Isolation of Different *Candida* Species from Clinical Samples and Comparison between Conventional and Hicrome Technique of Identification

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Abstract: *Introduction: Candida species are the most common cause of fungal infections worldwide.Candida species are the fourth leading cause of health care associated infections and the third most common cause of central line-associated bloodstream infections. Candida species are associated with the highest overall crude mortality of all nosocomial bloodstream infections comparable to that of Pseudomonas and exceeding that of Staphylococcus aureus infections. The vast majority of invasive Candida infections are caused by only four species which include C. albicans, C.glabrata, C.parapsilosis and C.tropicalis. Identification can be done by conventional method as well as by using chromogenic media.Aim: To isolate and identify the various candida species from clinical samples by both conventional and chromgenic method. Material methods: Various samples received in laboratory from patients of all age group and both sexes with suspected Candida infectionwere included in this study and the positive isolates were identified by conventional as well as chromogenic method. Results: Total 121 isolates of candida species were found of which 21-40 years was the most common age group and female predominance was seen. C.albicans was most commonly found followed by C.tropicalis. Hicrome agar for candida falsely identified C.parapsilosis as C.glabrata. Conclusion: Candida albicans is the main cause of candidiasis; however, non-albicans Candida species such as C. glabrata, C. tropicalis and C.parapsilosis are now frequently identified as human pathogens. Species identification using Hicrome Candida agar is rapid, technically simple, easy but for early identification of C. glabrata and C. parapsilosis both the corn meal agar and Hicrome Candida agar should be used.*

Keywords: Candida species, Hichrome candida agar.

1. Introduction

Candida species are the most common cause of fungal infections worldwide[1], the fourth leading cause of health care associated infections and the third most common cause of central line-associated bloodstream infections. They are associated with the highest overall crude mortality of all nosocomial bloodstream infections, comparable to that of *Pseudomonas* and exceeding that of *Staphylococcus aureus* infections.[2]The genus is composed of a heterogeneous group of organisms and more than 17 different *Candida* species are known to be the aetiological agents of human infection.[3]Among species of *Candida*, *C. albicans* is most often associated with serious fungal infections. Other *Candida* species also have emerged as clinically important opportunistic pathogens.[4]The vast majority of invasive *Candida* infections are caused by only four species which include *C. albicans, C.glabrata, C. parapsilosis and C. tropicalis*. The clinical manifestations of disease are extremely varied, ranging from acute, subacute and chronic to episodic. Involvement may be localized to the mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs, gastrointestinal tract or become systemic as in septicaemia, endocarditis and meningitis.[5]Identification of yeast pathogens by traditional methods like growth pattern on cornmeal agar, carbohydrate fermentation test, carbohydrate assimilation test are labour intensive and requires several days and specific mycological media. Chromogenic media contain chromogenic substrates which react with enzymes secreted by target microorganisms to yield colonies of varying colours.

2. Material and Methods

After approval from institutional ethical committee, present prospective study was conducted in tertiary care hospital. Various samples received in laboratory from patients of all age group and both sexes with suspected *Candida* infectionwere included in this study. Informed consent was taken from patients for sample collection. Clinical details were noted in the case record form.Patients who were on antifungal treatment were excluded. The specimens for laboratory investigation were collected from the clinically suspected cases of candidiasis under strict aseptic precautions. The various clinical specimens collected were oral swabs, ear swabs, vaginal swabs, urine, stool, CSF, sputum, blood, pus, nail scrapings etc. Two swabs/specimens were taken from each case. The specimens were transported immediately to the laboratory taking care not to dry out the specimens. One of the specimens was subjected for direct examination and the other for the culture. All the above samples were subjected to various mycological tests.

1) **Direct examination by KOH Mount**
2) **Gram stain**
3) **Culture on SDA(at 25°C and 37°C)**
4) **Growth on blood agar**
5) **Germ tube test for speciation:** A small portion of an isolate colony of the yeast to be tested was suspended in a test tube containing 0.5 ml human serum.The test tube was incubated at 35ºC for 2 hours.A drop of yeast–serum suspension was placed on a microscopic slide, overlaid with a coverslip and examined microscopically for

presence of germ tubes. **Observation:** Filamentous extension from yeast cell with no constriction at the neck was considered as germ tube.

