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Properties of *Candida albicans* Biofilms: An Update

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Abstract

Biofilm formation in *Candida albicans* is one of the important virulence factors causing infections refractory to antimicrobial agents. The factors responsible for increased resistance include complex architecture of biofilms, its matrix, increased efflux pumps and metabolic plasticity. Uses of various medical devices are associated with *C. albicans* biofilms. This review focuses on different aspects of *Candida albicans* biofilms. These include formation of four temporal stages, several important factors affecting biofilm formation, their resistance to commonly used antifungal agents including azoles and polyenes, the mechanism of resistance and alternative approaches inhibiting formation of biofilms (potential antibiofilm drugs). In conclusion, the detailed review of various aspects of *Candida* biofilms provide in depth insight of biology and pathogenesis caused by *Candida* biofilm. This detailed knowledge will certainly help in the better management of diseases associated with biofilm formation.

Keywords: *Candida albicans*; Biofilms; Resistance

Introduction

Amongst all, biofilm formation is one of the important virulence factors causing infections refractory to antimicrobial agents [1-3]. Biofilms are defined as communities of sessile organisms irreversibly associated with a surface, encased within a polysaccharide-rich extracellular matrix and exhibiting enhanced resistance to antimicrobial agents [4-6]. Biofilms are formed on both biotic and abiotic surfaces. Mature biofilms are much more resistant to antimicrobial agents and host immune factors in comparison to planktonic cells. The factors responsible for increased resistance include complex architecture of biofilms, its matrix, increased efflux pumps and metabolic plasticity [7,8].

Candida spp. is most common cause of fungal nosocomial bloodstream infections. Among *Candida* spp. *Candida albicans* is most prevalent species involved in nosocomial bloodstream infections. In spite of advanced antifungal therapy available, the mortality due to candidiasis was found to be 50 percent in adults and 30 percent in children [9,10].

Many studies have been done on bacterial biofilms, but biofilms of *Candida* spp. have been studied less. Use of various intravascular medical devices is associated with nearly half of the cases of nosocomial infections. Central venous catheters, urinary catheters, pacemakers, mechanical heart valves, joint prosthesis, contact lenses and dentures are all susceptible to *C. albicans* biofilm [11-14].

Central venous catheters are considered as the most common risk factor for development of candidemia [15]. Once the biofilm is formed on implanted medical devices, Candida biofilm has potential to seed disseminated bloodstream infections leading to invasive systemic infections. These medical devices associated infections may become disastrous. Also, management of these infections is difficult and costly.

The study of impact of Candida infections of medical devices including various vascular catheters, joint prosthesis, dialysis access (hemodialysis fistulas, hemodialysis grafts, peritoneal dialysis catheters), cardiac devices, central nervous system devices, urinary catheters, penile implants demonstrated differences in rate of infection, mortality, risk factors and species of Candida involved. Although removal of most of the devices to achieve cure in these patients had been suggested earlier [13], use of antibiotic-impregnated prosthesis, coating of catheters with antimicrobials, selective decontamination with topical non-absorbable antibiotics, preventive antibiotic lock technique, prophylactic peri-operative antibiotics, etc. have been recommended in recent European Society for Clinical Microbiology and Infectious Diseases (ESCMID) guidelines [16].

Recent advances in technology have developed novel approaches to investigate biofilms and to identify specific markers for biofilms. Specific genes with specific pattern of gene expression have been reported in *C. albicans* biofilms. Analysis of these genes can be performed by using techniques like

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micro-array analysis with probe. Alternatively Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), e.g. an RT-PCR assay can be used to analyse expression pattern of Agglutinin-Like Sequence (ALS) genes in *C. albicans* biofilms. In addition gene analysis, proteomic analysis of biofilm specific proteins can also be carried out e.g. phase-specific cell wall proteins expressed during the formation of biofilm cab be separated using two-dimensional gel electrophoresis which can be further digested to peptides and the digested peptides can be identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry [17].

In this review, different aspects of Candida biofilms are evaluated.

Formation of Biofilms

The biofilms formed *in vitro* are composed of fungal cells embedded in polysaccharide rich extracellular matrix.

Candida biofilm matrix is mainly composed of carbohydrates, proteins, phosphorus and hexosamines, where as Candida biofilm cell also consisted of yeast form cells (blastospores) and long tubular hyphae as well as pseudohyphae, and it associated with dimorphic switch between yeast and hyphal growth.

When formed *in vivo* or on *in vitro* devices like intravascular catheters, urinary catheters, these biofilms also contain host-derived biomolecules such as dead cells, fibrinogens, etc.

