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RESEARCH ARTICLE

SPECIES IDENTIFICATION, ANTIFUNGAL SUSCEPTIBILITY TESTING AND GENETIC VARIABILITY AMONG *CANDIDA* SPECIES ISOLATED FROM CLINICAL SAMPLES

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ABSTRACT

Candida albicans is an opportunistic pathogen that usually lives as commensal in the healthy human host. The most common infecting species is *C. albica*ns where as other non *albicans* species are C*. glabrata, C. parapsilosis, C. tropicalis* and *C. kruse*i also causes various clinical diseases. Candidemia is the fourth leading cause of bloodstream infections and carries 35–55% mortality. There is increase in the incidence of candidiasis cases has also been reported in immunocompromised individuals associated with non albicans *species*, such as *C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis.* The main risk factor is Invasive candidiasis, Urinary candidiasis, gastrointestinal candidiasis, Respiratory candidiasis and vulvo-vaginal candidiasis. MATERIAL AND METHOD: A total of 30 samples were collected from zonal hospital Solan and college students of SILB Solan. *Candida* species were isolated from 24 samples while 6 samples were found negative. Samples were further checked for phenotypic characterization such as Germ tube test, pseudohyphae production, Chlamydospore production, morphology on CHROM agar, sugar fermentation test, sugar assimilation test. Antifungal susceptibility testing is also done for the positive samples. Genotypic characterization is done by PCR and RFLP.

KEYWORDS: *C. parapsilosis, C. tropicalis, C. kruse*i and *C. glabrata*

INTRODUCTION:

Opportunistic fungal infections are widespread in immunosuppressed individuals and are a serious concern for the management of such individuals. In the past two decades, the frequency of invasive fungal infections and mortality has increased due to invasive mycoses (Rapp RP 2004). Numerous yeasts and moulds such as *Candida* spp. and Aspergillus spp. cause the most of the clinical diseases and common life-threatening infections. *Candida* species especially *Candida albicans* are the part of commensal flora (Cannon *et al*., 1995). The most common infecting species is *C. albica*ns where as other non *albicans* species are C*. glabrata, C. parapsilosis, C. tropicalis* and *C. kruse*i also causes various clinical diseases. These all approximately contribute 99% of all human clinical cases (Pfaller MA *et al.,* 2007*).* Candidemia is the fourth leading cause of bloodstream infections and carries a 35–55% mortality (Wisplinghoff H *et al.,*2004). As these organisms show a range of susceptibilities to existing antifungal drugs, distinguishing them is important for the selection of antifungal therapy. Despite highly active antifungal drugs, mortality remains high at 50–70% (Upton A *et al.,* 2007).

C. albicans has been considered the predominant etiologic agent of oral candidiasis *(*Stenderup, *A 1998). Candida albicans* is common and widespread opportunistic yeast Pathogen, it causes an increasing number of human cutaneous infections, oral candidiasis and vaginal candidiasis in recent years (McCullough MJ *et al.,* 1999). Over the last decade, an increase in the incidence of candidiasis cases has been reported in immunocompromised individuals associated with non albicans species, such as *C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis* (Carrillo-Muñoz *et al .,*2001). In 1995, *C. dubliniensis*, which is a species very, closely related to *C. albicans*, was isolated from cases of oral candidiasis in HIV-infected individuals (Sullivan D *et al.,* 1995). There is great heterogeneity reported among the individuals of the *Candida* isolates, *C. albicans* strains have been subdivided

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into different biological groups based upon genetic subtypes (Tamura M *et al .,*2001). Several studies are known to support the origin of genotypic differences among *C. albicans* isolates. The variation among different species might be correlated with their mechanism of virulence and pathogenicity (Lian CH *et al.,* 2004). Polymerase chain reaction (PCR) amplification and restriction enzyme digestion analysis are two of the most frequently used techniques in establishing the genotyping of *Candida* species (Iwata T *et al.,* 2006). The present study is designed to examine the phenotypic traits and to observe genetic variability in drug resistant *Candida* species.

In the present study, we selected restriction fragment length polymorphism (RFLP) as our typing method to compare the genotype of drug resistant *Candida* species isolated from different clinical cases. The study will help in characterization and to understood extent of genotypic variability among drug resistant *Candida* species.

METHODS:

A total of 30 samples (Urine and Throat) were collected from zonal hospital and SILB college students at Solan (H.P). Out of 30 samples, 22 were urine samples obtained from zonal hospital and 8 were throat samples collected from girls living in SILB hostel Solan. All the samples were collected in sterile air tight container. SDA was prepared for the isolation of *Candida* species from the various samples collected from the different places. samples were streaked on SDA slants under sterile condition and the slants were incubated at 25^0 C for 2-3 days. Various colonies of Candida were observed after incubation. 10% Glycerol were prepared and then transferred in small screw capped bottles. loopful of culture from already prepared slants into these small screw capped bottles containing glycerol and then stored at -20 ^{0}C. Isolated 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 observed as per methodology described by Arunaloke C *et al*., 2002.

GERM TUBE TEST:

Germ tube test was done for the presumptive identification of *candida albicans*. A very light suspension of the test organism was made in 0.5ml of sterile serum incubated at 37^0C for exactly 2 hrs. Placed one drop from the incubated serum on a slide with a cover slip. Observed the slide under microscope for production of germ tube. Germ tube represents initiation of hyphal growth arising from the yeast cell.

PSEUDOHYPHAE PRODUCTION:

Sterile needle yeast colonies were streaked on CMA plate supplemented with tween 80, sterile cover slip was placed over streaked colonies. The plates were incubated for 3-5 days at 25^0C. The plates were observed after 3-5 days under microscope to see the presence of pseudohyphae.

CHLAMYDOSPORE PRODUCTION:

The samples grown in SDA were seeded as 4 parallel streaks on petri plates containing corn meal agar supplemented with tween 80 and the plates were incubated at 30^0C for 3-5 days for the production of chlamydospore (Fisher F *et al*., 1998). The plates were visualized under optical microscope (Gatica JLM *et al*., 2002). The double walled rounded spores were observed as chlamydospore.

