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PREVALENCE OF BIOFILM PRODUCTION AMONGST CANDIDA SPECIES ISOLATED FROM CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL FROM NORTHEAST INDIA

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ABSTRACT

Context*:* *Candida species are component of normal flora of human beings. A variety of factors are known to predispose both superficial and deep seated candidiasis which act either by altering the balance of normal microbial flora of the body or by lowering the host resistance. Candida attributes its pathogenicity to its virulence factors**;** one of which is the biofilm production. The ability to form biofilms is associated with the pathogenecity and should be considered as an important virulence determinant during candidiasis.*

Aims:

1) To identify the spectrum of Candida species in clinical infections
2) To ascertain the prevalence of biofilm producing isolatesas
3) To identify their sensitivity pattern to available antifungal agents

Settings and Design:
The study is a Hospital Based prospective study carried out in the department of Microbiology of the Institute.

Methods and Material:
A total of 128 (One hundred and twenty eight) isolates of Candida obtained during the period from June 2013- May 2014 from different clinical specimens were recruited for the study. Identification was done by Wet mount, Gram staining, Germ tube formation, colour detection on Hichrome agar, chlamydospore formation on Cornmeal agar , biochemical tests and confirmation on Vitek 2 system. Biofilm production was observed on Sabouraud Dextrose Broth with 8% glucose by Test tube method.

Results:
Out of the 128 isolates, 28 (21.87%) were C.albicans while the rest 100 (78.13%) were Non-albicans Candida; the highest being C. tropicalis [40 (31.25%)] and C. glabrata [34 (26.56%)]. It was observed that C. albicans was less resistant to all the drugs as compared to non-albicans Candida species. Of the 28 isolates of C. albicans, only 12 (42.86%) were found to produce biofilms while out of the 100 isolates of Non-albicans Candida, 48 (48%) were able to form biofilms.

Conclusions:
Reduced susceptibility as well as frank resistance to drugs like azoles, as documented in our study, is an issue of crucial importance in treatment of immunocompromised patients with serious infections. Hence, antifungal susceptibility testing and detection of biofilm production is a promising tool for predicting the efficacy of a given agent in different clinical isolates.

Key-words: Azoles, Biofilm, Candida, Immunocompromised, Virulence

Introduction: Candidiasis is the commonest fungal disease found in humans affecting mucosa, skin, nails and internal organs. It is caused by various species of yeast like fungi belonging to genus *Candida* with *Candida albicans* as the representative species. It is found mainly as secondary infection in individuals with some underlying immunocompromised condition and rarely as the primary disease [1]

Candida species are component of normal flora of human beings. They are commonly found on the skin, throughout the gastrointestinal tract and female genital tract particularly higher in the vagina during pregnancy. A variety of factors are known to predispose both superficial and deep seated candidiasis. All these factors act either by altering the balance of normal microbial flora of the body or by lowering the host resistance.[1, 2]

During the last century two important predisposing factors have been noticed to be important. One was the advent of antibacterial antibiotics and their indiscriminate use and the other being the emergence of pandemics like AIDS, Malignancy, Diabetes mellitus and other immunocompromising disorders. Although *Candida albicans* remains the most common causative agent of both superficial and deep fungal infections, an increasing incidence of Non-*albicans Candida* has also been documented in the last few years. These species include *C. tropicalis, C. krusei, C. glabrata and C. parapsilosis* [1, 3]

Azoles antifungal agents have therapeutic activity against different Candida species. Amongst the azoles, Fluconazole shows that satisfactory tolerance has appeared and anti-fungal drug resistance is quickly becoming a major problem especially in immunocompromised patients. [4, 5] This resistance also favours the emergence of C. krusei and C. glabrata. [6]

Candida attributes its pathogenicity to its virulence factors**;** one of which is the biofilm production. A biofilm is a community of microorganisms and their extra cellular polymers that are attached to a surface.[7] Biofilms may help maintain the role of fungi as commensal and pathogen, by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms. Consequently, biofilm related infections are difficult to treat.[8] The biofilm production is also associated with high level of antimicrobial resistance of the associated organisms.[9] The potential clinical importance of species-level identification has been recognised as Candida species differ in the expression of

putative virulence factors and antifungal susceptibility.[10] Rapid identification of yeast species also guides early appropriate antifungal therapy

Aims and Objectives:

1) To identify the spectrum of *Candida* species in clinical infections
2) To ascertain the prevalence of biofilm producing isolates
3) To identify their sensitivity pattern to available antifungal agents.

