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RESEARCH ARTICLE

PHENOTYPIC DETECTION OF BIOFILMS IN CANDIDA SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMEN

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Abstract

Candida is one of the most frequently encountered opportunistic fungi that cause severe infection in humans because of its virulence factor. The ability of *Candida albicans* to form biofilms and adhere to host tissues and biomaterial surfaces is an important factor in its pathogenesis. One of the main characteristics of biofilms is their resistant to broad spectrum anti-microbial drugs. The aim of the study was to know the biofilm formation by various Candida species isolated from various clinical specimens. The study was carried out over a period of 1 year from January 2016 to December 2016 at the Department of Microbiology, Government Medical College and Hospital, Jammu. A total 120 Candida spp. were isolated from various clinical specimens. Speciation of Candida was done by standard yeast identification protocol and Candida CHROMagar. Biofilm formation was detected by Congo Red Agar, Tube method and microtitre plate method. Out of total 120 Candida spp. studied, biofilm production was seen in 63/100 (52.55%) isolates. While comparing all the three methods tube method proved more reliable, easy and more efficient. Antifungal efficacy of Coconut oil and Eucalyptus oil was also tested in this study against all Candida isolates. Eucalyptus oil was observed to be a better antifungal agent than Coconut oil in the present study. When coconut oil was tested against all *Candida albicans* isolates, the sensitivity of biofilm non producers was higher in comparison to biofilm producers.

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Introduction:-

A biofilm is a polysaccharide matrix that acts as a protective structural layer for the microorganism. By producing a biofilm, the micro-organism creates a shield to protect itself. (Sachin C Deorukhkar & Santosh Saini, 2013) Formation of a biofilm is a virulence factor of a microorganism. Biofilm is produced by both fungi and bacteria. In mycology, Candida spp. forms most common fungal biofilm which is extremely difficult to treat. (Nimet Yigit et.al., 2011). Candida spp. has an ability to adhere to the surface of commonly used medical devices. Candida spp. is the fourth most common cause of bloodstream infection (BSI) and the third most common cause of urinary tract infection (UTI). (Saroj Golia et.al, 2012) Formation of Biofilm exhibits increased resistance to commonly available antifungal therapies; these infections are very hard to treat. Most often, treatment of biofilm infections involves the removal of the infected medical devices (Pahwa N et.al., 2014). The majority of medically important Candida spp.

has now been shown to develop biofilm including *C. albicans* and Non albicans *Candida* which includes *C. krusei*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis* and *C. parapsilosis* (Serafino, A et.al.,2008 & Chander J.,2009). The Biofilms of *Candida* spp exhibits resistance to all available commonly used antifungal drug classes including the azoles (itraconazole, fluconazole, , voriconazole,), the echinocandins (caspofungin, micafungin, anidulafungin), the amphotericin B and flucytosine (Konopka K et.al., 2010). The formation of *Candida* biofilms carries important clinical repercussions because of their increased resistance to commonly available antifungal therapies and the ability of biofilms to withstand with host immune defences.(Khan MS et.al.,2012) *Candida* biofilms shows antagonistic impact on the health of the patients. The detection of biofilm becomes very necessary for the treatment of infection.(Ogbolu, D.O et.al., 2007) Various Phenotypic methods are used for the routine detection of Biofilms are - Microtiter Plate Method, Congo Red Agar and Tube adherence method. Fungal infections are treated with antifungal medications called antimycotics or antifungals. While antifungal are effective for many, some may experience side effects and rebound infection. To avoid side effects, one can use naturally occurring antifungals. (Ogbolu, D.O et.al., 2009) New studies shows that eucalyptus oil and coconut oil has powerful anti-fungal properties and it may be an effective treatment for biofilm. This antifungal efficacy of eucalyptus oil and coconut oil on the clinical *Candida* isolates was also tested in this study. The present study focused on-

1. Isolation and Identification of *Candida* spp. from various clinical specimens.
2. Detection of biofilm forming capacity of these isolates by various phenotypic methods.
3. Assessment of antifungal efficacy of plant oils on pathogenic *Candida* isolates.

Materials and Methods:-

The present study was conducted over a period of one year (January 2016 to December 2016) in the Department of Microbiology, Government Medical College & Hospital, Jammu, (J&K). The clinical isolates recovered from both outdoor and indoor patients and were identified by using standard microbiological protocols. Total 4150 clinical specimens were processed out of which 120 *Candida* species were recovered. Clinical specimens like Blood, Urine and Pus were included in the study. Samples were screened for budding yeast like cells with the help of Gram stain, and then inoculated on Sabourad's Dextrose Agar with Chloramphenicol at 37 °C for 24 hours. For initial speciation, Germ tube test was done followed by formation of Chlamyospore formation on Corn meal agar. Simultaneously *Candida* Spp. were inoculated on *Candida* CHROM agar and incubated at 37 °C for 24 hrs and the species were identified by colour of the colonies on CHROMagar media as per manufacturer's instructions (TM Media).

