Research Article

Study of biofilm formation as a virulence marker in *Candida* species isolated from various clinical specimens

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Abstract

Background: *Candida* species can be either commensals or opportunistic pathogens with the capacity to bring about several infections, extending from superficial to life-threatening. Nosocomial infections owing to *Candida* are also getting progressively significant. Early and precise diagnosis, correct treatment, and prohibition of candidemia owing to biofilms create a big task for microbiologists and clinicians worldwide. In addition to this is the emerging trend of antifungal drug resistance among the biofilm-producing strains of *Candida*.

Objective: To detect biofilm production in *Candida* species isolated from various clinical samples obtained from patients at a tertiary-care hospital in Ahmedabad.

Materials and Methods: A total of 67 *Candida* species (26 *Candida albicans* and 41 non*albicans Candida* species) isolated from various specimens (urine, sputum, endotracheal tube secretion, tissue, oral swabs, and other samples) were included in the study. The various *Candida* isolates were identified by using conventional methods, and their ability to produce biofilm was detected by the tube method and Congo red agar method.

Result: Of 67 *Candida* species, *Candida tropicalis* [38 (56.78%)] was the predominant species isolated. Biofilm positivity was seen with 46 (68.65%) isolates, and the biofilm production was observed more with non-*albicans Candida* species [30 (65.21%)] when compared with *C. albicans* species [16 (34.78%)]. Among the non-*albicans Candida* species, strong biofilm producers were *Candida parapsilosis* and *C. tropicalis*. Biofilm positivity was found to be higher in the tissue, endotracheal aspirate, and urine of *Candida* isolates when compared with isolates from other sites.

Conclusion: This study suggests an increasing prevalence of non-*albicans Candida* species in the various clinical samples isolated and shows them as strong biofilm producers when compared with *C. albicans* species. These data suggest that biofilm formation as a potential virulence factor might show a higher significance for non-*albicans Candida* species than for *C. albicans* and that the biofilm structure varies with the different species and strains of *Candida*, the nature of the colonized surface, and its localization. Thus, more remains to be determined about biofilms formed by the non-*albicans Candida* species, as they are now frequently encountered species in catheter-associated candidemias.

KEY WORDS: Candida, biofilm, non-*albicans Candida*, candidemia

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Introduction

Pathogenic fungi in the genus *Candida* have the ability to produce several infections extending from superficial to deep-seated mycoses. The *Candida* species have been identified as the fourth commonest reason of nosocomial invasive infections.[1]

Candida organisms are commensals; to act as pathogens, interruption of normal host defense is necessary. Therefore, general risk factors for *Candida* infections include immune-compromised states, diabetes mellitus, and iatrogenic factors such as antibiotic use, indwelling devices, intravenous drug use, and hyperalimentation fluids. Candidiasis has emerged as an alarming opportunistic disease, as there is an increase in the number of patients who are immune compromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy, or undergoing invasive surgical procedures and organ transplantation. 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.[2] One of the important factors contributing to the virulence of *Candida* is the formation of surface-attached microbial communities known as "biofilm."[3]

Structured microbial communities that are fixed to a surface and covered in a matrix of exopolymeric material are called as biofilms. A standard laboratory fungal model of biofilm formation includes two operational steps: (a) adhesion and (b) biofilm growth and maturation, and has three distinct developmental phases: early (0–11 h), intermediate (12–30 h), and mature (38–72 h). The detailed structure of mature *Candida albicans* biofilms consists of a dense network of yeast, hyphae, and pseudohyphae.[4]

The advantages of forming a biofilm include protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new traits.[2] This is of particular significance, because it is now estimated that a significant proportion of all human microbial infections involve biofilm formation. It has been estimated that some 65% of all human microbial infections involve biofilms. Biofilm formation helps the organism to evade host defenses, exist as a persistent source of infection, and develop resistance against antifungal agents. *Candida* species are frequently found in the normal microbiota of humans, which facilitates their encounter with most implanted biomaterials and host surfaces.[5] The resistance of biofilm-producing *Candida* species to antifungal agents represents a major challenge, especially in the design of therapeutic and prophylactic strategies. These factors constitute a clinical problem, resulting in high mortality and economic problem owing to prolonged hospital stay.[6]

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, sufficient literature is hard to find on medically relevant fungal biofilms, particularly in the prevailing scenario where immune-compromised conditions and nosocomial infections are on the rise. Consequently, further recognition and understanding of *Candida* biofilms is of major importance in the study of human candidiasis. Therefore, this study aims to provide insights on various aspects of *Candida* biofilms and their role in pathogenesis.

