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PREVALENCE OF VULVOVAGINAL CANDIDIASIS AMONG PRE AND POST-MENOPAUSAL DIABETIC AND NON-DIABETIC WOMEN

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ABSTRACT

The Study included 150 diabetics and non-diabetic, pre and post-menopausal women of the age group between 35-65yrs, with symptoms of vaginitis, who attended Gynaec OPD of SreeBalaji Medical College and Hospital, Chennai, and were screened for Vulvovaginal candidiasis. 100 were diabetic women and mentioned as study group and 50 were non-diabetic and mentioned as control group. In this study, pre-menopausal age group was between 35-45yrs and post-menopausal age group was between 45-65yrs. The higher prevalence of vaginal candidiasis was in post-menopausal age group (>45yrs), which contribute to 63.5%(out of 52 positive cases) and low prevalence rate in pre-menopausal age group (35 -45yrs) which was 36.5% (out of 52 positive cases). Diabetes mellitus was the common risk factor associated. Percentage of Candida isolated in diabetes was 42% and non-diabetes was 20%, and is one of the predisposing factors for non-albicans candida spp infection. The total prevalence of vulvovaginal candidiasis was 34.6% (out of 52 positive cases) which was similar to other studies. C. albicans was isolated in 50% from the 52 positive samples obtained, and was one of the predominant isolate among Candida species causing vulvovaginal candidiasis. Out of the 52 isolates, C. glabrata was isolated in 28.8%, C. tropicalis in 13.4% and C. krusei in 7.6%. Other common vaginal Candida species like C. parapsilosis and C. kefyr were not detected. Among Non-albicans group, C. glabrata was the predominant isolate found. Hi – Chrom agar was a comfortable rapid method for species identification, with a sensitivity of 98% and specificity of 100% Sugar assimilation test using Rapid identification kit is another method for speciation used here. Study showed, Hi-Chrom agar was the better method of speciation, compared to sugar assimilation test using Rapid identification kit. Hi-Chrom agar showed 100% sensitivity while SAT showed only 86.3% of sensitivity. Resistance to fluconazole in C. albicans showed 3.8% and among Non-albicans group, C. glabrata and C. krusei showed 100% resistance. For Itraconazole, C. albicans showed 69.2% sensitivity and Non-albicans group 60% of sensitivity. For Amphotericin B, 100% of the total isolates remained sensitive.

Keywords: Candida albicans, diabetics and non-diabetic, pre and post-menopausal women, itraconazole.

Introduction

The vaginal mucous membrane has normal physiological mechanism to prevent invasion by pathogenic microbes. Lactobacilli, a normal flora of vagina protect the vagina from the invasion of various pathogens. Despite the above defence mechanisms, the vaginal microflora is often disturbed due to various reasons. The vaginal micro- biota also changes during the life cycle of the female. The finer details of the composition and role of the vaginal flora are still a matter of debate. Vaginal candidiasis is one of the most common vaginal infections in women, in the fertile period and also the most frequent and important fungal disease of vagina. In majority of women, diagnosis of Vulvovaginal candidiasis is made at least once during their life time (1). In vaginal areas there exist a balance between the, normal vaginal flora and immune defence mechanism. When this balance is disturbed, colonization gets replaced by infection. There are multiple virulence factors by which Candida can cause cell damage leading to direct invasion of hyphae into epithelial tissue. During vaginal candidiasis, vagina is in a PH range of (PH 4-4.5); where as in Bacterial vaginosis PH level is raised (2). Among the many cause of vaginitis, Vulvovaginal candidiasis is the second most common, after bacterial vaginosis and it is diagnosed in up to 40% of women with vaginal complaints (3).

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The prevalence of Vulvovaginal candidiasis is highest among women in reproductive age group. Studies show around 55 percent of women in age group of 25yrs had at least one episode of physician diagnosed candidiasis, 75 percent of premenopausal women had at least one life ti me episode and 45 percent of postmenopausal women had two or more lifetime infection (4). Postmenopausal women have decreased estrogen production with thinning and inactivity of vaginal epithelium together with reduction in acidity and rise in PH. An estrogen deficient vagina can result in obvious problems such as discomfort and dyspareunia, and also can lead to an abnormal vaginal flora, which may lead to a variety of infections. One of the causes of recurrent vulvovaginal candidiasis is hyperglycemia. Vulvovaginal candidiasis is more common in diabetic women (5). Increased glucose levels in genital tissue enhance adhesion and growth of the yeast cells. Candida albicansbind to vaginal epithelial cells with greater affinity in diabetic patients than in non-diabetic patients, regardless of whether patients are premenopausal, postmenopausal or pregnant(6). Candida infection in vagina cause smelly, thick, white to yellow discharge along with itching, burning and swelling (7). Since the symptoms of vaginal candidiasis are not specific to the infection, diagnosis cannot be made based on the history and physical examination alone. 50% of patients with culture positive symptomatic vaginal candidiasis will have negative microscopy (1). Candida albicans is the most common species isolated from fungal vaginal infection in diabetics as well as in non- diabetics. Recently, vaginal infection with non C. albicans species has been reported with increasing frequency in non- diabetic groups possibly due to widespread and empirical use of antifungal drugs. In addition to diabetes, other risk factors for recurrent vaginal candidiasis are genetic, pregnancy, immune disorders, behavioral factors such as sexual activity, the use of high estrogen-dose oral contraceptives, excess antibiotic usage, obesity, drug addiction and HIV (8)

Numerous studies around the world showed that candida albicans is responsible for the largest number of symptomatic episodes of vaginal candidiasis than asymptomatic. Percentage of non-albicans is high in the recent decades and varies from 85-90%. Non-albicans species are most commonly represented by C. glabarta, C. tropicalis and C. krusei (9). Species identification is important for treatment of the candida infection, as the non-albicans species of candida is increasing now days. Hence the objective of the study was to document the frequency of vulvovaginal candidiasis, speciation of the isolates of candida using CHROM agar and sugar assimilation test using rapid identification kit and to determine the susceptibility profile of the candida species to antifungal agents such as Amphotericin B. Fluconazole and Itraconazole.

MATERIALS AND METHODS

Design of the study: Single centre, cross sectional and analytical study

Study Period: The work was carried out from JAN 2014 to JAN 2015, continuously over a period of 1 year.

Place of the study: Department of Microbiology, Central laboratory of SreeBalaji Medical College and Hospital, Chromepet, Chennai – 44.

Ethical consideration: Approved by Institutional Ethics Committee, SreeBalaji Medical College and Hospital, Chromepet Chennai – 44. Bharath University.

Statistical Analysis: Statistical analyses were carried out using statistical package for social sciences and EPI-Software by statistician. The proportional data of this cross sectional study were tested using Pearson's chi square analysis test and Binomial proportion test. The clinical and laboratory data thus obtained and analysed using the statistical package of the Microsoft office

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Excel 2007 Enterprise Edition Data were analyzed by SPSS statistical software and P value of <0.05 was considered significant. Mean values are reported as mean ± standard deviation.

Study Group: Study group include 100 women of age 35-65 of which, 50 were diabetic and 50 were non- diabetic, with symptoms of vulvovaginitis

Inclusion Criteria: This study includes 150 randomly selected diabetic and non-diabetic women of age 35 - 65years attending Gynaecology (OPD) of SBMC&H. Women were categorized as premenopausal {with regular or irregular menstrualperiods} and postmenopausal {absence of menses for at least 12 months} according to their menstrual status.

Exclusion Criteria: Those who had one or combination of the following were excluded

- Age less than 35 and more than 65 years
- Patients without symptoms of vulvovaginitis
- Pregnancy
- Hysterectomy
- Recent hospitalisation or surgery (<4months)
- Use of antifungal in the last 14days
- Malignancy and serious illness causing decrease in host immune status.

Details of study and control subjects: Study Subjects: 100

- Women with type 2 diabetes who were 35 - 65years of age were selected randomly from the outpatient section of the Gynaecology Department with or without diabetic history.
- All were married and parous
- They were on treatment with oral hypoglycaemic agents or insulin or both.
- They had symptom of vaginal itching, abnormal discharge from vagina, vaginal dryness and painful sexual intercourse.
- Diabetic history, medical and surgical history were assessed using the questionnaire

The subjects were categorized depending upon their menstrual status as

- Premenopausal: 35-45yrs
- Postmenopausal: 45-65yrs

The following laboratory data were included:

- Fasting blood sugar (FBS)
- Post prandial blood sugar (PPBS)
- HbA1C: Depending upon the HbA1C values, they were again categorized as:

HbA1C ≤7: Diabetes under control
HbA1C ≥7: Diabetes not under control

Those with conditions such as hypertension, arthritis and thyroid disease that were well controlled (these condition were prevalent among the study subject) were included in the study.

Control Subjects: 50

Women without diabetes were selected randomly from the patients who attend the Gynaecology OPD of SBMCH, with symptom of vaginal itching, abnormal discharge from vagina, vaginal dryness and painful sexual intercourse. All were interviewed and then non-diabetic status was confirmed by laboratory tests-FBS and PPBS. Distribution of control subjects was similar to study subject.

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Questionnaire:
A standardized questionnaire was used to collect the background data clinical details, co morbid conditions, parity, menstrual and sexual history and the complaints related to the vaginal infections.

Specimen Collection:
During the visit, the women were put in lithotomy position for gynaecological examination. A clean bi-valve speculum was inserted deep into the vagina. After proper inspection of the vagina, two high vaginal samples (vaginal discharge) were collected using sterile cotton swabs from lateral or posterior wall of the vagina. The swab was transported immediately to the microbiology laboratory and processed.

PH of the discharge:
The PH of the vaginal discharge was assessed using standard Litmus paper test.

Specimen Processing:

- First swab was used for preparing direct smear and Gram staining, for fungal identification
- Second swab was inoculated on slant of Sabouraud's dextrose agar supplemented with Gentamycin (0.006µg/ml) and well isolated colonies were used to plate on Hi-Chrom candida differential agar (Hi-media laboratories Mumbai). The plates and slants were incubated aerobically at 37°c for 24-48 hours.