Materials and Methods:

A total of 128 (One hundred and twenty eight) isolates of *Candida* obtained during the period from June 2013- May 2014 from different clinical specimens submitted to the Department of Microbiology were recruited for the study. Blood culture samples collected in blood culture bottles were incubated at 37^0C and were sub-cultured at 48 hrs, 5^{th} day and 10^{th} day onto Sabouraud dextrose agar (HiMedia, India) and blood agar plates. All other specimens were inoculated onto Sabouraud dextrose agar plates in addition to blood agar, chocolate agar and MacConkey agar (HiMedia, India). Identification was done by Wet mount, Gram staining, Germ tube formation [Fig2], colour detection on Hichrome agar [Fig 1], chlamydospore formation on Cornmeal agar [Fig 3, 4] and biochemical tests and confirmation on Vitek 2 system.

Biofilm production was observed on Sabouraud Dextrose Broth with 8% glucose by Test tube method. Biofilm formation was determined for all the isolates by using a method proposed by Branchini *et al*.[11] A loopful of organisms from the SDA plate was inoculated into a tube containing 10 ml Sabouraud's liquid medium supplemented with glucose (final concentration of 8%). The tubes were incubated at 37°C for 24 h after which the broth was aspirated out and the walls of the tubes were stained with safranin [Fig 5].

Antifungal susceptibility testing was performed by conventional method i.e Modified Kirby Bauer disc diffusion method as per CLSI guidelines. The study was done with prior approval from Institutional Ethical Committee.

Statistical analysis:

Data were analysed using the EPI Info 7 statistical software package. Chi-square test was used to see the association between two categorical variables. $P < 0.05$ was considered to be significant.

Results:

A total of 128 isolates of *Candida* species were obtained from different clinical specimens of patients visiting the Outpatient department (OPD) (6), patients admitted to In-Patient Department (IPD) (22) and Intensive Care Unit (ICU) (32) and patients diagnosed of malignancy (68) during the period from June 2013 – May 2014. Of these, 56 (43.75%) were from Blood, 28 (21.87%) from voided urine, 24 (18.75%) from urine of patients with in dwelling urinary catheter, 16 (16.67%) from Endotracheal Secretion and 4 (3.12%) from sputum. The distribution and percentage of different *Candida* species in these 128 isolates are given in [Table 1, Fig 6].

Accordingly, the species isolated were *C. albicans* [28 (21.87%)], *C. tropicalis* [40 (31.25%)], *C. parapsilosis* [10 (7.81%)], *C. glabrata* [34 (26.56%)], *C. krusei* [6 (4.68%)], *C. lusitaniae* [2 (1.56%)] and *C. keyfr* [8 (6.25%)] We observed that invasive candidiasis was more frequently caused by non-*albicans Candida* species as compared to *albicans.*

Resistance rates for amphotericin B (AMB), fluconazole 10 mcg(FLU), ketoconazole (KET), itraconazole (ITR), voriconazole (VOR) and miconazole (MIC) were 3.12%, 34.37%, 18.75%, 53.12%, 15.62% and 21.87% respectively. We observed that *C. albicans* was less resistant to all the drugs as compared to non-*albicans Candida* species [Table 2]. All the strains of *C. krusei* were found resistant to FLU.

Of the 28 isolates of C. albicans, only 12 (42.86%) were found to produce biofilms while out of the 100 isolates of Non-albicans Candida, 48 (48%) were able to form biofilms. There was found no significant correlation of Candida isolate with respect to biofilm production (p = 0.723). Amongst the Non albicans Candida, the highest incidence of biofilm production was found in C. glabrata [22 (64.70 %)] and C.

lusitanae [2 (100%)] [Table 3]. Our findings are consistent with the data published by Vinitha M *et al* [2011] [12]

The resistance rates to the different antifungal agents were also evaluated with respect to biofilm production by the different Candida isolates [Table 4]. The resistance rates of the antifungals were found to be a bit higher in the biofilm producing isolates compared to the non-biofilm producing isolates. The rate of Itraconazole resistance was found to be statistically significant (p= 0.0000000078) when compared with the biofilm production.