MORPHOLOGY ON CHROM AGAR:

The samples grown in SDA were seeded on chromgenic agar and incubated at 30^0 C for 48 hour. CHROM agar allows the selective yeast isolation and identifying colonies of *C.albicans, C.dubliniensis, C.tropicalis* and *C.krusei* by colour identification (Hospenthol DR *et al*.,2006).The strain were identified according to the colour of the colonies, *C.albicans or C.dubliniensis* as green colonies*, C.tropicalis* as steel blue colonies.

SUGAR FERMENTATION TEST:

Sugar fermentation test for various *Candida* species was done as described by Arunaloke C.*et.al 2002*. Pepared liquid fermentation medium containing peptone (1%), sodium chloride (5%), andrades's indicator (0.005%). Sterilized by autoclaving at 110^0 C for 10min. Added filtered sterilized sugar at the concentration of 2% to the medium. Poured into the sterile test tubes (approx. 5ml) and placed sterile Durham's tubes into each tube. Plugged the tubes with colour coded cotton plugs. Inoculum preparation was done by suspending heavy inoculums of yeast grown on sugar free medium. Inoculated each carbohydrates broth with approx. 0.1ml of inoculum. Incubated the tubes at 25^0 C up to 1 weak. Examined the tubes every 48-72hrs interval for the production of acid and gas (in Durhhams). Production of gas in the tube was taken as fermentation positive while only acid production may indicate that carbohydrate is assimilated.

SUGAR ASSIMILATION TEST:

The sugar assimilation test was performed for the assimilation of various sugars by *Candida species* described as per protocol described by Arunaloke C.*et.al*., 2002. Prepared a yeast suspension from a 24-48hrs old culture in 2ml of YNB by adding heavy inoculum. Added this suspension to the 18ml of molten agar and mixed well. Poured the entire volume in to a 90mm petri plate. Allowed the Petri plates at room temperature until the agar surface hardens. Placed the various carbohydrates-impregnated discs on the surface of agar plate. Sugar discs can be obtained commercially or can be prepared by punching 6mm-diameter disc from Whatman no. 1 filter paper. Sterilized the disc by placing them in hot air oven for 1hr. Add a drop of 10% filter sterilize solution to each disc. Dry the disc at 37^0 C and store at 4^0 C in airtight container. Incubated the plates at 37^0C for 3-4 days. The presence of growth around the disc is considered as positive for that particular carbohydrate.

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 inoculums suspension was inoculated within 15-20 minutes. .

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 was 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 + 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°C each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. An antimicrobial disk was dispensed onto the surface of the inoculated agar plate. The plates were incubated inverted position at 25^0 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.

EXTRACTION OF GENOMIC DNA:

Genomic DNA of the *Candida* species was extracted as per methodology described by Arunaloke C *et al.,* 2002

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF ERG 11 GENE:

Amplification of ERG11 gene was done by using polymerase chain reaction in five drug resistant *Candida* species. Cells were inoculated in fresh YPED medium with constant shaking at 30°C for overnight. Genomic DNA was isolated by using DNA extraction method and was used as template for amplification of coding region of ERG11 genes with the following primers: 5-GTTTCTACTGGATCCCATGG-3 and 5-TACATCTGTGTGTCTACCACC-3. PCR was carried out in 50µl volume containing 10 x PCR buffer 5µl, genomic DNA 2 µl, 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 analyzed by electrophoresis on 0.8% agarose gel and were visualized under transilluminator after staining the gel with ethidium bromide (ZHOU Yong *et al.,* 2011).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS:

Genetic variability among different *Candida* isolates was checked by employing RFLP analysis. Total amplified product of the of the five drug resistant *Candida* isolates were digested by using restriction enzymes EcoRI (Smith *et al*., 1989) .The 5 ul of of amplified product of the 5 *Candida* strains, i.e. two strains of *Candida albicans* (H2 and H15), two strains of *Candida tropicalis (*H1 and H7) and one strain of *Candida glabrata*(H6) were transferred into

the 1.5ml of fresh microfuge tubes. To each tube 5 ul of EcoRI assay buffer, 16 ul of molecular grade water and one 1ul of enzyme 40 U EcoRI was added. All the 5 tube was incubated overnight at 37^0C. After overnight incubation the digests was electrophoresed in 0.8% agarose gel contaning ethidium bromide in 0.5xTE buffer at 50 v for 4 hour and visualized under UV transilluminator to observe the band pattern (Bostock *et al*., 1993).

RESULTS:

A total of 30 samples were collected from zonal hospital Solan and college students of SILB Solan. Out of 30 samples, Different *Candida* species were isolated from 24 samples while 6 samples were found negative.

Table 1: Phenotypic Characterization 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 coloured with slightly mycelia 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 coloured with slightly mycelia 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 coloured with slightly mycelia 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 coloured with slightly mycelia 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 coloured with slightly mycelia border	_
18	*C.tropicalis* (H17)	Dark blue colony	Abundant pseudohyphae,pine forest arrangement.	Cream coloured with slightly mycelia 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 ON CANDIDA SPECIES ON DIFFERENT MEDIAS:

