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A Study of Candidal Biofilm in the Indwelling Devices

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I. Introduction

Indwelling Medical Devices (IMDs) are significant in patient care and due to their routine use in hospitals, critical patients easily become vulnerable to microbial colonization. Colonization of such devices by biofilm producing bacteria is a universal phenomenon and should not be ignored as it leads to resistant infections. A device-related infection (DRI) is an infection in a patient with a device (intravascular catheter, endotracheal tube or indwelling urinary catheter) that was in use for at least 48 hours before the onset of infection. Increased use of indwelling devices in hospitalized patient has increased the incidence of DRI, especially blood stream infections, originating from the microbial colonization of the intravascular catheter. Among DRI, common ones are catheter-related blood stream infections (CRBSI), followed by catheter associated urinary tract infections (CAUTI), and ventilator associated pneumonia (VAP), as seen in developed as well as developing countries.(1) Intravenous catheters are indispensable in modern medicine and are no longer restricted to hospital inpatients. There are a growing number of patients on 'home' intravenous therapy, predominantly for total parenteral nutrition or cancer chemotherapy. However, these devices are increasingly associated with sepsis and are now the commonest cause of all bloodstream infections. These infections cause significant morbidity and mortality. (2) Although the majority of implant infections are caused by gram-positive bacteria, mainly staphylococci, infections due to Gram-negative bacteria and fungi tend to be more serious. Fungal infections are most commonly caused by the pathogenic Candida species, particularly C. albicans, C. tropicalis and C. parapsilosis. These organisms are regarded as increasingly important nosocomial pathogens. (3) Candida organisms are commensals; and to act as pathogens, interruption of normal host defences is necessary. Therefore, risk factors for Candida infections include immune-compromised states, diabetes mellitus, and iatrogenic factors like antibiotic use, indwelling devices, intravenous drug use, and hyper alimentation fluids. Candida infection has emerged as an alarming opportunistic disease as there is an increase in number of patients who are immune-compromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation, requiring prolonged hospital stay. The virulence factors expressed by Candida species, to cause infections may vary depending on the type of infection, the site and stage of infection and the nature of the host response. One of the important factors contributing to the virulence of Candida is the formation of surface-attached microbial communities known as "biofilm". (4) Biofilm cells are organised into structured communities enclosed within a matrix of extracellular material. They are phenotypically different from planktonic or suspended cells; notably, they resist host defences and show a significantly decreased susceptibility to antimicrobial agents. (5) Infection with Candida , associated with indwelling medical devices can result in serious medical complications, expensive care and is noted as a frequent factor limiting the prolonged use of central venous catheters. (6) Urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilms on the inner or outer surfaces. The longer the urinary catheter remains in place, the greater is the tendency of these organisms to form biofilms and cause urinary tract infections. (7) Biofilm-associated organisms also show tolerance to antimicrobial agents. The implications of tolerance are that treatment of device associated infections with systemic antimicrobial agents is generally ineffective. (8) Until recently, C. albicans was by far the predominant species in most countries, causing up to two thirds of all cases of invasive Candida infection. However during recent decades, several countries around the world have witnessed a change in the epidemiology of Candida infections, characterized by a progressive shift from a predominance of Candida albicans to non-albicans Candida species(9). Biofilm production seems to be of more significance to non-albicans Candida than C. albicans. (10)

Aims and Objectives of study:

To study the biofilm forming capacity of Candida isolates from various indwelling devices and to speciate the Candida isolates obtained from the indwelling devices and detect the anti-fungal susceptibility pattern of these isolates. (Fluconazole, Flucytosine and Voriconazole, Caspofungin, Micafungin and Amphotericin-B) and to compare three different biofilm detection methods (CRA, TM, TCP), to detect the virulence factors of Candida species (Phospholipase, Proteinase, Biofilm)

Study Design:

Type of study: Descriptive study.

Sample size estimation: from reported cases of Candida infections of **5%.**
Taking p=5, and applying the formula n=4pq/d2, where in 'n' is sample size,
'p'=40,q=100-p, d is absolute allowable error= 5%.
By this formula our sample size to be taken is approximately 80.
Inclusion Criteria: Candida isolated from patients admitted in JSS hospital undergoing management with indwelling devices.
Exclusion criteria: Organisms other than Candida isolated from these indwelling devices.

Statistical methods applied

Both descriptive and inferential statistics were employed for data analysis.

Descriptive statistics

The Descriptive statistics procedure displays univariate summary statistics for several variables in a single table and calculates standardized values. Variables can be ordered by the size of their means alphabetically, or by the order in which the researcher selects the variables. In the present study following descriptive statistics have been employed

a. Frequencies
b. Percentages

Inferential statistics

Crosstabs (Cramer's V)

The Crosstabs procedure forms two-way and multi-way tables and provides a variety of tests and measures of association for two-way tables. The structure of the table and whether categories are ordered determine what test or measure to use. Cramer's V as a measure of association between rows and columns was employed.

II. Material & Methods

Patients admitted to JSS Hospital, with indwelling catheters, were included in the study over a period of two years from March 2013 to March 2015, after getting the ethical clearance from the Ethical Committee. A detailed history of these patients was taken specially with regard to the following - History of fever, Diabetes, Chronic infection, Smoking, Drug intake and Underlying conditions like immune compromised state, presence of indwelling devices. Samples were collected from IV catheter, Endotracheal aspirate, Urine. Samples were processed for Microscopy and Culture. Catheter and Endotracheal aspirates were used for microscopy. For Culture 5% sheep blood agar, Mac Conkey's agar, Sabouraud's dextrose agar with Chloramphenicol was used. All the samples were processed as per standard guidelines and both quantitative and semiquantitative analyses of samples were done. Intravenous catheter and Endotracheal aspirate was processed for semi quantitative culture and Urine from urinary catheter was processed for Quantitative culture. The organism was grown on Sabouraud's dextrose agar (SDA) at 37ºC for 24 hours.the organisms was inoculated in test tubes containing 2% carbohydrate solutions of dextrose, maltose, sucrose, and lactose. Andrade's indicator 0.005%, was used in all these carbohydrate solutions. Inverted durham's tubes are used for detection of gas. These tubes were incubated at 37ºC for 3-7 days. Growth of yeast around individual discs indicates assimilation of that compound. When the carbohydrate is not utilized, growth around disc is absent. Species differentiation was done on the basis of combined pattern of Carbohydrate fermentation test and Carbohydrate assimilation test according to a standard chart. Virulence testing of the following was also done as per the standard guidelines

1. Production of germ tube
2. Phospholipase estimation
3. Proteinase estimation
4. Adherence assay

Production of germ Tube - Strains of C.albicans produce germ tubes from their yeast cells when placed in a liquid enviornment and incubated at 37ºC for 1-2 hours. It is also known as Reynolds Braude phenomenon. Protinase test Protein substance (Bovine Serum Albumin) markedly influences the production of extracellular proteases by Candida species .Candida species when grown in a medium containing Bovine Serum Albumin causes hydrolysis of the substrate and hence proteolysis beyond the hinge of colonies which is visible as a clear halo. Adherence assay

Candida species when inoculated into SDB (Subourauds dextrose broth) medium that contains high glucose (8%) and protein(1%); adheres to the polystyrene tubes and plates and form biofilms.