Discussion:

Infection represents a frequent complication among patients admitted to tertiary care hospitals. In particular, the incidence of candidiasis has been increasing during the past years. Infections with these yeasts also have a direct impact on the choice of empiric antifungal therapy and clinical outcome. Prior knowledge of species distribution in clinical isolates and drug sensitivity pattern among species help the clinician to choose early empirical therapy. In this study, we observed that non-*albicans Candida* species had predominance over *C. albicans*, which is consistent with the published report from different parts of the world. [12, 15] *C. tropicalis* and *C. glabrata* were the most common isolates in all samples, followed by *C. albicans.* Furthermore, invasive *Candida* infection was mostly caused by non-*albicans Candida,* whereas the C. *albicans* was found mostly in endotracheal aspirates and mucosal surfaces. *Candida* species differ in their susceptibility to antifungal agents. For instance, most *Candida* isolates tested were susceptible to Amphotericin B. However, *C. parapsilosis, C. glabrata* and *C. lusitanae* isolates showed reduced susceptibility to Itraconazole. Similarly, C. parapsilosis, C. keyfr and C. krusei showed reduced susceptibility to Fluconazole. In fact all *C. krusei* isolates were found resistant to FLU. Moreover, around 93% of the biofilm producing isolates were resistant to Itraconazole and 47% were frankly resistant to Fluconazole, which points towards the potential role of these virulence factors in the emergence of resistant strains. Kothari *et al*., [13] from North India reported the susceptibility profile of *Candida* isolates as 92% were sensitive to AMB, 36% to FLU, 24% to ITR and 56% to VOR whereas in another study from South India [14] showed 100% sensitivity to VOR, 92% to AMB and 75% to FLU. Reduced susceptibility as well as frank resistance to drugs such as azoles, as observed in our study, is an issue of crucial importance in treatment of immune compromised patients with serious infections. Hence, antifungal susceptibility testing and detection of biofilm production is a promising tool for predicting the efficacy of a given agent in different clinical isolates.

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Table 1 : Candida isolates from different clinical samples

Specimen (n)	Albicans (%)	Tropicalis (%)	Glabrata (%)	Parapsilosis (%)	Krusei (%)	Lusitanae (%)	Keyfr (%)
Blood (28)	8 (14.28%)	24 (42.86%)	16 (28.57%)	2 (3.57%)	4 (7.14%)	0	2 (3.57%)
Voided urine (14)	4 (14.28%)	12 (42.86%)	6 (21.43%)	2 (7.15%)	0	0	4 (14.28%)
Catheterised urine (12)	4 (16.67%)	4 (16.67%)	8 (33.33%)	6 (25%)	0	2 (8.33%)	0
Endotracheal aspirate (8)	12 (75%)	0	0	0	2 (12.5%)	0	2 (12.5%)
Sputum (2)	0	0	4 (100%)	0	0	0	0

Table 2 : Resistance rates of Candida isolates to different antifungal agents

	FLU	AMB	VOR	KET	ITR	MIC
C.albicans	28.57%	1.56%	3.12%	28.57%	15.62%	0%
C.tropicalis	30%	5%	10%	20%	30%	30%
C.glabrata	23.53%	0%	23.53%	11.76%	76.47%	35.29%
C.parapsilosis	60%	0%	20%	20%	60%	20%
C.lusitanae	0%	0%	0%	0%	100%	0%
C.keyfr	50%	0%	0%	0%	0%	0%
C.krusei	100%	0%	33.33%	33.33%	33.33%	33.33%

Table 3 : Biofilm production amongst different Candida isolates

Isolate	Total No.	Total no. of Biofilm producing isolates	% of Biofilm producing isolates
C. albicans	28	12	42.86%
C. tropicalis	40	12	30%
C. glabrata	34	22	64.70%
C. parapsilosis	10	6	60%
C. krusei	6	2	33.33%
C. keyfr	8	4	50%
C. lusitanae	2	2	100%
Grand Total	128	60	46.87%