6) **Growth pattern on CMA for speciation**: Isolated colonies of Candida were picked up with inoculating wire.Three parallel cuts 1 cm apart were made into the surface of Cornmeal-Tween agar, holding the inoculating wire at about a 45-degree angle.A sterile coverslip was laid on the surface of agar, covering a portion of the inoculated streaks.The inoculated plates were incubated at 30ºC for 24-72 hours in a closed moisturized chamber.At the end of incubation period plates were examined microscopically (under 10x and 40x) at the edge of coverslip and the pattern of growth was observed to make a presumptive identification.

7) **Carbohydrate assimilation test**: Yeast nitrogen base agar was prepared. For use yeast nitrogen base agar medium was melted in a boiling water bath and allowed to cool to up to 47-48ºC. Yeast suspension was made from a 24-48 hours old culture in 4 ml of distilled water. The turbidity of suspension was adjusted to match no. 5 McFarland standards.The yeast suspension was poured in to molten yeast nitrogen base agar. It was mixed thoroughly by inverting bottles several times.Yeast –agar mixture was poured in to sterile Petri dish and allowed to solidify at room temperature.Carbohydrate discs purchased from HiMedia, Mumbai were evenly spaced on the plate. Inoculated plates were incubated at 25°C and examined by indirect light every other day for 14 days. Any amount of growth around a disc was considered as yeast assimilated that carbohydrate. Species were identified based on pattern of carbohydrate assimilation.

8) **Growth on chrome agar**: Isolated species were inoculated on HiCrome Candida differential agar to improve species identification based on coloured colony morphology. These agar plates were incubated at 37ºC for 48 hours. The species were identified by characteristic colony colour as per HiMedia technical data M1297 A

- *C. albicans*– Light green coloured smooth colonies
- *C. tropicalis* - Blue to metallic blue coloured raised colonies
- *C. glabrata* - Cream to white smooth colonies
- *C. krusei*- Purple fuzzy colonies

- *C. dubliniensis* - Dark green

3. Observation and Results

In present study, 121 isolates of *Candida species* were found. Species identification was done by both conventional method and Hicrome Candida differential agar.
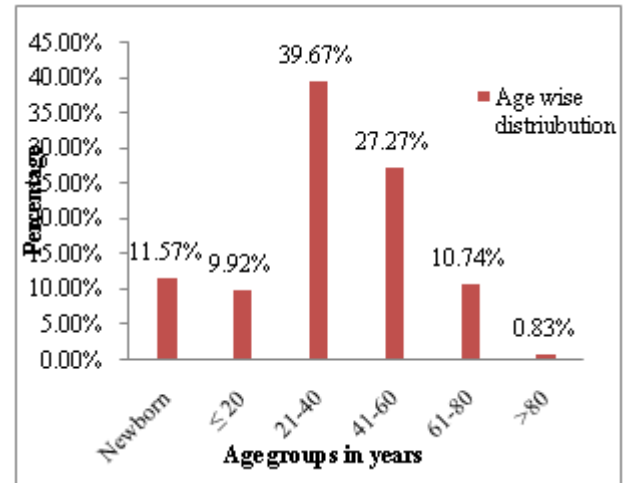

Figure 1: Age wise distribution of patients with *Candida* infection.

Table 1: Distribution of different species of *Candida*

Candida species	Number of isolates	Percentage (%)
C.albicans	62	51.24
C.tropicalis	29	23.97
C.glabrata	13	10.74
C.krusei	09	7.44
C.parapsilosis	05	4.13
C. dubliniensis	03	2.48
Total	121	100