Tissue Culture Method

Isolates from freshly sub-cultured plates were inoculated in Subarouds dextrose broth (SDB) with 8% w/v glucose and incubated for 24 hours at 37˚C in stationary conditions and then diluted to 1:100 with fresh SDB. Individual wells of sterile polystyrene 96 well flat bottom microtitre plates were filled with 200µl aliquots of diluted culture. Un-inoculated SDB served as a control to check sterility and nonspecific binding of media. Control strains were also inoculated in triplicate. The microtitre plate was incubated for 48 hours at 37˚C. After incubation contents of each well was removed by tapping the plates. After washing the wells for four times with 200µl of phosphate buffer saline (PBS pH 7.2), the floating planktonic bacteria were removed. The biofilms thus formed in plates were fixed using 2% w/v sodium acetate for 10 minutes and tainted with 0.1% w/v crystal violet for 10minutes. After washing thoroughly with de- ionized water to remove any excess stain, the plates were dried. Micro-ELISA auto-reader at the wavelength of 540 nm was used to measure the Optical Density (OD) of the stained adherent micro-organisms.(12,13,14) The OD540 value of sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD540 value from a control well was deducted from all test OD540 values. These OD540 values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate.

Congo Red Agar Method (CRA): Freeman et al (15) had described an alternative method of screening biofilm formation by Candida isolates. In the present study the Congo red agar (CRA) was optimized to get strong black pigmentation at 48hrs incubation and then for 2-4 days room temperature. Black coloured colonies with dry crystalline consistency interpreted as positive biofilm producing strains. Red coloured colonies-interpreted as negative for biofilm production.

The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods.(Parameters like sensitivity, specificity, negative predictive value, positive predictive value were calculated.(16) For flucytosine, isolates showing MIC's ≤4µg/ml were considered as susceptible, 8- 16µg/ml as intermediate and ≥32µg/ml as resistant. For amphotericin B, isolates showing a MIC of ≤1.0µg/ml were taken as susceptible and those with MIC>1µg/ml were considered as resistant.10, 11 MIC interpretative criteria were referred to those described in the CLSI document M 27-S3 for amphotericin B, 5-flucytosine

III. Results:

The results are in the tabular form as under

The samples collected from patients belonged to the wide range of age groups of 18 and above. It was found that maximum number of samples which yielded Candida, belonged to age group 31-45 years (41.3%), followed by 18-30 years (23.8%), and 46-60 years (22.5%) and more than 60 years (12.5%)

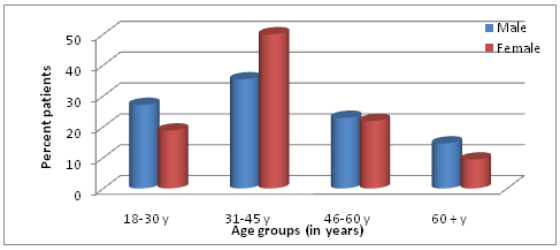
AGE GROUPS (YEARS)	Frequency (f)	%
18-30	19	23.8
31-45	33	41.3
46-60	18	22.5
>60	10	12.5
Total	80	100.0

TABLE 1: Age Wise Distribution of the Samples Collected (N=80)

TABLE 3: Age and sex distribution:

AGE (YEARS)	Frequency(f)	SEX		Total
	%	M	F	
18-30	**F**	13	6	19
	%	27.1%	18.8%	23.8%
31-45	**F**	17	16	33
	%	35.4%	50.0%	41.3%
46-60	**f**	11	7	18
	%	22.9%	21.9%	22.5%
>60	**F**	7	3	10
	%	14.6%	9.4%	12.5%
Total	**F**	48	32	80
	%	100.0%	100.0%	100.0%

Graph 1:



The male female sex ratio in our patients is 3:2. There is male predominance in all the age groups.

Table 4 : Duration Of Stay In Hospital

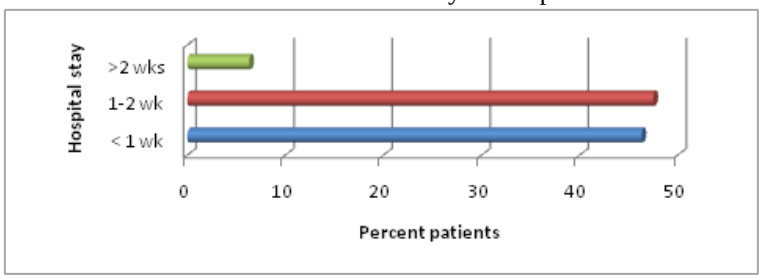


TABLE 4 shows that most of the patients in our study were admitted for an average 1-2 weeks (47.5%), followed by <1week (46.3%), and only 5 patients were admitted in the hospital for more than 2 weeks (6.3%).

Table 5:Co-Morbid Conditions In The Study Group:

	Number	**%**
SURGICAL	49	61.3
MEDICAL	25	31.3
TRAUMA	6	7.5
TOTAL	80	100.0

Table 5,shows that among the 80 patients in our study, 49 patients were surgical cases, 25 patients had medical co-morbid conditions, and 6were trauma cases.

Table 6:Associated Risk Factors In The Study Group:

RISK FACTORS	NUMBER	%
INDWELLING DEVICE	80	100
HTN	39	48.8
SMOKING	33	41.3
DM	36	45

Table 6 shows that all the patients in our study were associated with some indwelling devices, 48.8% were hypertensives, 41.3% were smokers, and 45% were diabetics.

