Speciation Of *Candida* Isolated From Cases Of Vaginal Candidiasis

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ABSTRACT

BACKGROUND

Vulvovaginal Candidiasis (VVC) is an exceedingly common mucosal infection of the lower female reproductive tract. Mostly, it is caused by *Candida albicans*. Yet, non-*albicans Candida* species are also on the rise. Also, increasing resistance to commonly prescribed antifungal agents is of serious concern.

METHODOLOGY

High vaginal swabs were collected from clinically suspected cases of VVC from patients attending outpatient department of Obstetrics and Gynecology of NMCTH. The swabs were subjected to microscopic analysis and culture. Germ tube test, CHROMagar, chlyamydospore formation test and sugar assimilation test were applied for identification of the isolated *Candida* spp. Antifungal susceptibility testing of the isolates to fluconazole was done by disk-diffusion method.

RESULTS

The rate of culture positive cases of VVC was 46.3%. Most number of cases belonged to the age group of 18-27 years (43.6%). Adhibasi-janajati and married women were most commonly affected (55.9% and 62.3% respectively). Abnormal vaginal discharge with burning and itching were the most common symptoms. *C. albicans* accounted for the majority of the isolated *Candida* spp. (57.8%), followed by *C. glabrata* (26.5%) and *C. tropicalis* (9.8%), *C. krusei* (3.0%), *C. parapsilosis* (2.0%) and *C. keyfr* (0.9%). A total of 34.3% of isolated *Candida* spp. were resistant to fluconazole.

CONCLUSION

Both *C. albicans* and non-*albicans Candida* species were responsible for acute cases of VVC and RVVC. A majority of non-*albicans Candida* species were found to be resistant suggesting rise in their prevalence and resistance to fluconazole.

KEYWORDS

Candidiasis, Vulvovaginal, *Candida*, Fluconazole

BACKGROUND

Vulvovaginitis is a cumbersome condition characterized by abnormal vaginal discharge with irritation of vulva, vagina, or both.[1, 2] These generally account for 90% of all infective and non-infective cases.[2, 3] In Nepal, one in three women need gynecological consultation for abnormal vaginal discharge.[3]

Many studies have shown than 75% of female population will have at least one episode of vulvovaginal candidiasis (VVC)[1, 2, 4-8] and, 40-50 % will have recurrent episode during their lifetime.[1, 5, 8-11] The condition of recurrent VVC (RVVC) is defined as three or more episodes per annum.[11] In contrast to isolated episodes, RVVC often constitutes a management problem, which can be disabling for affected women.[12]

Despite several treatment modalities and application of new effective drugs, VVC is a complex and considerable problem in gynecology and obstetrics. The disease is a source of great physical and psychological discomfort. During the recent years, studies have found that the involved *Candida* species are changing.[13] In the study of Heydati et al, *Candida albicans* and non-*albicans Candida* species were responsible for 42.5% and 57.5% of VVC cases, respectively.[1] According to the study of Barakoti et al, *C. albicans* was isolated in 56% of VVC patients, while in the remaining cases, the disease was caused by other *Candida* species.[14] Non-*albicans Candida* species are often associated with recurrent, severe and complicated vulvovaginal candidiasis.[15] Many of them have decreased susceptibility to antifungal agents. *C. glabrata* and *C. krusei*

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have been found to be inherently resistant to azole group of drugs, particularly to fluconazole.[16, 17]

The frequent empiric prescription of fluconazole for sporadic VVC infection without speciation of the affecting *Candida* strain, coupled with over-the-counter availability of topical azole agents and the widespread use of a low-dose weekly fluconazole regimen for recurrent VVC infection, combines to create ideal conditions for emergence of fluconazole-resistant *Candida* strains causing RVVC infection to evolve, emerge, and spread. Therefore, this study was focused to determine the species of *Candida* responsible for VVC and their susceptibility to fluconazole, the commonly used antifungal drug.

OBJECTIVES

The study was conducted to identify different species of *Candida* from cases of vaginal candidiasis in Nepal Medical College Teaching Hospital (NMCTH) and analyze susceptibility of isolated *Candida* species against fluconazole.

