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Original Research Article

A Study of Biofilm Production and Antifungal Susceptibility of Clinical Isolates of *Candida* Species

Rahul P. Dhale[1*], M. V. Ghorpade[2*], C. A. Dharmadhikari[3#]

[1]PhD Student, [2]Professor, [3]Professor,
[*]Department of Microbiology, Krishna Institute of Medical Sciences Deemed University, Karad, Dist. Satara, Maharashtra, India.
[#]Department of Microbiology, Tatyasaheb Kore Dental College and Research Centre, New Pargaon, Dist. Kolhapur, Maharashtra, India.

Corresponding Author: Rahul P. Dhale

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ABSTRACT

Purpose: The biofilm formation of an organism can be considered as virulence factor, which turns sensitive organisms into the resistant one for antimicrobial agents. *Candida* biofilms are observed in blood, mucosal surface and most medical devices, such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters i.e. nonliving objects in patient's body. This study was designed to characterise speciation of *Candida*, biofilm production and antifungal activity after biofilm formation.
Materials and Methods: Speciation of *Candida* was done by Dalmau plate technique on corn meal agar, also sugar assimilation and fermentation test were performed by using 2% concentration of sugars. Quantitative measurement of biofilm formation was assessed by microtitre plate assay for 425 *Candida* isolates using XTT {2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} reduction assay. Antifungal susceptibility was done to biofilm forming and non biofilm forming *Candida* using Percentage Transmission (%T) assay and confirmation by XTT reduction assay.
Results: Out of 425 strains, XTT reduction assay gave 72 biofilm positive strains accounting for 16.94 %. Out of 72, 56 strains became resistant to amphotericin B and 41 strains became resistant to fluconazole after induced biofilm production which is significant. Conclusion: The biofilm formation of an organism can be considered as virulence factor, which turns sensitive organisms into resistant organisms for antimicrobial agents. *Candida* biofilm formation is observed in blood, mucosal surface and most medical devices in patient's body.

Key words: Biofilm, XTT reduction assay, antifungal resistance, medical devices.

INTRODUCTION

A biofilm is a complex aggregation of microorganisms growing on a solid substrate. Biofilms are characterised by structural heterogeneity, genetic diversity, complex community interactions, and an extra cellular matrix of polymeric substance. [1]

Candidiasis is an infection of the skin, mucosa and rarely of the internal organs by *Candida albicans* and by other *Candida* species (yeast like fungus). *Candida* speciation can be done by identified of growth characteristics and sugar assimilation/fermentation tests. *Candida* species are frequently found in the normal microbiota of humans, which facilitates their encounter with most implanted biomaterials, blood and host surfaces. Devices such as stents, shunts, prosthesis, implants endotracheal tubes, pacemakers and various types of catheters, to name a few have all been shown to support colonization and biofilm formation by *Candida*, and are often associated with high-level antifungal resistance and act as virulence factor. [2-4]

The main objective of this study was to find out the prevalence of biofilm forming clinical isolates of *Candida*, identify the species and determine the antifungal susceptibility of these isolates. As these antifungal resistant *Candida* species after biofilm production may cause severe infections difficult to eradicate, this study assumes significance.[2] It is also very significant because this is one of kind study in which antifungal testing is done before and after biofilm production. The present study will help us in understanding the significance of biofilm formation and antifungal susceptibility of biofilm forming *Candida* isolates.

MATERIALS AND METHODS

Clinically diagnosed 425 cases as UTI (Urinary Tract Infections), Renal failure, Meningitis, Cellulites, Septicemia, Ketoacidosis, Pneumonia, Respiratory infections, Skin infections, Abscess, Diabetes, RVD (Retro Viral Diseases), Heart diseases, Oral lesions, Dental caries etc. were processed to isolate *Candida*. During the period of Sept. 2008 to Jan. 2013, specimens were collected from Krishna Hospital Karad & Private Medical Laboratories in an around Kolhapur, Sangli (MS) and from Belgaum (KS) by conventional method.