Table 7: Haematological Parameters In The Study Group::

	CRITERIA	FREQUENCY	PERCENTAGE(%0
HB	**<10gm/dl**	50	62.5
TLC	**>11000 / cumm**	54	67.5
LYMPHOCYTES	**>40%**	4	5.0
NEUTROPHILS	**>70%**	41	51.3

Table7, shows that in our study 62.5% patients were anaemic, 67.5% patients had TLC count more than >11,000, 5% patients had lymphocytosis,and 51.3% had neutrohils >70%

Table 8:Ward Wise Distribution Of The Samples:

DEPARTMENT	**FREQUENCY (f)**	**%**
TCW	2	2.5
MICU	10	12.5
RICU	11	13.8
NEPHROLOGY	3	3.8
MEDICINE	13	16.3
ICCU	4	5.0
UROLOGY	1	1.3
NEUROSURGERY ICU	16	20.0
SURGERY	7	8.8
SICU	12	15.0
PULMONOLOGY	1	1.3

Total	80	100.0

Graph 3:

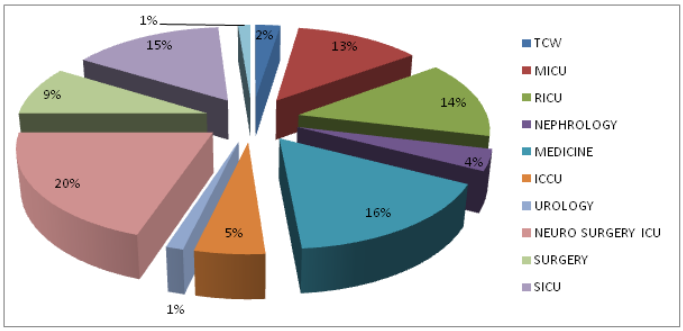


Table 8, and Graph 3, shows that maximum number of samples were received from Neurosurgery ICU (17), and minimum from Pulmonology ward (1).

Table 9 shows that total of 850 indwelling devices, samples received during the course of study. Out of these, 500 were from urine from Catheterised patients, 200 ET secretion, 150 from intravascular catheter tips.

Table 9:

Samples	Total Number	Culture Positive	Culture Negative	Candida Positives (Out Of All Culture Positives)	
Urine From Catheterised Patients	500	400	32	Number	%
				68	17.0%
Et Secretions	200	170	20	10	5.88%
I.V. Catheter Tips	150	68	80	2	2.94
Total	850	638	132	80	25.82

Table 10, shows that ,68 urine samples from catheterised patients (85%), 10 Endotracheal tubes secretions (12.5%), and 2 , I.V catheter tips (2.5%) were Candida culture positive

Table 10: Samples Collected From The Study Group:

SAMPLE	**NUMBER**	**%**
URINE	68	85.0
ET SECRETION	10	12.5
I.V.CATHETER TIP	2	2.5
Total	80	100.0

Table 11, and Graph 4, shows that in our study Candida tropicalis (46.3%) was the predominant isolate , followed by C.albicans (28.8%), C.famata (11.3%), C.parapsillosis (5%), C.glabrata (3.8%), C.krusei (2.5%), and C.lusitaneae (2.5%)

Table 11 : Candida Species Isolated In Our Study:

Species	**Number**	**%**
C.famata	9	11.3
C.tropicalis	37	46.3
C.albicans	23	28.8
C.glabrata	3	3.8
C.parapsillosis	4	5.0
C.krusei	2	2.5
C.lusitaneae	2	2.5
Total	80	100.0

Graph 4:

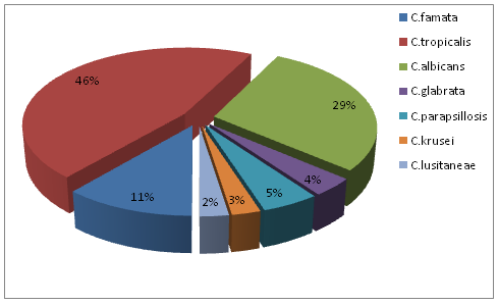


Table 12 and Graph 5 ,shows 57% Germ tube negative isolates ,which were confirmed to be Non-albicans Candida. And 29% germ tube positive isolates, which were confirmed as Candida albicans .

Table 12 : Germ Tube Positivity Among Candida Spp.:

Result	Frequency (F)	%
Negative	57	71.3
Positive	23	28.8
Total	80	100.0

Graph 5:

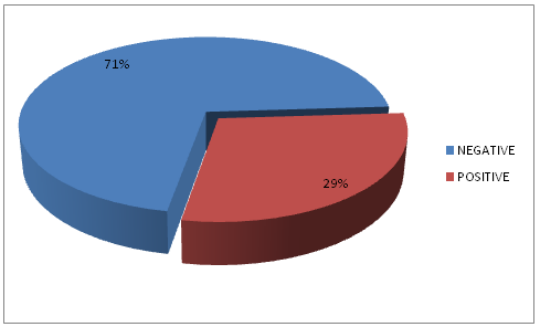


Table 13:Phopholipase Assay:

VALUES(P_z)	Frequency(f)	%
<0.7	9	11.3
0.71-1	10	12.5
>1	61	76.3
Total	80	100.0

- Phospholipase zone is the ratio of colony diameter and diameter of the white zone of precipitation around phospholipase positive colonies.
- P_z <1.00 +ve
- P_z <0.7 -more virulent.

Graph 6 :

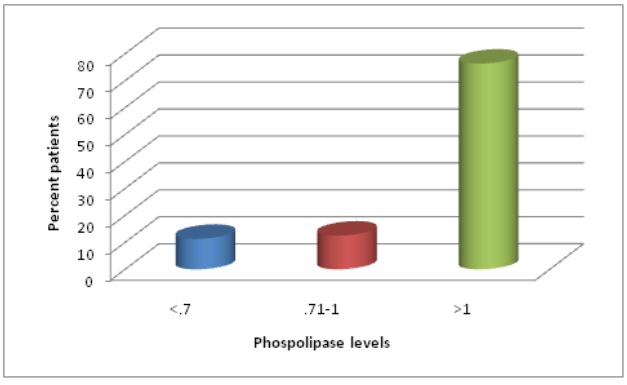


Table 13 and Graph 6, shows that 80 isolates of Candida 76.3% showed no phospholipase activity, 12.5% were positive for phospholipase, and 11.3 % were more virulent.

Table 14: Phospholipase Assay In Reference To Candida Species

Candida species	Frequency (f)	PHOSPHOLIPASE VALUE			Total
	% of Candida	<0.7	0.71-1	>1	
C.famata	F	0	0	9	9

	%	.0%	.0%	100.0%	100.0%
C.tropicalis	F	0	0	37	37
	%	.0%	.0%	100.0%	100.0%
C.albicans	F	9	10	4	23
	%	39.1%	43.5%	17.4%	100.0%
C.glabrata	F	0	0	3	3
	%	.0%	.0%	100.0%	100.0%
C.parapsillosis	F	0	0	4	4
	%	.0%	.0%	100.0%	100.0%
C.krusei	F	0	0	2	2
	%	.0%	.0%	100.0%	100.0%
C.lusitaneae	F	0	0	2	2
	%	.0%	.0%	100.0%	100.0%
Total	F	9	10	61	80
	%	11.3%	12.5%	76.3%	100.0%

According to the Table 14,only Candida albicans showed phospholipase activity (9+10),i.e.82.60% of all C.albicans.where as all non-albicans candida were negative for phospholipase activity.

Graph 7:.

