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Detection of Biofilm among the Clinical Isolates of Candida Species and Their Antifungal Susceptibility at Tertiary Care Hospital, Western Rajasthan

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A B S T R A C T

Candida species are part of the normal flora of humans. A number of variables are known to promote both superficial and deep-seated candidiasis, and they work either by disrupting the balance of the body's normal microbial flora or by reducing host resistance. Candida's pathogenicity is attributed to its virulence factors, one of which is biofilm development. The ability to produce biofilms is linked to pathogenecity and should be regarded a key virulence characteristic during candidiasis. ***Aim***: To detect biofilm formation among the clinical isolates of Candida species using modified Tissue Culture Plate Method and antifungal susceptibility pattern in Candida species withbiofilm production. This study was a prospective study conducted during a period of 1year on the samples received in microbiology lab,Dr. S.N. Medical College, Jodhpur for culture sensitivity test.In this study,155(05.17%) Candida species strains were isolated,out of 2995 various clinical specimens.There is an increase prevalence of non Candidaalbicans 101(65.16%) species [*C. tropicalis*55 (35.48%), *C. parapsilosis*19 (12.26%), *C. krusei*14 (09.03%), *C.* and *C.kefyr* 04 (02.58%)] isolated from various clinical samples and showed strong biofilm producers compared to *C. albicans*54(34.84%) species. Out of 155 Candida strains tested 118 (76.13%) were found to be biofilm producers. The positivity was more with urine samples 77 (82.8%) followed by sputum samples 16 (72.72%) andblood 13 (50%) isolates. TCP (Tissue culture plate) method detects 40% as Strong biofilm producers (4+, 3+), 27.74% as Moderate biofilm producers (2+), 08.39% as Weak biofilm producers (1+) and 23.87% strains were biofilm negative. There was high resistance pattern among biofilm producers in comparison with non-biofilm producers. The majority of the resistance of biofilm producing isolates was belonging to fluconazole (91.43%) followed by itraconazole (86.44%), voriconazole (83.05%) and ketoconazole (71.19%).In this study, amphotericin-B was found effective against biofilm producing Candida Species. Non-biofilm producing Candida species strains were comparatively much more sensitive to these antifungal agents. Reduced susceptibility to medications like azoles, as revealed in our work, is a critical concern in the treatment of immunocompromised patients with serious illnesses. As a result, antifungal susceptibility testing and biofilm identification is a potential approach for predicting a given agent's activity in diverse clinical isolates.

Keywords: Candida, biofilm, antifungal.

INTRODUCTION

Recent advances in research technology have allowed researchers to study bacteria and fungi in their natural environment, and over 95% of bacteria existing in nature are in biofilms [1]. *Candida* species are found as normal flora in healthy individuals and are known to cause opportunistic infections with high rates of mortality, especially in immunocompromised individuals [2]. *Candida* spp. cause systemic diseases which are the fourth leading cause of nosocomial bloodstream infections in modern hospitals. The most challenging clinical problem is the increased rate of non-*Candida albicans*isolation and the rapidly growing resistance of *Candida* species [3]. *Candida albicans*is the most prevalent among *Candida* spp., which causes both superficial and systemic infections [4].

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 [5]. One of the important factors contributing to the virulence of candida is the formation of surface attached microbial communities known as "biofilm. Biofilms are structured microbial communities that are connected to a surface and contained in an exopolymeric material matrix. The benefits of producing biofilm include environmental protection, nutritional availability, metabolic cooperation, and the acquisition of new characteristics. Biofilm formation helps the organism to evade host defenses, exist as a persistent source of infection and develop resistance against antifungal agents [6].

The resistance of biofilm producing Candida species to antifungal agents represents a major challenge especially in the design of therapeutic and prophylactic strategies. One of the primary reasons for this is the influence on treatment, as antifungal medicine frequently fails and surgical intervention is required. These factors constitute a clinical problem, resulting in high mortality as well as economic problem due to prolonged hospital stay [7].

The role of bacterial biofilms in disease has been investigated in detail over a number of years and considerable literature is available on their structure and properties. However, significant literature on medically relevant fungal biofilms is difficult to come by, especially in the current climate with immune-compromised diseases and nosocomial infections on the rise.

Aim & Objectives

1. To detect biofilm formation among the clinical isolates of Candida species using modified Tissue Culture Plate Method.
2. Antifungal susceptibility pattern in Candida species with biofilm production.