Materials and Methods

In this study, a total of 108 clinical isolates of *Candida* were collected from patients attending a tertiary-care hospital, Ahmedabad, Gujarat, India.

Of the 67 *Candida* isolates, 23 were obtained from urine, 17 from sputum, nine from endotracheal secretion, four from blood, four from tissue samples, eight from oral cavity, and two from others (fluids, stool, etc.) [Table 1]. The urine isolates were obtained from catheterized patients with symptoms of urinary tract infections, the respiratory samples from pulmonary tuberculosis cases, the bloodstream isolates from ICU patients with catheter-related septicemias, and tissue samples from patients with a diabetic foot or chronic nonhealing wound. The *Candida* isolates obtained were further identified by conventional methods such as gram stain (direct microscopy), germ tube test, Sabouraud dextrose broth, microscopic morphology on cornmeal agar, and sugar assimilation tests.[7] Culture on CHROMagar was also used for identification of the species. Biofilm producer *C. albicans* (ATCC 10231) and non-biofilm producer *Staphylococcus. epidermidis* (ATCC 12228) reference strains were used as controls in this study.

Biofilm Formation

Biofilm production was detected by two methods—tube method and Congo red method.

Tube method

As described by Branchini et al.,[8] a loopful of organisms from Sabouraud dextrose agar (SDA) plate was inoculated into polystyrene tube (Falcon conical tube) containing 10 mL of Sabouraud dextrose broth supplemented with glucose (final concentration, 8%). The tubes were then incubated at 37°C for 24 h after which the broth was aspirated out gently. The tubes were then washed once with distilled water and then stained with 1% safranin. The tubes were then kept still for 7 min. Safranin was then removed, and the tubes were examined for biofilm production. Biofilm production was read independently by two different observers. The adherent biofilm layer was scored visually as negative, weak positive, or strong positive as described by Shin et al. In this study, all positive results, including weak or strong, were considered as positive.

Congo red method

Freeman et al.[9] had described an alternative method of screening biofilm formation by *Candida* isolates, which requires the use of a specially prepared solid medium—brain–heart infusion (BHI) broth supplemented with 8% glucose and Congo red. The medium was composed of BHI (37 g/L), glucose (80 g/L), agar no.1 (10 g/L), and Congo red stain (0.8 g/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 min separately from

Table 1: *Candida* species isolated from different clinical samples (*n* = 67)

No. of *Candida* species isolated	Urine	Sputum	Tissue	ET secretion	Oral swabs	Blood	Others
C. albicans (26)	5	14	1	2	4	0	0
C. tropicalis (38)	17	3	3	7	4	2	2
C. parapsilosis (1)	1	0	0	0	0	0	0
C. guilliermondii (2)	0	0	0	0	0	2	0

Table 2: Biofilm production by various *Candida* species

Candida sp.	Total no. of isolates, *n* (%)	No. of biofilm positive by tube method, *n* (%)	No. of biofilm positive by Congo red method, *n* (%)
C. albicans	26 (38.80)	16 (61)	16 (61)
C. tropicalis	38 (56.73)	29 (65)	24 (63)
C. parapsilosis	1 (1.49)	1 (100)	1 (100)
C. guilliermondii	2 (2.98)	0 (0)	0 (0)
Total	67	46 (68.65)	41 (61.19)

Table 3: Biofilm production in various clinical samples by tube method

Nature of specimen	Total no. of isolates, *n* (%)	No. of biofilm positive by tube method, *n* (%)	No. of biofilm positive by Congo red method, *n* (%)
Urine	23 (34.32)	16 (69.56)	14 (60.86)
Sputum	17 (25.37)	10 (58.82)	10 (58.82)
ET secretion	9 (13.43)	8 (88.88)	7 (77.77)
Oral swabs	8 (11.64)	5 (62.5)	4 (50)
Tissue	4 (5.97)	4 (100)	3 (75)
Blood	4 (5.97)	2 (50)	2 (50)
Others	2 (2.98)	1 (50)	1 (50)
Total	67	46 (68.65)	41 (61.19)

other medium constituents and then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24–48 h at 37°C. Positive result was indicated by red colonies, and negative results were indicated by white or very light pink-colored colonies.

Statistical analysis

The analyzed results were expressed as percentages for the description of *Candida* isolates according to species and various clinical samples. Statistical significance of prevalence of biofilm production in non-*albicans Candida* higher than *C. albicans* was tested by χ^2-test.

