol/L $MgCl_2$ 5µl, 10pmol/L each primer 2.5µl and 3U/µl Taq polymerase 2.5µl. Amplification was performed in thermal cycler for 1 cycle of 4 min. at 94°C and then for 35 cycles, each of which consisted of 30s at 94°C, 1min. at 55°C and 1min. at 72°C; this was followed by 1 final cycle of 10 min. at 72°C. The PCR products were then analysed by electrophoresis on 0.8% agarose gel (containing 0.5µg/ml of ethidium bromide) and visualized under UV transilluminator. (ZHOU Yong *et al*., 2011).

Sequencing of amplified products and determination of genetic variability in ERG 11 gene of drug resistant species:

The PCR amplified products were sequenced by using the same forward and reverse primers used for the identification of ERG11 gene by Xcleris laboratories Pvt Ltd Ahmadabad. The variability in ERG 11 gene was detected by comparing the nucleotide sequences with the homologous gene sequences from NCBI.

RESULTS:

A total of 30 samples (Urine and throat) were processed at Department of Microbiology SILB which were collected from UTI infected patients (urine) from Zonal hospital Solan and college students (throat) of SILB. 24 samples were found positive for *Candida* infection and 6 were found negative.

Table 1: Detail of sample collection.

Sample No.	Sample source	Sample type	*Candida* infection
1	Hospital	Urine	+
2	Hospital	Urine	+
3	Hospital	Urine	+
4	Hospital	Urine	+
5	Hospital	Urine	+
6	Hospital	Urine	+
7	Hospital	Urine	+
8	Hospital	Urine	+
9	Hospital	Urine	+
10	Hospital	Urine	+
11	Hospital	Urine	+
12	Hospital	Urine	+
13	Hospital	Urine	_
14	Hospital	Urine	+
15	Hospital	Urine	+
16	Hospital	Urine	+
17	Hospital	Urine	+
18	Hospital	Urine	+
19	Hospital	Urine	+
20	Hospital	Urine	+
21	Hospital	Urine	+
22	Hospital	Urine	_
23	Student	Throat	_
24	Student	Throat	_
25	Student	Throat	_
26	Student	Throat	+
27	Student	Throat	_
28	Student	Throat	+
29	Student	Throat	+
30	Student	Throat	+

Total 30 samples were collected from UTI infected patients (urine) and students (throat). 24 samples were found infected with *Candida* and 6 were found negative.

Phenotypic characteristics:

All the *Candida* isolates showed growth on Saboraud Dextrose agar (SDA). Colonies were white to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology showed spherical to sub spherical budding yeast cells or blastoconidia. Out of the 24 isolates 12/24 yielded several shades of green colonies on CHROM agar *Candida* suggestive of *C. albicans* (Fig.1). Only 6/24 of the isolates developed a distinctive dark blue color on CA typical of *C. tropicalis* (Fig.1). 2/24 of the isolates developed a pink color suggestive of *C. parapsilosis* (Fig.1). Other *Candida* species gave colonies

with colors as described in Table 2. Germ tube test indicated that 12/24 of the *C. albicans* were germ tube positive (Figure 2) and 12/24 were germ tube negative. *C. albicans* produced abundant chlamydospores and pseudohyphae with clusters of spores (Figure 3) on CMA. *C. tropicalis* formed blastoconidia singly and long pseudohyphae on CMA. *C. parapsilosis* formed blastoconidia along curved pseudohyphae and giant mycelial cells. *C. guilliemondii* formed fairly short, fine pseudohyphae and clusters of blastoconidia at septa. *C. krusei* formed pseudohyphae with cross-matchsticks or tree-like blastoconidia. *C. glabrata* did not form any pseudohyphae but small, oval, single terminal budding, non encapsulated yeast cells. Different *Candida* spp. showed variation in production of acid production as described in Table 3 (fig 4). In sugar assimilation test showed different zones of precipitation around different sugars disc by different *Candida* spp. as described in Table 4 (fig 5). *C.albicans* showed the sugar assimilation around glucose, maltose, sucrose, galactose, xylose and trehalose disk. *C. tropicalis* showed the sugar assimilation around the glucose, maltose, sucrose, galactose, xylose, trehalose and cellobiose disc; *C. guiliermondii* showed the sugar assimilation around the glucose, maltose, sucrose, galactose, xylose, trehalose, melbiose ,raffinose, cellobiose and raffinose disk; *C.krusei* showed the sugar assimilation around the glucose disk only; *C.parapsilosis* showed the sugar assimilation around the glucose, maltose, sucrose, galactose, xylose and trehalose disk, and *C.glabrata* showed the sugar assimilation around the glucose, maltose and trehalose disk.

Table 2: Phenotypic characteristics of isolated *Candida* spp.