Table 4: Resistance rates of antifungals compared to Biofilm production

Anti fungal Agent	Biofilm Positive		Biofilm Negative	
	Sensitive	Resistant	Sensitive	Resistant
Fluconazole	32	28	52	16
Amphotericin B	58	2	66	2
Voriconazole	44	16	64	4
Ketoconazole	40	20	64	4
Itraconazole	4	56	56	12
Miconazole	44	16	56	12

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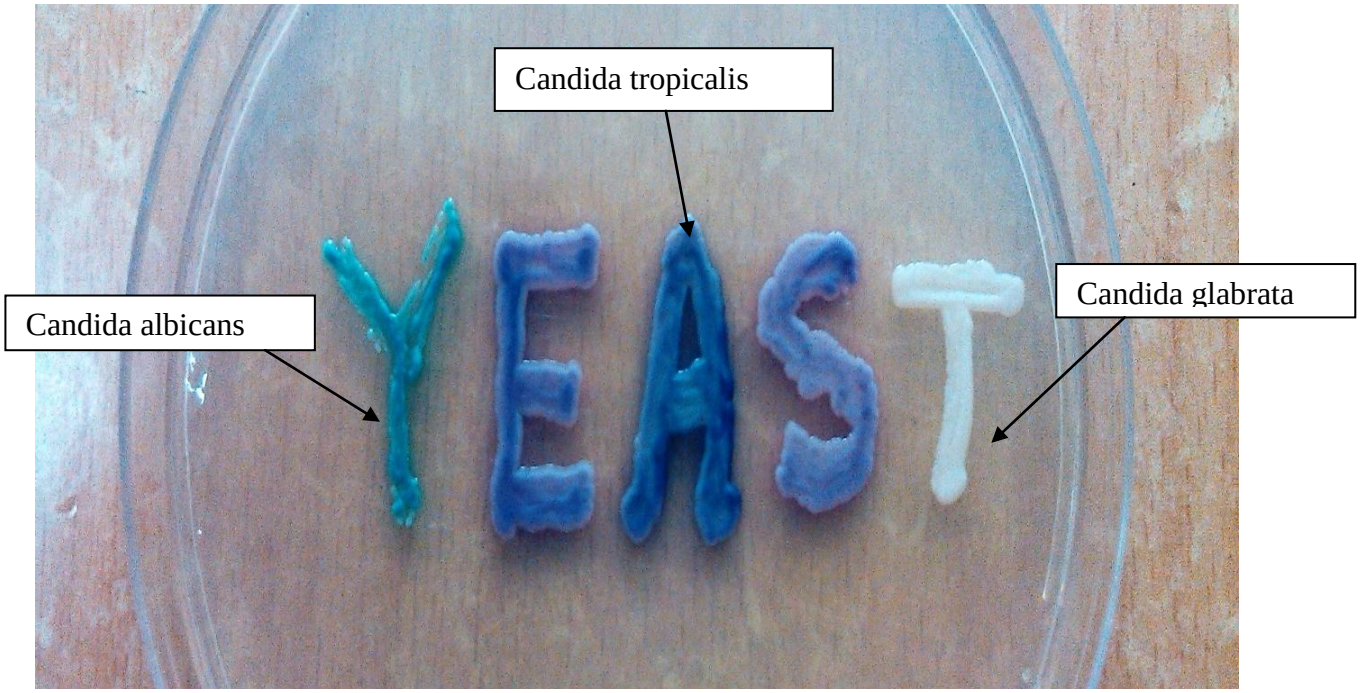