Material and Methods

The present study was a prospective study conducted during a period of 1year on the samples received in microbiology lab, MDM hospital, Dr. S.N. Medical College, Jodhpur for culture sensitivity test. Total 2995 various clinical specimens (urine, blood, sputum, indwelling medical devices, tracheal (swabs and devices), pus/ear swab, various body fluids) were included in the study after obtaining ethical clearance from the Institutional Ethics Committee.

Identification of genus *Candida* was done by colony morphology, Gram-staining, germ tube test, 0.1% Glucose agar test, Sugar fermentation test, Sugar assimilation test, CHROM agar according to standard microbiological techniques [8].

Biofilm production was determined using modification of tissue culture plate method (TCP method) described by Shin *et al*., [9]. Biofilm production was detected by Percentage Transmission method or percent transmittance (%T) by measuring optical density (%T) in microtitre plate with Spectrophotometric reader at 405 nm with a microtiter plate reader (ErbaLisaScan® EM).Biofilm production by each isolate was scored as either negative (%Tbloc, < 5), 1+ (%Tbloc, 5 to 20), 2+ (%Tbloc, 20 to 35), 3+ (%Tbloc, ≥35). We categorized Tbloc percentage 1+ as weak, 2+ as moderate and 3+ as strong biofilm producer. Each isolate was tested in triplicate.

All the isolates were subjected to the antifungal susceptibility test according to CLSI document M 44 – A2 by disk diffusion testing method for yeasts. Muller Hinton agar supplemented with 0.5µg/ml Methylene Blue Dye and 2% Glucose (MHMB) was used for sensitivity testing. The inoculated plates were incubated at 37^0C for 24 hours or longer [10]. Statistical analysis was performed by using Statistical Package for SPSS. Data were analysed for Mean, Median & P value. Statistical significant was defined the P value < 0.05.

RESULTS

In this present study, 155(05.17%) Candida species strains were isolated, out of 2995 various clinical specimens like urine 93(06.97%), blood 26 (12.56%), sputum 22 (14.76%), indwelling medical devices 05 (06.10%), tracheal (swabs and devices) 03 (01.17%), pus/ear swab 02 (00.37%), various body fluids 02 (03.57%) etc. We observed an increase prevalence of non Candidaalbicans 101(65.16%) species [*C. tropicalis*55 (35.48%), *C. parapsilosis*19 (12.26%), *C. krusei*14 (09.03%), *C.* and *C.kefyr* 04 (02.58%)] isolated from various clinical samples (Fig 1) and showed strong biofilm producers compared to *C. albicans*54(34.84%) species. Majority of the patients belonged to 0-10 years age group (23.23%) followed by 41-50 years of age group (15.48%). The male and female patients ratio of Candidiasis was 1.38: 1 respectively.

Along with biofilm production, an increase in resistance of these Candida species isolates to the routinely used antifungals, make them difficult to treat.

In this study, Out of 155 Candida strains tested 118 (76.13%) were found to be biofilm producers. The positivity was more with urine samples 77 (82.8%) followed by sputum samples 16 (72.72%) and blood 13 (50%) isolates (Table 1). TCP method detects 40% as Strong biofilm producers (4+, 3+), 27.74% as Moderate biofilm producers (2+), 08.39% as Weak biofilm producers (1+) and 23.87% strains were biofilm negative (Table 2).

The susceptibility pattern difference of Candida species to ketoconazole, fluconazole, itraconazole and voriconazole was found to be statistically significant (P value = 0.00) whereas it isstatistically non-significant with respect to amphotericin-B (P value = 0.50).

There was high resistance pattern among biofilm producers in comparison with non-biofilm producers. The majority of the resistance of biofilm producing isolates was belonging to fluconazole (91.43%) followed by itraconazole (86.44%), voriconazole (83.05%) and ketoconazole (71.19%) (Table 3). In this study, amphotericin-B was found effective against biofilm producing Candida Species. Non-biofilm producing Candida species strains were comparatively much more sensitive to these antifungal agents. Results are summarized as frequency tables, and percentages were worked out.