Strain No.	Isolated organism	CHROMagar	CMA with tween 80	SDA	Germ tube
1	*C.tropicalis* (H1)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
2	*C.albicans* (H2)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
3	*C.albicans* (H3)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
4	*C.krusei* (H4)	Whitish pink colony	Elongated yeast, abundant pseudohyphae (match stick like appearance)	White to cream coloured, butyrous	_
5	*C.parapsilosis* (H5)	Pink/violet colony	Giant hyphae, blastospores at nodes	Cream coloured to yellowish, glistening and soft, mostly smooth or winkled	_
6	*C.glabrata* (H6)	Light pink colony	Yeast only	White to cream coloured, soft, glossy and smooth	_
7	*C.parapsilosis* (H7)	Pink/violet colony	Giant hyphae, blastospores at nodes	Cream coloured to yellowish, glistening and soft, mostly smooth or winkled	_
8	*C.albicans* (H8)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
9	*C.albicans* (H9)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
10	*C.tropicalis* (H10)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
11	*C.guilliermondii* (H11)	Whitish blue colony	Scant pseudohyphae with chains of blastoconidia	White to cream coloured, butyrous	_
12	*C.tropicalis* (H12)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
14	*C.guilliermondii* (H13)	Violet colony	Scant pseudohyphae with chains of blastoconidia	White to cream coloured, butyrous	_

15	*C.tropicalis* (H14)	Blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
16	*C.albicans* (H15)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
17	*C.tropicalis* (H16)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
18	*C.tropicalis* (H17)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream-colored with a slightly mycelial border	_
19	*C.albicans* (H18)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
20	*C.albicans* (H19)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
21	*C.albicans* (H20)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
26	*C.albicans* (S21)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
28	*C.albicans* (S22)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
29	*C.albicans* (S23)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+
30	*C.albicans* (S24)	Green colony	Terminal chlamydoconidia	White to coloured, smooth, soft	+

Morphology of Candida species on different agar.

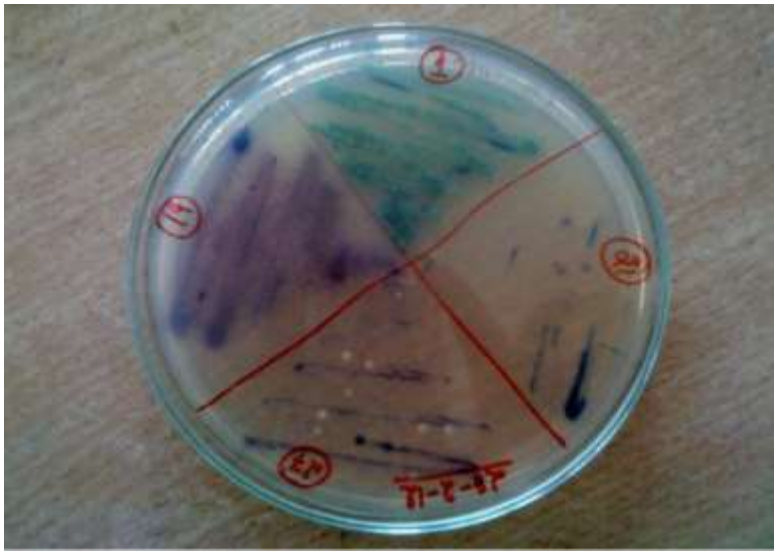


Figure 1: CHROM agar plate, showing *Candida* colonies of different colour.

Table 3: Results of Sugar fermentation test.

Strain No.	Species	Sugar fermentation			
		Glucose	Maltose	Sucrose	Lactose
H1	*C.tropicalis*	A	A	A	-
H2	*C.albicans*	A	A	-	-
H3	*C.albicans*	A	A	-	-
H4	*C.krusei*	A	-	-	-
H5	*C.parapsilosis*	A	-	-	-
H6	*C.glabrata*	A	-	-	-

H7	*C.parapsilosis*	A	-	-	-
H8	*C.albicans*	A	A	-	-
H9	*C.albicans*	A	A	-	-
H10	*C.tropicalis*	A	A	A	-
H11	*C.guilliermondii*	A	-	A	-
H12	*C.tropicalis*	A	A	A	-
H13	*C.guilliermondii*	A	-	A	-
H14	*C.tropicalis*	A	A	A	-
H15	*C.albicans*	A	A	-	-
H16	*C.tropicalis*	A	A	A	-
H17	*C.tropicalis*	A	A	A	-
H18	*C.albicans*	A	A	-	-
H19	*C.albicans*	A	A	-	-
H20	*C.albicans*	A	A	-	-
S21	*C.albicans*	A	A	-	-
S22	*C.albicans*	A	A	-	-
S23	*C.albicans*	A	A	-	-
S24	*C.albicans*	A	A	-	-

C. tropicalis showed the acid production in glucose, sucrose and maltose where as the *Candida albicans* showed the acid production in glucose and maltose, *Candida guilliermondii* showed the acis production in glucose and sucrose and the rest of the other *Candida* spp. showed the acid production in glucose only.