Fig 1 : Candida isolates on HiChrome Agar

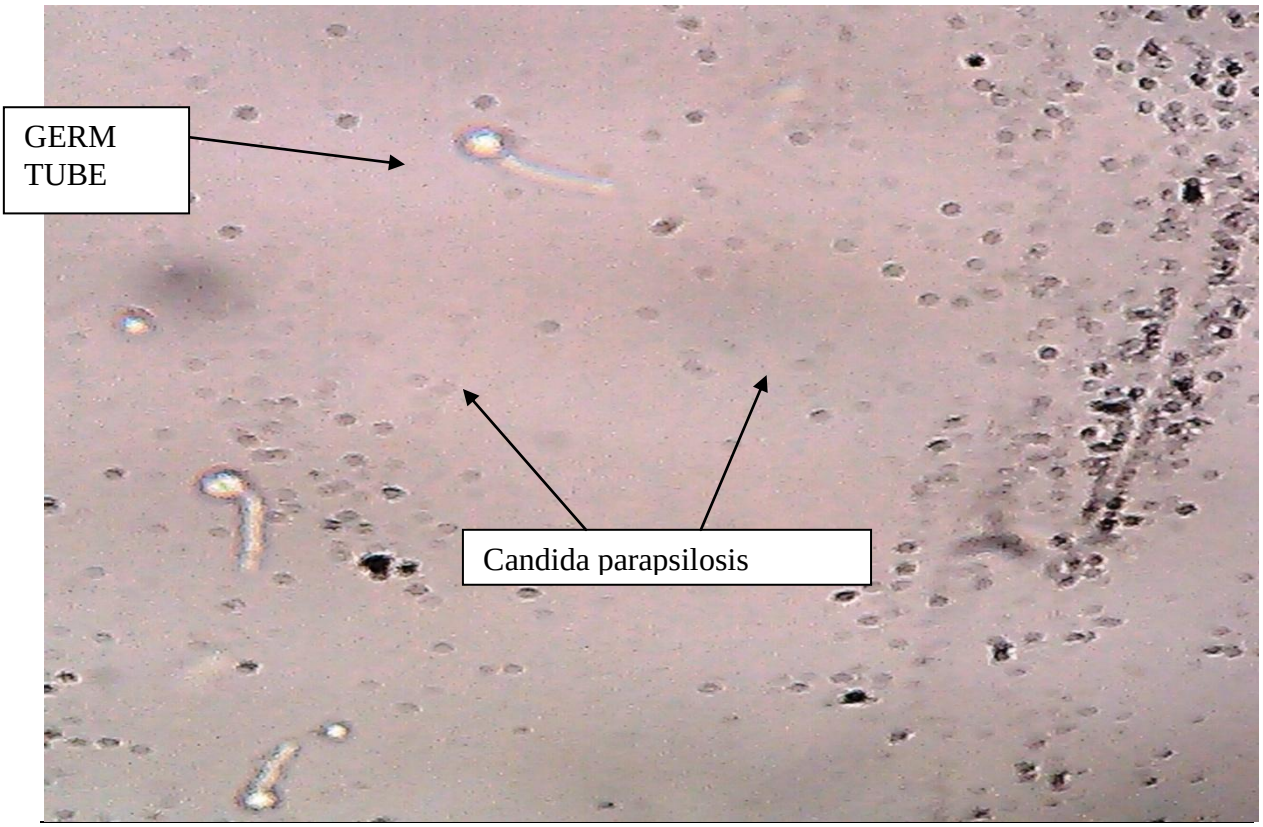

Fig 2 : Positive Germ Tube Test

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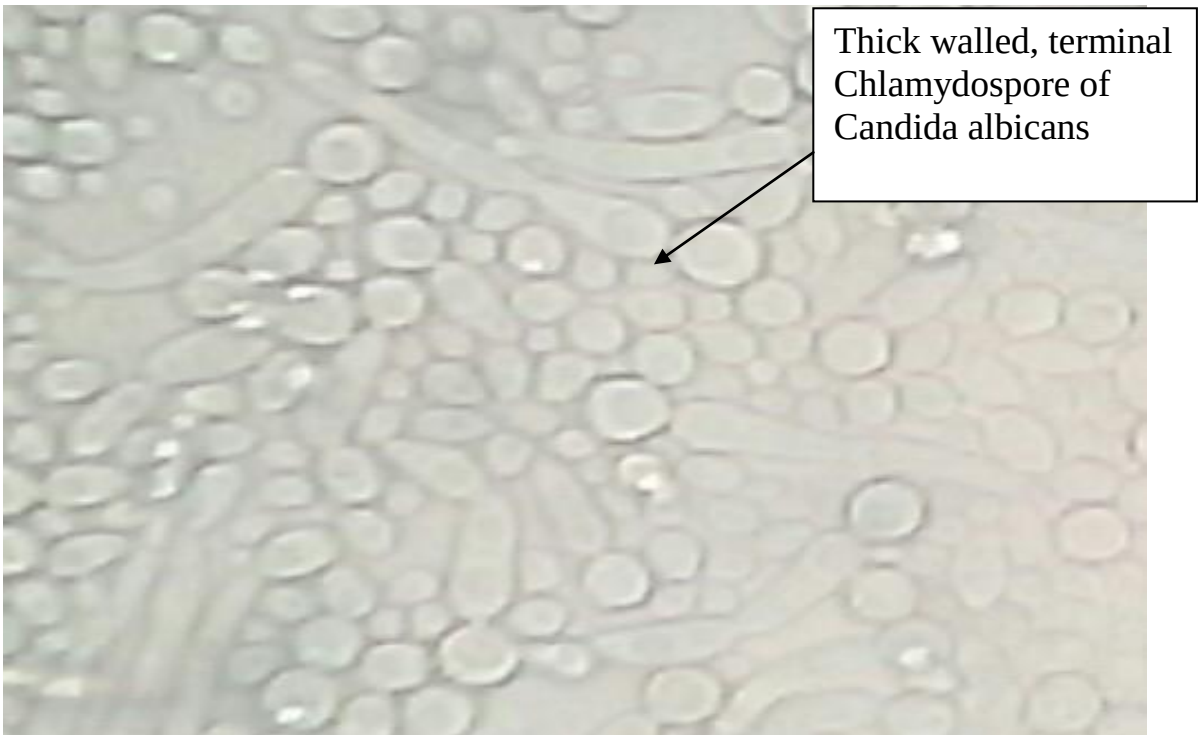