Formation of biofilms occurs in a sequential process. *C. albicans* biofilm formation comprises four temporal stages, *viz* adherence to surface, proliferation to form a basal layer of anchoring cells, growth of pseudohyphae and hyphae concomitant with production of extracellular matrix material and slow dispersal of yeast form of cells from biofilm to seed new sites [18-21].

The process of adherence to biomaterials is mediated by non-specific factors such as hydrophobicity of cell surface and electrostatic forces as well as by specific legends (adhesions on fungal cell surface) such as serum proteins (fibrinogen and fibronectin) and salivary factors. As per the recent study the specific attachment to biomaterials may also be mediated by cell surface proteins encoded by members of the ALS family of adhesion-producing genes and EAP1 [22]. This initial attachment of yeast cells to the substrate is followed by cell division, proliferation of these yeast cells and development of biofilm.

Various workers used scanning and transmission electron microscopy to study the morphology of biofilms formed by *Candida* spp. [20,23]. The structure of biofilms varies depending on the substrate on which it forms. These differences may be attributed to differences in substrate properties and nutritional conditions. The fluorescent and confocal microscopies are also used to reveal the structure of biofilms.

Factors affecting biofilm formation

The various factors affecting biofilm formation include:

Fluid flow: Physiological conditions such as fluid flow at the site of infection influences and modulates the nutrient exchange and structural integrity of biofilms [24-26]. Candida biofilms formed under flow produces more extracellular matrix compared to those formed under static conditions.

Substrate: Several studies demonstrated the role of substrate in modulating the ability of Candida to form biofilm. Different substrates can greatly influence the architecture, morphology and thickness of biofilm. It has been found that biofilms formed by *Candida albicans* was slightly increased on latex or silicone elastomer as compared to polyvinyl chloride. It was substantially decreased on polyurethane or 100% silicone [20,27].

Nutrients: Nutrients particularly sugars, lipids and serum are crucial determinants of biofilm forming ability of Candida. The biofilm forming ability has been found to be increased in presence of sucrose [28], glucose [20], lipid emulsion [29] and human serum [30].

Species variability: The ability to form biofilm may vary within the strains of different Candida spp. The biofilms formed by *C. parapsilosis*, *C. pseudotropicalis* and *C. glabrata* were significantly less than more pathogenic *C. albicans* [20]. It has been reported that the amount of biofilms formed on catheter by *C. parapsilosis*, *C. glabrata* and *C. tropicalis* is less in volume as compared to biofilms formed by *C. albicans* and also, there is difference in architecture between these [31]. These results demonstrate that the biofilm forming ability, structure and matrix composition are highly dependent on species of Candida. In general, *C. albicans* produces quantitatively larger and qualitatively more complex biofilm than other species.

Microbial cohabitants: Studies on the interaction between *C. albicans* and bacteria in polymicrobial biofilm show that secretion of signaling molecules that influence the behavior of one species towards other, by direct cell to cell physical contact and by alteration of local environment such as change in pH and oxygen concentration. These interactions are clearly of great importance and interest with relevance to human health. The ability of Candida to form biofilm is also affected by presence of additional *Candida* species or of different bacterial cohabitants, e.g. two common oral fungi i.e. *C. albicans* and *C. tropicalis* bind to *Streptococcus gordonii* while two other *Candida species* i.e. *C. krusei* and *C. kefyr* do not [32]. It has also been found that the presence of lactobacillus on fibre and uroepithelial cells affect the ability of biofilm formation by Candida [33]. A reduction in biofilm formation of *C. albicans* when this fungus is added to preformed biofilms of non-albicans Candida and bacteria [34]. The studies on interaction between *Pseudomonas aeruginosa* and *C. albicans* showed that *P. aeruginosa* formed a dense biofilm on *C. albicans* filaments and killed the fungus. On the contrary, *P. aeruginosa* neither bound nor killed the yeast form of *C. albicans* [35]. In general, co-culturing of *C. albicans* with bacteria reduces the biofilm forming ability of *C. albicans* [36].

The host products like salivary adhesins as well as microbial proteins have been proposed to be involved in the mechanisms of these interactions in biofilms. Fatty acid signaling molecule and trans-2-decenoic acid (SDSF) produced by *S. mutans* could affect biofilm architecture [37]. Several virulence factors of *P. aeruginosa* including homoserine lactones and phenazine (pyocyanin) are involved in inhibition of Candida biofilm [35,38,39]. These earlier studies demonstrate that the interactions between fungus-fungus and fungus-bacteria play critical role in modulating the ability of *C. albicans* to form biofilm.