Table 4: Results of Sugar assimilation test

Strain No.	Species	Glu	Mal	Suc	Lac	Gal	Mel	Cel	Ino	Xyl	Raf	Tre	Dul
H1	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H2	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H3	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H4	*C.krusei*	+	-	-	-	-	-	-	-	-	-	-	-
H5	*C.parapsilosis*	+	+	+	-	+	-	-	-	+	-	+	-
H6	*C.glabrata*	+	+	-	-	-	-	-	-	-	-	+	-
H7	*C.parapsilosis*	+	+	+	-	+	-	-	-	+	-	+	-
H8	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H9	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H10	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H11	*C.guilliermondii*	+	+	+	-	+	+	+	-	+	+	+	+
H12	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H13	*C.guilliermondii*	+	+	+	-	+	+	+	-	+	+	+	+
H14	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H15	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H16	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H17	*C.tropicalis*	+	+	+	-	+	-	+	-	+	-	+	-
H18	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H19	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
H20	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
S21	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
S22	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
S23	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-
S24	*C.albicans*	+	+	+	-	+	-	-	-	+	-	+	-

Glu– glucose, mal- maltose, suc- sucrose, lac- lactose
Gal- galactose, mel- mellibiose, cel- cellobiose, ino- inositol
Xyl- xylose, raf- raffinose, tre- trehalose, dul- dulcitol
C.albicans showed the zones of precipitation around glucose, maltose, sucrose, galctose xylose and trehalose disc, *C.tropicalis* showed the zones of precipitation around the glucose, maltose, sucrose, galctose xylose, trehalose and cellobiose disc, *C.guiliermondii* showed the zones of precipitation around the glucose, maltose, sucrose, galctose xylose, trehalose, melbiose ,raffinose, cellobiose and raffinose disc, *C.krusei* showed the zones of precipitation around the glucose disc only, *C.parapsilosis* showed the zones of precipitation around the glucose, maltose, sucrose, galctose xylose and trehalose disc, and *C. glabrata* showed the zones of precipitation around the glucose,maltose,and trehalose disc.

Antifungal susceptibility:

Out of 24 isolates, 9 were found resistant to fluconazole and 12 were found resistant to voriconazole. Two isolates *C.tropicalis* (H1) and *C.tropicalis* (H12) were resistant to both the antifungal agents. The diameters of zones of inhibition (in mm) were measured for the interpretation of results (Table 5).

Table 5: Results of antifungal susceptibility testing

Strain	Species	Fluconazole		Result	Voriconazole 1mg	Result
		10mg	25mg			
H1	*C.tropicalis*	0	0	R	0	R
H2	*C.albicans*	26mm	21mm	S	0	R
H3	*C.albicans*	36mm	36mm	S	0	R
H4	*C.krusei*	17mm	14mm	S	40mm	S
H5	*C.parapsilosis*	28mm	25mm	S	0	R
H6	*C.glabrata*	15mm	10mm	S	0	R
H7	*C.parapsilosis*	0		R	42mm	S
H8	*C.albicans*	32mm	26mm	S	0	R
H9	*C.albicans*	21mm	17mm	S	37mm	S
H10	*C.tropicalis*	20mm	21mm	S	0	R
H11	*C.guilliermondii*	40mm	35mm	S	42mm	S
H12	*C.tropicalis*	0	0	R	0	R
H13	*C.guilliermondii*	0	0	R	47mm	S
H14	*C.tropicalis*	18mm	13mm	S	0	R
H15	*C.albicans*	35mm	17mm	S	0	R
H16	*C.tropicalis*	20mm	21mm	S	45mm	S
H17	*C.tropicalis*	21mm	18mm	S	0	R
H18	*C.albicans*	0	0	R	37mm	S
H19	*C.albicans*	0	0	R	38mm	S
H20	*C.albicans*	25mm	20mm	S	0	R
S21	*C.albicans*	0	0	R	43mm	S
S22	*C.albicans*	0	0	R	36mm	S
S23	*C.albicans*	0	0	R	36mm	S
S24	*C.albicans*	36mm	29mm	S	50mm	S

R- Resistant; S- sensitive

The two of azole resistant isolates *C.tropicalis* (H1) and *C.tropicalis* (H12) were selected for DNA extraction. The PCR amplification of ERG 11 gene of these two isolates was done with the help of suitable primers and both the amplified products were sequenced.