Result

Among the 67 *Candida* isolates, 41 (61.19%) were non-*albicans Candida* species and 26 (28.81%) *C. albicans*. Among the non-*albicans Candida* species, the most common isolate was *C. tropicalis* 38 (56.73%). The *Candida* isolates obtained from different clinical samples are shown in Table 1.

Of 67 *Candida* species tested, 46 (68.65%) were found to be biofilm producers. Biofilm production was found to occur most frequently among non-*albicans Candida* [30 (65.21%)] than in *C. albicans* [16 (34.78%)].

The results of biofilm production were also analyzed with respect to the site of infection. The biofilm positivity was observed more with tissue, endotracheal aspirate, and urine isolates, and it was less with isolates from sputum and blood [Table 3].

Discussion

Biofilms are a collection of microorganisms surrounded by the slime they secrete. The ability to form biofilm is associated with the pathogenicity and, as such, should be considered as an important virulence determinant during candidiasis.[10] In our study, we evaluated 67 *Candida* isolates from various clinical samples, namely urine, blood, tissue, sputum, oral swabs, endotracheal aspirates, etc. Our data showed predominance of non-*albicans Candida* species [41 (61.19%)]

when compared with *C. albicans* [26 (28.81%)]. In this study, 68.65% of the *Candida* isolates tested were found to be biofilm producers. Biofilm production was found to occur most frequently among non-*albicans Candida* species (65.21%) than *C. albicans* (34.78%). Among the non-*albicans Candida* species, the biofilm positivity occurred most frequently among isolates of *C. parapsilosis* (100%), followed by *Candida tropicalis* (65%) [Table 2]. Our study also showed a correlation between biofilm productions by the various *Candida* isolates with respect to their source of isolation. The biofilm positivity was observed more with tissue, endotracheal aspirate, and urine isolates, and it was less with isolates from sputum and blood [Table 3]. The tissue isolates were obtained from chronic nonhealing diabetic foot, and culture mostly yielded *C. tropicalis*. The urine isolates were obtained from catheterized patients with symptoms of urinary tract infections, and the cultures yielded mainly *C. tropicalis*. The *Candida* species isolated from endotracheal tube also showed 66.67% of biofilm positivity. These devices become colonized by the *Candida* species that forms biofilm, the detachment of which can result in candidemia. Indwelling catheters, therefore, represent a major risk factor associated with nosocomial *Candida* infections.[11] Devices such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and various types of catheters have all been shown to support colonization and biofilm formation by *Candida*.[5] The less biofilm producers were the isolates from respiratory tract (58.82%) that were obtained from pulmonary tuberculosis cases.

Our data showed predominance of non-*albicans Candida* species [41 (61.19%)] when compared with *C. albicans* [26 (28.81%)]. Studies by Mujika et al.[12] and Shin et al.[13] also indicate a trend toward an increasing prevalence of infections caused by species of non-*albicans Candida*. In this study, 68.65% of the Candida isolates tested were found to be biofilm producers. This finding is in concordance with studies conducted by Muni et al.,[10] (64%) and Mohandas and Ballal[2] (73%). Biofilm production was found to occur most frequently among non-*albicans Candida* species (65.21%) than *C. albicans* (34.78%). Similar findings have been reported by Girish and Menon[14] and Muni et al.[10] Among the non-*albicans Candida* species, the biofilm positivity occurred most frequently among isolates of *C. parapsilosis* (100%), followed by *C. tropicalis* (65%) [Table 2]. *C. tropicalis* have also been recognized as strong slime producers by many studies (Dag et al.,[15]; Mohandas and Ballal;[2] and Vinitha and Ballal[15]).

Statistical analysis for the prevalence of biofilm production more in non-*albicans Candida* than *C. albicans*, tested by χ^2-test shows $p < 0.0001$, which is statistically significant. In our study, we found five samples positive by tube method but negative by Congo red method. This may be owing to less sensitivity of Congo red agar method as studied by Oliveira and Cunha.[19] They found tube method (82% sensitive) and Congo red agar method (73% sensitive) in samples using PCR as a reference.

Candida biofilms may help maintain the role of fungi as commensals and pathogen, by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms. Consequently, biofilm-related infections are difficult to treat.[18] Hence, the study emphasizes the need for an effective antibiofilm treatment that requires improved knowledge of the pathogen itself and of the host response to adhesion and biofilm formation, the properties of the substrates onto which the biofilm develop and the interactions within microbial communities.

Conclusion

To conclude, biofilm formation as a virulence factor might show a higher significance for non-*albicans Candida* species than for *C. albicans*, and this ability to form biofilms is intricately linked with the ability of the organisms to adhere, colonize, and subsequently cause infection in susceptible individuals.

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