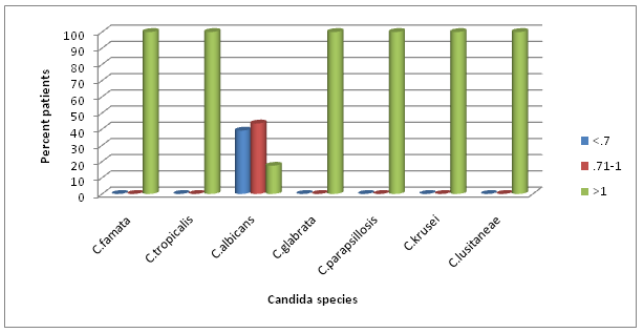
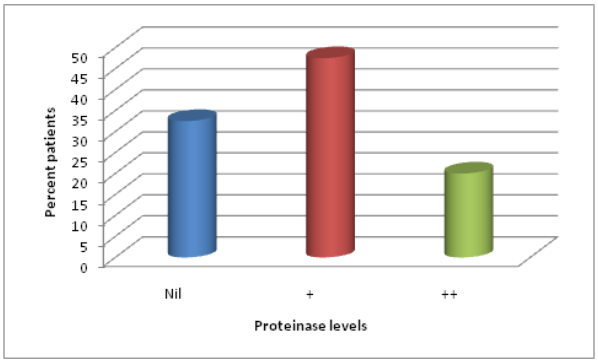


Table14 and Graph 7, shows that in our studies only C.albicans showed phospholipase activity , none of the other species showed phospholipase enzyme production

Table 15:Proteinase Assay:

RESULT	Frequency (f)	%
Nil	26	32.5
+	38	47.5
++	16	20.0
Total	80	100.0

Graph 8:



NIL- when no visible halo is present
(+) when visible proteolysis is limited to 1-2 mm around the colony.
(++) when the zone of proteolysis is > 2 mm from the margin of the colony

Table 15,and Graph 8, shows that in our study proteinase activity was seen in 67.5% (47.5+20.0)of patients isolates . Rest 32.5% were negative for proteinase activity.

Table 16 :Proteinase activity with reference to Candida spp.

Species	Frequency (f)	PROTEINASE ACTIVITY			Total
	% of Candida	Nil	+	++	
C.famata	f	5	3	1	9
	%	55.6%	33.3%	11.1%	100.0%

C.tropicalis	f	11	22	4	37
	%	29.7%	59.5%	10.8%	100.0%
C.albicans	f	8	6	9	23
	%	34.8%	26.1%	39.1%	100.0%
C.glabrata	f	2	1	0	3
	%	66.7%	33.3%	.0%	100.0%
C.parapsillosis	f	0	3	1	4
	%	.0%	75.0%	25.0%	100.0%
C.krusei	f	0	1	1	2
	%	.0%	50.0%	50.0%	100.0%
C.lusitaneae	f	0	2	0	2
	%	.0%	100.0%	.0%	100.0%
Total	f	26	38	16	80
	%	32.5%	47.5%	20.0%	100.0%

Graph 9:

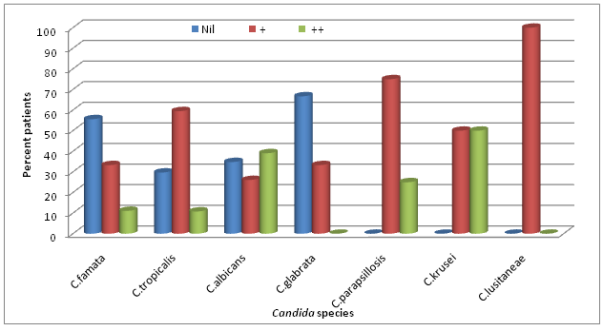


Table 16 and Graph 9, showed the presence of proteinase activity in C.albicans (65.21%), C.tropicalis 70.20%(22+4), C.glabrata 33.3%, C.parapsillosis 100%, C.krusei and C.lusitaneae 100%, which says proteinase activity was more in Non-albicans Candida as compared to Candida albicans

Table 17 :Biofilm Detection By Congo Red Agar (Cra) Method:

Result	Number	%
Negative/Weekly +Ve	72	90.0
Positive	8	10.0
Total	80	100.0

Graph 10:

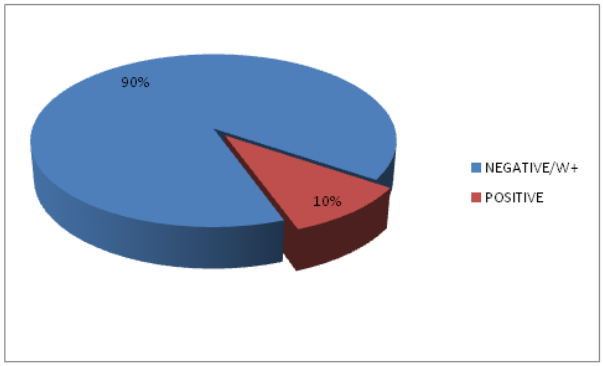


Table 17, and Graph 10, shows that in CRA method of biofilm detection 10% were positive , 2.5% were weekly positive and 87.5% were negative for biofilm formation.

Table 18 : CRA method with reference to Candida species

	Frequency (f)	CRA		Total
	% of Candida	NEGATIVE/WEEKLY+VE	POSITIVE	
C.famata	F	8	1	9
	%	88.9%	11.1%	100.0%
C.tropicalis	F	34	3	37
	%	91.9%	8.1%	100.0%
C.albicans	F	19	4	23
	%	82.6%	17.4%	100.0%
C.glabrata	F	3	0	3

	%	100.0%	.0%	100.0%
C.parapsillosis	F	4	0	4
	%	100.0%	.0%	100.0%
C.krusei	F	2	0	2
	%	100.0%	.0%	100.0%
C.lusitaneae	F	2	0	2
	%	100.0%	.0%	100.0%
Total	F	72	8	80
	%	90.0%	10.0%	100.0%

Graph 11:

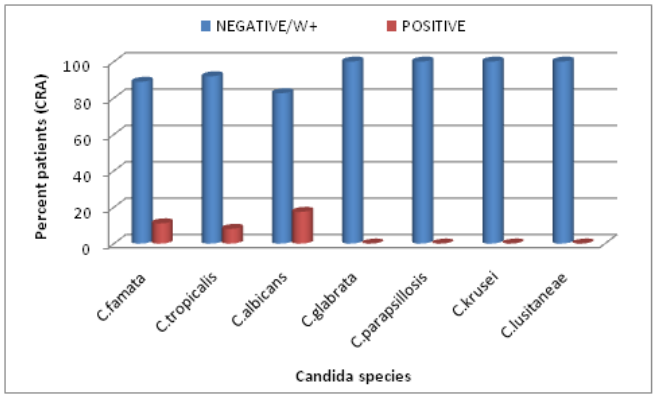


Table 18, and graph 11,shows that by CRA method only 3 (8.1%) of C.tropicalis , and C.albicans 4(17.4%) were positive. other non albicans Candida spp. were negative for biofilm production