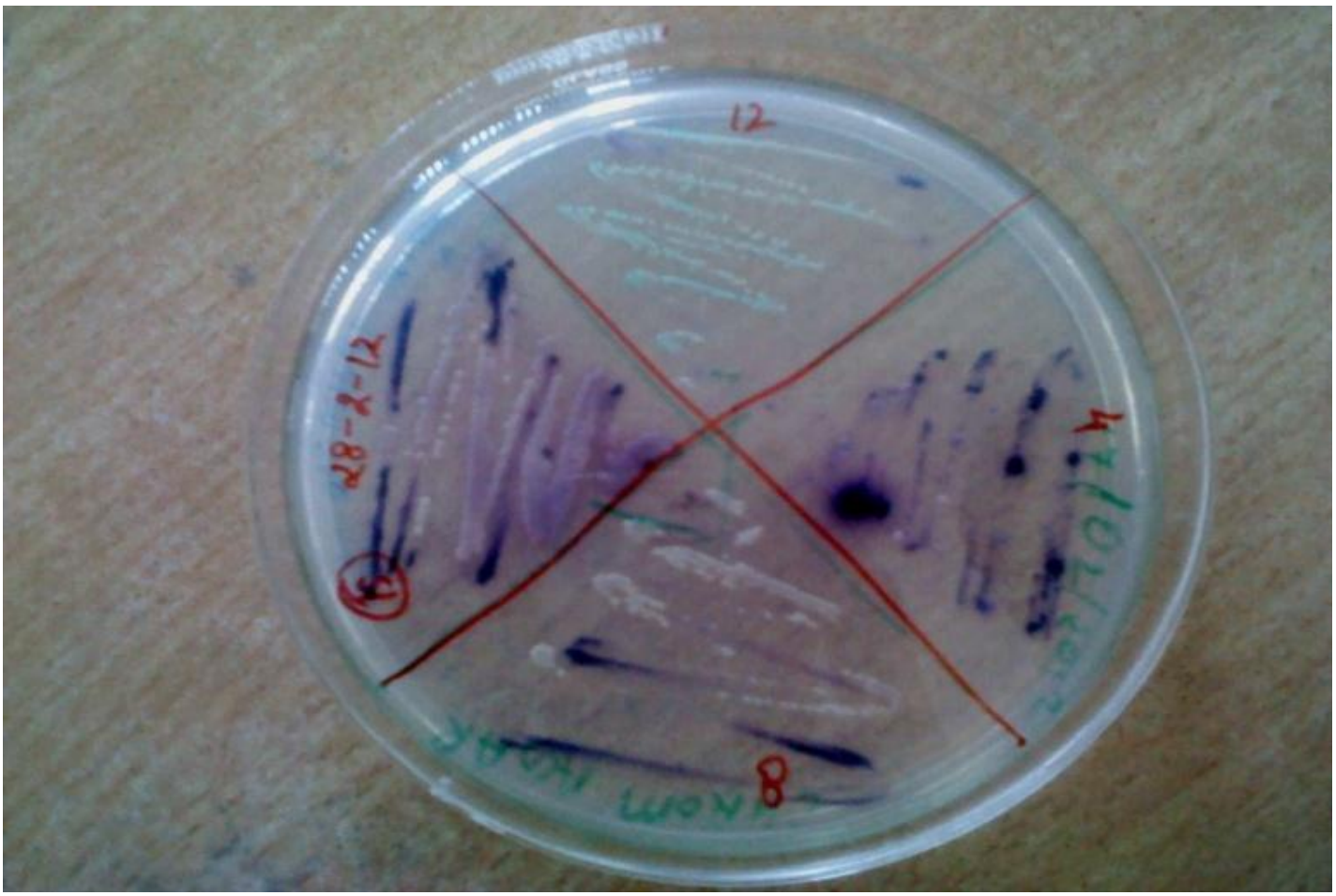

Figure 1: CHROM agar plate showing green coloured colonies of *Candida albicans* and blue coloured colonies of *Candida tropicalis*.