METHODS

This research was a descriptive cross-sectional study carried out in Department of Obstetrics and Gynecology and Department of Microbiology in NMCTH. All women (>=18 years) presenting with abnormal vaginal discharge clinically suspected of vaginal candidiasis were included. Women presenting during menstruation and/or not willing to participate were excluded. The patients filled out a consent form to participate in the research. Patient's information and history were taken by using proforma enquiring about their age, ethnicity, marital status, vulvovaginal symptoms, and presence of pregnancy and consumption of OCPs. Patients with three or more discrete attacks of VVC per year were considered as having RVVC.

Sample collection and Laboratory diagnosis

Vaginal discharge was taken using sterilized speculum and sterile swabs. Two swabs were collected per patient, one for direct microscopic examination and the other for fungal culture. For each sample, a slide was prepared for Gram staining and the sample for fungal culture was inoculated into Sabouraud's Dextrose Agar (SDA) media which was incubated at 37^{0}C for 2 to 3 days.

Identification of *Candida* species was done on the basis of morphology and colony color on CHROMagar, germ tube test, chalmydospore formation on cornmeal agar, and sugar assimilation test with Yeast Nitrogen base (YNB) agar (Hi-Media, Mumbai, India).

For sugar assimilation test, the colonies grown on SDA were inoculated in saline to make a heavy suspension. The suspension was incubated at room temperature for about 24 hours to exhaust any carbohydrate reserves. A lawn culture was made on the YNB agar plate. The sugar disks (Glucose, Sucrose, Lactose, Trehalose, Raffinose and Cellobiose) obtained from Hi-Media, Mumbai, were placed on the agar plate and incubated at 30^{0}C for 24 to 72 hours. Most of the isolates showed increased growth around the carbohydrate disks. Incubation for up to a week was required for few isolates.

Antifungal susceptibility test[18-22]

Mueller Hinton Agar (MHA) with glucose (2%) and methylene blue (0.5 µg/L per ml) was used. For preparation of Mueller Hinton with glucose and methylene blue (MHA-GMB), flooding procedure was done. Briefly, the GMB solution was prepared by adding 200 µl of a stock methylene blue solution (5 mg/ml) to 100 ml of a 40% glucose solution. The GMB solution was dispensed into screw-capped tubes (1.5 ml for 100-mm-diameter plates) and then sterilized by autoclaving. The tubes with GMB solution was then stored at 5 to 8°C until used. The day before testing, GMB-containing tubes was allowed to warm to room temperature, and at the same time MHA plates were dried in a 35°C incubator for 1 to 2 hr. The dried agar surface was then flooded with the GMB solution, and that solution was allowed to absorb overnight at room temperature.

Antifungal susceptibility testing was done by disk diffusion method. The inoculum was prepared by picking 5 distinct colonies of *Candida* species from SDA and emulsifying in normal saline. The turbidity was adjusted with 0.5 McFarland standard using Wickerham card. A disk of fluconazole (25 µg) was placed on each inoculated MHA-GMB plate. It was then incubated at 35^{0}C for 24 hours. If the growth was not clearly visible, the plates were re-incubated for another 24 hours.

Data analysis

Chi-square test was performed using the SPSS software (version 16) and differences were considered significant at $P < 0.05$.

RESULTS

A total of 220 patients clinically suspected of VVC were studied during a period of 12 months at NMCTH, Attarkhel, Nepal. Fig. 1 shows the rate of culture proven vaginal candidiasis. The mean age of VVC patients was 29.2 years. The highest frequency of VVC was found between 18-27 years of age group (43.6 %) followed by 28-37 years (39.5 %). There was no significant correlation between age and VVC (p=0.296) (Table 1). The highest prevalence of culture positive cases was seen among Janajati group (55.9%) followed by the group of Brahman/ Chhetri. However, no significant correlation was found (Table 2). The highest number of culture positive VVC cases were found among

married women (72.4%) (Table 3) where a significant correlation was found. Abnormal vaginal discharge concomitant with burning and itching (48.2%) was the most prevalent symptom in patients with VVC (Table 4).