Biofilm Formation- Biofilm formation was detected by three methods as described below.

Microtitre plate method;-

Biofilm formation was determined by using 96-well pre-sterilized polystyrene microplates (HiMedia) For each isolate, a suspension from an overnight culture on SDA was prepared in sterile distilled water and adjusted to 1 McFarland. Each well of the microplate was filled with 180 µl of Sabouraud dextrose broth (HiMedia, India) supplement with 8% glucose and then 20 µl of the standard suspension of tested isolates was inoculated. Microplates were covered and incubated at 37°C for 24 hours. The medium in wells was removed and washed three times with sterile phosphate buffer solution (PBS). Microtitre plates were stained with 1% Safranin for 5 minutes and then percentage transmittance (%T) was read at 630 nm by an ELISA reader. All tests were done in triplicates and mean were calculated. Finally, adherent biofilm layers were scored as either negative; weak (+) (percentage transmittance (%T ≤ 20)); moderate, (++) (%T = 20-35); strong (+++) (%T =36-50) and very strong (++++) (%T ≥ 50).

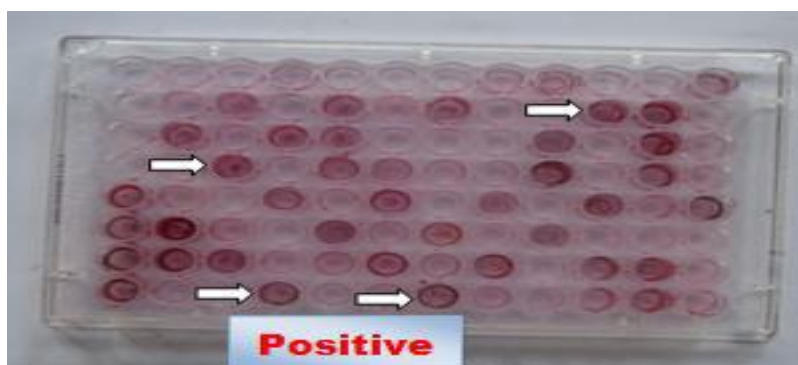


Fig 1:- Biofilm formation by *Candida* species (Microtitre plate method)

Congo red Agar Method:-

Congo Red Agar (CRA) is a simple and qualitative method for detecting biofilm production described by Freeman et al using Congo Red Agar medium. CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L and Congo Red indicator 8 g/L. Firstly Congo red stain was prepared as a concentrated aqueous solution separately from the other medium constituents and autoclaved at (121°C for 15 minutes) and then added to the autoclaved brain heart infusion agar with sucrose which is cooled at 55 °C. CRA plates were inoculated with test organisms and incubated at 37 °C for 24 hr aerobically. Black colonies with a dry crystalline consistency will indicate biofilm production. The experiment was performed in triplicate and repeated three times.



Fig 2:- Biofilm formation by *Candida* species (Congo Red Agar)

Tube Method:-

A loopful of organisms from the surface of SDA plate was inoculated into tube containing 10ml of Sabouraud Dextrose Broth supplemented with glucose. The tubes were incubated at 35°C for 48hours. After incubation, the culture supernatants were decanted and the tubes were washed with phosphate buffer saline (pH 7.3) and the dried tubes were stained with 1% Safranin. Excess stain was removed by washing with de-ionized water. Tubes were then dried by positioning them invertedly. Tubes were then observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall of the test tube.



Fig 3:- Biofilm formation by *Candida* species (Tube method)

Antifungal Efficacy Testing of Eucalyptus Oil and Coconut Oil:-**Preparation of Impregnated Paper Discs**

The commercially available extracts of Eucalyptus oil and coconut oil were used. A 0.04 ml of 100% concentration of the Eucalyptus oil and coconut oil extracts was impregnated into the discs. The impregnated discs were left to dry

for 24 hours in the incubator at 37°C. After drying, the discs were transferred back into the sterile container and stored.

Antimicrobial Sensitivity Test:-

Antimicrobial experiments on *Candida* species were carried out on SDA plates. The test strain were streaked on the plates using sterile wire loop and allowed to dry for 15 minutes. Sterile impregnated discs were applied onto the inoculated plates. The plates were then incubated at 37°C for 24–48 hours. Zones of inhibition were assessed after the period of incubation.