Direct Microscopical Examination:
The first swabs were used for performing Grams stain. A thin smear was prepared on a clean glass slide by rolling the swab on the slide. After air drying and heat fixing the smear, it was stained with gram stain. Gram stained smear was examined under oil immersion to look for the presence of Gram positive budding yeast cell with or without pseudohyphae (54).

Culture:

Examination of culture:

ON SDA:
After 24 hours, the SDA slants were examined for the presence of growth. White to cream-colored, pasty and smooth colonies appears. These colonies morphologically resembling the members of genus candida were subjected to gram staining and LCB mount. On microscopic examination they appeared as gram-positive budding yeast like cells. Subsequently the Germ tube test was done and the isolates were classified as Germ tube test positive and negative. The germ tube positive samples were further incubated at 45°C.

SPECIES IDENTIFICATION: The various *Candida spp,*
were identified based on following tests.

Germ Tube Test:

- A small portion of the colony was isolated suspended in a test tube containing 0.5 ml of rabbit or human plasma or serum
- The test tube was incubated at 37°C for 2 hours.
- A drop of the yeast suspension was placed on a microscope slide, overlaid with a cover slip and examined microscopically for the presence of germ tubes, which are long tube like projection from yeast cells.
- Isolates producing germ tubes were presumptively identified as C. albicans or C. dubliniensis.

Growth at 42°C:

- Germ tube positive isolates were subculture to SDA and incubated at 42°C.

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- Isolates of C. albicans grow, while C. dubliniensis do not grow at 42°C

CANDIDA SPECIES	**MACROSCOPIC FEATURES**
C.albicans and C. dubliniensis	Colonies are cream colored, pasty and smooth.
C.trpoicalis	Colonies are cream to off-white in colour, glistening to dull, soft, smooth or wrinkled with a mycelia fringe.
C. kefyr	Creamy, dull, soft and smooth colonies.
C. krusei	Flat, dull, dry and wrinkled colonies.
C. parapsilosis	Creamy colonies developing in a lacy pattern.
C.guillermondii	Thin, flat, glossy, cream to pink, smooth or wrinkled colonies.
C. glabrata	Glistening, smooth, creamy coloured colonies.

CANDIDA SPECIES	**MICROSCOPIC FEATURE**
C. albicans, C.parapsilosis	Ovoid, elliptical or round blastoconidia seen.
C. guillermondii ,C. tropicalis	Ovoid or elliptical blastoconidia seen.
C. kefyr ,C. krusei	Elongated, slender oval blastoconidia seen.

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Candida HI- CHROM agar:

After 24 hours, growth on CHROM agar plates from isolated colony on SDA was examined. The colour of the colonies produced on HI CHROM agar was interpreted and species identified as per manufacturers chart (55). Hi- Chrom agar takes only 48 hours for species identification and it is a comfortable alternative to conventional methods that take 96-120 hours. Hi-Chrom agar has several advantages like rapidity, direct identification of species, thus making it very useful in early identification and thereby early initiation of appropriate antifungal treatment. Hi-Chrom agar helps in reducing the time for diagnosis, thereby reducing the duration of hospital stay and cost of treatment.

Candida species	**Colour on HI-Chrom agar**
C. albicans	Light green colour colonies.
C. dubliniensis	Dark green colonies.
C. tropicalis	Steel blue colonies with a pink halo.
C. glabrata	Pink to Purple colonies
C. parapsilosis	Cream colored colonies.
C. guilliermondii	Cream to Pale pink or purple colonies
C. krusei	Pale pink, dry rough colonies with spreading, pale edges.

SUGAR ASSIMILATION TEST:

Sugar assimilation test was done using Rapid Identification Kit KB006 from Hi-media Mumbai. KB006 Kit is a standardized colorimetric identification system utilizing twelve conventional biochemical tests. The tests are based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes which are indicated by a spontaneous colour change in the media (56).

Preparation of Inoculum: Inoculum is prepared from picking 2-3 well isolated colonies from 24hours culture SDA slants and make a homogenous suspension in 2-3ml sterile saline

- Open the kit aseptically, Inoculate each well with 50 µl of the above Inoculum by surface inoculation method. Alternatively, the kit can also be inoculated by stabbing each individual well with a loopful of Inoculum
- Incubate at 22-25ºc for 24-48 hours.
- Interpret results as per the standards given in the identification index.

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Well No	Test	Principle	Original colour of the medium	Positive Reaction	Negative Reaction
1	Urease	Detects urease enzyme	Orangish yellow	Pink	Orangish yellow
2	Melibiose	Melibiose utilization	Pinkish Red/Red	Yellow	Red/Pink
3	Lactose	Lactose utilization	Pinkish Red/Red	Yellow	Red/Pink
4	Maltose	Maltose utilization	Pinkish Red/Red	Yellow	Red/Pink
5	Sucrose	Sucrose utilization	Pinkish Red/Red	Yellow	Red/Pink
6	Galactose	Galactose utilization	Pinkish Red/Red	Yellow	Red/Pink
7	Cellobiose	Cellobiose utilization	Pinkish Red/Red	Yellow	Red/Pink
8	Inositol	Inositol utilization	Pinkish Red/Red	Yellow	Red/Pink
9	Xylose	Xylose utilization	Pinkish Red/Red	Yellow	Red/Pink
10	Dulcitol	Dulcitol utilization	Pinkish Red/Red	Yellow	Red/Pink

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11	Raffinose	Raffinose utilization	Pinkish Red/Red	Yellow	Red/Pink
12	Trehalose	Trehalose utilization	Pinkish Red/Red	Yellow	Red/Pink

Species	Urease	Melibiose	Lactose	Maltose	Sucrose	Galactose	Cellobiose	Inositol	Xylose	Dulcitol	Raffinose	Trehalose
Candida albicans	-	-	-	+	+	+	-	-	+	-	-	+
Candida glabrata	-	-	-	+	-	-	-	-	-	-	-	+
Candida tropicali	-	-	-	+	+	+ *	+	-	+	-	-	+ *
Candida krusei	+ *	-	-	-	-	-	-	-	-	-	-	-

* Strain variation

Antifungal Susceptibility Test:

Antifungal Susceptibility Testing for Candida isolates was done by,

- Disc diffusion method, as per CLSI Guidelines on Antifungal Susceptibility testing in M-44A document.

Medium: Mueller Hilton Agar with 2% glucose and 0.5 µg methylene blue dye adjusted to a pH of 7.2 -7.4was used.

Inoculum preparation:

Organisms were sub cultured and incubated at 35°C to ensure purity and viability.

Inoculum was prepared by direct colony suspension method. Five colonies of approximately 1 mm diameter from a 24 -hour old culture was picked and suspended in 5 ml of sterile normal saline.

Suspension was vortexed and adjusted spectrophotometrically at 530nm to 0.5 McFarland's standard containing, 1x 106 to 5 x 10 6 cells/ ml.

Inoculation of test plate:

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- Sterile cotton swab stick was dipped into the suspension, rotated several times and pressed firmly against the inside wall of the tube to remove excess fluid.
- It was streaked evenly over the entire agar surface 3 times, rotating each time at an angle of 60°C, to ensure an even distribution of inoculum.
- Application of disks to inoculated plates:
- Antimicrobial discs were dispensed onto the surface of an inoculated agar plate by means of a sterile forceps and pressed down.
- The discs were evenly distributed on the plate with a distance of 2.5 cm from centre to centre of the discs.
- Plates were inverted and incubated at 37°C within 15 minutes.
- Reading and Interpretation:
- Results were read at 20 -24 hours, when semi- confluent growth had formed. In case of insufficient growth, it was read at 48 hours.
- Zone of inhibition was measured at the point where there is prominent reduction in growth.
- QC strains used to ensure quality control are:
- Candida albicans ATCC 90028
- Candida tropicalis ATCC 750.

Zone diameter, (nearest whole in mm)

Antifungal agent Disc Content	**Resistant Zone Diameter**	**Susceptible– Dose Dependent**	**Sensitive**
Fluconazole,25µg	<14mm	15-18mm	>19mm
Itraconazole,10µg	<11mm	12-19mm	>20mm
AmphotericinB,100U	<10mm	Not applicable	>10mm

The interpretive criteria for the Fluconazole disk test were those published in the CLSI guidelines M44. Break points for Itraconazole (10µg) and Amphotericin B (100U) are not available. The response was interpreted according to the study by Negri et al (57). A quality control was performed in all batches in accordance with the CLSI document M27- A2 by using QC strains (58).

RESULTS

This cross-sectional study was carried out during the period between JANUARY 2014 to JANUARY 2015 in SreeBalaji Medical College and Hospital, Chromepet, Chennai. Speciation of fungal isolates using vaginal swabs taken from 150 vulvovaginitis patients was done and their antifungal susceptibility testing was done by disk diffusion method.

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TABLE: 1Mean age of Study Subject

Mean age	Study Group		Control Group	
	Diabetic women with symptoms of vulvovaginitis (n = 100)		Non-diabetic with symptoms of vulvovaginitis (n = 50)	
	Pre-menopausal	Post-menopausal	Pre-menopausal	Post-menopausal
	37	55	35	53

Out of the above 150 cases, 100 diabetic patients were mentioned as a Study group and 50 Non-diabetic patients were mentioned as a Control group. Approximate age group of Pre-menopausal women was between 35 -45years and a Post-menopausal woman was between 45-65years. Among 100 diabetic patients, the mean age group of pre- menopausal women were 37 and post-menopausal women were 55 years. In case of the 50 Non- diabetic patients, mean age group of pre –menopausal women were 35 and post-menopausal were 53 years.

TABLE: 2 Age Distribution of Study Subject Based on menstrual status

Menstrual status	Study Group n =100	Control group n = 50	Total no of patients
Pre-menopausal (35-45yrs)	40	20	60
Post-menopausal (45-65yrs)	60	30	90
	100	50	150

Out of 100 diabetic patients, 40 were in pre- menopausal age group and 60 were in post-menopausal age group. In case of 50 non –diabetic patients, 20 were in pre- menopausal age group and 30 were in post-menopausal age group.