Sabouraud's dextrose agar was used to isolate *Candida* species. On Corn meal agar, Dalmau plate technique was used for speciation of *Candida*. Further confirmation was done by sugar fermentation/assimilation tests with bromothymol blue indicator, using 2% concentration of glucose, maltose, sucrose, lactose, galactose, and trehalose. [5]

For antifungal sensitivity, suspensions were prepared from individual colony grown on Sabouraud's dextrose agar, in 5 ml of sterile 0.85% saline to a density of a 0.5 McFarland's nephalometer standard tube no.3 (approximately 10^7 cells/ml) followed by a 1:20 dilution in Sabouraud's broth. Initially all *Candida* strains were screened for biofilm production (with 100 µl of this suspension) by XTT reduction assay. [3,4] Then antifungal sensitivity test was done for all *Candida* by using Amphotericin B (Himedia laboratories Pvt. Ltd. Mumbai) in DMSO (Dimethyl sulfoxide) (Himedia laboratories Pvt. Ltd. Mumbai) and Fluconazole (Dynamicro India and Himedia laboratories Pvt. Ltd. Mumbai) of final concentration 10.5 mg/L (mean concentration). 42 mg/L antifungal agents were mixed with 200 µl (approx. 10^7 cells/ml) suspension in each well. [6-11] Before biofilm formation antifungal sensitivity was done for all *Candida* species by broth dilution method. After biofilm formation antifungal sensitivity was done by the same, broth dilution method and biofilms were confirmed by XTT reduction assay. [3,7, 10-12]

OBSERVATIONS AND RESULTS

Table No. 01. *Candida* species isolated and their biofilm positivity by XTT reduction assay.

Sr. No.	Biofilm producible *Candida* species	Total species	Biofilm positive	Percentage (%)
1	*Candida albicans*	190	49	25.78 %
2	*Candida guilliermondii*	18	03	16.66 %
3	*Candida famata*	83	11	13.25 %
4	*Candida krusei*	17	02	11.76 %
5	*Candida lusitaniae*	17	02	11.76 %
6	*Candida keyfr (C. pseudotropicalis)*	11	01	09.09 %
7	*Candida parapsilosis*	37	02	05.40 %
8	*Candida glabrata*	47	02	04.25 %
9	*Candida tropicalis*	04	00	00.00 %
10	*Candida dubliniensis*	01	00	00.00 %
	Total	425	72	16.94 %

Table no. 02. Conversion of Amphotericin B sensitive strains to resistant.

72 Biofilm producing *Candida* strains		
Candida strains sensitive to Amphotericin B before Biofilm production	*Candida* strains sensitive to Amphotericin B after Biofilm production	*Candida* strains resistant to Amphotericin B after Biofilm production
58/72	02/58 (03.44%)	56/58 (96.55 %)

Table no. 03 shows, conversion of Fluconazole sensitive strains to resistant.

72 Biofilm producing *Candida* strains		
Candida strains sensitive to Fluconazole before Biofilm production	*Candida* strains sensitive to Fluconazole after Biofilm production	*Candida* strains resistant to Fluconazole after Biofilm production
46/72	05/46 (10.86%)	41/46 (89.13%)

Table No. 04. Biofilm positive *Candida* strains from various clinical specimens.

Sr. No.	Specimen	Total no.	Biofilm positive strains	Percentage (%)
1	Catheter tip	12	08	66.66 %
2	Tips and tubes (Suction/ OVC/ UVC/ Endotrachial etc.)	05	02	40.00 %
3	Cervical swab	06	02	33.33 %
4	Blood	71	21	29.57 %
5	Pus	17	4	23.52 %
6	Oral swab	51	10	19.60 %
7	Vaginal swab	12	02	16.66 %
8	Sputum	78	09	11.53 %
9	Stool	27	03	11.11 %
10	Urine	126	10	07.93 %
11	Other (wound swab /skin scrap etc.)	20	01	05.00 %
	Total	425	72	16.94 %

Significantly very high proportion of Biofilm positive cases were detected from Catheter tip (χ^2=35.889, p<0.001). [For purpose of this analysis four specimens; Catheter tip, Blood, Oral swab and others (i.e. remaing all together) were cosidered].