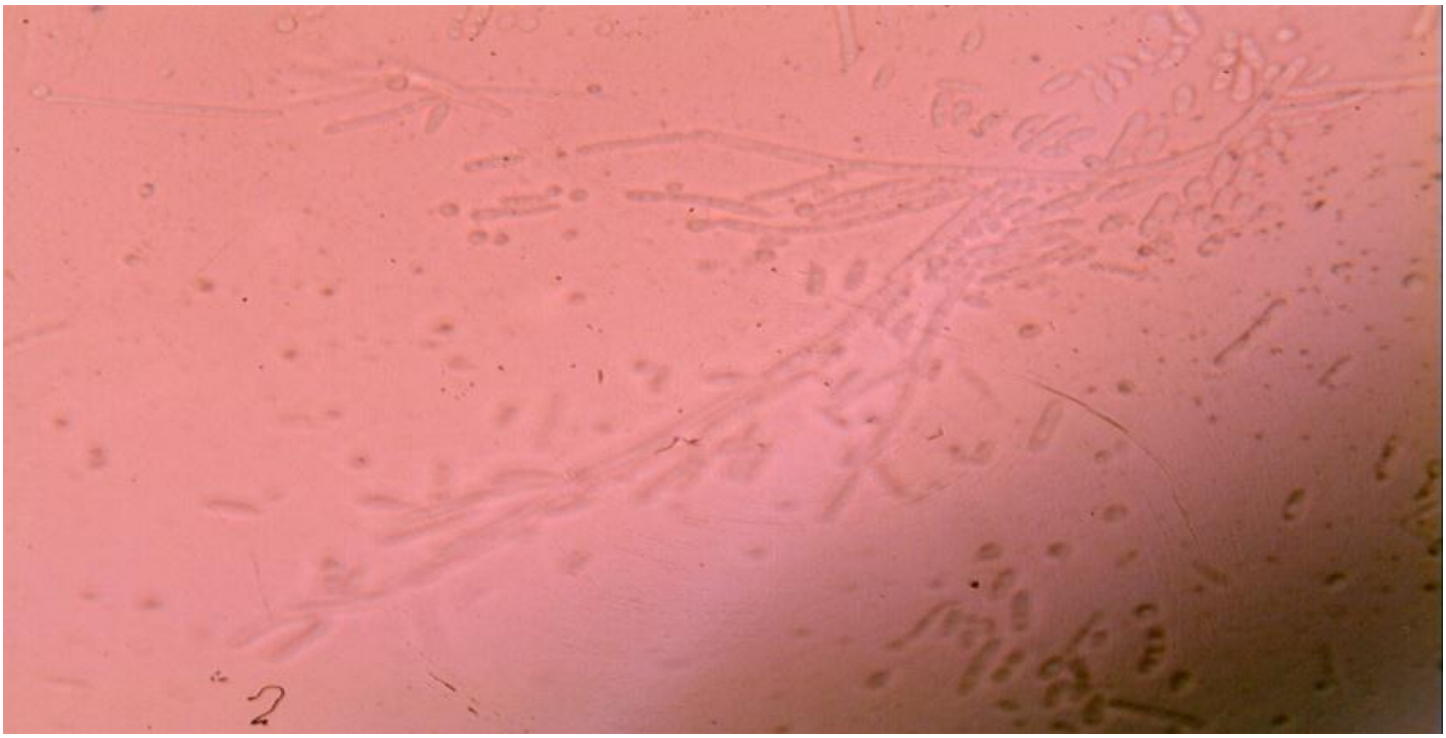

Figure 2: *Candida krusei* showed budding cell broadly ellipsoidal to cylindrical. Pseudomycelium often present, robust.

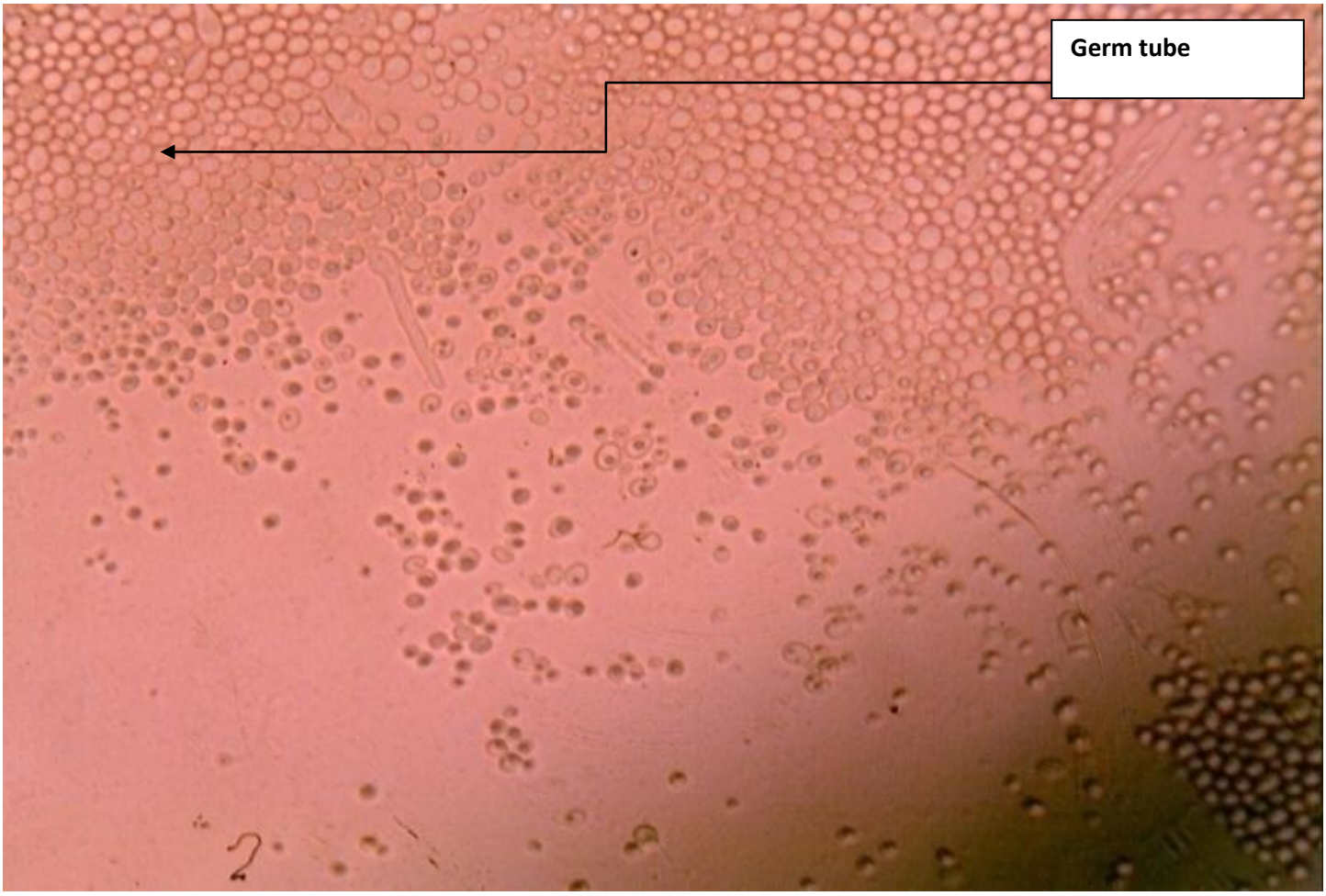

Figure 3: - Germ tube formation by *Candida albicans*.

Table 2: 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 whereas 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.