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TABLE: 3Prevalence of *Candida* species versus age

Menstrual status with age	Study group	Control group	Prevalence of vaginal infection in percentage
	Diabetic with *Candida* infection n = 42(42%)	Non-diabetic with *Candida* infection n = 10(20%)	
Pre-menopausal (35-45yrs)	15	4	19(36.5%)
Post-menopausal (45-65yrs)	27	6	33(63.5%)

In our study, among the study and control group of 150 patients, 52 patients were positive for vaginal candidiasis. 19 out of 52 , with percentage of 36.5% were in pre-menopausal age group and 33 patients with percentage of 63.5% were in post-menopausal age group

TABLE: 4Gram staining findings in direct smear

Microscopic Examination	Diabetic (Study group) n = 100	Non- Diabetic (Control group) n = 50
Positive	42(42%)	10(20%)
Negative	58(58%)	40(80%)

High vaginal swab collected from all symptomatic patients of both diabetic and non-diabetic group, processed for direct microscopic examination and gram staining for fungus identification. Among study group 42 (42%) patient's showed positive microscopic finding for Candida species while 58 (58%) were negative. In case of non diabetic group, 10 (20%) patients showed positive finding for Candida species while 40 (80%) were negative. The positive sample for Candida species were further processed for fungal culture on Sabouraud's dextrose agar.

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TABLE: 5Diabetic status of study subjects

Parameters	**Diabetic patient (study group) n = 100**		**Non-Diabetic patient (control group) n = 50**	
Menstrual status	**Pre-menopausal n = 38**	**Post-menopausal n = 62**	**Pre-menopausal n = 27**	**Post-menopausal n = 23**
Symptom Yes	38	62	27	23
Duration	7yrs	12yrs	-	-
HbA1C	12	13	5.9	6.2
FBS	220	260	98	100
Type of diabetes Type 1	8	12	-	-
Type 2	30	50	-	-
Treatment	Insulin	Insulin	-	-

In our study, 100 Diabetic patients are mentioned as study group and 50 non-diabetic patients are mentioned as control group. Out of 100 diabetic patients, 38 are in the age group of pre-menopausal (35-45yrs) and 62 are in the age group of post-menopausal (45-65yrs). In Non-diabetic patients, 27 are in pre-menopausal age group and 23 are in post-menopausal age group. In our study, patients who came with symptoms of vulvovaginitis were included in both study and control group. The proforma showed the diabetic status of the study subject as follows:
The **mean duration of diabetic status** in the pre and post-menopausal age group patients is 7 - 12years The**mean HbA1C** values for the pre and post menopausal diabetic patients are 12 & 13, while in case of non-diabetic patients mean HbA1C values for pre and post menopausal patients are 5.9 & 6.2. The Mean Fasting Blood Sugar (FBS) level for the study group of the pre and post-menopausal patients are 220 & 260mg/dl. In control group, the mean fasting blood sugar level of pre and post-menopausal patients are 98 & 100mg/dl. In our study group, 8 pre-menopausal patients belong to Type 1 Diabetes mellitus and 30 are of Type 11 Diabetes mellitus. In case of post- menopausal patients, 12 belong to Type 1 diabetes mellitus and 50 are of Type 11 Diabetes mellitus. All the diabetic patients mentioned above in our study are under treatment with insulin.

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TABLE: 6Identification of *Candida albicans*from Germ tube test

Germ tube test	**Study group with candida infection (n = 42) 42%**		**Control group with Candida infection (n = 10) 20%**	
Menstrual Status	**Pre-menopausal n = 15**	**Post-menopausal n = 27**	**Pre-menopausal n = 4**	**Post – menopausal n = 6**
Positive	8(53.3%)	13(48.1%)	2(50%)	3(50%)
Negative	7(46.6%)	14(51.8)	2(50%)	3(50%)

Among the 42 diabetic candidiasis patients, 8 out of 15 pre-menopausal patients were positive for germ tube test and 7 were negative, and 13 out of 27 post-menopausal patients were positive for germ tube test and 14 were negative. In case of non diabetic candidiasis patients, 2 out of 4 pre-menopausal patients were positive for germ tube test and 2 were negative, and 3 out of 6 post-menopausal patients were positive for germ tube test and 3 were negative.

TABLE: 7Distribution of *Candida species* in Hi- chrome agar

***Candida Species* in chrome agar**	**Diabetic with *Candidiasis* infection (n = 42) 42%**				**Non- Diabetic with *Candidiasis* infection (n = 10) 20%**				**Number of different *Candida* species isolated**
Menstrual Status	**Pre-menopausal (n = 15)**		**Post-menopausal (n = 27)**		**Pre – menopausal (n = 4)**		**Post-menopausal (n = 6)**		
	n	**%**	**N**	**%**	**N**	**%**	**n**	**%**	

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C.albicans	8	53.5	13	48	2	50	3	50.2	**26(50%)**
C.glabrata	5	33.3	**7**	25.5	2	50	1	16	**15(29%)**
C.tropicalis	1	6.6	5	18.5	0	0	1	16.6	**7(13.4%)**
C.krusei	1	6.6	2	7	0	0	1	16.6	**4(7.6%)**

Out of 42 diabetic candidiasis patients and 10 non diabetic candidiasis patients, 26 belonged to C. albicans, 16 isolates were C.glabrata, 7 isolates were C.tropicalis and 4 isolates were C.krusei. Candida albicans was the predominant species in both the diabetic and non diabetic group. Among Non-albicans, Candida glabrata was the predominant isolate found.

TABLE: 8
Comparison of methods in identification of *Candida Species*

Total no of *Candida isolated* n = 52	**METHOD USED**			
	Hi -Chrom Agar		**Sugar Assimilation Test**	
Species	**N**	**%**	**n**	**%**
C.albicans	26	50	25	48
C.glabrata	15	29	10	19.2
C.tropicalis	7	13.4	7	13.4
C.krusei	4	7.6	3	5.7
No of species isolated, out of 52 positive sample	52	**100%**	45	**86.3%**

Hi-Chrom agar was the best method of Candida speciation as it identified all Candida Species from the 52 isolated samples was 100%. The ability of sugar assimilation test using Rapid identification kit (KB006) to identify the different Candida species from the 52 isolated samples was only 86.3%, rest were unidentifiable by this method. This shows Hi chrome agar is more sensitive method for Candida speciation.

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TABLE: 9
Antifungal susceptibility to Fluconazole

Species & no of isolates (n)	Susceptible n (%) <8µg/ml		Susceptible Dose Dependent n (%), 16-32µg/ml		Resistance n (%), >64µg/ml	
	n	%	N	%	N	%
C.albicans(26)	20	76.9	5	19.2	1	3.8
C.glabrata(15)	-	-	-	-	15	100
C.tropicalis(7)	4	57	2	28.5	1	14
C.krusei(4)	-	-	-	-	4	100

By Disk Diffusion Method, 24(46%) Candida isolates were sensitive and 21(40.3 %) isolates were resistance to Fluconazole.

TABLE: 10
Antifungal Susceptibility to Itraconazole

Species & no of isolates (n)	Susceptible n (%) <0.125µg/ml		Susceptible Dose Dependent n (%), 0.25-0.5µg/ml		Resistance n (%), >1µg/ml	
	n	%	n	%	N	%
C.albicans(26)	18	69.2	2	7.6	6	23
C.glabrata(15)	10	67	2	13	3	20
C.tropicalis(7)	5	71.4	0	-	2	28.5
C.krusei(4)	3	75	0	-	1	25

By Disk Diffusion Method, 36(69%) Candida isolates were sensitive and 12(23%) isolates were resistance to Itraconazole.

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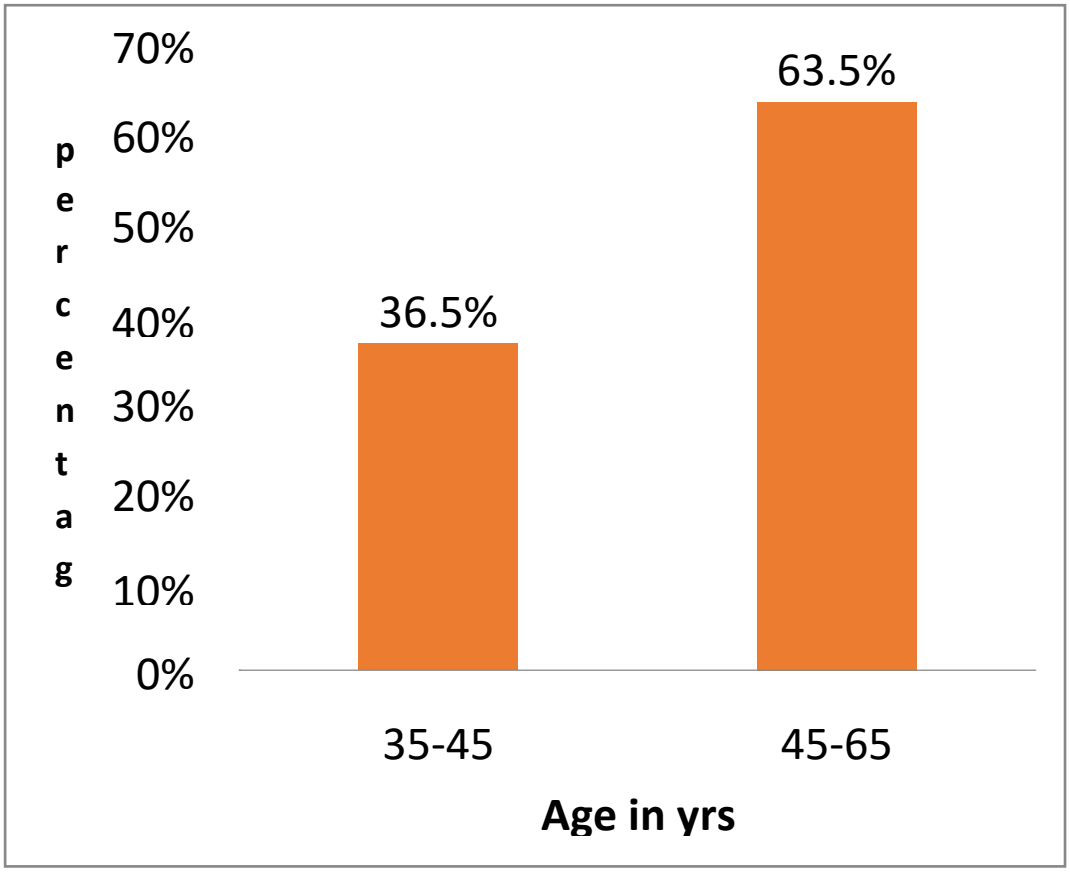
TABLE: 11
Antifungal Susceptibility to Amphotericin B

Species & no of isolates (n)	**Susceptible n (%) <1µg/ml**		**Resistance n (%), >1µg/ml**	
	n	**%**	**n**	**%**
C.albicans(26)	25	96.1	1	3.8
C.glabrata(15)	15	100	-	-
C.tropicalis(7)	6	85.7	1	14.2
C.krusei(4)	3	75	1	33.3

By Disk Diffusion Method, 94% of the Candida isolates were highly sensitive to amphotericin B.