Table No. 05. Logistic Regression model to predict biofilm positivity:

Specimen	β	S.E.	Wald	df	Sig.	Exp(B) (O.R.)	95.0% C.I. for EXP(B)	
							Lower	Upper
Specimen			34.760	10	.000			
Catheter tip	3.638	1.195	9.269	1	.002	38.000	3.654	395.211
Tips & Tubes (Suction/ OVC/ UVC / Endotracheal etc.)	2.539	1.373	3.418	1	.064	12.667	.858	186.905
Cervical swab	2.251	1.343	2.812	1	.094	9.500	.684	131.997
Blood	2.077	1.058	3.851	1	.050	7.980	1.002	63.523
Pus	1.766	1.175	2.260	1	.133	5.846	.585	58.431
Oral swab	1.533	1.085	1.998	1	.158	4.634	.553	38.855
Vaginal swab	1.335	1.286	1.078	1	.299	3.800	.306	47.211
Sputum	.908	1.085	.699	1	.403	2.478	.295	20.802
Stool	.865	1.195	.524	1	.469	2.375	.228	24.701
Urine	.493	1.078	.210	1	.647	1.638	.198	13.538
Constant	-2.944	1.026	8.236	1	.004	.053		

Logistic regression analysis was carried out by coding biofilm production (positivity) '1' and biofilm non production (negativity) '0'. Considering this variable as dependent variable and Specimen and *Candida* Species independent variables, the logistic regression analysis was conducted. Wald statistics revealed that variable Specimen was significantly identifying the biofilm positivity. Amongst various Specimens Catheter tip and Blood were significantly identifying the positivity.

Logistic regression analysis was carried out to detect specimens significantly associated with biofilm production.

Table No. 06. Predictive ability of Logistic regression model.

Observed	Predicted		
	Biofilm		Percentage Correct
	Negative	Positive	
Negative	238	115	67.4
Positive	25	47	65.3
Overall Percentage			67.1

a. The cut value is 0.180

Logistic regression model shows out of 353 non biofilm formation species, 238 were correct negative for biofilm formation while out of 72 biofilm positive species 47 were correct biofilm positive.

DISCUSSION

The nature of biofilm structure and the physiological attributes of biofilm forming organisms confer an inherent resistance to antimicrobial agents like antifungals, antibiotics, disinfectants, or germicides.

Candidiasis has emerged as a significant medical problem because of advance in modern medicine owing to indiscriminate long term use of antibiotics, cytotoxic therapies, immunosuppressive drugs, and AIDS related complexes. [13,14-16]

The formation of *Candida* biofilms carries important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defenses. [16-20]

In the present study *Candida albicans* was major isolate i.e. 190/425 (44.70%) followed by C. *famata* 83/425 (19.52%), C. *glabrata* 47/425 (11.05%), C. *parapsilosis* 37/425 (08.70%), C. *guilliermondii* 18/425 (04.23%), C. *lusitaniae* 17/425 (04.00%) isolates etc. We had more isolates of *Candida albicans* than that of non *Candida albicans* which is in contrast to the studies of Vinitha M [21] et al, 34 species of *Candida* were isolated from blood samples, which include *C. albicans* 7/34 (20.58%), *C. glabrata* 4/34 (11.76%), *C. parapsilosis* 4/34 (11.76%), *C. guilliermondii* 2/34 (5.88%), *C. krusei* 13/34 (38.23%), *C. tropicalis* 2/34 (05.88%), and *C. keyfr (pesudotropicalis)* 2/34 (5.88%).