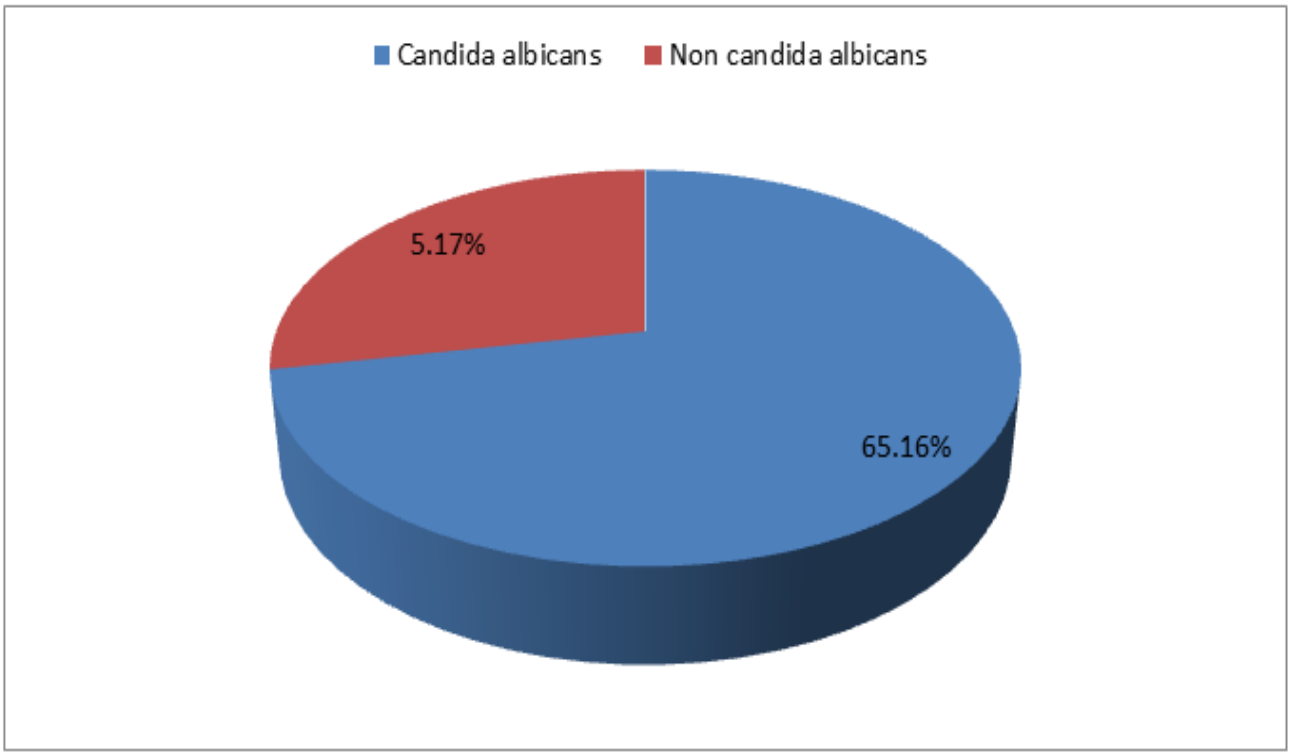

Fig 1: Distribution of Candida albicans& non candida albicans

Table-1: Biofilm production in Candida species from different clinical samples

Type of specimens	Total no. of isolates	Biofilm Producers (%)	Biofilm Non Producers (%)
Urine	93	77 (82.8%)	16 (17.2%)
Blood	26	13 (50%)	13 (50%)
Sputum	22	16 (72.72%)	06 (27.27)
Indwelling medical device	05	03	02
Tracheal	03	03	00
Pus/Ear swab	02	02	00
Fluid	02	02	0
Throat swab	02	01	0
Conjuctival swab	01	01	0
Total	155	118	37
Percentage	100	76.13	23.87

(Chi-square = 15.720; p value = 0.0466)

Table-2: Screening of the Candida species isolates for biofilm formation by Tissue Culture Plate Method

Candida Species Isolated	Tissue Culture Plate Method			
	Strong 3+	Moderate 2+	Weak 1+	Negative
C.albicans	22 (35.4%)	17 (39.5%)	04 (30.7%)	11 (29.7%)
C.glabrata	01 (1.61)	04 (9.3%)	00	04 (10.8%)
C.krusei	09 (14.5%)	03 (6.9%)	01 (7.6%)	01 (2.7%)
C.parapsilosis	07 (11.2)	04 (9.3%)	01 (7.6%)	07 (18.9%)
C.tropicalis	20 (32.2)	15 (34.8%)	07(53.8%)	13 (35.1%)
C.kefyr	03 (4.8)	00	00	01 (2.7%)
Total (%)	62 (40%)	43(27.74%)	13(08.39%)	37 (23.87%)

Table-3: Comparison of antifungal susceptibility pattern in Candida species with or without biofilm production