GRAPH: 1 **Prevalence of vaginal candidiasis with respect to menstrual status and age**



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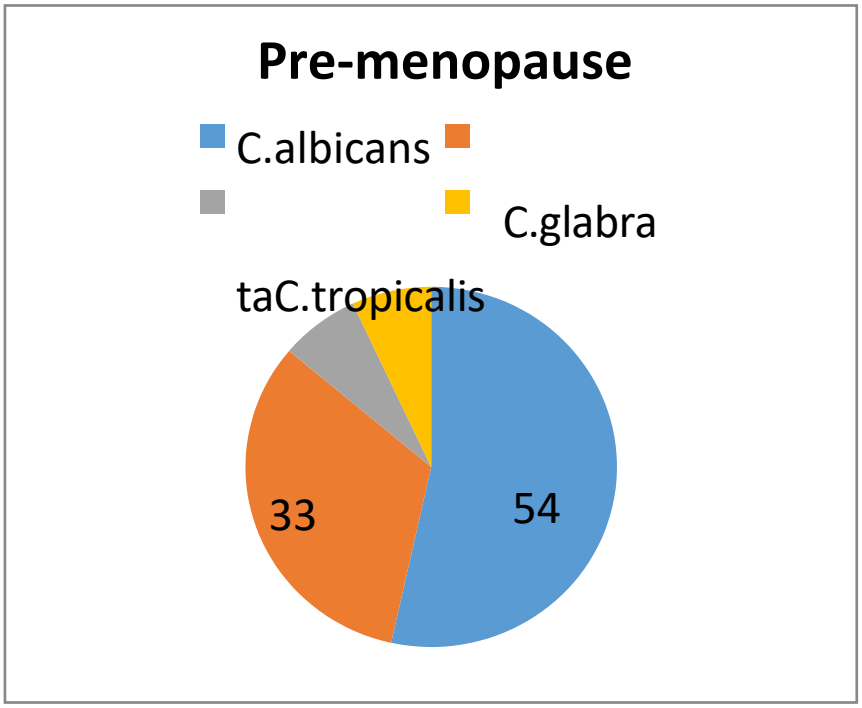
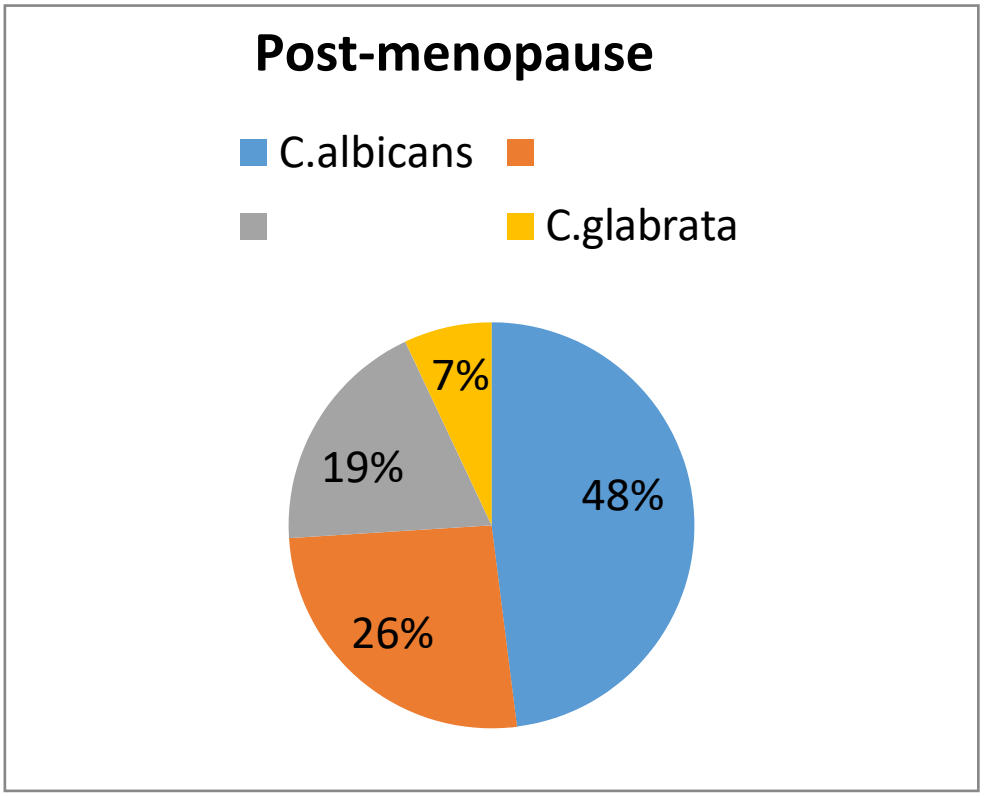
PIE CHART: 1 Distribution of *Candida species* in Diabetic group (Hi-Chrom agar)



Pie chart: 1 shows, distribution of candida species as follows in pre-menopausal diabetic subjects: C.albicans 53.5 (54%), C.glabrata 33%, C.tropicalis 6.6(7%) and C.krusei 6.6 (7%).

PIE CHART: 2 Distribution of *Candida species* in Diabetic group (Hi-Chrom agar)



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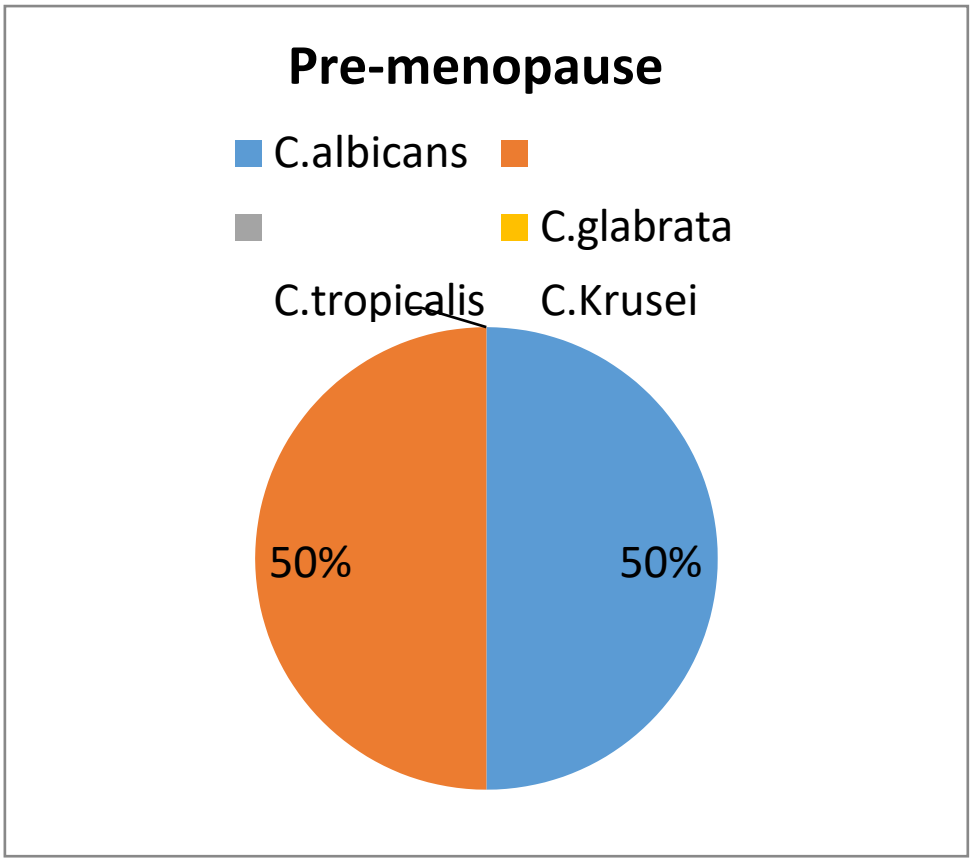
Pie chart: 2 shows, distribution of candida species as follows in post-menopausal diabetic subjects:
C.albicans 48 %, C.glabrata 25.5 26%), C.tropicalis 18.5(19%) and C.krusei 7%.

PIE CHART: 3 Distribution of *Candida species* in Non Diabetic group



Pie chart: 3 shows, distribution of candida species as follows in pre-menopausal Non- diabetic subjects:
C.albicans 50%, C.glabrata 50 %, C.tropicalis 0% and C.krusei 0%.