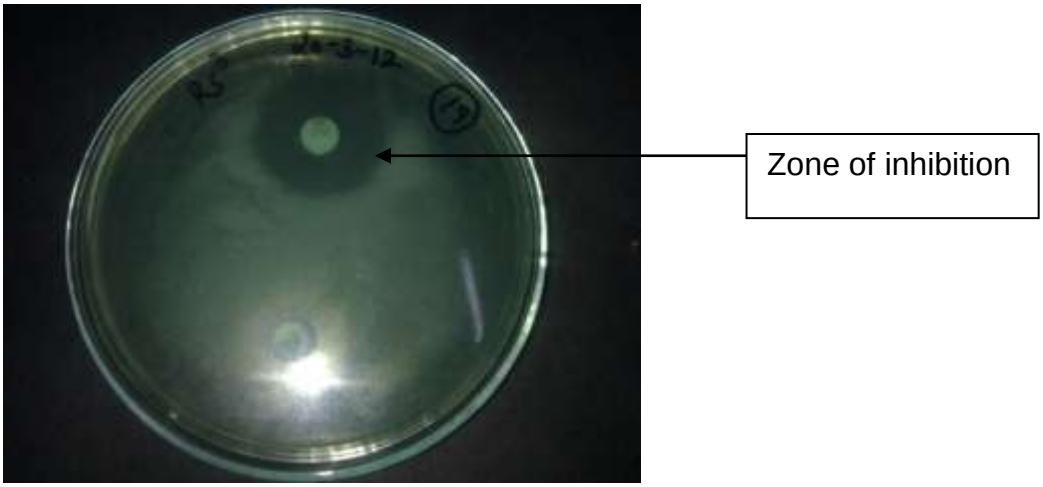


Figure 2: Zone of inhibition around fluconazole disc (25mg) for *Candida* species.

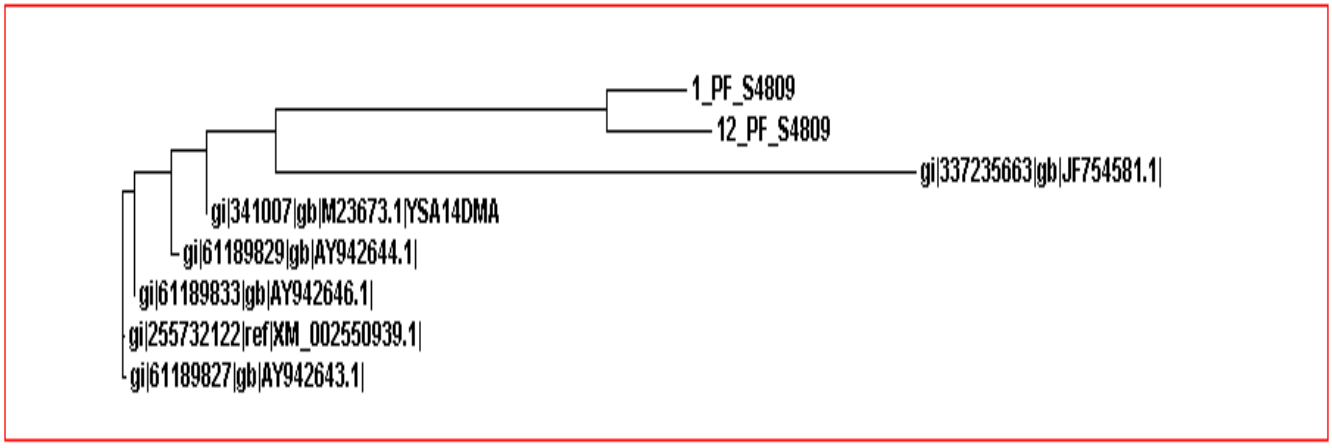
Table 6: Results of PCR amplification and sequencing of ERG11 gene in drug resistant *Candida* species.

Organism	Azole susceptibility	Primer sequence	Length of amplified product	Nucleotide Sequence of amplified product	
				Forward	Reverse
C. tropicalis (H1)	R	5'GTTTTC TACTGGA TCCCATG G-3' 5'TACATC TGTGTCTA CCACC-3'	500bp	Sample-1 F-CGCATGTTGGGTAAGGTGT TATTTATGATTCGTCTCAAGT ATAGATTATAGGAATTTCTA TGAGCGGCGTCTCTGCTCCG ACAACGGAGTCTTGCCAAAC CTGGGAACCCAAAATTGAAT GGGGTGAAACCTTTATTTTG TTAACGATCTTTTTTTCAAAA CCTCTGACGAAAACCTGTCT CATTTTAGCACCATATAAAC TACGCCAAGCATTGAAAATT GTGATGTCTGGTTGAGTTTT CATAACGATAGCATGACCAA GGTTTCTTTCCGTGGTTTTG AAACTAACATCGTTAACAAA ATAATTCAGAACTTCCCCTCT GATCTTTGGAACATAGGTTT TGAAAGAATCAGTAATCAA AGCAAATTTAGCAAACTTCT TTTGTTCCATTAATCTTAAGT TTGGACAATCATAAATAACA CCTTTACCAAAAACAGGAAT AGTCAAATGGGTATAAGCTC CTTCCAGCAAAA	Sample-1 R-GCTGTTTTGGGTATGGTGTTAT TTAAGATTCCTTCAAGTAATGA TCATAGGAATTTCTATGATCAC CGTCTCTTCTCCGATAACGAGG TAATGGCAAGTTTGGGAAAAC AAAATTGATTGGGGTGAAACC TTTATCCTAGTCAACAAACTTTT GATCAAATGATCTGTCGAAACT CTTTCTCATTTCATCACCATATA AACTACGAGAAGCATTGAAAA TAGTGATGTCTGGTTGAGTTTT CATAACACTAGCATCACCAGGT TTCTTTCCGTGGTTTTGAAACTA ACATCGTTACAAAATAATTCAG AACTTCTTCTCTGATCTTTGGAA CACAGGTTTTGAAAGAATCAGT AATCAAAGCAAATTTAGCAAAC TTCTTTTGTTCCATTAATCAGAG AGTTTGGACAATCATAAATAAC ACCTTTACCAAAAACAGGAATA GTCAAATGGGTATAAGTTCCTT CCACCAAAAA
C. tropicalis	R	5'GTTTTC	500bp	**Sample-12-F-**	**Sample-12-R-**