Fig 3 : Candida albicans on Corn Meal Agar

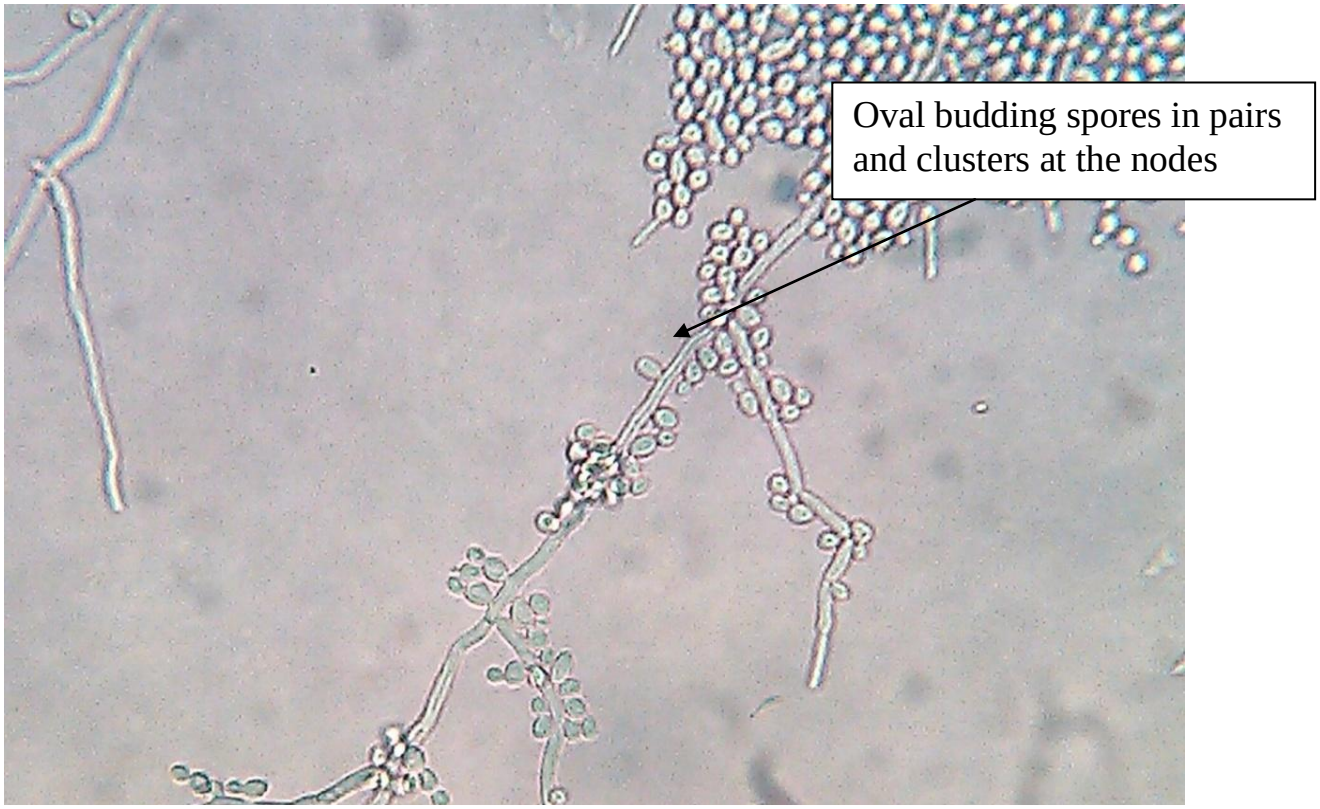

Fig 4 : Candida tropicalis