PIE CHART:4 Distribution of *Candida species* in Non-diabetic group (Hi-Chrom agar)

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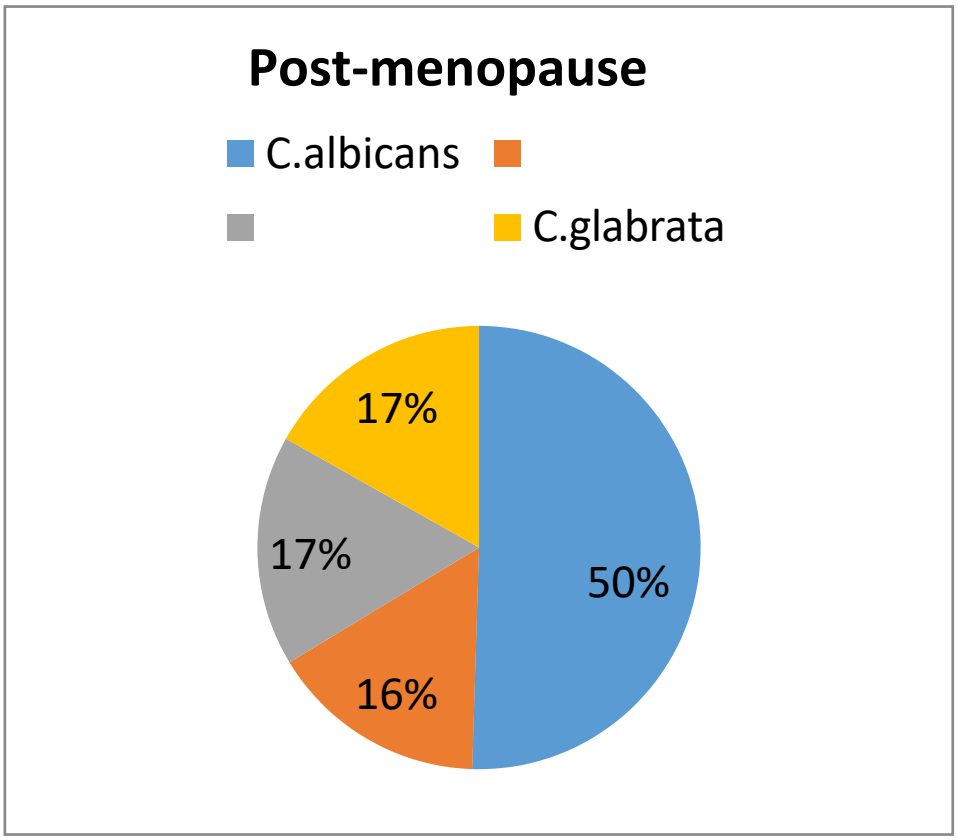
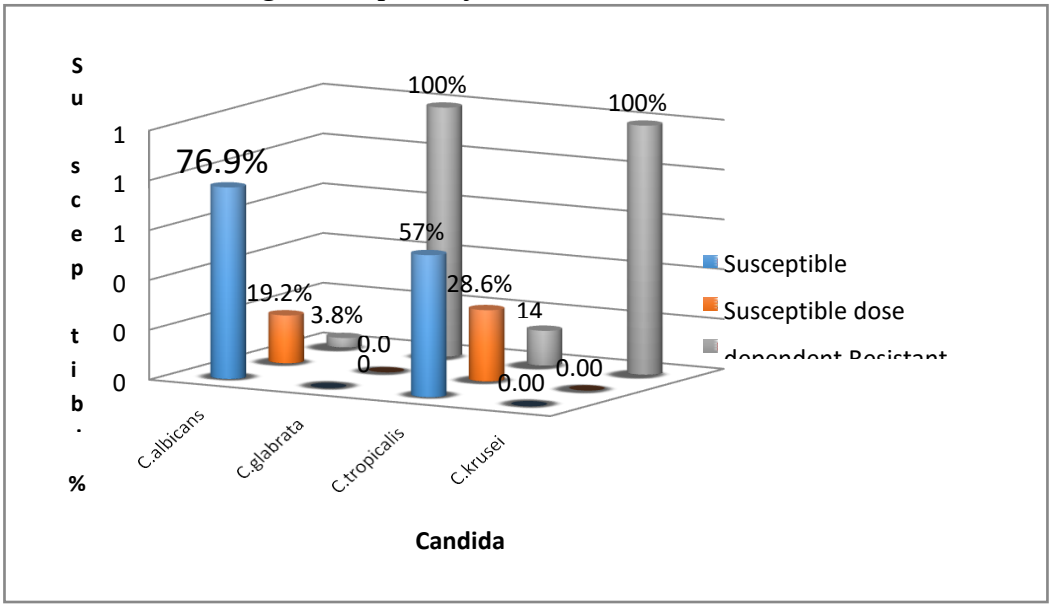


Pie chart: 4 shows, distribution of candida species as follows in post-menopausal Non- diabetic subjects:
C.albicans 50.2 (50%), C.glabrata 16 %, C.tropicalis 16.6(17%) and C.krusei 16.6 (17%).

GRAPH: 2 Antifungal susceptibility to Fluconazole



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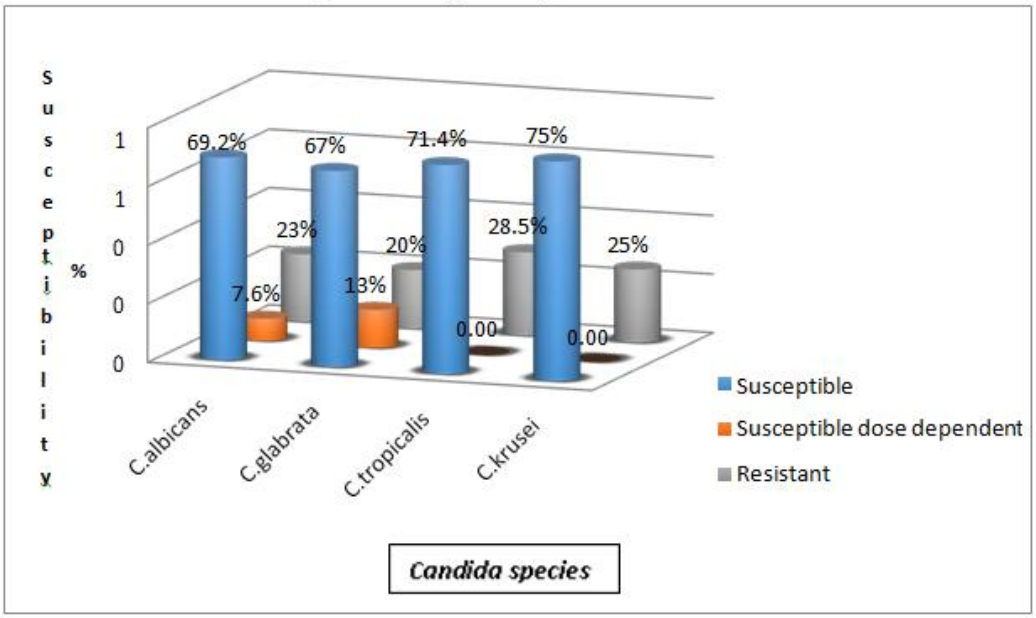
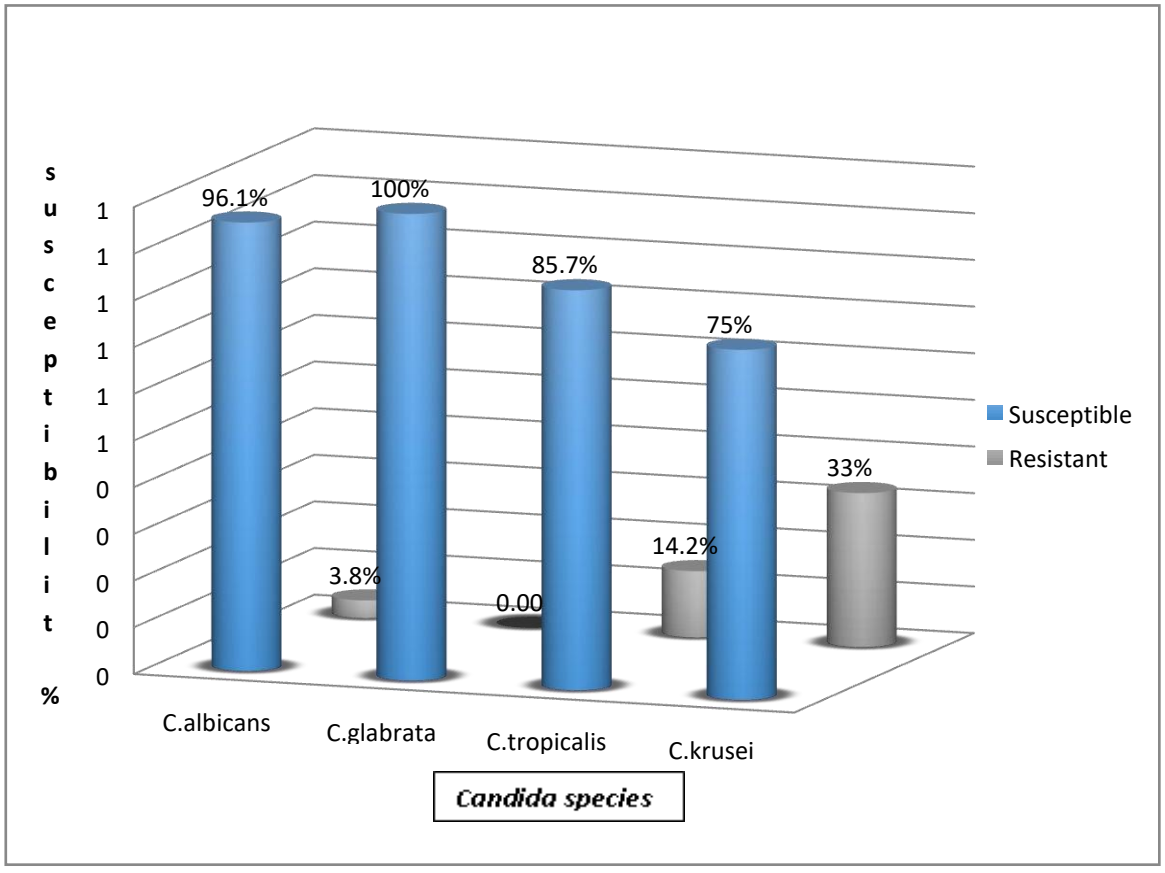
GRAPH: 3 Antifungal susceptibility to *Itraconazole*



GRAPH: 4 Antifungal Susceptibility to Amphotericin B

Figure: 1



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Candida species in SDA Slant and SDA Plate