Total number of *Candida* species isolated was 121. Out of 121 isolates, *Candida albicans* (51.24%) was the most common species. Among NAC, *C.tropicalis* (23.97 %) was most common followed by *C. glabrata* (10.74%), *C.krusei* (7.44 %), *C. parapsilosis*(4.13 %) *and C.dubliniensis* (2.48 %).
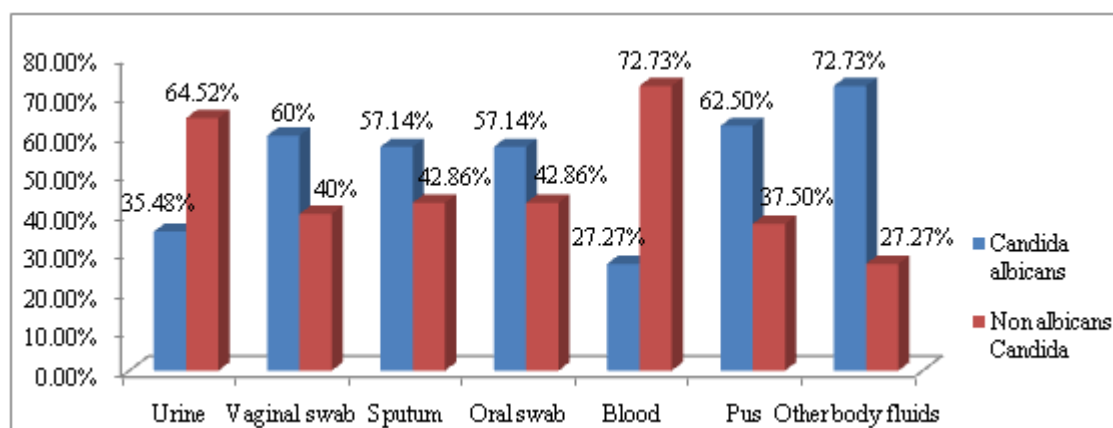

Figure 2: Distribution of *C. albicans* and non *albicans Candida* among different clinical samples

Among urine and blood samples non-*albicans Candida* were more common than *C. albicans* whilein vaginal swab, sputum and oral swab sample *C. albicans* was more common than non-*albicans Candida*.
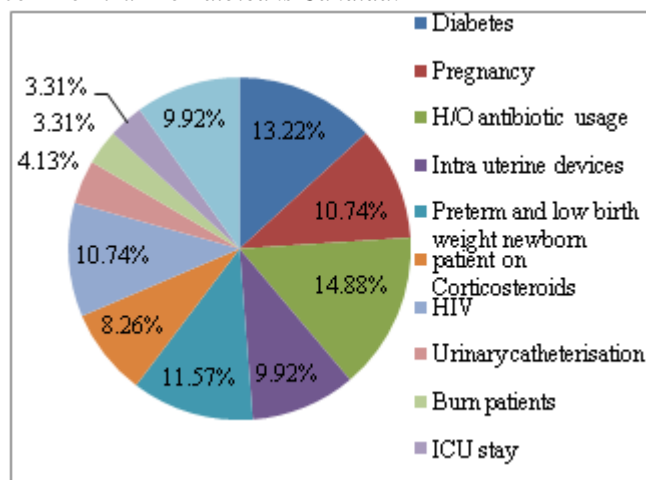

Figure 3: Various predisposing factors in patients having *Candida* infection.

Table 2: Identification of various species of *Candida* by conventional method and Hicrome Candida differential agar

Candida species	Conventional method	Hicrome agar
C.albicans	62	62
C.tropicalis	29	29
C.glabrata	**13**	**18**
C.krusei	9	9
C.parapsilosis	**5**	**-**
C.dubliniensis	3	3

In our study conventional method was considered as reference method for speciation. It included of germ tube formation, growth pattern on Cornmeal-Tween agar and carbohydrate assimilation test. Approximately 7 to 14 days were required for species identification using these methods. However some species were presumptively identified using conventional method within 72 hours, while using Hicrome Candida agar species were identified within 48 hours. All species were correctly identified by Hicrome Candida differential agar as compared to conventional methods, except 5 species of *C.parapsilosis* (identified by conventional method) which were identified by Hicrome Candida agar as *C. glabrata*.