Table 19 :Biofilm Detection By Tube Method(Tm):

Result	Frequency (F)	%
NEGATIVE / WEEKLY +VE	35	43.8
STRONG +VE	23	28.8
MODERATE +VE	22	27.5
Total	80	100.0

Graph 12:

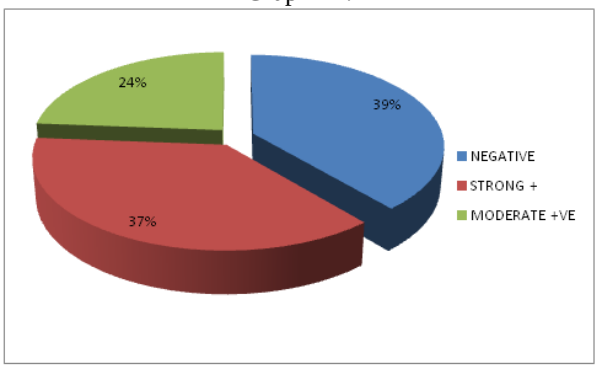


Table 20 : Biofilm by TM method with reference to Candida spp.

Species	Frequency (F) % Of Candida	TM NEGATIVE/WEEKLY +VE	 STRONG +VE	 MODERATE +VE	Total
C.Famata	F	4	4	1	9
	%	44.4%	44.4%	11.1%	100.0%
C.Tropicalis	F	13	13	11	37
	%	35.1%	35.1%	29.7%	100.0%
C.Albicans	F	12	6	5	23
	%	52.2%	26.1%	21.7%	100.0%
C.Glabrata	F	2	0	1	3
	%	66.7%	.0%	33.3%	100.0%
C.Parapsillosi	F	3	0	1	4

s	%	75.0%	.0%	25.0%	100.0%
C.Krusei	F	0	0	2	2
	%	.0%	.0%	100.0%	100.0%
C.Lusitaneae	F	1	0	1	2
	%	50.0%	.0%	50.0%	100.0%
Total	F	35	23	22	80
	%	43.8%	28.8%	27.5%	100.0%

Table 19 & 20, and Graph 13 ,shows 56.3% (28.8%+ 27.5%) isolates were positive for biofilm formation, where as 43.8% were non biofilm forming Candida. Candida albicans showed 47.8% positivity.

Graph 13:

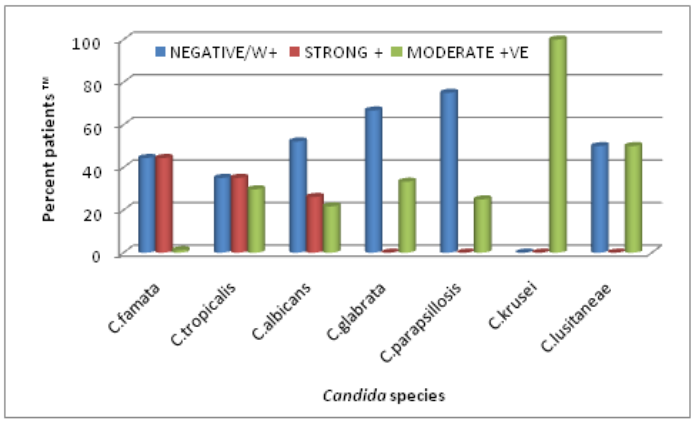


Table 21 :Biofilm Detection By Tissue Culture Plate (Tcp) Method:

Result	Number	%
NEGATIVE	31	38.8
STRONG +	30	37.5
MODERATE +VE	19	23.8
Total	80	100.0

Graph 14:

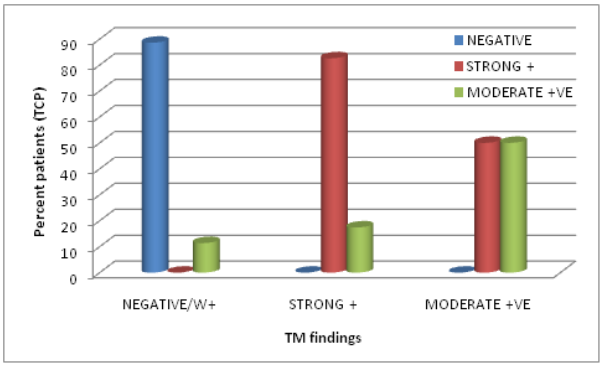
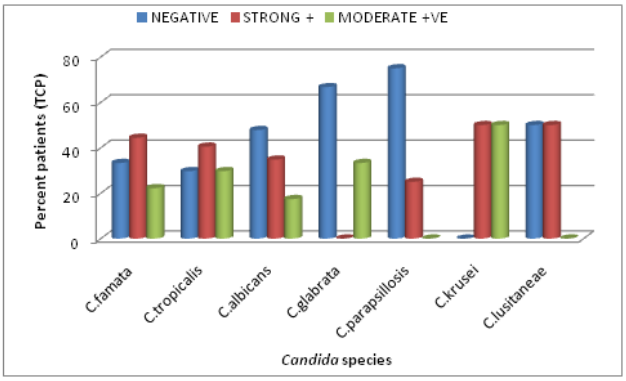


Table 22: TCP method with reference to Candida species

Candida species	Frequency (f)	TCP			Total
	% of Candida	NEGATIVE	STRONG +VE	MODERATE +VE	
C.famata	f	3	4	2	9
	%	33.3%	44.4%	22.2%	100.0%
C.tropicalis	f	11	15	11	37
	%	29.7%	40.5%	29.7%	100.0%
C.albicans	f	11	8	4	23
	%	47.8%	34.8%	17.4%	100.0%
C.glabrata	f	2	0	1	3
	%	66.7%	.0%	33.3%	100.0%
C.parapsillosis	f	3	1	0	4
	%	75.0%	25.0%	.0%	100.0%
C.krusei	f	0	1	1	2
	%	.0%	50.0%	50.0%	100.0%
C.lusitaneae	f	1	1	0	2
	%	50.0%	50.0%	.0%	100.0%

TOTAL	f	31	30	19	80
	%	38.8%	37.5%	23.8%	100.0%

Graph 14:



According to the table 21 & 22 and graph 13 & 14, 61.3% of Candida isolates were biofilm positive by TCP method, and 38.7% were negative by this method