Result:-

Out of 4150 samples, only 120 (2.89%) were culture positive for *Candida* spp. Out of 120 *Candida* spp. isolated, 58 (48.33%) were identified as *Candida albicans* and 62 (51.66%) were identified as Non-*albicans* *Candida* species. All isolates were studied for biofilm production. Out of total 120 *Candida* species studied, biofilm production was seen in 63/120 (52.55%) isolates.

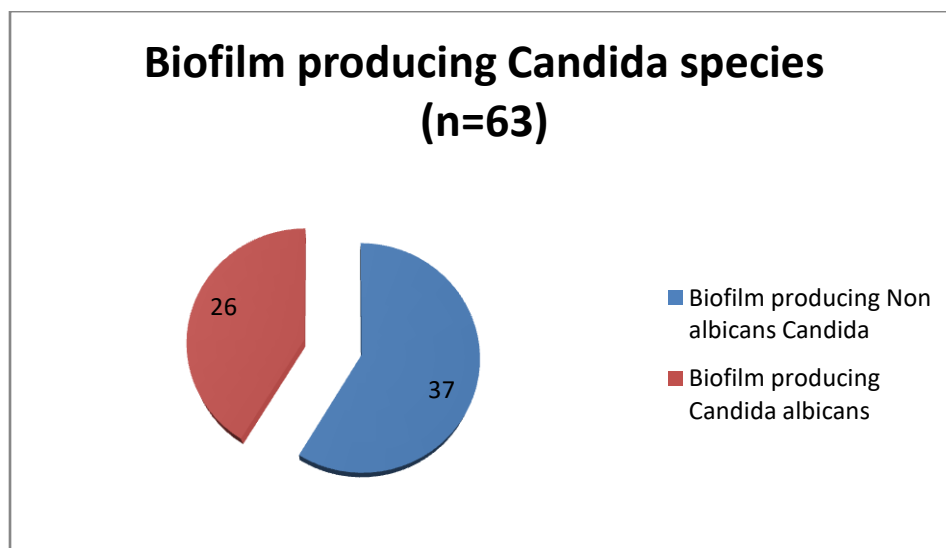


Fig 4:- Biofilm producing *Candida* species

Table 1:- Incidence of *Candida* spp. in Clinical Samples

S.No.	Specimen (n=120)	No. of <i>C. albicans</i> isolates (n=58)	No. of Non albicans <i>Candida</i> isolates (n=62)
1.	Blood(n=59)	27	32
2.	Urine(n=36)	12	24
3.	Pus(n=25)	19	6

Table 2:- Biofilm producers in *Candida albicans*

Specimen (n=120)	No. of <i>Candida albicans</i> isolates	Biofilm (+)	Biofilm (+)%	Biofilm (-)	Biofilm (-)%
120	58	26	44.82%	32	55.17%

Table 3:- Biofilm producers in Non albicans *Candida*

Specimen (n=120)	No. of Non albicans <i>Candida</i> isolates	Biofilm (+)	Biofilm (+)%	Biofilm (-)	Biofilm (-)%
120	62	37	59.67%	25	40.32%

Table 4:- Effect of Coconut Oil on *Candida albicans*

Specimen (n=58) Candida albicans	Sensitive to Coconut Oil	Sensitive	Resistant to Coconut Oil	Resistant
Biofilm producers (n=26)	7	26.92%	19	73.07%
Biofilm non producers (n=32)	12	37.5%	20	62.5%

Table 5:- Effect of Eucalyptus Oil on *Candida albicans*

Specimen (n=58) Candida albicans	Sensitive to Eucalyptus Oil	Sensitive	Resistant to Eucalyptus Oil	Resistant
Biofilm producers (n=26)	14	53.84%	12	46.15%
Biofilm non producers (n=32)	23	71.87%	09	28.12%

Table 6:- Effect of Coconut Oil on Non albicans Candida

Specimen (n=62) Non albicans Candida	Sensitive to Coconut Oil	Sensitive	Resistant to Coconut Oil	Resistant
Biofilm producers (n=37)	08	21.62%	29	78.37%
Biofilm non producers (n=25)	11	44%	14	56%

Table 7:- Effect of Eucalyptus Oil on Non albicans Candida

Specimen (n=62) Non albicans Candida	Sensitive to Eucalyptus Oil	Sensitive	Resistant to Eucalyptus Oil	Resistant
Biofilm producers (n=37)	12	32.43%	25	67.56%
Biofilm non producers (n=25)	17	68%	8	32%