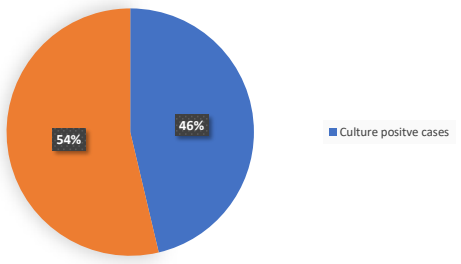

Fig. 1: Rate of culture positive cases of VVC among clinically suspected cases

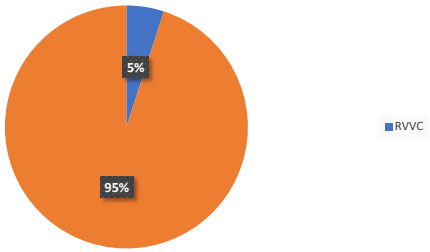

Fig. 2. Prevalence of RVVC among women with VVC

Table 1: Age wise distribution of vaginal candidiasis

Age group	N*	%	P value
18-27	96	43.6	0.296
28-37	87	39.5	
38-47	35	16.0	
48-57	2	0.9	
Total	220	100.0	

N Total number of clinically suspected cases of VVC*

Table 2: Distribution of VVC among different ethnic groups

Ethnicity	N*	%	P value
Brahman/Chhetri	66	30.0	0.067
Madhesi	1	0.5	
Dalits	30	13.6	
Aadhibasi Janajati	123	55.9	
Total	220	100.0	

N Total number of clinically suspected cases of VVC*

Table 3: Distribution of VVC according to marital status

Marital status	N*	%	P value
Married	137	62.3	0.000
Unmarried	83	37.7	
Total	220	100.0	

N Total number of clinically suspected cases of VVC*

Table 4: Vulvovaginal symptoms in patients with VVC

Symptoms	N*	Percent	P value
Burning and itching	82	37.3	0.247
Abnormal vaginal discharge	32	14.5	
Burning, itching and abnormal vaginal discharge	106	48.2	
Total	220	100.0	

N Total number of clinically suspected cases of VVC*

Overall 62 patients with clinical VVC had an underlying factor (pregnancy and consumption of OCPs) while risk factors among rest of the patients could not be revealed. A total of 38 were pregnant, and 24 women were consuming oral contraceptive pills for at least last 2 months (Table 5).

Table 5: Frequency of VVC with risk factors

Risk factors	N*	%
Pregnancy	38	17.0
OCP	24	11.0
Not revealed	158	72.0
Total	220	100.0

N Total number of clinically suspected cases of VVC*

Not revealed Includes the category of those patients who were reluctant to confide on the above risk factors such as hygiene habits, sexual exposure, frequency of sexual exposure, exposure to steady or non-steady partners and abortion. These could not beruled out in this particular subset of the study population.*

Among 220 clinically suspected cases, 11 (5%) were found to manifest recurrent episodes (Fig. 2). Among 38 pregnant women and 24 women consuming OCPs, 2.2% and 1.4% of them respectively, gave history of RVVC (Table 6).

Table 6: Distribution of risk factors associated with RVVC

Risk factors	N*	Total n RVVC (%)
Pregnancy	38	5 (2.2)
OCP	24	3 (1.4)
Not revealed*	158	3 (1.4)
Total	220	11 (5.0)

N Total number of clinically suspected cases of VVC*

Not revealed Includes the category of those patients who were reluctant to confide on the above risk factors such as hygiene habits, sexual exposure, frequency of sexual exposure, exposure to steady or non-steady partners and abortion. These could not be ruled out in this particular subset of the study population.*

C. albicans was the predominant species (57.8%). C. glabrata was predominant among non-albicans Candida species (26.5% of total 102 isolated species) followed by C. tropicalis (9.8%), C. krusei (3%), C. parapsilosis (2%) and C. keyfr (0.9%), shown in Table 7.

Table 7: Different species of Candida isolated from total cases of candidiasis

Isolated species	Total n	Percent (%)
Candida albicans	59	57.8
Candida glabrata	27	26.5
Candida tropicalis	10	9.8
C. krusei	3	3.0
C. parapsilosis	2	2.0
C. keyfr	1	0.9
Total	102	100.0

Overall, antifungal susceptibility profile of *Candida* species to fluconazole was found to be 65.7% susceptible and 34.3% resistant (Fig. 3). Most of the isolates of C. albicans were sensitive. Highest resistance was found in C. krusei (intrinsically resistant) and C. keyfr (Table 8).