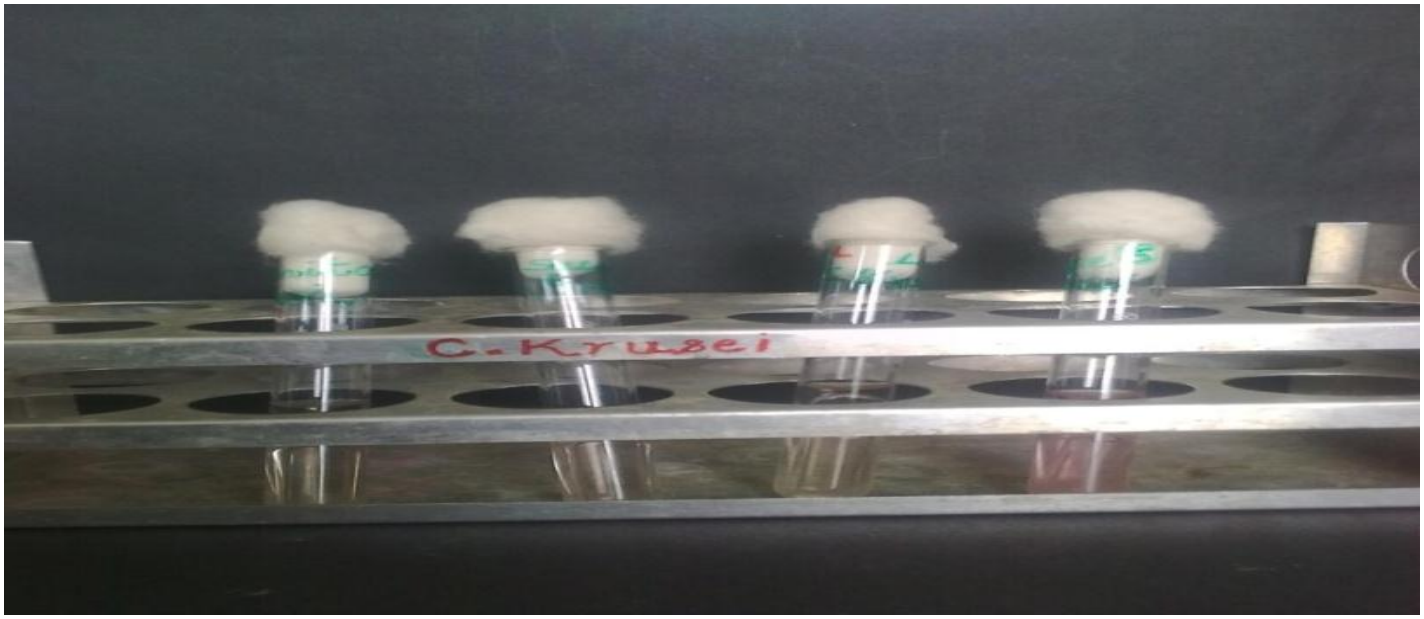

Figure 4: - Results of sugar fermentation test for *C krusei*.

Table 3-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

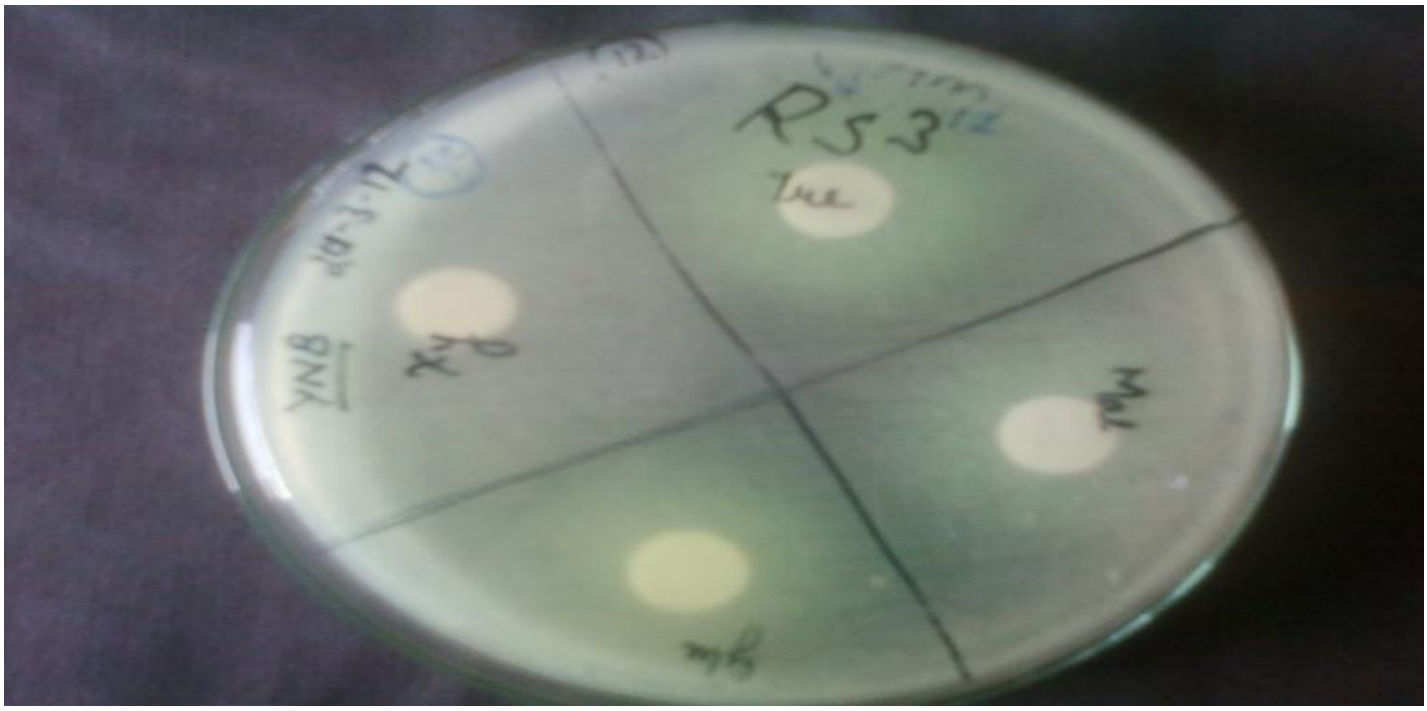

Figure 5:- Results of sugar assimilation test showing the zones of precipitation around different sugar discs for *C.glabrata*.

Table 4: Results of Antifungal Susceptibility Testing.