Table 3: Performance of Hicrome Candida differential agar as identification medium compared with conventional method

Candida species (121)	True positive	True negative	False positive	False negative
C.albicans	62	59	0	0
C.tropicalis	29	92	0	0
C.glabrata	13	103	5	0
C.krusei	9	112	0	0
C.dubliniensis	3	118	0	0

Five species of *C. parapsilosis* were identified as *C. glabrata* by Hicrome Candida agar which were considered as false positive.

Table 4: Sensitivity and specificity of Hicrome Candida differential agar for each species.

Species	Sensitivity (%)	Specificity (%)
C.albicans	100	100
C.tropicalis	100	100
C.glabrata	100	95.37
C.krusei	100	100
C.dubliniensis	100	100

We obtained 100% sensitivity and specificity of HiCrome Candida differential agar for *C. albicans, C. tropicalis, C. krusei* and *C. dubliniensis* but sensitivity and specificity of Hicrome Candida differential agar for *C. glabrata* was 100% and 95.37% respectively.

4. Discussion

The incidence of infections caused by *Candida* species has increased considerably over the past three decades, mainly due to the rise of the AIDS epidemic, an increasingly aged population, higher numbers of immunocompromised patients and the more widespread use of indwelling medical devices. *Candida albicans* is the main cause of candidiasis; however, non-*albicans Candida* species such as *C. glabrata, C. tropicalis* and *C.parapsilosis* are now frequently identified as human pathogens.[6]

Total 121 *Candida* species were isolated from various clinical samples. In present study, most of the *Candida* isolates (39.67%) were predominant in the age group of 21-40 years (figure 1) which correlates with study conducted by DharwadS et al[7] and Jaggi T et al.[8]

We found female preponderance in our study (figure 2) which was concordant with the study conducted by Sajjan AC et al[9] and Dharwad S et al.[7] This is supported by higher percentage of vaginal swab sample included in our study.

	Candida Albicans (%)	Candida Tropicalis (%)	Candida Glabrata (%)	Candida Krusei (%)	Candida parapsilosis(%)	Candida Dubliniensis (%)
Pfaller et al[10]	65.3	7.2	11.3	2.4	6.0	0.1
Sajjan et al[9]	65	24.3	-	10.7	-	-
Mondal et al[11]	42.5	27.7	17.5	4.6	7.4	-
Our study	**51.24**	**23.97**	**10.74**	**7.44**	**4.13**	**2.48**

Among the various clinical isolates of *Candida* species we obtained *C.albicans* (51.24%) as the most common isolate followed by *C. tropicalis* (23.97%), *C. glabrata* (10.74%), *C. krusei* (7.44%), *C. parapsilosis* (4.13%) and *C.dubliniensis* (2.48%) (Table1). While non-*albicans Candida* were 48.76% (figure 3). In respect to predominance

Paper ID: SUB154893

3097

of *C. albicans* isolates and distribution of species, similar results were found in study conducted by Pfaller MA et al,[10]Sajjan AC et al[9] and Mondal S et al.[11]

Factors like increased use of antifungal drugs, use of broad spectrum antibiotics, long term use of catheters and increase in the number of immunocompromised patients contributes to the emergence of non-*albicans Candida* species.[12]

Among non-*albicans Candida* we found *C. tropicalis* as a most common isolate which was concordant with study conducted by Xessi I et al[13], ShivprakashaS et al[14] and Pethani JD et al.[15]Higher isolation of *C. tropicalis*may be because of its endogenous transmission and also as it is considered to develop systemic infection in 50-60% of colonised patients.[16]Considering the predisposing factor in association with *Candida* infection, a study done by Arora D et al [17] found intra venous catheter as most common risk factor followed by prolonged antibiotic and immunosupression. Also study conducted by Sajjan AC et al[9] found diabetes as most common risk factor followed by pregnancy and drug intake. While in our study (figure6), we found antibiotic usage (14.88%) as most common risk factor followed by diabetes (13.22%).