on Corn Meal Agar

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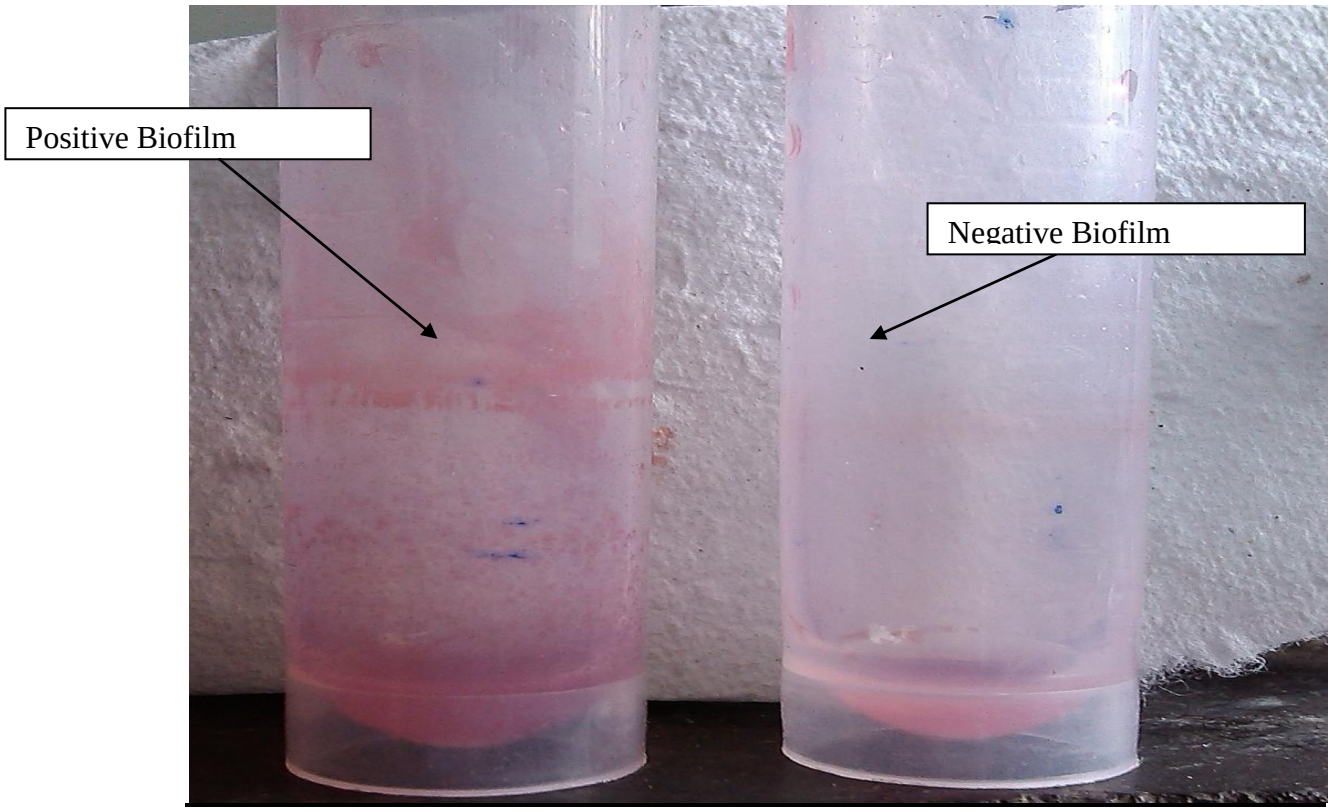