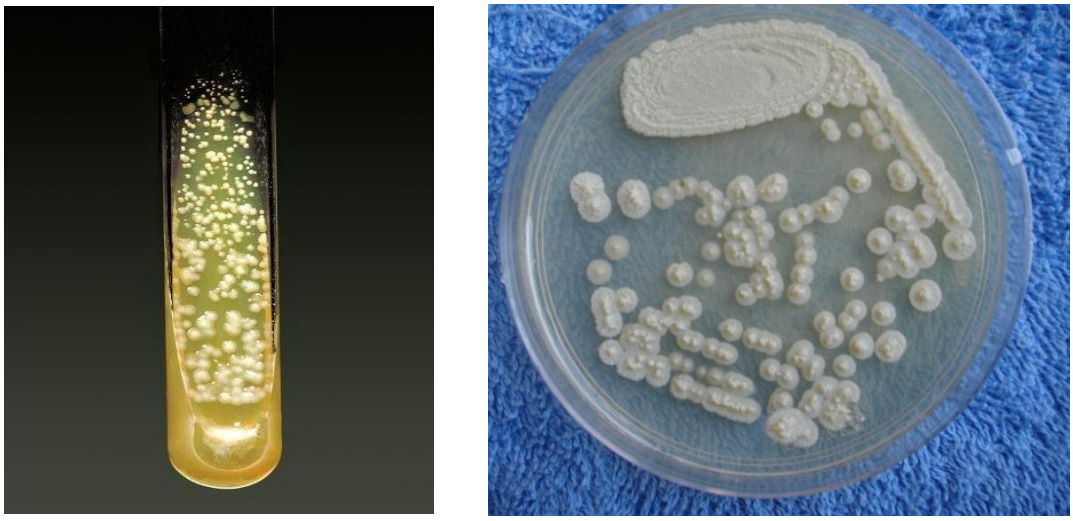

Figure: 2
***Candida Species* in Gram Staining**

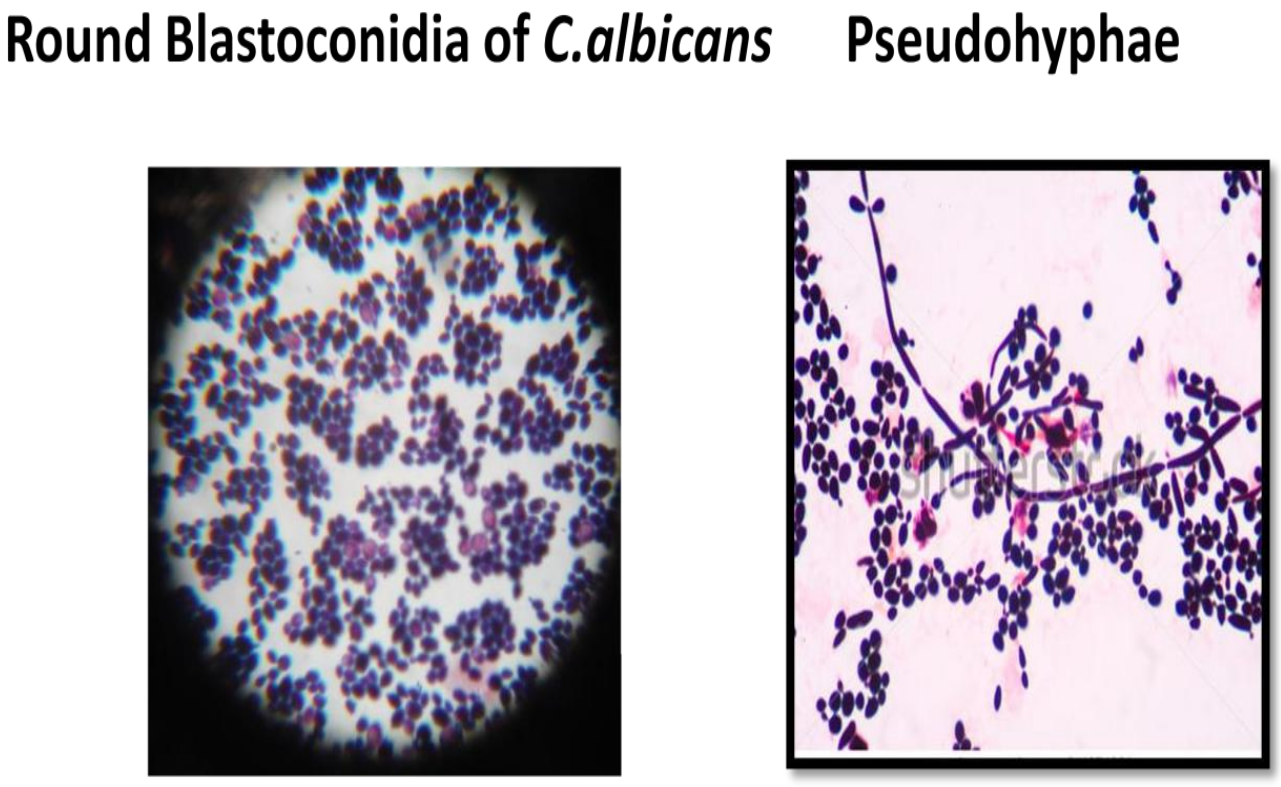

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Figure: 3
Germ tube formation of *C.albicans*

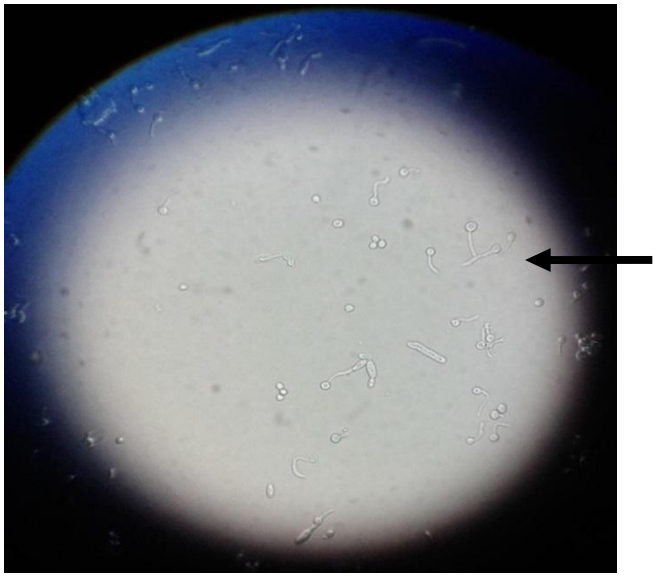

Figure: 4
Candida species in Hi Chrome Agar

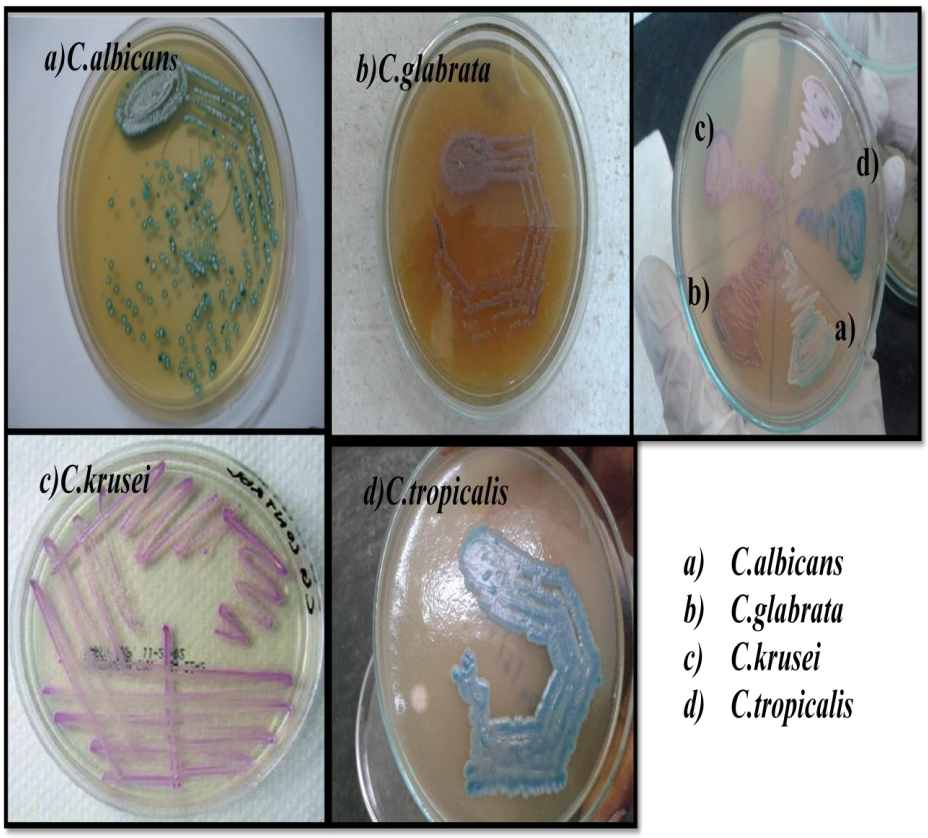

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Figure: 5
Sugar Assimilation Test using Rapid Identification Kit