(H12)		TACTGGA TCCCATG G-3' 5'TACATC TGTGTCTA CCACC-3'		CGTTTAGGTATGGTGTTATT TATAGATTCGTCTCAAGTCA AGATTATAGGAATTTCTATG AGCGGTGTCTCTTCTCCGAC AACGGGGTCATGGCAAATT TGGGAACCCAAAATTGATTG GGGTGAAACCTTTATTTTGT TAACAATCTTTTGTTCAAAA CCTCTGACGAAAACCTGTCT CATGTTAGCACCATATAAAC TCAACCAAGCATTAAAAATT TCGATGTCTGGTTGAGTTTT CATAACAATAGCATGACCAA GAGTTCTTTCCGTGGTTTTG AAACTAACATCGCTGACAAA ATAATTCAGAACTTCCCCTCT GATCTTTGGAACCCAGGTTT TGAAAGACTCAGTAATCAAA GCCATTTAGCAATTTCTTTTG TTCCATTAATCTTACGTTTGG ACAATCATAAATAACACCTT TACCAAAAACAGGAATAGT CAAATGGGTATAAGCTGCTT CAGCAA	ACGGTCTGGGTCATGGTGTTAT TTAAGATTCGTCCCAAGTCTAG ATTAGAGGAATTTCTATGAGCG CTGTCTCGTCTCCGACAACGAG GTCATGGCAAATTTGGGAACC CAAAATTGATTGGGGTGAAAC CTTTATTTTGTTAACAAACTTTT GTTCAAATCCTCTGTCGAAACT CTGTCTCATTTCAGCACCATAT AAACTACGCCAAGCATTGAAA ATAGTGATGTCTGGTTGAGTTT TCATAACAATAGCATCACCAAG GTTTCTTTCCGTGGTTTTGAAA CTAACATCGCTAACAAAATAAT TCAGAACTTCCCCTCTGATCTTT GGAACATAGGTTTTGAAAGAA TCAGTAATCAAAGCAAATTTAG CAAACTTCTTTTGTTCCATTAAT CTAGAGTTTGGACAATCATAAA TAACACCTTTACCAAAAACAGG AATAGTCAAATGGGTATAAGTT CCTTTCAGCAA

Genetic variability in ERG11 gene:

The ERG11 gene of two drug resistant *Candida* was amplified and sequenced. The obtained ERG 11 sequences of the isolates were compared with the homologous sequences with which maximum similarity was observed. Two of the drug resistant *Candida* species isolated during the study has shown maximum similarity with *Candida tropicalis* strain ATCC 13803; *Candida tropicalis* MYA-3404 & *Candida tropicalis* strain IHEM 10264. The isolate *Candida tropicalis* (H1) showed 91% similarity and isolate *Candida tropicalis* (H12) showed 89% similarity with the above species. The mutations were determined on the basis of the homology of the native *Candida* isolates with the other *Candida* isolates with which they were showing maximum similarity. Conserved regions were selected for observing the extent of mutations in the ERG 11 gene of drug resistant *Candida* species. FASTA format of native and homologous sequences were taken and then processed in Clustal W for dendrogram construction. Variation in ERG 11 gene were studied by multiple sequence alignment of the obtained ERG 11 sequences of the native isolates with the homologous sequences with which maximum similarity was observed. The native species showed variations like addition or deletion of nucleotides at specific position when compared with homologous species. Variation was also observed between the ERG11 genes of the two native isolates.



Dendrogram of *Candida* species.

1_PF_S4809= ***Candida tropicalis* (H1) ****
gi|61189833= ***Candida tropicalis*** strain IHEM 10264
gi|255732122= ***Candida tropicalis*** MYA-3404
gi|337235663 = ***Candida tropicalis*** strain ATCC 13803
gi|61189829 = ***Candida tropicalis*** strain IHEM 21233
gi|61189827 = ***Candida tropicalis*** strain IHEM 21232
gi|341007 = **Candida *tropicalis*** cytochrome P450
12_PF_S4809= ***Candida tropicalis* (H12)****
***** Represents the native isolates

As observed in dendrogram, the two native isolates i.e *Candida tropicalis* (H1) and *Candida tropicalis* (H12) grouped together where as other strains were grouped separately. This may be due to the two native isolates had originated from the similar geographic region where as the other homologous strains originated in different regions.