Table 8: Antifungal susceptibility pattern of Candida species

Isolated species	Total n	Sensitive (%)	Resistant (%)	p value
C. albicans	59	41 (69.5)	18 (30.5)	0.091
C. glabrata	27	16 (59.2)	11 (40.8)	
C. tropicalis	10	8	2 (20.0)	
C. krusei	3	0	3 (100.0)	
C. parapsilosis	2	1 (50.0)	1 (50.0)	
C. keyfr	1	0 (0.0)	1 (100.0)	
Total	102	66 (64.7)	36 (35.3)	

DISCUSSION

Different studies have shown the rate of culture positive cases of VVC ranging from as low as 2.6% to as high as 72.7% in clinically suspected cases of VVC.7, [23-29] This variation in the rate of culture positivity may be due to inaccuracies in pathogen detection and diagnosis, developing drug resistance, incompletion in therapy, over the counter use of medicines and lack of proper health habits.[1, 30] In this study, the prevalence of VVC was 46.3% which is similar to the study done by Guzel et al in Turkey (43.2%).[29]

VVC is common in women of reproductive age.[31] In the present study, the age group 18-27 had the highest frequency of VVC followed by the age group of 28-37 years. This age is the most reproductively active age group; sexual act and change in hormonal milieu during pregnancy are the leading predisposing factors for this age group.[3] Sexual intercourse may facilitate movement of *Candida* into the vagina. Our result is similar to the findings from study done by Tellapragada et al[32] from India, Padhye et al[33], Kandel et al[28] and Barakoti et al[14] from Nepal. No significant correlation was not found between age group and occurrence of the disease (p=0.296) which is concordant with the finding of Hedayati et al.[1]

The frequency of clinically suspected VVC was found to be highest among *Adhibasi Janajati* group (55.9%) followed by *Brahmin/Chhetri* (30%). However, there was no significant correlation between ethnic aggregation and VVC (p=0.067). Shrestha et al[3] found the highest prevalence of vaginitis among *Indo-Aryans* in Paropakar maternity hospital in Thapathali. Also, majority of women were *Brahmin* (33%) in Tribhuvan University Teaching Hospital, Maharajgunj, Kathmandu.[34] However, the ethnic aggregation and predisposition to the vaginal infection is not genetically determined and may be strongly determined by the behavioral factors, socioeconomic awareness, health awareness, hygiene, etc.[3]

Out of 220 cases of VVC, 62.3% women were married which is similar to the finding of Agarwal et al (90%) in eastern Nepal.[35] Similarly, 90.2% and 94.8% of married women presented with acute VVC and RVVC respectively in Turkey.[29] There was a significant correlation between marital status and VVC cases (p=0.000). More frequent involvement in sexual activity among married women may be the reason behind high prevalence of VVC and other vaginitis.[36]

McClelland et al stated that though vulvovaginal pruritis without discharge is the most specific clinical presentation, it correctly predicts VVC in only 38% of cases.[37] Also, French et al pointed out that presence of a thick, curdled-appearing discharge suggests diagnosis of candidiasis because it is rarely present in BV or trichomoniasis.[38] This study found that 48.2% of patients presented with complains of burning, itching and abnormal vaginal discharge. However, there was no statistically significant correlation between symptoms and VVC (p=0.247).The findings are similar to Aslam et al[39] in Lahore, Pakistan.

Overall, in our study, 17 % of were pregnant and 11% were consuming OCPs for at least the last 2 months. However, history regarding sexual habits and hygiene could not be elicited from most of our study subjects. Therefore, other predisposing factors for VVC could not be analyzed. Common factors contributing to VVC were pregnancy, oral contraceptive use and antibiotics found by Nwadioha et al[26] in Jos, Nigeria. Padhye38 found that 1.7% of Nepalese women with complain of abnormal vaginal discharge were consuming OCPs. The incidence of VVC among women consuming OCPs was higher irrespective of type of oral contraceptive consumed.[40] In our study, most of the pregnant women were in 3rd trimester of pregnancy which is similar to the finding of Olowe et al.[41] Increase in hormonal influences and alteration of vaginal pH, decrease in anti-*Candida* activity of neutrophils due to elevated progesterone may increase the risk of VVC in pregnancy.[26,

[30, 39] However, Sobel et al[5] stated that a precipitating factor is not found in most patients with acute VVC.