Our study compares well with study of Vinitha M [22] et al, who studied 111 isolates of *Candida*, out of which 49/111 (44.14%) were *Candida albicans*, 7/111 (06.30%) *C. glabrata*, 4/111 (03.60%) *C. guilliermondi*, 2/111 (01.80%) *C. kefyr*, 35/111 (31.53%) *C. krusei*, 5/111 (04.50%) *C. parapsilosis* and 9/111 (08.10%) *C. tropicalis*. In the study of Tumbarello M [23] et al, out of 294 *Candida* isolates, *Candida albicans* were 168 (57.10%), *C. parapsilosis* 64 (21.70%), *C. tropicalis* 28 (09.50%) and *C. glabrata* 26 (08.80%). Tortorano AM [17] et al studied 59 *Candida albicans* blood stream isolates. Tumbarello M [24] et al studied 207 *Candida* blood stream isolates, and they found that, *C. albicans* was most commonly isolated 122 (58.90%), followed by *C. parapsilosis* 47 (22.70%), *C. tropicalis* 20 (09.60%) and *C. glabrata* 11 (05.30 %). While Pruthi V et al [18] isolates 100 different microorganisms from 86 clinical cases (Intrauterine devices)

composed of 20 *Candida albicans* and 12 *Candida dubliniensis* isolates.

In the present study, total 72/425 *Candida* species showed biofilm production, in which 49/190 (25.78 %) *Candida albicans* showed biofilm production. Followed by C. *guilliermondii* 03/18 (16.66%), *C. famata* 11/83 (13.25%), *C. krusei* and *C. lusitaniae* 2/17 (11.76%) also *C. keyfr, C. parapsilosis, C. glabrata* species showed biofilm formation activity (Table No. 1). Girishkumar CP [13] et al observed biofilm positivity in *C. albicans* 11/18 (61.11%), in *C. guilliermondii* 2/3 (66.67%), in *C. glabrata* 5/6 (83.33%), in *C. tropicalis* 23/24 (95.83%) while in *C. parapsilosis* 6/6 (100%) from blood stream and oral isolates. V. Pruthi et al (2003) [18] found that, 20/20 (100 %) *Candida albicans* and 12/12 (100 %) *C. dubliniensis* isolates shows biofilm positivity. In the study of Vinita M [21] et al, among 34 *Candida* isolates from blood specimen of 120 catheter related ICU patients, 42.85% *C. albicans* and 63.33% *non Candida albicans* species shows biofilm positivity. So not only *Candida albicans* but *Candida non albicans* species also form biofilm which has become an emerging problem in management of infectious diseases. From the above comparison it is clear that the biofilm positivity of different species in our study slightly varies with those of other studies.

In the study of Shin JH [4] et al, bloodstream *Candida* isolates 58/101 (57.00%) and 83/259 (32.00%) from other clinical isolates shows biofilm positivity. Girishkumar CP [13] et al studied 58 *Candida* isolates from immmunoconpramised patients, in which 48 *Candida* were biofilm producer includes 30/36 (83.3%) blood stream isolates and 18/22 (81.8%) oral isolates. They also found that, biofilm producing blood stream isolates were significantly more among non-*C.albicans Candida* (93.1%) in comparison to *C. albicans* (42.9%). Tortorano A M [17] et al found that, *Candida albicans* isolates from blood stream infections shows 23/59 (39.00%) biofilm positivity. V. Pruthi et al (2003) [18] found that *Candida albicans* and *C. dubliniensis* showed 100 % biofilm positivity from Intrauterine devices. Tortorano A M [23] et al found that, 80/294 (27.2%) biofilm positivity from Candidemia patients. Thus it can be seen that our present study shows contrasting results as compares to other studies like Shin JH [4] et al, Girishkumar CP [13] et al, Tortorano A M [17] et al, Vinita M [21] et al, Vinita M [22] et al, etc.