Multiple sequence alignment of ERG11 gene sequences of native isolates with homologous sequences obtained from NCBI.

```
1_PF_S4809                      -----ATAG----GTTTTGAAAGAATCAGTAATCAAA--GCAAATTTAGC 373
12_PF_S4809                     -----CCAG----GTTTTGAAAGACTCAGTAATCAAA--GCCA-TTTAGC 370
gi|61189833|gb|AY942646.1|      -----ATGGTGATGTTTT------TTCATTTAT------GTTA-TTGGGT 784
gi|255732122|ref|XM_002550939. -----ATGGTGATGTTTT------TTCATTTAT------GTTA-TTAGGT 267
gi|337235663|gb|JF754581.1|     TCTCAATGG----GTTCATAACTTTTCTGAAAATAGAATGCAA---TGGC 352
                                      *    ***         **    *       *  *     *
```

There may be addition of A in *Candida tropicalis* **(**H1) at position 331, 367 & 348, 349; there may be addition of C, A at position 327, 328, 344 & 345 in *Candida tropicalis* **(**H12).

```
1_PF_S4809                      AAAC---TTCTTTT-GTTCCATTAATCTTAAGTTTGGACAATCATAAATA 419
12_PF_S4809                     AA-T---TTCTTTT-GTTCCATTAATCTTACGTTTGGACAATCATAAATA 415
gi|61189833|gb|AY942646.1|      AAAG---TTATGA-----CTGTTTAT-TTGGGTCCAAAAGGTCACGAAT- 824
gi|255732122|ref|XM_002550939. AAAG---TTATGA-----CTGTTTAT-TTGGGTCCAAAAGGTCACGAAT- 307
gi|337235663|gb|JF754581.1|     ATGTGCATTCTCAAAGTTTCTTTAATAGTGTTGTTAACCAATGGTAATT- 401
                                *      ** *          ** **  *           *    * *
```

There may be addition of A, C at position 372,373; also addition of T at 382, 383; addition of GTT at 385, 386, 387; addition of C at 396; addition of GG at 405, 406 and addition of A at 419 in *Candida tropicalis* (H1). There may be deletion of A at place 368; addition of T at 369, 378,379; addition of GTT at 381,382,383; addition of C at 392; addition of GG at 401,402 & addition of A at 415 in *Candida tropicalis* (H12).

```
1_PF_S4809                      ACACCTTTACCAAAAACAGGAATAGTCAAATGGGTATAAGCTCCTTCCAG 469
12_PF_S4809                     ACACCTTTACCAAAAACAGGAATAGTCAAATGGGTATAAGCTGCTTC-AG 464
gi|61189833|gb|AY942646.1|      --TCATTTAC----------AAT-GCTAAAT---TATCCGATGTTTC--- 855
gi|255732122|ref|XM_002550939. --TCATTTAC----------AAT-GCTAAAT---TATCCGATGTTTC--- 338
gi|337235663|gb|JF754581.1|     -----TTTGCAAGTCTTCGTAA--GTCAAATCG-TTCAAGTCACCAC--- 440
                                     *** *          **  *  ****   *    *      *
```

There may be addition of A, C at 420,421; also addition of CAAAAACAGG at 330-339; addition of A at 343; addition of G at 353 & addition of CAG at 467-469 in *Candida tropicalis* (H1). There may be addition of A, C at 416,417; also addition of CAAAAACAGG at 326-335; addition of A at 339; addition of G at 349 & addition of AG at 463, 464 in *Candida tropicalis* (H12).

```
1_PF_S4809                      CAAAATTTTTGGTGGAAGGAACTTATACCCATTTGACTATTCCTGTTTTT 519
12_PF_S4809                     CAA-----TTGCTGAAAGGAACTTATACCCATTTGACTATTCCTGTTTTT 509
gi|61189833|gb|AY942646.1|      ---------TGCTG-AAGAAGCTTATACCCATTTGACTACTCCTGTTTTT 895
gi|255732122|ref|XM_002550939. ---------TGCTG-AAGAAGCTTATACCCATTTGACTACTCCTGTTTTT 378
gi|337235663|gb|JF754581.1|     -------CCTTTT--------CTTTCAACAAGTTGGTCA-----ATTCTT 470
                                      *  *        ***  * * * ***   *      ** **
```