Women suffering from three to four attacks of VVC within a year are often diagnosed with RVVC.[12] Women encountering RVVC are subjected to greater discomfort and a greater cost.[6]

In this study, RVVC was diagnosed based on the clinical history. The patients were accessed for the risk factors and the species of *Candida* isolated from their vaginal discharge. A total of 11 (5%) women gave a history of 3-4 similar episodes in the past 12 months. A prevalence of 15.5% of RVVC among culture positive cases of VVC among Flemish patient population was reported by Vos et al.[42] Hedayati et al[1] found a prevalence of 24.2% of RVVC among Inranian patients with candidal vulvovaginitis. The difference in the incidence in our study may be because RVVC was diagnosed based entirely on patients' recollection of the past signs and symptoms. The diagnosis of the condition made by conventional means by health providers is often false and is also often misdiagnosed by the affected woman herself.[12]

C. albicans was the predominant species in this study, accounting for 57.8% of the isolates. This species accounted for more than half of the isolates identified in studies around the world.[5, 26, 43] Narayankhedkar et al[2] in India detected 54.5% of C. *albicans* and 45.5% were species other than C. *albicans* in cases of VVC. A total of 56% and 65.3% of C. *albicans* was isolated by Barakoti et al[14] and Kandel[28] in NMCTH and Bharatpur, Nepal respectively. *C. albicans* is able to adhere to vaginal epithelium more readily than other *Candida* species, which might explain the predominance of this species over others. Also, *C. albicans* constitutes a part of normal vaginal flora.[44]

In this study, 42.2% of isolates were non-*albicans Candida* species which is almost of the same number as that of isolated *C. albicans*. The finding is in agreement with the findings of Barakoti et al[14] where 44% of the total isolates were non-*albicans Candida* species. In his study, *C. glabrata* and *C. tropicalis* accounted for 12% of isolated spp. each, which were followed by *C. parapsilosis* (8%) and *C. krusei* (4%). Hedayati[1] observed around 22% of C. glabrata and 16.4 % of *C. dubliniensis*. *C. glabrata* (26.5%) was predominant non-*albicans Candida* species in this study followed by *C. tropicalis* (9.8%), *C. krusei* (3%), *C. parapsilosis* (2%) and *C. keyfr* (0.9%). *C. glabrata* (35%) was the second most common species by Guzel et al.[23] He also found *C. krusei* (3.8%), *C. parapsilosis* (0.9%) and *C. tropicalis* (0.9%) as causative agents of VVC. However, the rate of *C. keyfr* isolation in this study was less than the above reports (0.9%). All these findings suggest that non-*albicans Candida* species are emerging as important pathogens in VVC.

Antifungal susceptibility testing to fluconazole was done for all 102 isolates by disc diffusion method. A total of 64.7% of isolates were found susceptible while 35.3% were resistant. Elfecky45 found 22.2% of total resistant *Candida* isolates to fluconazole in VVC cases. Khadka et al[21] detected resistance among 20% of *Candida* spp. obtained from different clinical samples at Tribhuvan University Teaching Hospital. Mondal et al[20] in Birgunj, found 18% of resistant species in total isolates. Though disc diffusion is considered simple and reliable for fluconazole susceptibility testing, fluconazole MICs should be determined for strains found to be resistant by the disc test as the test does not differentiate if the isolates are truly resistant or susceptible-dose dependent.[46]

CONCLUSION

Both *C. albicans* and non-*albicans Candida* species were responsible for acute cases of VVC and RVVC. A majority of non-albicans Candida species were found to be resistant suggesting rise in their prevalence and resistance to fluconazole. Therefore, speciation of *Candida* species and analysis of their susceptibility to antifungals is highly recommended for best therapeutic approach.

Abbreaviations

µg Microgram; µl Microliter; AIDS Acquired Immunodeficiency Syndrome; CMA Cornmeal Agar; Fig Figure; gm Gram; IUCD Intrauterine Contraceptive Device; MDR Multidrug Resistance; MHA Muller Hinton Agar; MHA-Muller Hinton agar with glucose and methylene blue GMB; mL Milliliter; NMCTH Nepal Medical College Teaching Hospital; OCP Oral Contraceptive Pills; RVVC Recurrent vulvovaginal candidiasis; SDA Sabouraud's Dextrose Agar; UV Ultravoilet; VVC Vulvovaginal candidiasis

Conflict of interests

We declare that we do not have any conflict of interest.