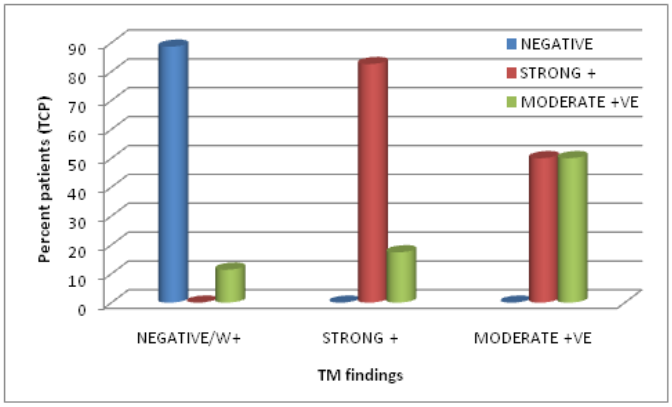
Table: 23comparison Of Tm And Tcp:

		Frequency (F)	TCP			Total
		% Of Candida	NEGATIVE	STRONG +VE	MODERATE +VE	
TM	NEGATIVE/WEEKLY +VE	F	31	0	4	35
		%	88.6%	.0%	11.4%	100.0%
	STRONG +VE	F	0	19	4	23
		%	.0%	82.6%	17.4%	100.0%
	MODERATE +VE	F	0	11	11	22
		%	.0%	50.0%	50.0%	100.0%
Total		F	31	30	19	80
		%	38.8%	37.5%	23.8%	100.0%
Test Statistics: CV=0.686, P=0.000						

TP (a) =45
FN (b)= 4
FP (c)=0
TN (d)=31

1. Sensitivity of TM = a /a+b =TP/TP+FN
   At 95% Confidence interval (CI) = 80.40 to 97.73%
2. Specificity of TM =d/c+d= TN/ FP+TN
   At 95% Confidence interval (CI)= 88.78 % to 100.00%
3. Positive Predictive Value (PPV) = a/a+c = TP/TP+FP
   At 95% Confidence interval (CI)=92.13% to 100 %
4. Negative Predictive Value (NPV)=d/b+d =TN/TN+FN
   At 95% Confidence interval (CI)=73.26% to 96.80 %

Graph 15:



The above Table 23 and graph 15, shows P value of 0.000 between TM and TCP methods, which shows a high significance and states that TM is highly sensitive and equally good method as TCP.

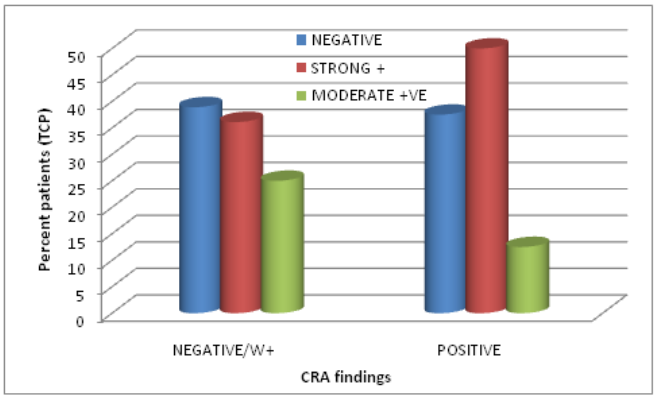
Table 24:Comparison Of Cra With Tcp

	Result	Frequency (f)	TCP			Total
		% of Candida	NEGATIVE	STRONG +	MODERATE +VE	
CRA	NEGATIVE/W+	f	28	26	18	72
		%	38.9%	36.1%	25.0%	100.0%
	POSITIVE	f	3	4	1	8
		%	37.5%	50.0%	12.5%	100.0%
Total		f	31	30	19	80
		%	38.8%	37.5%	23.8%	100.0%
Test statistics CV=0.103, P=0.655						

TP (a) =05
FN (b)= 44
FP (c)=03
TN (d)=28

1. Sensitivity of TM = a /a+b =TP/TP+FN
   At 95% Confidence interval (CI) = 3.40% to 22.23%
2. Specificity of TM =d/c+d= TN/ FP+TN
   At 95% Confidence interval (CI)= 74.25% to 97.96%
3. Positive Predictive Value (PPV) = a/a+c = TP/TP+FP
   At 95% Confidence interval (CI)=24.49% to 91.48 %
4. Negative Predictive Value (NPV)=d/b+d =TN/TN+FN
   At 95% Confidence interval (CI)=38.89% to 27.62 %

Graph 16:



The above Table 24,and Graph 16, shows P value of 0.655 between CRA and TCP methods, which shows a poor significance and states that CRA is not as good method as TCP and so should not be used in place of TCP method in biofilm detection.

Anti-fungal susceptibility :in VITEK–2 compact systems

Table 24:Resistance pattern of Biofilm producing Candida(%)

		Anti fungals						
Candida spp.	**Total isolates**	**Fluconazole**	**Flucytosine**	**Variconazole**	**Caspofungin**	**Amphotericin**	**Micafungin**	
		n (%)	**n (%)**	**n (%)**	**n (%)**	**Bn (%)**	**n (%)**	
C.albicans	11	07 (63.63)	08 (72.72)	02 (18.18)	02 (18.18)	01 (9.09)	02 (18.18)	
C. tropicalis	26	20 (74.07)	20 (76.92)	08 (29.62)	01 (3.70)	03 (11.11)	01 (3.70)	
C.famata	06	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	
C.parapsillosis	01	01 (100)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	
C.glabrata.	01	01 (100)	01 (100)	00 (00)	00 (00)	00 (00)	00 (00)	
C.krusei.	02	02 (100)	00 (00)	00 (00)	00 (00)	01 (50)	00 (00)	
C.lusitaneae	01	00 (00)	00 (00)	00 (00)	01 (100)	00 (00)	01 (100)	

On observation the resistance pattern among biofilm producing C. albicans, more than 60% and 70% are resistance to Fluconazole and Flucytosine while almost 20% are resistance to Voriconazole and Caspofungin. The resistance for the same antifungal was seen more in C. tropicalis where almost 75%, 80% and 30% resistance was seen to Fluconazole, Flucytosine and Voriconazole but in case of Caspofungin the resistance was quite low . The antifungal susceptibility results showed highest resistance to Fluconazole , Flucytosine and Voriconazole although Caspofungin, Micafungin and Amp-B showed good efficacy.