Candida products: Studies based on target gene disruption, microarray-based transcriptomics, proteomics and genomics demonstrate that several genes, proteins and metabolites play significant roles in maintenance of biofilm phenotype by Candida. In first proteomic analysis of Candida biofilm, it was found that protein; alcohol dehydrogenase was involved in modulating the biofilm. Candida genes implicated in biofilm formation include ACE2,

YWP1, HWP1, LL34 (RIX7), ALS3, GAL10, VPS1, SUR7, GUP1, PEP12, TPK1/2, NRG1 (transcriptional repressor) and its target BRG1 (GATA family transcription factor), UME6 (transcriptional regulator), HGC1 (a cyclin-related protein), SUN41 (a putative cell wall glycosidase), EFG1, STV1 and VPH1 (Golgi/vacuolar subunits of vacuolar proton-translocating ATPase isoforms), CEK1 (map kinase), BCR1, SPT20, SAC1 (PIP phosphatase) and the novel transcriptional factors such as Ndt80, Rob1 and Brg1 [40-58].

Extracellular matrix production is controlled by additional factors. β-1,3 glucan is the major component of biofilm matrix. The zinc-responsive transcriptional factor Zap1 negatively regulates β-1,3 glucan. Glucan transferases (Bg12 and Phr1), glucoamylases (Gca1 and Gca2) and exo-gluconase, Xog1 are positive regulators of β-1,3 glucan production [59,60]. Furthermore, recent studies have shown that *C. albicans* biofilms were resistant to killing by neutrophils and also don't trigger the production of Reactive Oxygen Species (ROS) [61].

Additionally, quorum sensing molecules such as 3R-Hydroxy-Tetradecaenoic Acid (3R-HTDE) [62], farsenol [63] and cis-2-dodecenoic acid (BDSF) [64] also play significant role in Candida biofilm formation.

Certain metabolic processes such as carbohydrate assimilation, amino acid metabolism and intracellular transport [65] and glycolytic flux and hypoxia adaptation [66] have been suggested to have critical role in Candida biofilm formation. The mechanisms by which these proteins, genes modulate the biofilm formation are currently being investigated.

Antifungal Susceptibility Profile of Candida Biofilms

Although there is variation in susceptibility pattern of Candida biofilms to various antifungal agents, resistance has been frequently reported against commonly used antifungal agents. Candida biofilms have been found to be resistant to commonly used antifungal agents including azoles and polyenes [67-69]. In contrast to biofilms, planktonically grown *C. albicans* has shown susceptibility to most of the antifungal agents. In some earlier reports, amphotericin B, echinocandins and voriconazole have been reported to be active against Candida biofilms [70,71].

Scanning electron microscopy and confocal scanning laser microscopy demonstrated that caspofungin affected cellular morphology within biofilms. Coating of biomaterial with caspofungin had an inhibitory effect on subsequent development of *C. albicans* biofilm. Aminocandin, a newer echinocandin also showed antibiofilm properties [72]. In a recent study on time-lapse microscopic observation of effect of micafungin and fluconazole on Candida biofilm formed on silicon disks in RPMI medium, both drugs have shown inhibitory effect on Candida biofilm with relative differences in time of their action on biofilms [73].

Currently several agents are under investigation as potential antibiofilm drugs for Candida biofilm. These include chlorhexidine, sodium hypochlorite, zosteric acid, filastatin, EDTA/ethanol catheter lock solutions, gention violet and essential oils [74-79]. Additionally physical interventions such as low-level laser [80], photodynamic therapy [81] and antimicrobial coating of catheters [13] have also been proposed as possible therapeutic alternatives. However, more detail studies are warranted to determine efficacy of these agents against Candida biofilms.

Mechanisms of Resistance of Candida Biofilms

Various models developed, allowed detailed understanding of mechanisms of resistance to *C. albicans* biofilms. The methods include studying alterations in drug target involving changes in membrane sterol, membrane localized drug efflux pump assay at functional and transcriptional level and reduced or limited drug penetration through biofilms.

The alteration in sterol composition is linked to antifungal resistance. The modulation in the level of sterols may contribute to drug resistance in phase-specific manner [82]. The role of efflux pump in biofilm associated resistance has also been reported [83]. Persister cells are another contributor to drug resistance property of biofilm. These are phenotypic variants of a minor subset of metabolically dormant yeast cells. Persister cells are extremely resistant to antifungal drugs [84].