Name of Anitfungal	Biofilm producer isolates		Biofilm Nonproducer isolates	
	Sensitive	Resistant	Sensitive	Resistant
Ketoconazole	34 (28.81%)	84 (71.19%)	32 (86.49%)	05 (13.51%)
Fluconazole	09 (08.57%)	96 (91.43%)	32 (88.89%)	04 (11.11%)
Itraconazole	16 (13.56%)	102 (86.44%)	27 (72.97%)	10 (27.03%)
Amphotericin-B	93 (78.81%)	25 (21.19%)	31 (83.78%)	06 (16.22%)
Voriconazole	20 (16.95%)	98 (83.05%)	29 (78.38%)	08 (21.62%)

DISCUSSION

In this study, Biofilm forming ability of each isolate was detected using Tissue Culture Plate method as described by Shin *et al*., [9].The percentage transmittance (%T) value was measured for each isolate and subtracted from the %T value for the reagent blank to obtain a measure of the amount of light blocked while passing through the wells (%Tbloc). Biofilm production by each isolate was scored as negative (%Tbloc<5), weak as 1+ (%Tbloc 5 to 20), moderate as 2+ (%Tbloc 20 -35), strong as 3+ (%Tbloc 35-50) and 4+ (%Tbloc>50). C. albicans ATCC 90028 was used as control. The data so obtained was entered in excel worksheets and analysed using suitable statistical methods.

For the purpose of statistical analysis in this study, strong, moderate and weak biofilm producers were considered as the biofilmproducers whereas negative biofilm producers were taken as the non. In the present study 118 (76.13%) of the 155 Candida isolates tested were found to be biofilm producers which could be further classified as 62% strong producers (43% as 4+ and 19% as 3+), 43% as 2+ or moderate producers and 13% as 1+ or weak producers. The rest of 37% Candida species isolates were found as negative or non biofilm producer by this method. This finding is in concordance with studies conducted by Muni *et al*., [11] (64%) and Mohandas *et al*., [12](73%). biofilm producers.

The biofilm positivity was more in urine samples (77) followed by sputum samples (16) isolates. A single strain of Candida species isolated from both throat swab and conjunctival swab was also found to be biofilm producer Biofilm production was found to occur most frequently among non Candidaalbicans species (63.56%) than C. albicans (36.44%). Similar findings have been reported by Girish*et al.*, [13] and Muni *et al*., [11]. Among the non Candidaalbicans species, the biofilm positivity occurred most frequently among isolates of C. tropicalis (35.59%) followed by C. krusei (11.02%), C. parapsilosis (10.17%), C. glabrata (04.24%) and C. kefyr (02.54%). C. tropicalis and C. krusei have also been recognized as strong slime producers by many studies (Dag *et al*., [14], Mohandas *et al*., [12] and Vinitha*et al*., [15].

Antimicrobial resistance is an innate feature of biofilms that, in addition to the increasing rates of reported antimicrobial resistance amongst clinical strains, may further complicates patient treatment.

In this study, the antifungal susceptibility pattern of biofilm producers is far more resistant as compared to the non biofilm producers which results higher degree of resistance to fluconazole in biofilm producers (91.43%) as compared to biofilm non producers (11.11%) as reported by several authors136 whereas amphotericin-B (78.81%) showed maximum efficacy in biofilm producers followed by ketoconazole (28.81%), voriconazole (16.95%) and itraconazole (13.56%).

On the other hand, non biofilm producing candida species strains are comparatively much more sensitive to these antifungals i.e. fluconazole (88.89%), ketoconazole (86.49%), amphotericin-B (83.78%), voriconazole (78.38%) and itraconazole (72.97%).

Possible resistance mechanism involves restricted penetration of drug through biofilm matrix, phenotypic changes resulting from a decreased growth and nutritional limitation, expression of resistance genes induced by contact with a surface and persistence of a small number of cells.

CONCLUSION

The present study suggests an increasing prevalence of non Candidaalbicans species in the various clinical samples isolated and also shows them as strong biofilm producers compared to C. albicans species. Along with biofilm production, an increase in resistance of these Candida species isolates to the routinely used antifungals, make them difficult to treat. Therefore, knowledge of the local species distribution of Candida through presumptive identification, followed by confirmation and an effective anti-biofilm treatment, is essential to initiate early empirical therapy, especially in an ICU setup, which harbors a lot of immunocompromised patients and other patients susceptible for acquiring various fungal infections. It is increasingly obvious that infections caused by Candida species are an escalating clinical problem, and with a limited arsenal of antifungals and a growing menace of biofilms, a lot has to be done for proper disease management.

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