Table 25: Resistance pattern of Non-Biofilm producing Candida(%)

Candida spp.	**Total isolates**	**Fluconazole**	**Flucytosine**	**Variconazole**	**Caspofungin**	**Amphotericin**	**Micafungin**	
		n (%)	**n (%)**	**n (%)**	**n (%)**	**Bn (%)**	**n (%)**	
C.albicans	12	2 (18)	3(27)	00 (00)	00 (00)	00 (00)	00 (00)	
C. tropicalis	11	4 (36)	3(27)	01 (09)	01 (09)	01 (09)	01 (09)	
C.famata	03	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	
C.parapsillosis	03	1 (33.3)	1 (33.3)	00 (00)	00 (00)	00 (00)	00 (00)	
C.glabrata.	02	1 (50)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	
C.krusei.	00	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	
C.lusitaneae	01	00 (00)	01 (00)	00 (00)	00 (00)	00 (00)	00 (00)	

The resistance pattern among non- biofilm producing C. albicans, more than 18% and 27% are resistance to Fluconazole and Flucytosine while no resistance seen to Voriconazole and Caspofungin. The resistance for the same antifungal was seen more in C. tropicalis where almost 36%, 27% resistance was seen to Fluconazole, Flucytosine but in case of Caspofungin ,Micafungin and voriconazolethe resistance was quite low (9%). This shows that resistance to most commonly used antifungal drugs was more in case of biofilm producing Candida as compared to non biofilm producers.

IV. Summary And Discussion:

Biofilms are studied in a wide range of scientific disciplines including biomedicine, water engineering, and evolutionary biology . Biofilms are the most common mode of bacterial growth in nature and are also important in clinical infections, especially due to the high antibiotic resistance associated with them. In contrast to the extensive literature describing bacterial biofilms , little attention has been paid to medically relevant fungal biofilms . Biofilm is of particular significance, since it is now estimated that a significant proportion of all human microbial infections involve biofilm formation. Transplantation procedure, immunosuppression, the use of chronic indwelling devices, and prolonged intensive care unit stay increased the prevalence of fungal disease. Fungi most commonly associated with such disease are in the genus Candida.(26) Trauma patients often face an abrupt disruption of epithelial barriers allowing deepseated contamination from various pathogens either from the environment or from their own flora, such as Candida spp. Also, established risk factors for the development of candidiasis such as central venous catheters (CVC),total parenteral nutrition (TPN) and antibiotic therapy are inevitable in this particular population.(18) Candida produces large quantities of viscid slimy material in glucose containing solutions. The ability to form extensive biofilm on catheters and other prosthetic devices, also contribute to the prevalence of this organism as an aetiological agent of intravascular nosocomial infection. Therefore, study of biofilm production of Candida is both interesting as well as a clinically important exercise.(10) A total of 80 patients samples including indwelling devices, blood and urine were collected and processed. Majority of our samples were urine sample from catheterized patients, followed by ET secretion and I.V. catheter tips. These were mainly collected from ICU'S and wards. In this study we got 66.25% of cases from different ICUs which was comparatively more than general wards 33.75%. And most of the patients had hospital stay for 1-2 weeks followed by <1 week. As, most of the patients were from ICU's with average stay of 1-2 weeks, the Health care workers have to emphasise more .In the present study, all the isolates were finally confirmed by performing germ tube test , chlamydospore formation, sugar assimilation and

fermentation reactions. Sugar assimilation and fermentation tests are therefore considered to be important in identifying Candida species especially non-albicans Candida, but it is a tedious procedure.

Germ tubes ,which mark the onset of hyphal growth, and are induced by contact with serum ,are particularly involved in the pathogenesis of Candida infection. The formation of germ tube is accompanied by an increased adherence to epithelial cells . In our study, germ tube formation was observed in 28.8% of Candida strains , which were identified as C.albicans, whereasremaining strains (71.3%) failed to produce germ tube(NAC).

A study by Alhussaini M S et al (2013), observed germ tube production in 27/50 (54%) of Candida strains, which were identified as C.albicans and remaining strains failed to produce germ tube which were identified as non albicans Candida(NAC).(19) The study performed by Mohammadi P et al. (2012) also concluded that Glucose, Galactose, Maltose &Trehalose were used by all the yeasts as far as the identification of Candida species. are concerned which was close to our study.(20) Kumar A et al. (2014) also observed from their study that most species of

Candida assimilated glucose, galactose, maltose, trehalose and sucrose,the finding which was also at par with our study.(21) Among 80 isolates from all the indwelling devices and urine Candida tropicalis (46.3%), followed by C.albicans (28.8%), C.famata (11.3%), C.parapsillosis (5%), C.glabrata (3.8%), C.krusei (2.5%), and C.lusitaneae (2.5%) . that is, Non -albicans candida were 71.2%, where as C.albicans were only 28.8 %. In our study Candida tropicalis were 46.3%, and C.albicans were 28.8%. In our study male population were more 35.4%and they were more in the age group 31-45 years. The major risk factors were indwelling devices(urinary catheter),followed by HTN, DM, and smoking. A study by J.K. Oberoi et al(2012), New Delhi, states that there is a shift from Candida albicans to non-albicans Candida species causing fungaemia.(9) Nadeem SG et al. (2010, Pakistan) in their study showed isolation of C.albicans to be maximum 201 (41.2 %) followed by C.tropicalis 140 (28.7%), C.parapsilosis 32 (6.5%), C.krusei 30 (6.1 %), C. glabrata 21 (4.3 %) and C. guilliermondii 21 (4.3 %) which was contradictory to our finding.(22) On the contrary, Kobayashi CC et al (2004, Brazil) in their study showed Candida albicans was isolated in 35.6% and C.tropicalis (22%) was the second most frequent species isolated .Most patients were women (57.8%) with a mean age of 48.7 years. The principal risk factors that were observed in patients with candiduria included antibiotics therapy (100%), urinary catheterization (84.4%), surgical procedure (66.7%), female sex and extended hospitalization.(23). In our study we found biofilm forming Candida albicans 52.2%, which was quite low as compared to non- albicans Candida, specially C.tropicalis, which gave 70.27% biofilm positives. Similarly, In a study by Vinitha Mohandas et al (2011),a total of 81 (73%) out of 111 Candida species isolates obtainedfrom the clinical isolates produced biofilm. Only 51% (25 of 49) of C.albicans isolates produced biofilm, which was significantly lower than the percentage of all non-albicans Candida species isolates producing slime.(24). Similarly, In a study by Saroj Golia et al(2012),Out of 108 Candida species tested 71(65.74%) were found to be biofilm producers. Biofilm production was found to occur most frequently among non-albicansCandida44 (61.97%) than Candida albicans 27(38.03%).(4) Saurabh Muni et al also found that The biofilm positivity was found more with Non albicans Candida species (78.9%) as compared to Candida albicans (54.8%) .(10) Jong Hee Shin et al in their study also says that Only 8% (11 of 146) of C. albicans isolates produced biofilms, which was significantly lower than the percentage of all non-Candida albicans Candida species isolates producing biofilms.(25) On the contrary, D. M. Kuhn in his study mentions that C.albicans produces quantitatively larger and qualitatively more complex biofilms than other species, in particular to C.parapsillosis.(26) In our study with CRA method of biofilm detection, the sensitivity and specificity of CRA method was evaluated by using TCP method as a gold standard .We observed Sensitivity 80.40 to 97.73%, Specificity 88.78 % to 100.00%, PPV 92.13% to 100 %, and NPV was 73.26% to 96.80 %(At 95% Confidence interval). Similar to our study, a study by Ilknur Dag et al (2010, Turkey) found that by the congo red method, classification of existing biofilm in Candida species was problematic. It is known that, Congo red has interaction with various polysaccharides, however it shows high affinity to chitin and glucan (Roncero and Duran, 1985). Congo red not only binds to the carbohydrates of extracellular matrix (ECM) generated by the Candida but also to chitin and glucan present in the cell wall; therefore, we conclude that the interaction of the Congo Red with extracellular matrix and the cell wall composition could limit its use in the evaluation of fungal biofilm formation. So we cannot recommend CRA test as a general screening for biofilm formation of Candida species.(27)Whereas , a study by Naveen Saxena et al.(2014, Kota) for Biofilm production of Candida isolates by Congo Red Agar Method (CRA). They subjected 120 Candida isolates for biofilm production & detected 38.33% as biofilm positive and 61.66% as biofilm negative. C.albicans were found to be the most common species 32 (80%). The sensitivity and specificity of CRA method was evaluated by using microtiter plate method as a gold standard. Out of total biofilm positive Candida, 21.73% were strong biofilm producers and 78.27% were weak biofilm producers. They gave the opinion that CRA method is a quantitative and reliable method for the detection of biofilm forming microorganisms and this method can be recommended as a general screening method for detection of biofilm producing Candida in laboratories.(81) If we compare and correlate TCP method then we found that our study is correlated with Vinitha et al (2011)(79) in which a total of 81(73%) out of 111 Candida species isolates obtained from the clinical isolates produced biofilm. We