There may be addition of CAA at 470-472; addition of AATTT at 473-477; addition of G at 481 & 484 in *Candida tropicalis* (H1). Similarly there may be addition of CAA at 466-468; addition of A at 480 in *Candida tropicalis* (H12).

```
1_PF_S4809                      GTGTTCCAAAGATCAGAGAAGAAGTTCTGAATTATTTTGT-AACGATGTT 663
12_PF_S4809                     ATGTTCCAAAGATCAGAGGGGAAGTTCTGAATTATTTTGTTAGCGATGTT 653
gi|61189833|gb|AY942646.1|      ATGTTCCAAAGATCAGAGAAGAAGTTTTGAATTATTTTGTTAACGATGTT 1039
gi|255732122|ref|XM_002550939.  ATGTTCCAAAGATCAGAGAAGAAGTTTTGAATTATTTTGTTAACGATGTT 522
gi|337235663|gb|JF754581.1|     AT---CAAAACACCAATTAAAAGGTTAGCAATTTCTTGATCAGTCATTTT 603
                                 *   * *** * **     * ***   ****  **  * *   ** **
```

There may be addition of G at 614; also addition of C at 640 & deletion of T at 654 in *Candida tropicalis* (H1). There may be addition of GG at 628, 629; addition of C at 636 & addition of G at 652 in *Candida tropicalis* (H12).

```
1_PF_S4809                      AGTTTCAAAACCACGGAAAGA---AACCT-GGTGATGCTAGTGTTATGAA 709
12_PF_S4809                     AGTTTCAAAACCACGGAAAGA---AACCTTGGTGATGCTATTGTTATGAA 700
gi|61189833|gb|AY942646.1|      AGTTTCAAAACCAAGGAAAGA---GACCATGGTGTTGCTAGTGTTATGAA 1086
gi|255732122|ref|XM_002550939.  AGTTTCAAAACCAAGGAAAGA---GACCATGGTGTTGCTAGTGTTATGAA 569
gi|337235663|gb|JF754581.1|     AACACCATCTTTATAAGTAGAGTTAACCAACAAGGAATCA-----ATCAA 648
                                 *    **     *     ***    ***     *     *     ** **
```

There may be addition of C at 673; addition of AA at 684, 685; addition of T at 688; deletion of T at 689 & addition of GA at 693, 694 in *Candida tropicalis* (H1). Similarly, addition of C at 669; addition of AA at 680, 681; addition of GA at 689, 690; addition of T at 684, 694 in *Candida tropicalis* (H12).

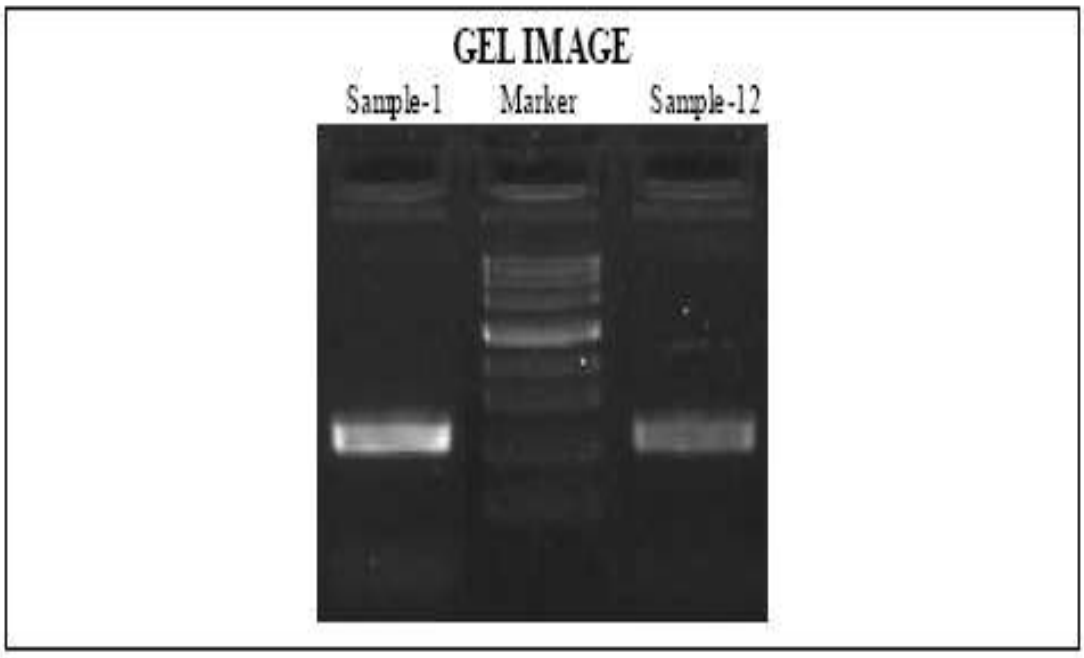


Figure 3: Amplified product on 0.8% agarose gel.