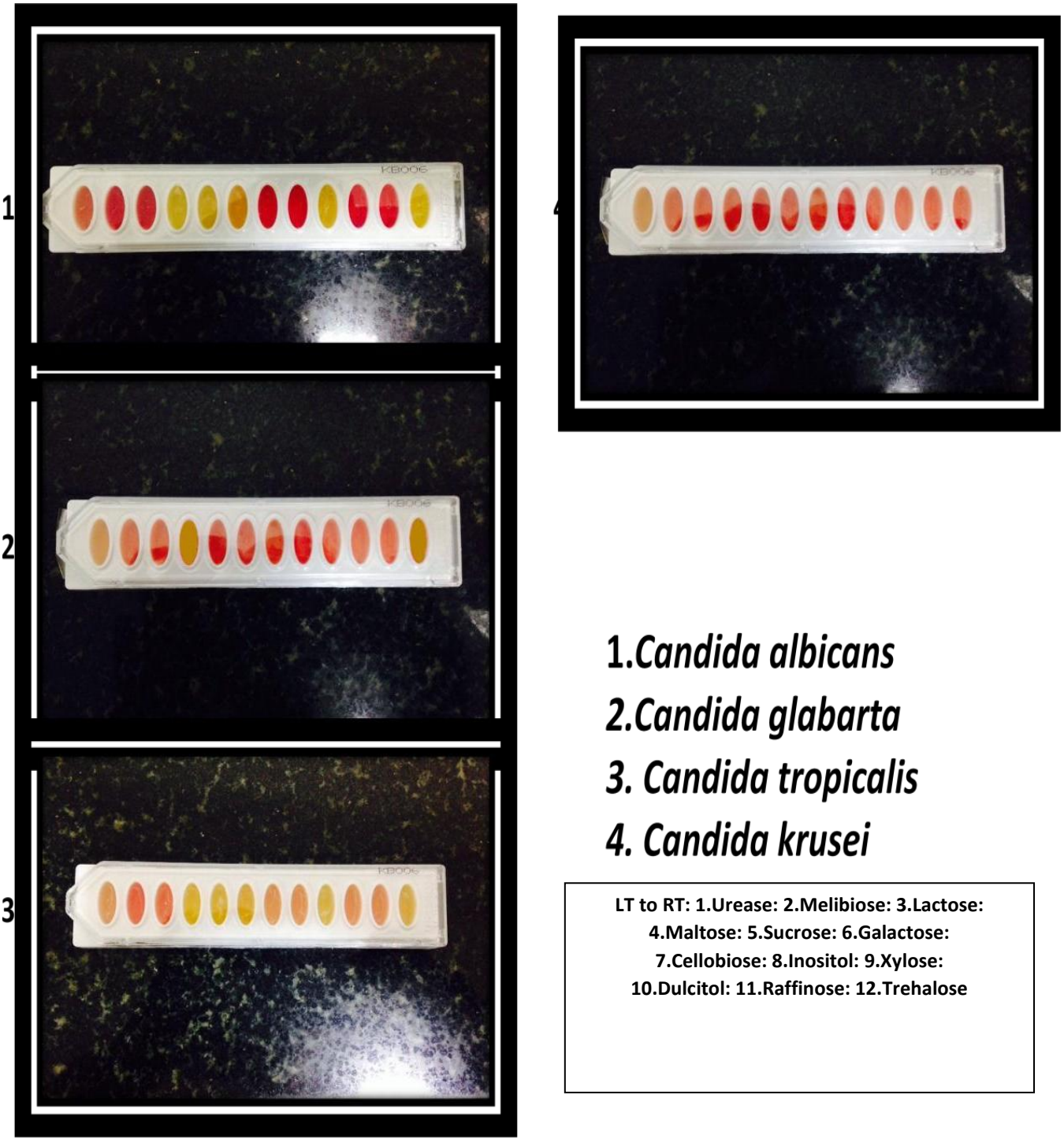

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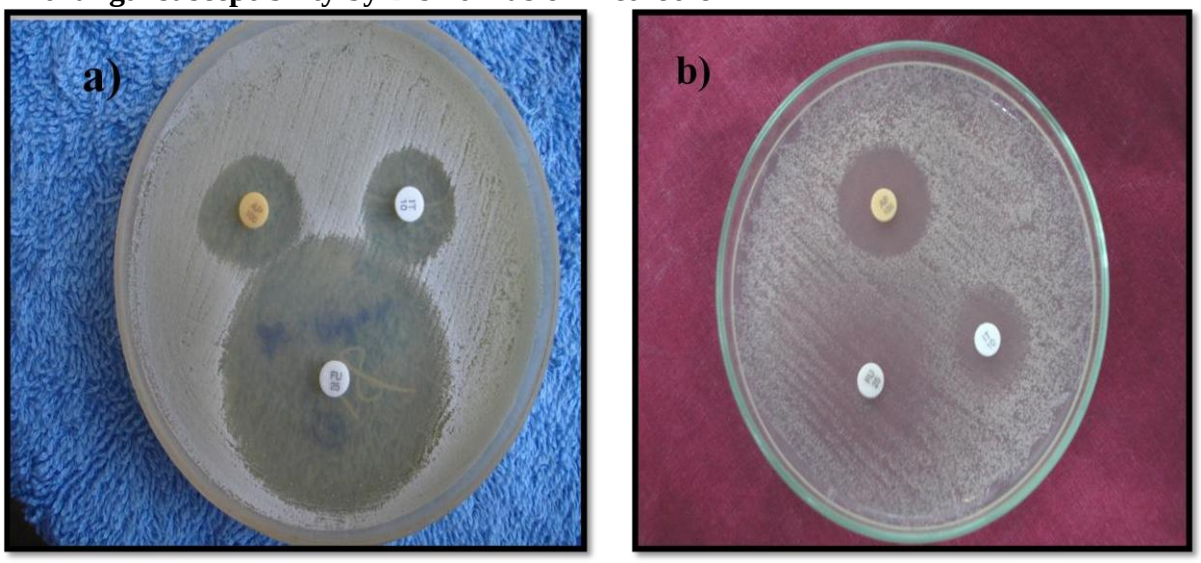
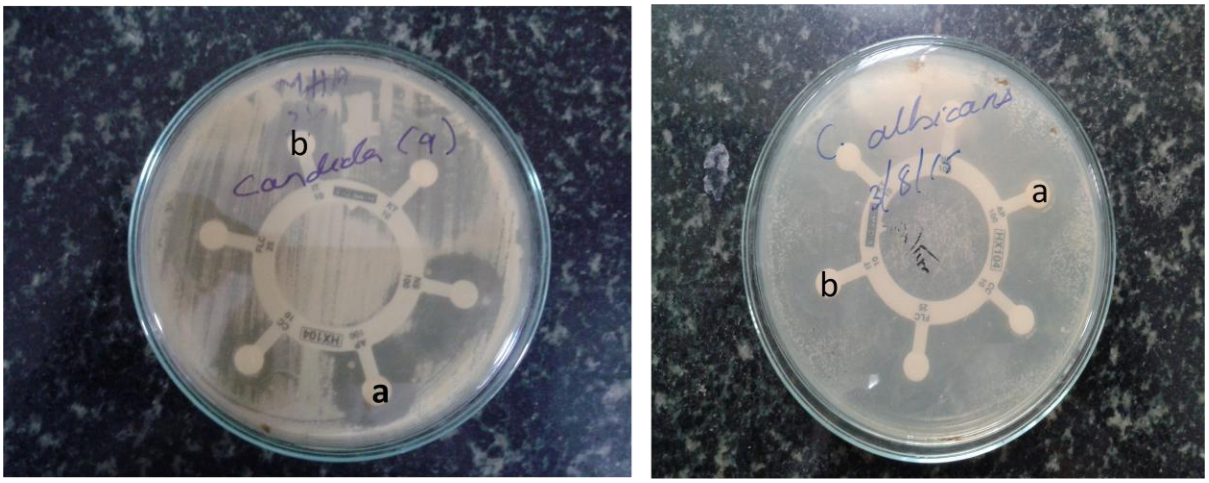
Figure: 6
Antifungal susceptibility by Disk diffusion method on MHA



a) **Fluconazole sensitive strain**
b) **Fluconazole resistance strain**



a) **Amphotericin B sensitivity**
b) **Itraconazole sensitivity**

DISCUSSION

Vaginitis is a common condition for which women of all age groups attend the gynaec OPD every day. Vulvovaginal candidiasis is a cause of significant morbidity in women population in the reproductive age group. Clinical importance of species level identification is important as they differ in expression of virulence factors and antifungal susceptibility. Current study was

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undertaken to speciate the Candida isolates from patient attending Gynaecology OPD of SreeBalaji Medical College and Hospital and to find their antifungal susceptibility pattern. The study also concentrated on the changes observed in the species distribution, the shift towards Non-albicansspecies in our hospital. In this study out of 150 patients with symptoms of vulvovaginitis, 100 were diabetic and mentioned as study group and 50 were non-diabetic and mentioned as control group. Vaginal candidiasis is an extremely common infection in 60-70% women during their reproductive age at least once in their lives (59). In our study, we included the patient with age group between 35 -65years. Approximate age group of pre-menopausal women was between 35 -45 yrs and post-menopausal between 45-65yrs. The mean age group of pre-menopausal diabetic group were 37yrs and non-diabetic group were 35yrs, as well as the mean age group of post-menopausal diabetic group were 55yrs and non-diabetic group were 53yrs (Table: 1). Among study. Group 40 out of 100 were in pre-menopausal age group and 60 were in post-menopausal age group, in case of control group 20 out of 50 were in pre-menopausal age group and 30 were in post-menopausal age group (Table: 2). The present study shows the higher prevalence of vaginal candidiasis in post-menopausal age group contributing to 63.5%(>45yrs) compared to pre-menopausal age group which was only 36.5%(35 -45yrs) (Table: 3/ Graph:1), this was similar to study done by Alo et al reported that prevalence of vaginal candidiasis was more in higher age group (>45yrs), compared to those between younger age group (<35yrs)(60). This outcome agreed with Akroth et al and Willacy and Jackson et al who reported peak vaginal infection between higher age group 45 -70yrs (61-62). But study done by Deepababin et al does not correlate with our study, were they showed highest frequency of vaginal candidiasis in age group of (26 -35yrs), followed by age group of (18-25yrs), low frequency between the age group of (>40yrs). The reason for the disparity can be , the age group taken in this study(35-65yrs), does not consist of women of reproductive age group (18-35yrs) (63).According to age distribution, one interesting trend was noticed upon comparing the distribution of Candida spp for the different age groups. Prevalence of Non-albicansspp were noted more in women of post-menopausal age group compared to pre-menopausal, and it correlates well with the study done by John-paulvermitsky et al, were the study shows > 18% of Non-albicans isolated in women of menopausal age and older (>51yrs), compared to <4.8% for young adults between <30yrs and <11.8% for middle aged women between 30-50yrs, the possible explanation includes changes in patients physiology, hormone balance and decrease in immune function as age advances (64).