For differentiation among different species of *Candida* conventionally germ tube test, growth pattern on cornmeal agar and sugar assimilation tests are being used which are technically difficult, time consuming and difficult to interpret which may take 72 hours to two weeks for species identification.[18, 19] Chromogenic agar is technically simple, easy to interpret and rapid method to differentiate among different *Candida* species. It facilitates the detection and identification of *Candida* species and provides result in 24-48 hours. Among the newer tests, chromogenic agar is rapid and cost effective as compared to other expensive systems like API systems, Vitek 2 ID system and molecular methods.[20]

We obtained 100% sensitivity and specificity of HiCrome Candida differential agar for *C.albicans, C.tropicalis, C.krusei and C.dubliniensis* but sensitivity and specificity of Hicrome Candida differential agar for *C.glabrata* was 100% and 95.37% respectively (table 5). However our study correlates with following studies showing high sensitivity and specificity of chromogenic agar.

Species	C.albicans		C.tropicalis		C.glabrata		C.krusei	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
YucesoyM et al[21] (2003)	99.4%	100%	97%	100%	98.9%	100%	100%	100%
Daef Eet al[22]	100%	98.9%	100%	100%	100%	99%	100%	100%
YucesoyM et al[23] (2005)	100%	100%	100%	100%	90%	100%	100%	99.5%
Our study	100%	100%	100%	100%	100%	95.37%	100%	100%

For *C. glabrata*, specificity of Hicrome Candida agar was 95.37%, as 5 species of *C.parapsilosis*(identified by

conventional method) were identified by Hicrome Candida agar as *C. glabrata*. Shettar SK et al [24] reported that on Hicrome Candida agar*C.parapsilosis* gave same cream colour as that of *C.glabrata*. Ghelardi E et al [25] studied Chromogenic Candida agar (CCA, Oxoid, basingtoke, UK) for identification of *Candida* species. According to their study, this medium didn't allow discrimination of *C. glabrata* and *C.parapsilosis*.

This may be because *of C. glabrata, C. kefyr, C. parapsilosis*and *C.lusitaniae*appear as a variety of beige/brown/yellow colours due to the mixture of natural pigmentation and some alkaline phosphatase activity.[26]*C.glabrata*and *C. parapsilosis* can be easily differentiated from growth pattern on Cornmeal agar as *C. glabrata* doesn't produce pseudohyphae. Thus, the combination of Cornmeal agar and Hicrome Candida agar can be used for early identification of *C. glabrata*.[24]

Conclusion

Although our study showed *C. albicans* as most common species causing candidial infection, non-*albicansCandida* are rising among various clinical samples. Species identification using Hicrome Candida agar is rapid, technically simple, easy to interpret as compared to the time consuming, technically demanding and difficult to interpret conventional methods. Hicrome Candida differential agar can be reliably used for identification for *C. albicans*, *C. krusei*, *C. tropicalis, and C. dubliniensis* but for early identification of *C. glabrata* and *C. parapsilosis* both the corn meal agar and Hicrome Candida agar should be used.To avoid simultaneous use of cornmeal agar and Hicrome Candida agar, we recommend Hicrome Candida agar to be improved further to differentiate other non-*albicans Candida* species.