have got 49(61.25%) out of 80 Candida species isolates obtained from the clinical isolates produced biofilm ,with high resistance pattern among biofilm producers. In our study by TM method, we have got 45(56.25%) biofilm positives which includes both moderate and strongly positives, and 35 (43.75%) negative for biofilm. TM when compared to TCP method we found Sensitivity and specificity, were 91.8%and 100%, respectively and the positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 100%. So we can say that The TM correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminate between weak and biofilm negative isolates due to the variability in observed results by different observers Similarly , in a study byIlknur Dag etal (2010, Turkey)Sensitivity and specificity of the TM as compared to standard TCP method, were 68 and 98%, respectively and the positive predictive value (PPV) was 97% and the negative predictive value (NPV) was 83%.(27) According to an Indian study by M.Bhatt et al (2015) there was no major discrepancies observed between visual and spectrophotometric reading, for Candida species. This related well with our study as well where TM showed high sensitivity and specificity when compared to TCP method.(28). In a paper on Staphylococcus aureus by M Gogoi et al The TCP, TM and CRA detected 61.7%, 41.7% and 18.2% of biofilm producers, respectively which co relates with our study.(29) According to our results by TCP method , we conclude that the TCP method is a reliable and practical method for determining the biofilm formation of clinical Candida isolates. However, because the polystyrene tubes or plates may not reflect exactly the ability to form a biofilm in vivo, clinical decisions must be given carefully. Proteinase and phospholipase secretion has been implicated as potential virulence factors for some Candida species responsible for catheter related candidemia in intensive care unit (ICU) patients with indwelling devices.(24). According to our study only Candida albicans showed phospholipase activity (9+10),i.e.82.60% of all C.albicans.where as all non-albicans Candida were negative for phospholipase activity.But on the other hand proteinase activity was shown by(9+6).15 C.albicans (65.21%), C.tropicalis 70.20%(22+4), C.glabrata 33.3%, C.parapsillosis 100%, C.krusei and C.lusitaneae 100%, which says proteinase activity was more in Non-albicans Candida. A study by Amit Kumar et al(2011,Himachal Pradesh) ,Protease activity was determined during the study and two isolates i.e. Candida albicans and Candida tropicalis were strongly positive for protease activity, whereas others species were mild positive. Phospholipase activity was reported in all the isolates.(83The phospholipase and proteinase activities of C. albicans isolates were found to be higher than those of non-albicans Candida isolates as shown by Fatma Mutlu Sariguzel et al (2015,Turkey).(30) Another study by Concetta et al(2012,Italy), also showed phospholipase activity in all strains of C.albicans and 48% of them exhibited protease activity whereas in non albicans Candida, none of them showed phospholipase activity and only one strain of C.parapsilosis was found to be positive for protease activity.(31) Our result also indicate that even though all the isolated strains were pathogenic, not all strains of Candida produced proteinase and phospholipase as virulent factors. The virulence of Candida species is attributed not to a single factor but to a combination of several factors, like proteinase, phospholipase , biofilm production etc.(32) According to our study, the biofilm forming Candida spp. are more resistant to antifungals , as compared to non-albicans Candida. Especially more resistance is seen to fluconazole, flucytosine and voriconazole . Although Caspofungin, Micafungin and Amp-B showed good efficacy to biofilm and non-biofilm forming Candida. A study by Mary Ann et al (2004,USA) also states , biofilm-associated infections are difficult to treat, which emphasizes the need to develop antimicrobial drugs that show activity against biofilm-associated organisms and specifically target biofilm-associated infections. The novel classes of agents, namely the lipid formulation of amphotericins and the echinocandins, have been shown to have unique activities against the resistant Candida biofilms.(33)

V. Conclusion

The study demonstrates that biofilm formation and consequent infections of medical indwelling devices is a serious problem in hospitals in this era. Despite the large number of antimicrobial agents available, it is extremely difficult to eradicate microorganisms from biofilms as a high degree of resistance is demonstrated by most of organisms isolated. Baring a few studies abroad, there are virtually no reports to date of studies with Candida biofilm detection by three methods (CRA,TM and TCP) in our country. Several studies of the epidemiology of Candida infections have been carried out but definitive relationships between strains, types, and properties such as

Pathogen city , commensalism and infectivity have not been established. Hence , the purpose was to study the spectrum and association of Candida with biofilm formation and to establish the role of virulence factors in Candida in the causation and persistence of the disease. Our data indicates that the TCP is an accurate and reproducible method for screening and can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of Candida species along with susceptibility testing to reduce resistance pattern. Accurate species identification is important for the treatment of Candida infections, as most of the non-albicans Candida are inherently resistant to anti-fungal agents especially to azoles. Hence rapid identification and speciation of Candida species is essential in guiding appropriate anti-fungal therapy. However, no single phenotypic test is highly effective in identifying Candida species. Therefore, a combination of tests is sometimes

necessary for the identification. Virulence factors in Candida species may indicate the invasiveness of the infections. But further studies need to be done in this field to establish their role in infections.

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