Fig 5 : Positive and Negative Biofilm production

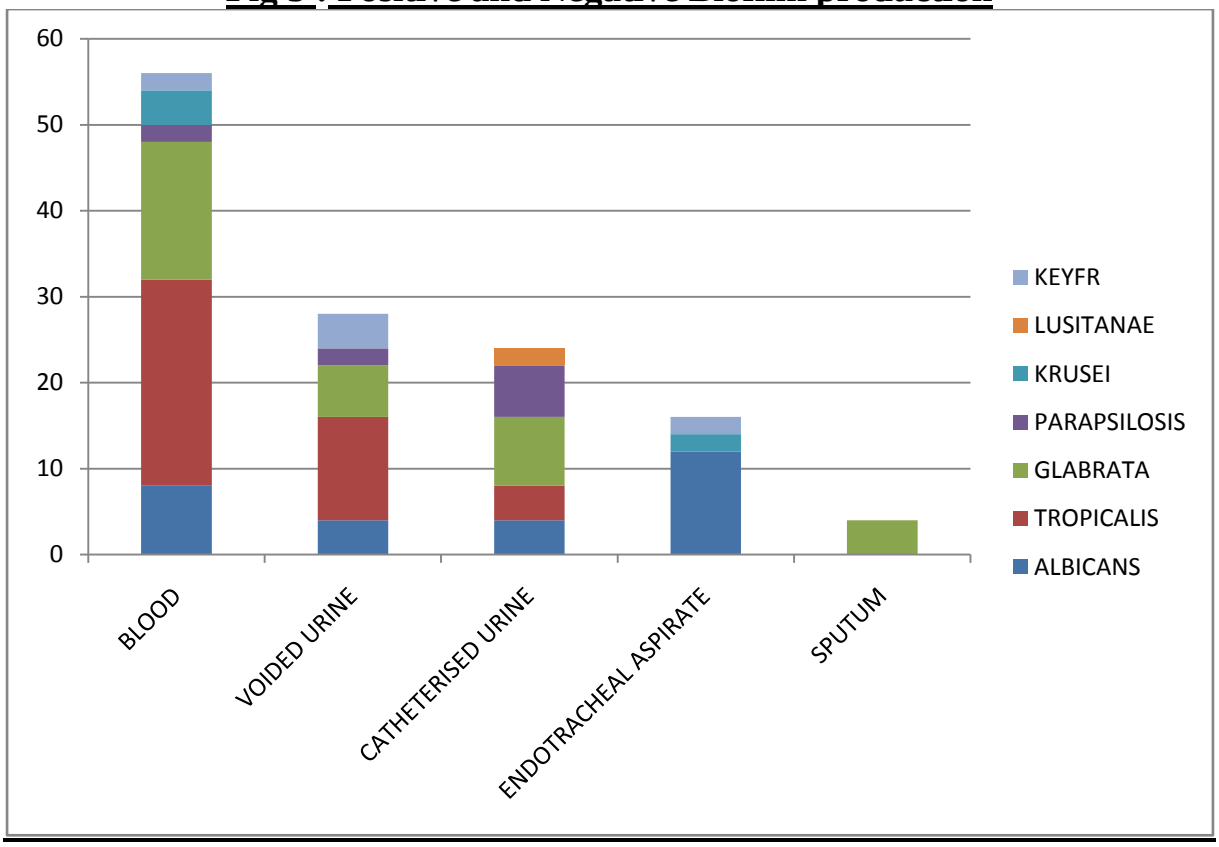

Fig 6 : Candida isolates from different clinical samples