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Paper ID: SUB154893

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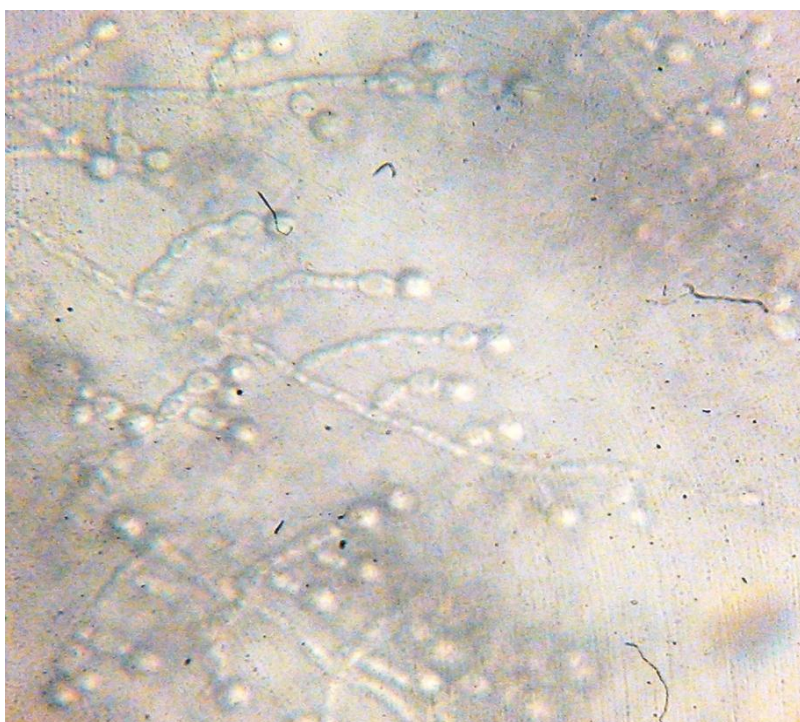
Photograph 1: Growth pattern of C.albicans on Corn meal-Tween agar
(Pseudohyphae with terminal Chlamydospores.) (40x)
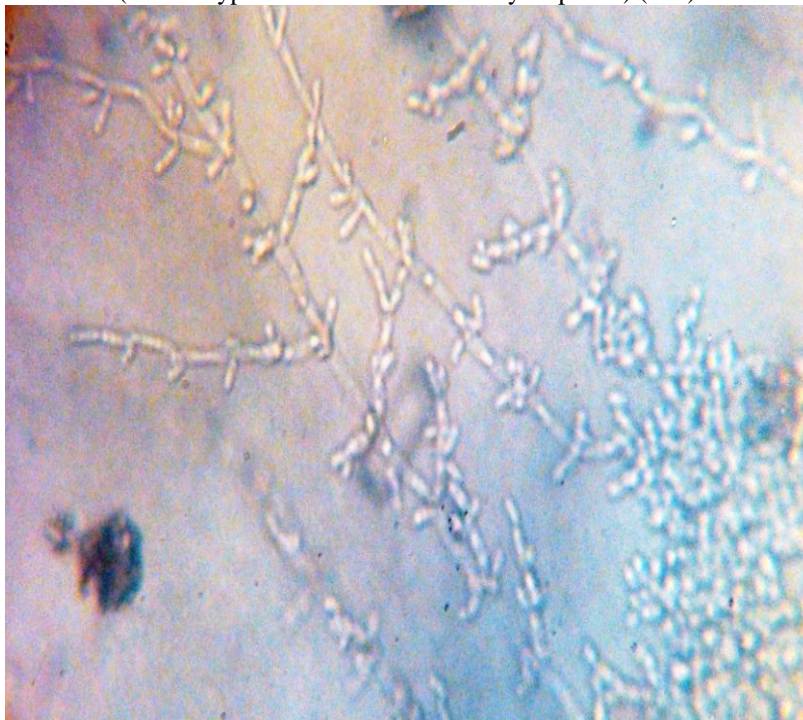
Photograph 2: Growth pattern of *C.tropicalis* on Corn meal-Tween agar
(Abundant pseudohyphae often radiating with clusters of blastoconidia at the centre)(40x)
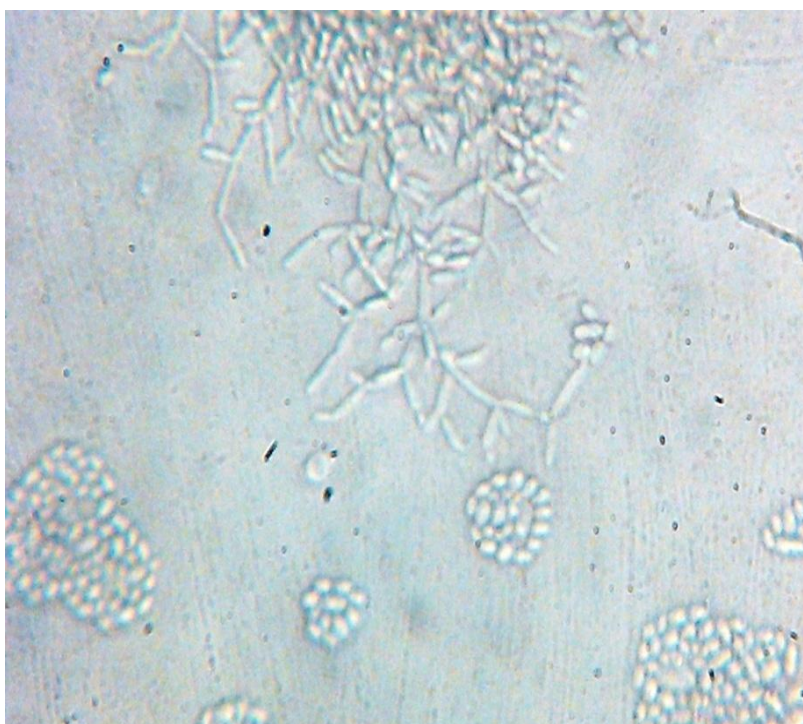
Photograph 3: Growth pattern of C.krusei on Corn meal- Tween agar
(Extensive branched pseudomycelium with chains of elongate cells giving cross matchstick or tree-like appearance) (40x)