We have carried out antifungal testing before biofilm formation and after biofilm formation. As per the result in our study it is clear that after biofilm formation the isolates become more resistant to antifungal agents. In the present study, out of 425 strains, 308 strains were sensitive and 117 strains were resistant to amphotericin B, while 323 species was sensitive and 102 strains were resistant to fluconazole including biofilm producible (before biofilm production) and non biofilm producible *Candida* species. Out of 117 *Candida* species resistant to amphotericin B, 14 strains (out of 72 biofilm producing) were already resistant to amphotericin B before biofilm production. Out of 102 *Candida* species resistant to fluconazole, 26 strains (out of 72 biofilm producing) were already resistant to fluconazole before biofilm production.

In total 72 biofilm producible *Candida* species, 58/72 strains were sensitive to amphotericin B before biofilm production. Out of 58 strains 2/58 (03.44%) *Candida* strains remained sensitive after induced biofilm production, while 56/58 (96.55%) strains become resistant to amphotericin B after induced biofilm production which is significant. (Table No. 2)

In total 72 biofilm producible *Candida* species, 46/72 strains were sensitive to fluconazole before biofilm production. Out of 46/72 strains 05/46 (10.86%) *Candida* strains remained sensitive after induced biofilm production, while 41/46 (89.13%) strains become resistant to fluconazole after induced biofilm production which is significant. (Table No. 3)

Subha TS [25] et al found that *Candida* biofilms are 30-4000 times more resistant to antifungal drugs than planktonic cells. Baillie GS [26] et al found that amphotericin B and fluconazole requires 20 times more the MIC, also in the study of Perumal P [19] et al observed 10-20 fold greater MIC to inhibit the *Candida* biofilms. In the study of Al-Fattani [15] et al, *Candida* biofilms showed highly resistance to amphotericin B and fluconazole despite the high drug concentration used (30 times than MIC).

In the present study *Candida* species isolated from Catheter tip which could form biofilm i.e. 08/12 (66.66%), Tips 02/05 (40.00%), cervical swab 02/06 (33.33%), Blood 21/71 (29.57%), Pus 4/17 (23.52%) etc. shows maximum biofilm formation activity, while oral swab, sputum, stool, urine etc. shows minimum biofilm formation activity (Table No.4, 5 & 6).

It is difficult to compare our findings with those of others authors because of the limitation in carrying out the work as different authors have studied only prevalence rate of biofilm forming *Candida*, characterisation of *Candida* along with predisposing factors. But the present study has done by all of the above factors apart from antifungal sensitivity before and after biofilm formation.

Biofilm becomes an emerging problem in management of infectious diseases. So in clinical diagnosis, infections of *Candida* should be investigated for biofilm production, which can be considered as an important virulent factor. [27-29] Use of this methodology to detect biofilm formation should be helpful for the selection of antifungal agents active against biofilms and for the screening of new effective antifungal agents to combat Biofilm-associated infections. [3,7,11,30-32]

CONCLUSION

We can conclude that, the biofilm production is a newer concept, associated with pathogenic weapon of *Candida* and can be considered as virulence factor, which turns sensitive *Candida* into the resistant one for antifungal agents. *Candida* biofilms are observed in most medical devices, such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters i.e. nonliving objects in patient's body.

To face this problem there is a need to find out newer antifungal agents or to increase the concentration of antifungal agents which in turn may be harmful to the patients. Molecular studies on biofilm formation have begun to shed light on the driving forces behind the transition to the biofilm mode of existence, including quorum sensing, which in the future may offer a potential therapeutic avenue. Future studies should focus on in vivo-grown biofilms and the determination of the biofilm-forming capacity of *Candida* species and also investigate the use of new materials and other preventive strategies that could be employed to inhibit biofilm formation. Research on newer technologies has demonstrated that surface modifying agents having antibiofilm properties when incorporated in biomedical device materials can inhibit biofilm formation of *Candida* and it should be included in routine laboratory investigation. In-depth knowledge of ultrastructure of microbial biofilms and the use of novel treatment therapies will lead to reduction in device-

related infections caused by *Candida*. In this direction further studies would highlight and follow an effective strategy for prophylaxis and treatment of *Candida* biofilms.

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