Lane 1:- sample *Candida tropicalis* (H1)
Lane 2:- 1Kb marker
Lane 3:- sample *Candida tropicalis* (H12)

Discussion:

In the recent years the number of serious opportunistic yeast infections, particularly in immunocompromised patients has increased significantly (Richardson MD *et al*., 2003). *Candida* species accounts for over 80% of such infections. In the present study *Candida* species were collected from urine sample of patients of zonal hospital Solan and their phenotypic and genotypic characters were studied. *C. albicans* was the most frequently isolated yeast pathogen accounting for 86.8 % of the isolates. This is in line with previous investigations where *C. albicans* accounted for up to 80 % of the candidiasis infection (Wade JM., 1993). In the current studies 50% of the isolates were *Candida albicans.* Previous studies have reported other non *albicans Candida* as emerging significant pathogens (Coleman DC *et al*., 1988; Moran GP *et al*., 2003). Earlier *C. albicans* was the most frequently isolated yeast pathogen; however other non albican *species* were also known to associate with Candidiasis. These species include *C. glabrata*, *C. krusei, C. parapsilosis, C. guilliermondii* and *C. tropicalis.* In current

study 50% of samples were found infected with these non *C. albicans* species.

The ability to easily differentiate between *C. albicans* and other *Candida* species was found difficult in routine laboratory practice (Sullivan D *et al*., 1998; Ruhnke M *et al*., 1997). The detection and identification of microorganisms depend on the availability of easy to perform screening and cost- effective methods. In the present study phenotypic traits such as chlamydospore production, pseudohyphae formation, germ tube production, biochemical pattern and morphological examination of *Candida* species on CHROM agar has been studied. This helped in understanding identification and differentiation of *Candida* species up to species level. Different *Candida* species showed the different coloured colonies on CHROM agar which helps in the identification of different *Candida* species. Since *C. albicans* is the yeast species most often isolated from clinical material, most clinical laboratories approach yeast identification by applying simple rapid tests such as germ tube formation to distinguish *C. albicans* from other species which require more extensive testing for proper identification (Pincus DH *et al*., 1999). In present study it was found that 50% of the isolates were germ tube positive.

Several methods for identification of *C. dubliniensis* and discrimination from *C. albicans* have been reported. They include formation of dark green colonies on CHROM agar *Candida* (Pincus DH *et al*., 1999; Tintelnot K *et al*., 2000), no or strictly reduced growth at 45°C and lack of ability to assimilate xylose (Gales A.C *et al*., 1999). In our investigation, none of *C. dubliniensis* was isolated, all the *C. albicans* isolates grew at both 37°C. Azole antifungals are widely used for therapy and prophylaxis of *Candida* infections. A better understanding of the mechanisms of resistance to these agents as well as early detection of resistance is essential for patient management. Azole resistance is often due to a combination of factors including increased expression of efflux pumps and missense mutations in ERG11 (White TC *et al*., 1998; Morschhauser J., 2002; Perea S *et al*., 2001; Chau AS *et al*., 2004). The latter have been linked to clinically-relevant increases in the MICs, not only to fluconazole, but also to the newer azoles voriconazole (Morschhauser J., 2002; Perea S *et al*., 2001; Sanglard D *et al*., 2002; Chau AS *et al*., 2004). In current study, results of antifungal susceptibility testing showed that various species of *Candida* were found resistant to number of antifungal agents such as voriconazole and fluconazole. Out of 24 isolates, 9 were found resistant to fluconazole and 12 were found resistant to voriconazole. Two isolates *C.tropicalis* (H1) and *C.tropicalis* (H12) were resistant to both the antifungal agents.

Fluconazole, voriconazole and amphotericin B are the drugs of choice used for the treatment of fungal infection. However, due to continue use of these drugs most of the *Candida* sp. become resistant to these drugs. In all fungal species, ERG11 is the gene encoding ERG11p or lanosterol 14α- demethylase, an essential enzyme for ergosterol synthesis. In previous studies, it was observed that antifungal susceptibility depend upon the regulation of ERG11 gene in *C. albicans* (Lamb *et al.,* 1997; Perera S *et al*., 2001; Lupetti A *et al.,* 2002) and also in *C. glabrata* and *C. dubliniensis* clinical isolates (Perera S *et al.,* 2002; Marichal P *et al.,*1997; I. Casalinuova A *et al.,*1999). Recently, mutations G487T and T916C were found in an induced fluconazole-resistant strain of *C. albicans* along with T395A (F83Y) and neither of them was present in the fluconazole susceptible parent strain or any other susceptible strain. These data imply that G487T and/or T916C might be correlated with fluconazole resistance in *C. albicans* (Jiang WS *et al*., 2006). In present study, disk diffusion method (CLSI M44-A protocol) was employed to find azole drug resistant species. The amplification and sequencing of ERG 11 gene of selected drug resistant species were done. The dendrogram was formed with Clustal W tool. In present study mutations were seen from multiple sequence alignment. Addition and deletion of various nucleotides were observed at different positions between native and homologous species. Even mutations were observed in the two native isolates isolated during the study.

The current study helps in understanding susceptibility pattern and mechanism of drug resistance *Candida* species at genetic level.

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