Sr. No.	Strain No.	Species	Voriconazole	Amphotericin B	Results of Voriconazole	Results of amphotericin B
1.	H1	*C.tropicalis*	0mm	6mm	Resistant	Resistant
2.	H2	*C.albicans*	0mm	4mm	Resistant	Resistant
3.	H3	*C.albicans*	0mm	16mm	Resistant	Sensitive
4.	H4	*C.krusei*	40mm	9mm	Sensitive	Resistant
5.	H5	*C.parapsilosis*	0mm	7mm	Resistant	Resistant
6.	H6	*C.glabrata*	0mm	8mm	Resistant	Resistant
7.	H7	*C.parapsilosis*	42mm	9mm	Sensitive	Resistant
8.	H8	*C.albicans*	0mm	0mm	Resistant	Resistant
9.	H9	*C.albicans*	37mm	0mm	Sensitive	Resistant
10.	H10	*C.tropicalis*	0mm	17mm	Resistant	Sensitive
11.	H11	*C.guilliermondii*	42mm	15mm	Sensitive	Sensitive
12.	H12	*C.tropicalis*	0mm	10mm	Resistant	S-DD
13.	H13	*C.guilliermondii*	47mm	0mm	Sensitive	Resistant
14.	H14	*C.tropicalis*	0mm	0mm	Resistant	Resistant
15.	H15	*C.albicans*	0mm	0mm	Resistant	Resistant
16.	H16	*C.tropicalis*	45mm	11mm	Sensitive	S-DD
17.	H17	*C.tropicalis*	0mm	0mm	Resistant	Resistant
18.	H18	*C.albicans*	37mm	16mm	Sensitive	Sensitive

19.	H19	*C.albicans*	38mm	8mm	Sensitive	Resistant
20.	H20	*C.albicans*	0mm	10mm	Resistant	S-DD
21.	S21	*C.albicans*	43mm	0mm	Sensitive	Resistant
22.	S22	*C.albicans*	36mm	15mm	Sensitive	Sensitive
23.	S23	*C.albicans*	36mm	15mm	Sensitive	Sensitive
24.	S24	*C.albicans*	50mm	14mm	Sensitive	Sensitive

Significant number of isolates were found resistant to two antifungal used. 50% isolates were resistant to the voriconazole and 60% isolates were resistant to the amphotercin B where as 13% isolates found semi dose dependent against amphotercin B.

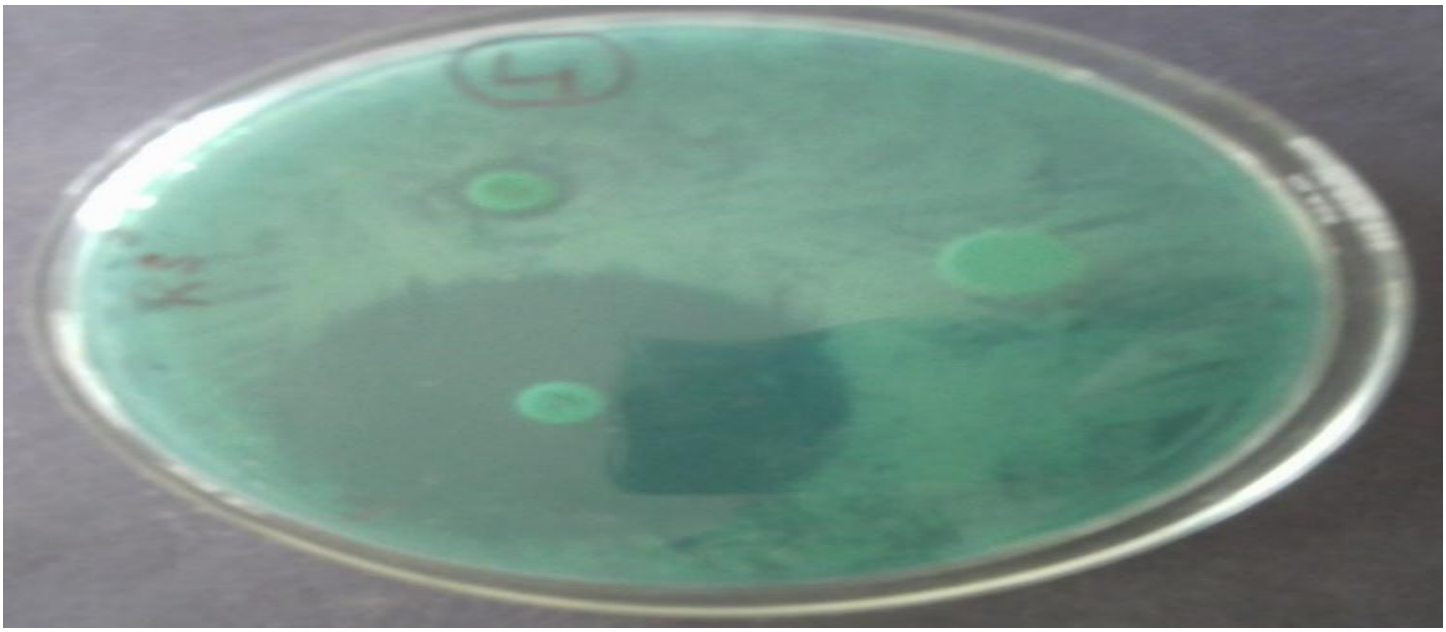

Figure 6: Zone of inhibition around the antifungal discs (voriconazole and amphotercin B) for *Candida albicans.*