Antibiofilm Strategies: Perspectives, Research and Development

A better understanding of mechanism of formation of biofilm and its maintenance could lead to development of new antifungals that specifically target a particular state of biofilm. The susceptibility of Candida biofilms to the current therapeutic agents remains low, with exception of echinocandins. These agents are applied in different approaches as alternative methods. The perspectives of usage are:

Lock therapy approach

This approach is currently recommended and employed in treating catheter related bloodstream infections particularly in use of long term catheters according to the guidelines of the Infectious Disease Society of America [85]. The lock therapy involves the instillation of high doses of antimicrobial agents from 100-1000 fold the Minimum Inhibitory Concentration (MIC) directly into the catheter in order to 'lock' it for certain period of time from hours to days [86]. Although lock therapy does not promise to achieve 100 percent biofilm inhibition, few reports are promising for potential use of lock technique to treat infected catheters. The results of use of lock therapy seems promising but not yet convincing on cost effective point of view as huge dosages are still needed to eradicate fungal growth.

Material coating and novel anti-biofilm surfaces

A developing field of research focuses on the usage of modified materials or coated surfaces. This prevents adherence and biofilm development. Surface characteristics of implants such as surface roughness, chemistry, free energy can influence the type and feature of biofilm [87]. For example, in *Candida albicans*, the adhesion to denture material is increased if its roughness is enhanced [88]. Thus, surface modifications and surface coating with antifungal agents help to inhibit the Candida biofilm.

Quorum sensing molecules and natural by-products

Several quorum sensing molecules and natural by-products have been shown to influence the Candida biofilm formation. The examples are physical and chemical signals from oral bacterium Streptococcus gordonii modulates adhesion and biofilm formation by *C. albicans*.

Farnesol, a sesquiterpene and signaling molecules produced by *C. albicans* repress biofilm formation *in vitro* [89]. Tyrosol

accelerates hypha production in early stage of biofilm development [90]. Homoserine lactone produced by *Pseudomonas aeruginosa* represses *C. albicans* filamentation [35]. Also phenazine produced by *Pseudomonas* has exhibited antifungal activity against *C. albicans*. In a different approach, Valle, et al. demonstrated the polysaccharides produced by commensal organisms antagonize biofilm formation [91].

The identification and alteration of the communication signals would certainly help in better understanding of species co-existence and permit a better control of biofilm [92]. Targeting quorum sensing molecules in research could help in finding the novel anti-biofilm agents [93].

Host response to biofilm: Perspective of Immunotherapy

Although immune response is complex, immunotherapy has great potential against infections [94]. Little is known about the interaction between human phagocytes and Candida biofilm. However, immunotherapeutic treatment against candidiasis has been undertaken [95].

C. albicans and *C. parapsilosis* biofilm were found more susceptible to the additive effects of phagocytic host defense and echinocandin anidulafungin than to each separately and to the combination of the azoles, voriconazole with phagocytes [96,97].

Dectin 1, a receptor for β-glucan found on phagocytes is required for fungal killing and induction of early inflammatory response. This finding is of great interest for biofilm recognition by immune system as β-1,3 glucan are found in high amounts in extracellular matrix of Candida biofilm *in vitro* and *in vivo* [98].

Immunotherapeutic strategies such as vaccination, anti-candida antibodies and cytokine therapy are under investigation to treat Candida infection [99]. However, their application to treat Candida biofilm is still in preliminary stage. In relation to this, antibody based approach was studied which showed to have reduced adhesion and biofilm formation *in vitro* [100]. *In vivo* studies of antibody approach remain to be investigated in context of biofilm.

Experimental Models of Candida Biofilm

Microbial biofilm formation is multistep process involving physical, chemical and biological changes [101]. To study the versatility of Candida biofilm in humans, it is necessary to develop *in vitro* and *in vivo* models, which provide similar situation for formation of biofilm. It is also necessary to develop models that demonstrate common and specific characteristics of Candida biofilm morphology. Various models have been studied to investigate the properties of biofilm *in vitro* [19]. These range from simple assays with catheter discs to more complex flow systems such as the perfused biofilm fermenter or reactor and shear stress rotating disc system [66,102]. Subsequently various *in vitro* models have studied biofilm formation on variety of articles like plastics, microtitre plate, biofilm chips formed on glass slide, Calgary film devices, microporous membrane cellulose filters, acrylic strips, voice prosthesis, catheter discs, contact lenses and tissue culture flasks. Although biofilm formation takes place on variety of substrates, clinically relevant substrates like catheter, denture acrylic strips, voice prosthesis and contact lenses show similarity to clinical settings under physiological conditions than those formed on non-physiologically relevant substrates. *In vivo* models not only include abiotic substrates like catheters in rat, mouse and rabbit, denture in rat, contact lens in mouse but also biotic surfaces such as oral cavity, oropharyngeal mucosa, tongue and vaginal mucosa in mouse [18,103-107].

Conclusion

The detailed review of various aspects of Candida biofilms provide in depth insight of biology and pathogenesis caused by Candida biofilm. This detailed knowledge will certainly help in the better management of diseases associated with biofilm formation.

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