Paper ID: SUB154893

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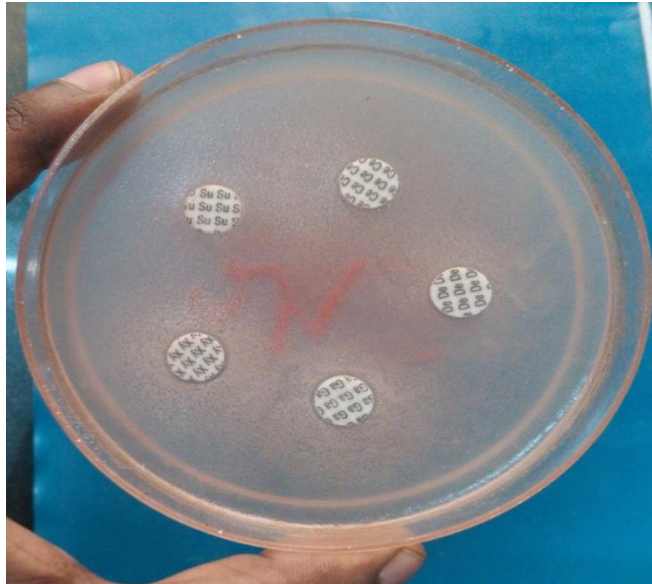
Photograph 4: Carbohydrate assimilation test
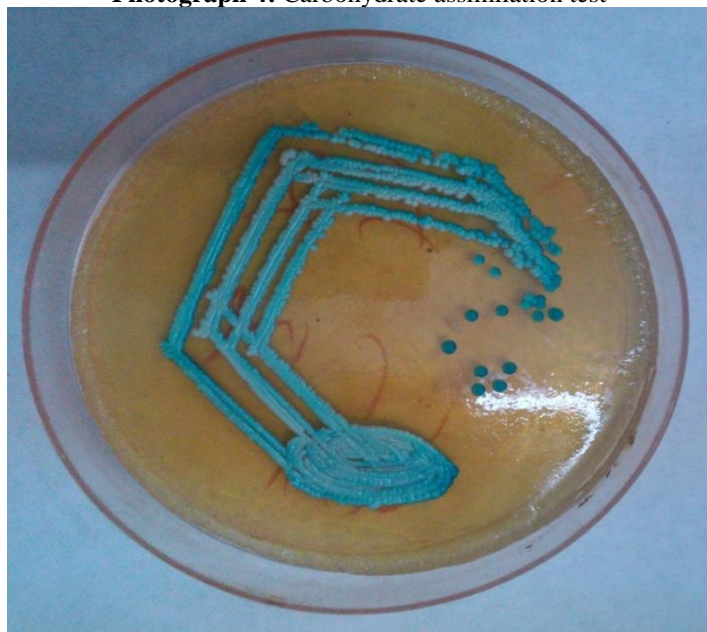
Photograph 5: *C.albicans* showing green colonies on Hicrome Candida agar

Photograph 6: *C.tropicalis* showing blue colonies on Hicrome Candida agar

Paper ID: SUB154893

3101