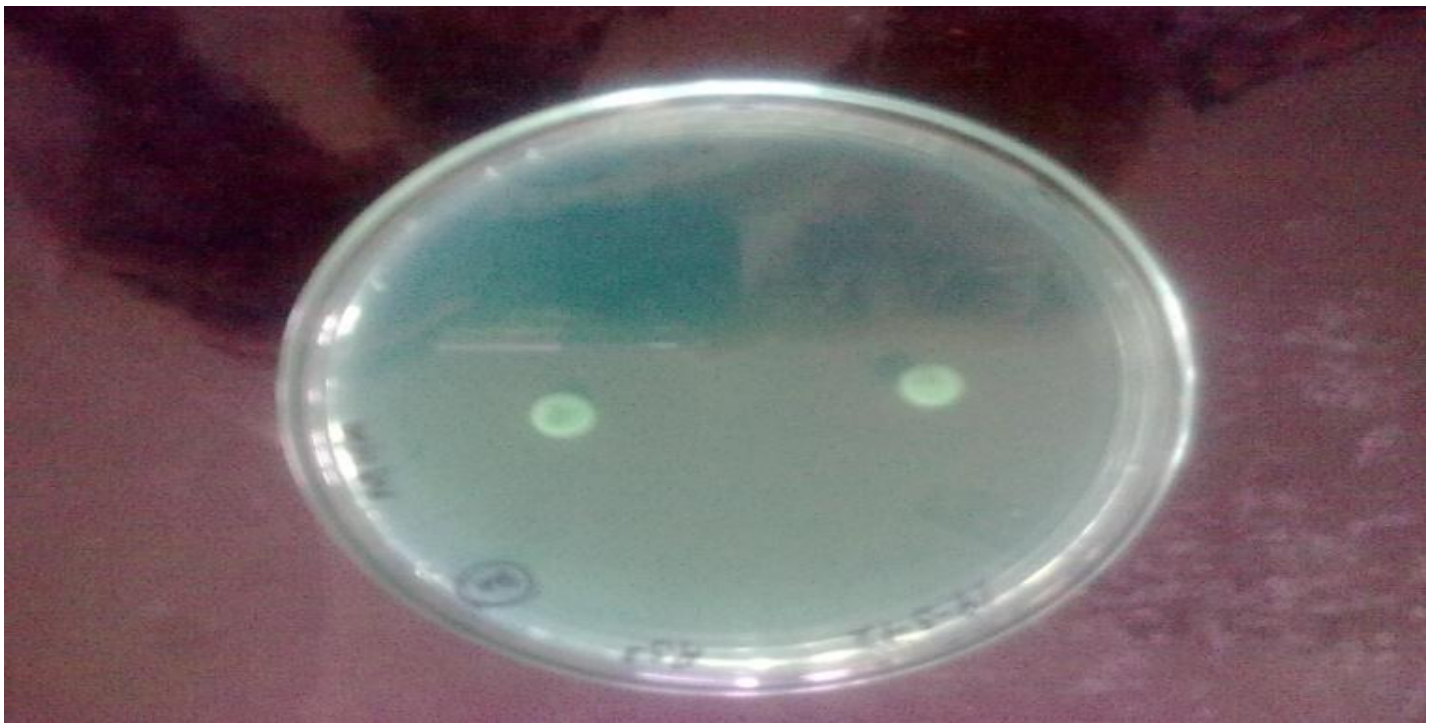

Figure 7: Plate showing fluconazole ane amphotercin B resistant *Candida albicans.*

Table 5: RESULTS OF RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS OF AMPLIFIED ERG 11 GENE.

Strain No.	Species	Restriction Enzyme	Restriction site	Size of bands
H1	*Candida tropicalis*	EcoRI	GAATTC	410bp 300bp 200bp
H2	*Candida albicans*	EcoRI	GAATTC	300bp 200bp
H6	*Candida glabrata*	EcoRI	GAATTC	340bp 280bp 200bp 170bp
H8	*Candida albicans*	EcoRI	GAATTC	410bp 300bp 200bp
H15	*Candida tropicalis*	EcoRI	GAATTC	410bp 300bp 200bp

The drug resistant *Candida* isolates, which were resistant to both the antifungal agent were selected for RFLP analysis. A total of 4 polymorphic RFLP bands of different sizes were detected. The results shows that the RFLP profile of 3 bands (410,300 and 200bp) were the most common and the profile of 4 bands were only found in one isolate. Generally, for most strains, the specific enzyme digestion product corresponded to its genotype. Several strains of similar species showed different RFLP profiles. For example, *Candida albicans* (H_2 and H_8) had different RFLP profiles with 2 and 3 DNA bands. While *Candida tropicalis* (H_1 and H_{15}) had similar band pattren respectively, Strain *Candida glabrata* (H_6) showed 4 bands. Comparison of the RFLP profiles showed that the restriction profiles of the most of the strains were identical, whereas only two species *Candida glabrata* (H_6) and *Candida albicans* (H_2 and H_8) showed different band pattern. Moderate degree of variation was observed among drug resistant *Candida* isolates.