Authors' contributions

AG was responsible for study design, supervision of work and guidance. AG was contributed to laboratory work and data analysis. RB was contributed to writing and manuscript preparation. All authors read and approved the final manuscript.

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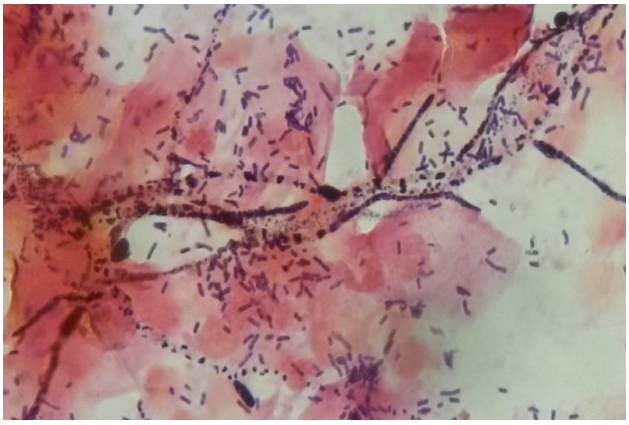
Ethical approval and consent to participate

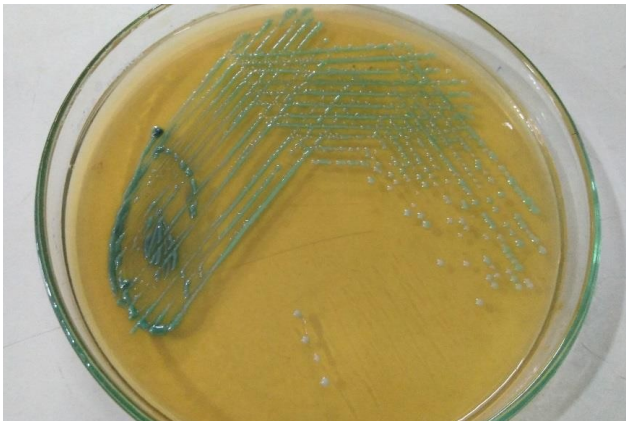
The ethical approval for study was taken from Institutional Review Committee, Nepal Medical College Teaching Hospital, Attarkhel before sample collection.

PHOTOGRAPHS

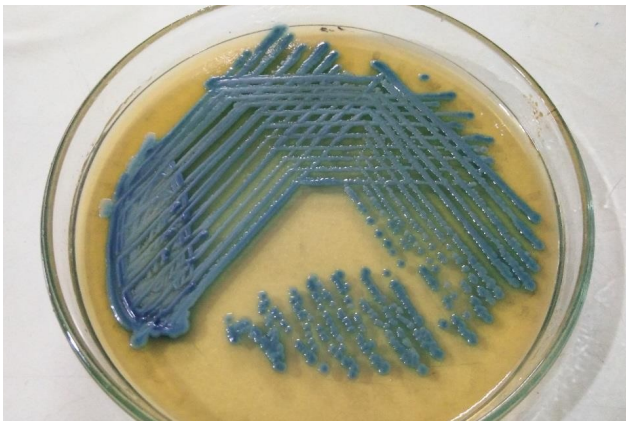
Photograph no. 1. Wet mount of vaginal discharge showing pseudohyphae and yeast cells



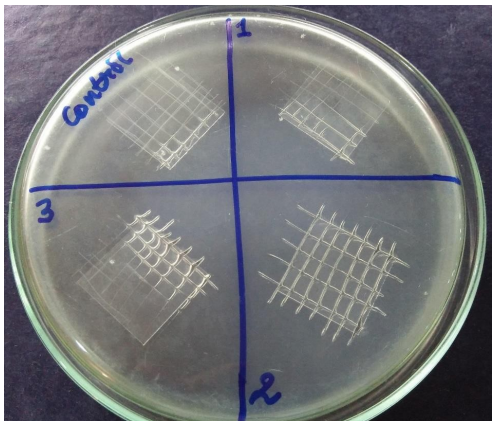
Photograph no.2. Grams stain of vaginal discharge showing pseudohyphae and yeast cells