Discussion:-

The present research work was conducted with aim to study the biofilm production by various *Candida* spp. isolated from various clinical samples at Government Medical College and Hospital, Jammu, India. It is increasingly obvious that infections caused by *Candida* spp. are an escalating clinical problem, and with a limited arsenal of antifungal and a growing menace of biofilms, a lot has to be done for proper disease management. In the present study, biofilm production was found to occur most frequently in Non-albicans *Candida* than *C. albicans*. This finding is in contrast to an earlier report that suggested that pathogenic Non-albicans *Candida* were more likely to produce virulence factor biofilm than *C. albicans*. (Calderone RA et.al.,2001) In the present study, Out of 4150 samples, only 120 (2.89%) were culture positive for *Candida* spp. Out of 120 *Candida* spp. isolated, 58 (48.33%) were identified as *Candida albicans* and 62 (51.66%) were identified as Non-albicans *Candida* species. All isolates were studied for biofilm production. Out of total 120 *Candida* species studied, biofilm production was seen in 63/120 (52.55%) which is in agreement to the findings of the studies conducted by Mythreyl SR. et al.(Mythreyi Shekar Rishpana et.al., 2015) In our study we found 44.82% of *C. albicans* were biofilm producers which correlate well with the findings of Melek Inci et al. (Melek Inci et.al., 2012) and Saurabh M et al. (Saurabh M et al., 2012) Similarly, 59.67% of Non-albicans *Candida* were biofilm producers which is agreement to the findings of S. Golia et al. (Golia, S. et.al.) and Sahar Ali M et al.(Sahar Ali M et al., 2013) In our study we found that Non albicans *Candida* was more biofilm produces as compared to *C. albicans*.

Since Biofilms have been bethinking as a virulence factor bestowing the Candidal infection, a reliable method for their diagnosis is necessary. Three different methods were used for the evaluation of biofilm i.e. Congo red agar,

Microtitre plate method and Test tube adherence method. Out of these three different methods, Test tube method is fast, simple, reliable, and reproducible method. Tube method was commonly used for early detection of Biofilm production in routine use. The findings of present study showed that it is the most sensitive method for detection of biofilms which is in concordant with the study conducted by Oliveira and Cunha et.al.(Oliveira et.al.,2011) Biofilms have great impact on public health because biofilm-associated *Candida* exhibit dramatically plummet susceptibility to antimicrobial agents. This susceptibility may be intrinsic (as a natural outcome of growth in the biofilm) or acquired (due to transfer of extra chromosomal elements to susceptible organisms in the biofilm). It is likely that biofilms evade anti microbial challenges by multiple mechanisms. Some researches indicate that plants oils are used as antifungals because of their broad spectrum antifungal activity. (Nisha VJ.et.al., 2015)

Plant oils are increasingly claimed to Antifungal. Efficacy of Coconut oil and Eucalyptus oil was also tested in this study against all *Candida* isolates. Selected oils have been suggested to have potent antimicrobial activity.

The disc diffusion assay is a standard method widely used for the rapid screening of natural products for antifungal activity. Plant oils were screened using this very convenient assay method. The results indicate that caution is needed, since different oils have different diffusion rates on agar plates. This may contribute to variations in the size of the inhibitory zones, leading to erroneous conclusions regarding their antifungal activity. Hence this assay was run in triplicate and the average zone size was considered for interpretation. Both the oils were effective and showed anti-*Candida* activity at low concentrations; however Eucalyptus oil showed better antifungal activity as compared to coconut oil.(Epka, O.D.,et.al., 1996)

When coconut oil was tested against *Candida albicans* isolates, sensitivity of 26.92% was of biofilm producers *Candida albicans* whereas 37.5% biofilm non producers *Candida albicans*. Biofilm producers *Candida albicans* showed 53.84% sensitivity to Eucalyptus oil whereas non biofilm producers *C.albicans* showed 71.87% .When coconut oil was tested against all Non albicans *Candida* isolates, the sensitivity of biofilm producers was 21.62% and biofilm non producers was 44%. Whereas in the case of Eucalyptus oil 32.43% sensitivity was obtained against biofilm producers and 68% of biofilm non producers Non albicans *Candida* which was in concordant with the study conducted by M. Bansal et.al.(M. Bansal, et.al.,2016) This shows that Biofilm producers *Candida* spp. resist the antifungal activity of plant oils. This can be due to failure of oil to penetrate the full depth of the Biofilm.

The results presented in this study clearly demonstrate the antifungal potential of the selected plant oils. Eucalyptus oil may be used as anti-biofilm agent at low concentrations. However, before they are considered for use as topical preparations, a careful exploration of their undesirable effects needs to be undertaken.

Conclusion:-

Biofilm is one of the known virulence factors of *Candida* spp. Early detection of Biofilm production may be useful and apply for clinical decision because of its suggestive property for potential pathogenic capacity of *Candida* isolates. The results of the present study emphasize the role of biofilm in *Candida albicans* as well as non-albicans *Candida* as a virulence factor. It also encourages the need for further examination of the efficacy of plant oils against other forms of systemic and superficial fungal infections.

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