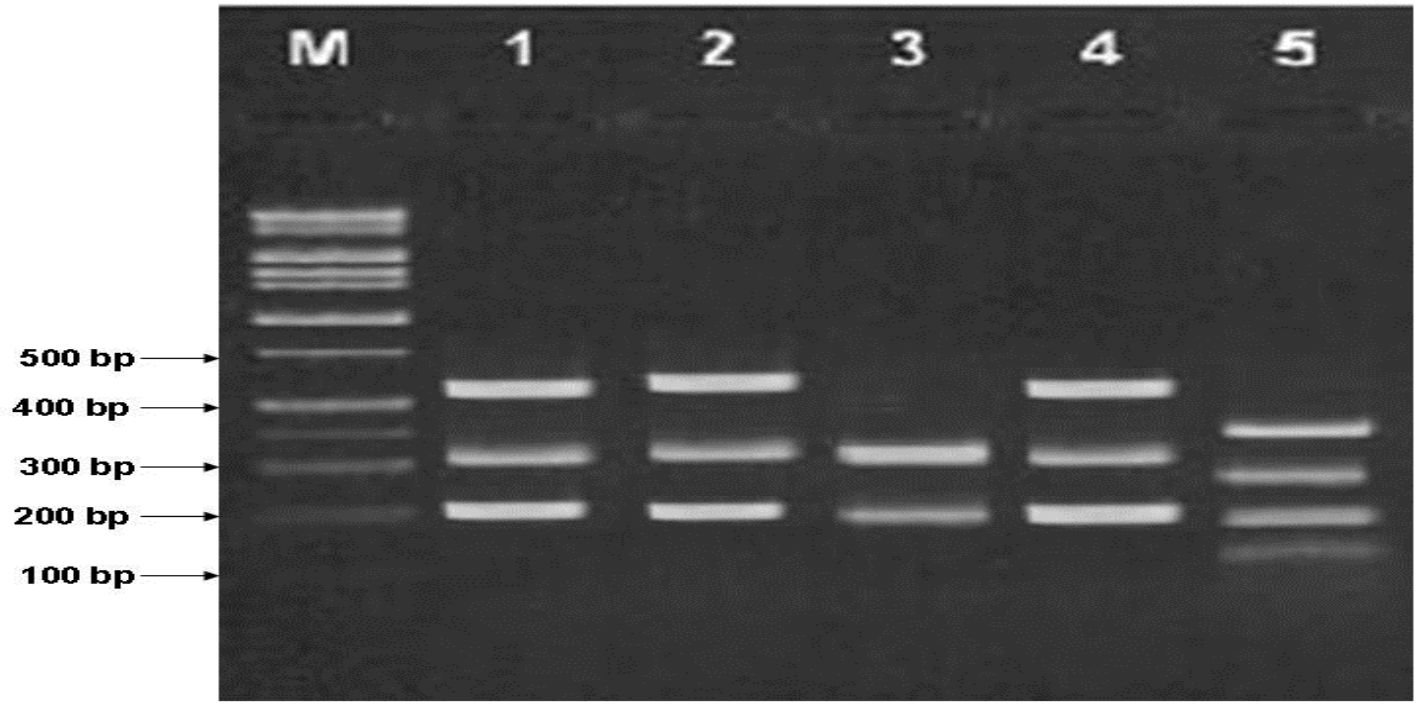

Figure 8: RFLP band pattern of amplified product while digesting with EcoR1 restriction endonuclease enzyme. M=1Kb marker, lane-1 =*Candida tropicalis* (H1), lane-2 *Candida tropicalis* (H15), lane 3= *Candida albicans* (H2), lane-4= *Candida albicans* (H8) and lane-5 = *Candida glabrata* (H6).

DISCUSSION:

Candida is yeast and the part of the normal microflora of human body. *Candida* is known as opportunistic fungal pathogens as it causes infection when person become immunocompromised, immunonosuppressed or diabetic. In immune compromized individuals *Candida* causes diseases like oral thrush, intestinal candidiasis, vaginal thrush and onchomycosis (Chamaine 2005, Bennett JE *et al.,* 1992,). In the present study *Candida* species were isolated from the UTI patients of zonal hospital Solan and college students of SILB. Farina *et al .,*1999, studied the prevalence of *Candida* infection during the period of 1988 to 1997 at the regional hospital of Bergamo In Italy 168 cases of fungaemia were studied, out of which 70% cases were due to the *Candida* species and this shows the dominance of *Candida* species in immunocompromised host. *Candida* is a leading pathogen causing the different types of candidiasis such as vaginal, respiratory, urinary and invasive candidiasis etc. since 1990, it has become clear that *Candida* species continue to become an important etiological agent of nosocomial infection. The *Candida* species have been typed and classified using a wide variety of techniques and proportion of such infection is increased due to the involvement of non albicans species (Fridkin *et al .,* 1996).To prevent and control the nosocomial infection, identification of *Candida* species upto the species level is most important (Pfaller *et al .,*1997). In the present study phenotypic traits such as chlamydospore production, pseudohyphae formation, germ tube production has been studied. *C.albicans* showed the terminal chlamydoconidia, C*. guiliermondii* showed the chain of blastoconidia with pseudohyphae, *C.glabrata* formed the yeast only, *C.tropicalis* formed the abundant pseudohyphae having the pine forest arrangement terminal chlamydospore in cluster, *C.parapsilosis* formed the giant hyphae and blastospore at nodes on corn meal agar. Germ tube production test was positive only for the *Candida albicans.* Fluconazole, voriconazole and amphotericin B are the choice of drugs used for the treatment of fungal infection. However, due to continue use of these drugs, most of the *Candida* sp. developed resistance against these drugs. Casalinuova IA *et al.,* 1999 studied that fluconazole; voriconazole and amphotericin B were the drugs of choice used for fungal infection. Most of the *Candida sp.* Are now becoming resistant due to their continuous use. In the current study it was found that maximum number of *Candida* isolates were resistant to voriconazole and amphotericin B. 50% strain were resistant to the voriconazole and 60% strains were resistant to the amphotercin B where as 13% strains were interpreted as semi dose dependent against amphotercin B. Results of Antifungal susceptibility testing shows that various species of *Candida* becomes resistant to a number of antifungal agents such as voriconazole and fluconazole. Xiao-dong S *et al.,* 2008 studied that genotypes of strains isolated from cutaneous and vaginal infections were significantly different within these two body site. They studied the mechanisms that influence genotype of colonizing of *C. albicans* in cutaneous and vagina of the patients.

A number of different methods of detecting variations in genetic sequences in *Candida* strains have been developed, including RFLP analysis(Smith *et al.,*1989).In the present study the DNA of the 10 drug resistant *Candida sp.* were isolated and ERG 11 of these *Candida sp* was amplified by PCR. RFLP of the amplified product was done with restriction enzyme EcoRI. The RFLP technique with the restriction enzyme EcoRI used in this study proved to be a reliable, sensitive method for assessing the genetic relatedness of different *Candida* species. A total of 4 polymorphic RFLP bands of different sizes were detected. The results shows that the RFLP profile of 3 bands (410,300 and 200bp) were the most common and the profile of 4 bands were only found in one isolate. Several strains of similar species showed different RFLP profiles Genotypic studies have the potential to provide more reproducible classifications of organisms. These studies will help us in understanding identification of *Candida* species, pathogenicity pattern and genetic relatedness between different species.

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