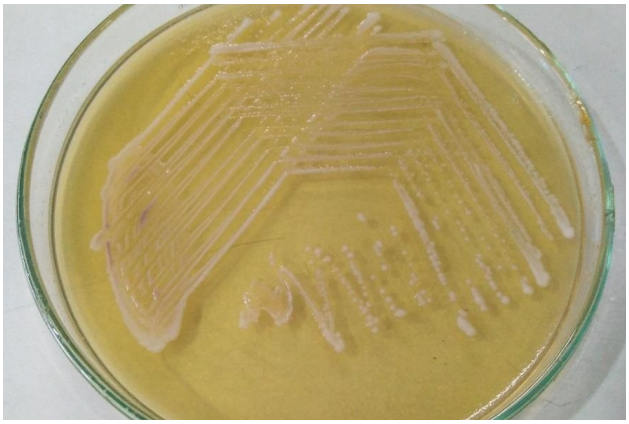
Photograph no.3. Light green colonies of C. albicans in CHROMagar



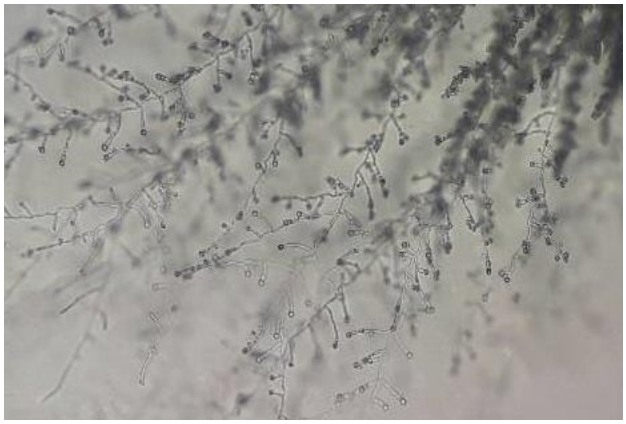
Photograph no.4. Metallic blue colonies of C. tropicalis in CHROMa



Photograph no.7. Inoculation of isolated Candida species on cornmeal agar

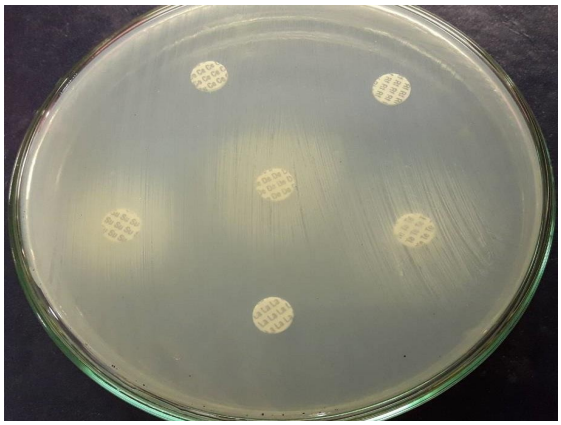


Photograph no.5. Light cream colored colonies of C. glabra

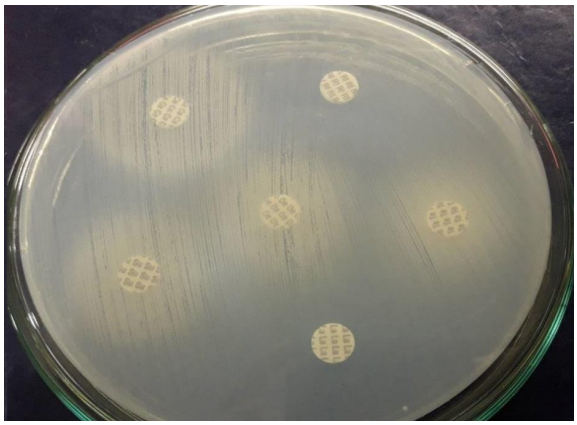


Photograph no.8. Cornmeal agar showing terminal chlamydospores (under 10X magnification) in C. albicans

Photograph no.6. Purple colored colonies of C. krusei on CHROMagar



Photograph no.9. Sugar assimilation pattern of C. albicans



Photograph no.10. Sugar assimilation pattern of C. tropicalis

Photograph no.11. Antifungal susceptibility testing to fluconazole by disk diffusion method