Out of 100 diabetic patients, 42 (42%) were infected with vaginal candidiasis, among 50 non-diabetic patient 10 (20%) were infected with vaginal candidiasis, identified b y Direct Gram staining (Table: 4/ Fig: 1 & 2). There seems to be a significant link between hyperglycemia and vaginal candidiasis. The possible etiology of recurrent infection is the poor management of glucose level in diabetic women that impair several aspect of humoral host defence, such as decreased random motion of neutrophils, chemotaxis, phagocytosis and microbial killing (69). Diabetes is a proven pre-disposing factor for vulvovaginal infection especially vaginal candidiasis both in pre and post menopausal age group (65). In our study, the mean duration of diabetes mellitus was 12 ± 6yrs and their mean fasting blood sugar level was 191 ± 67mg/dl. Hyperglycemia limits Neutrophils function among persons with uncontrolled diabetes, including neutrophils ability to phagocytose and kill Candida. With the oxidative killing ability of neutrophils hindered, diabetics may not be able to clear pathogens. Hyperglycemic individual may also have increased risk for Candida colonization because their secretion contains glucose, which can serve as nutrients for Candida organisms. Fucose (6-deoxy Galactose) is a vaginal

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epithelial cell receptor that aids in adhesion of Candida to vaginal epithelial cells. Fucose contains isomer of glucose and acts as one form of receptor site for Candida adhesion in diabetes(66). The mean glycosylated hemoglobin level in our study population was 7/3 ± 2.58%, which correlates with study done by Ella M De Leon et al, has got similar HBA1c level (67). Women with abnormal HbA1C level were almost three times more likely to be colonized with Candida than those with normal HbA 1C level (68) (Table: 5)

In this study, total prevalence of vaginal candidiasis in both pre and post-menopausal age groups was 34.6%(52 out of 150 were positive for vaginal candidiasis) which was compared to similar studies by Saporiti et al (33.1 %) and Omar A A et al (39%) (70, 71), but the prevalence was higher than the studies by Jindal et al (23.4%), Odds Fc et al (15 - 20%) (72), Momani et al (18 -25%) (73). In this study the most common species isolated were C.albicans (50%), and confirmed by germ tube test (Table: 6) (Figure 3), followed by C.glabrata (29%), C.tropicalis (13.4%) and C.krusei (10%) which correlates with the study done by Gultekin et al in which C.albicans (53%), C.glabrata (34.5), C.tropicalis (15.2%) &C.krusei (8.3%) (74). Hi-Chrom agar was used for Candida speciation in our study. The methods of identification of Candida by corn meal agar and sugar assimilation test are time consuming; On CMA it takes around (24-72h) and in case of sugar assimilation test it may take around (72h-2wks). Besides, these procedures are labour intensive and take longer time to determine the diagnosis. Several chromogenic substrate containing culture media have been developed. The advantages are, it should support the growth of yeast but not of bacteria. It should facilitate the recognition of specimen containing mixture of yeast species and exposure of the fungi to the differential indicator substances should not affect their viabilities for subsequent subculture. CHROM agar is a chromogenic substrate containing culture medium which fulfils this entire requirement. CHROM agar candida is a differential medium being widely used to differentiate candida species. These chromogenic media yield colonies of different colours secondary to chromogenic substance that react with enzymes secreted by the organisms (75). A major advantage of CHROM agar is the ability to detect mixed cultures of yeast in clinical specimens (76) (Figure: 4).

C.albicans was identified as light greencolour colonies in CHROM agar, as compared to other studies like Baradkar et al(77). Differentiation between light green and a dark green colony posed a problem and was found to be subjective. Other tests like germ tube test, growth at 45°c and chlamydospores formation on CMA and SAT (sugar assimilation test) need to be done only in case of ambiguity. In this study C.glabrata, produced cream to white colonies as mentioned in Hi-media, but it needs to be highlighted that C.parapsilosis too were of the same colour. The strains of C.parapsilosis can be easily differentiated from C.glabrata which does not produce pseudohyphae on CMA. In this study C.tropicalis, produced purple, fuzzy and steel blue colour colony as mentioned in Hi-media. Study done by Yucesoy et al, revealed that all C.krusei isolates produced rough, fuzzy spreading big pink coloured colonies on CHROM agar. In our study shows the morphology of the colonies of C.krusei to be large, fuzzy, rough, and pink. Rapid identification of C.krusei with chromogenic media is important because it exhibit innate resistance to fluconazole.

The species of Candida isolated in our study, by using CHROM agar was C.albicans with 50% (26 cases), C.glabrata with 28.8% (15 cases), C.tropicalis with 13.4% (7 cases) and C.krusei with 7.6% (4 cases), which correlates with study done by Faraji et al, which reported that C.albicans with 62/5% (20 cases), C.glabrata with 18/7% (6 cases), C.tropicalis with 9/4% (3 cases) and C.krusei with 9/4% (3 cases) (78) (Table:7/ Pie chart 1-4) Though C.parapsilosis (2.7%) and c. kefyr(2.7%) has been obtained in the study done by Sasikala et al, which was

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similar to study done by Mohanty et al, were C.parapsilosis (2.6%) and C.kefyr (3.6%), in our study these species was not detected (0%) (85, 86). In this study, sugar assimilation test was done using Rapid identification kit from Hi-media Mumbai [KB006 Hi Candida TM Identification Kit] .KB006 is a standardized test system that can be used for identification and differentiation of Candida species. Each KB006 Kit is a standardized colorimetric identification system utilizing twelve conventional biochemical tests. The test is based on the principle of PH changes which are indicated by a spontaneous colour change in the media (Fig 4). Candida species isolated by using SAT Rapid identification was C.albicans with 48% (25 cases), C.glabrata with 19.2% (10 cases), and C.tropicalis with 13.4% (7 cases) and C.krusei with 5.7% (3 cases) with a total sensitivity of 86.3% which was similar to study done by AnjanaGopi et al (79) (Table: 8/ Fig: 5).

In this study we observed that, Hi- Chrom agar was the best method of Candida speciation as it identified all Candida species with 98% sensitivity and 100% specificity, which was similar to studies done by Yucesoy et al which showed 97% sensitivity and 100% specificity and Willinger et al showed 98.8% sensitivity and 100% specificity for all Candida species, as compared to sugar assimilation test using Rapid identification kit (Table: 8) (80, 81).

In this study, the most isolated Candida species from the symptomatic patients was C.albicans, which accounts for 80% the of total vulvovaginal candidiasis (82). It shows that C.albicans is more prevalent than other Non-albicans species (83). Recent studies show an increase frequency of Non-albicans species causing vulvovaginal candidiasis. It is important to emphasize that in the past three decades there has been an increasing percentage of infection caused by Non-albicans species of Candida, particularly C.glabrata, C.tropicalis, andC.krusei and it is resistant to conventional therapy (84). In our study, among the Non-albicans group, we observed that C.glabrata was the most common species present in the vaginal isolates (28.8%); this was similar to the study done by Sasikala et al where showed C.albicans was present in 51.8% and C.glabrata was present in 29.1% of the patient with candidiasis (84). Report from other workers like Mohanty et al, Gowsami et al and Mendezan F V et al are also similar to our study (85, 87, and 88). In vitro Antifungal susceptibility testing is becoming important because of the emergence of new Non-albicans strains and increased inherent and acquired resistance to azoles and Amphotericin B. So agar based antifungal susceptibility testing is an alternative to the Microdilution method. It is easy to perform and inexpensive for routine laboratories CLSI M44 - A Disc diffusion testing with glucose methylene blue Muller Hinton Agar is a very convenient method for antifungal susceptibility testing (89).

The study shows, for Fluconazole, the overall susceptibility rate for C.albicanswas 76.9% and C.tropicalis was 57%: Susceptible dose dependent for C.albicans 19.2% and C.tropicalis 28.5%: Resistance for C.albicans was 3.8% and C.tropicalis 14%. In our study C.glabrata&C.krusei showed resistance to fluconazole, as similar to study done by Sobel et al (Table: 9/Graph:2/Fig: 6) (90). Candida krusei and C.glabrata is known for its intrinsic resistance to fluconazole (91). For Itraconazole over all susceptibility rate for C.albicans was 69.2%, for C.glabrata 67%, for C.tropicalis 71.4% and for C.krusei 75%. Susceptible dose dependent for C.albicans was 7.6% and C.glabrata 13%. Resistance for C.albicans was 23%, for C.glabrata 20%, for C.tropicalis 28.5% and for C.krusei 25% (Table: 10/Graph: 3/Fig: 6). Antifungal susceptibility testing to Amphotericin B shows minimal resistance pattern to different Candida species, it has high susceptibility rate. Over all susceptibility rate for C.albicans was 96.1%, for C.glabrata was 100%, for C.tropicalis was 85.7% and for C.krusei was 75%. Resistance for C.albicanswas 3.8%, for C.glabrata 0% percentage, for C.tropicalis 14.2% and for C.krusei 33.3% (Table: 11/Graph: 4/Fig: 6). Our finding shows an overall susceptibility rate for Amphotericin B to be 94% which

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correlate with the study done by Saldanha et al and Noake et al were Candida species are more susceptible to Amphotericin B (92% (92, 93).

CONCLUSION

The epidemiological profile of vaginal candidiasis varies from country to country as well as within the country depending upon so many factors like socioeconomic and health factors. In this study like similar ones done earlier, diabetes is one of the risk factor for vulvovaginal candidiasis, as the genital tissue nature in diabetics enhances yeast adhesion and growth. Candida binds to the vaginal epithelial cell with greater propensity in diabetic patients than in non-diabetic patients. It is recommended that these diabetic patients need to observe better blood sugar control and hygienic habits, as they have a poor immune status. Constant surveillance of vulvovaginal candidiasis is important as C.glabrata is more invasive, and can lead to fatalcandidemia. Identification of Candida to species level is essential, as it can give an idea to the clinician about empirical therapy in emergency situation. The emergence of fluconazole resistance in Non-albicans candida may caution against its use as a prophylactic agent in hospitals. However C.glabrata and C.krusei exhibit intrinsic resistance to fluconazole and cannot be used. One of the causes of frequent isolation of Non-albicans species from Vulvovaginitis patients may be the increased use of tropical azole agents.
So the present study emphasizes the need for doing antifungal susceptibility tests for all Candida isolates, to control the emergency and spread of new resistance strains in the future. This study also provides the baseline information on the prevalence and antifungal susceptibility pattern of Candida isolates among diabetic and non diabetic patients with vulvovaginal infection in our region. The diagnosis of genital candidiasis should not be on clinical ground alone as similar types of manifestation may be produced in other infective or allergic conditions. Therefore, laboratory support should be sought for confirmation of the diagnosis. As the transmission of the infection may be through sexual activity, it is taken as one of the sexually transmitted disease. Therefore simultaneously diagnosis and treatment of the sexual partner is necessary to prevent re-infection.

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