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BIOFILM AND RELATED ANTIMICROBIAL RESISTANCE

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ABSTRACT

In recent decades, fungal pathogens have become a serious hazard to humanity due to their rising association with high rates of morbidity and mortality rate. Concerningly, limited availability and developing antimicrobial resistance to antimycotics available have increased the frequency of illness. The pathogen requires essential virulence traits, such as biofilm formation, in order to survive and flourish in the surrounding environment. Fungal biofilm is a complex architecture where cells breed, multiply and flourish in extracellular matrix (ECM). Fungal cells get adhered to biotic and abiotic surface and form biofilm which not only provide suitable microenvironment for their survival but also protect the cells from harsh environment insults. Considering the significant significance of biofilm, this review focus on the fundamental idea of biofilm, its composition and formation majorly of Aspergillus and Candida. Further it also elaborates the role of extracellular matrix in pathogenesis and ECM related antimicrobial resistance mechanisms.

Keywords: Aspergillus, Candida, Biofilm, antimicrobial resistance, mechanism, ECM.

INTRODUCTION

The worldwide incidences of fungal infections are ever increasing. The high prevalence of fungal infections is majorly seen in tropical regions due to its warm and humid climatic condition, delivering all the favorable conditions to the pathogen necessary for their survival (Hsu, L. Y., *et al.,* 2012). Fungal infections range from superficial involving cutaneous mycoses and subcutaneous mycoses to invasive type which could lead to serious life-threatening systemic infection, if remain untreated (Hsu, L. Y., *et al.,* 2012). Superficial mycoses is among the cutaneous and sub-cutaneous infections affecting skin, hair, nails, causing inflammation to some extent and if remain untreated for long time, fungal pathogen penetrates deep into the tissue resulting in critical condition like blood stream infection. Invasive fungal infection is primarily predominated in critically ill patients in intensive care units (Ostrosky-Zeichner, L., *et al.,* 2017). Recent reports from the US health care network highlighted the global invasive fungal infection (IFI) scenario, indicating that 3154 patients were the source of a total of 3375 invasive fungal infection episodes, with candidiasis being the most prevalent (55%) and *Coccidioides* spp. coming in second place (25.1%), then *Aspergillus* spp. (8.9 percent) (Webb, B. J., *et al.,* 2018). Although *Candida* spp. is the prime cause of serious fungal infections, however, dimorphic molds, invasive molds, and infections brought on by *Cryptococcus*

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and *Pneumocystis* also significantly increase the burden of disease (Vallabhaneni, S., *et al.,* 2016). These pathogenic fungi are primarily responsible for causing severe infections in hospitalized patients with low immunity.

The major sources of infections in hospitals are indwelling catheters, parenteral nutrition, immunosuppressant, and surgical procedures, along with evolution of antifungal resistant clinical strains. All these factors together act as predisposing conditions for fungal infections in weak immune system (Alangaden, G. J. 2011; Limper, A. H., *et al.,* 2017; Lockhart, S. R., & Guarner, J. 2019). Filamentous fungi including *Aspergillus,* is the main causative agent of pulmonary and sinus related diseases giving rise to a critical condition known as Invasive Aspergillosis (IA) (Meersseman, W., *et al.,* 2004; Hope, W., *et al.,* 2013). Further infections by and *fusarium* spp. and endemic fungi *Histoplasma capsulatum* are seen to re-emerge in North and South America among HIV+ and immunocompromised population taking tissue necrosis antagonists (Lockhart, S. R., & Guarner, J. 2019). Member of *Candida* spp. including *C. albicans* are a part of healthy human mucosal flora inhabiting gastrointestinal and genitourinary tracts and are of great medical importance. In women they are the main cause of opportunistic fungal infections including vulvovaginal candidiasis (Brandolt, T. M., *et al.,* 2017). Candidemia is the blood stream infection condition caused by *Candida* spp., it is the fourth most leading cause of blood stream infection and third most leading cause for morbidity among ICU patients (Magill, S. S., *et al.,* 2014). About 40% - 60% mortality rate of ICU admitted patients is associated with Invasive Candidiasis (IC) (Groth, C. M., & Dodds-Ashley, E. S. 2016).

When a fungal pathogen first enters a host, it begins to adjust in the host's microenvironment this is facilitated by several modification in pathogen's morphology, biofilm formation and alteration of gene expression profile (Gulati, M., & Nobile, C. J. 2016). Biofilm formation is one of the most crucial factors that enhances pathogen's pathogenicity that helps it adapt to the host's microenvironment. Species including *Candida*, *Aspergillus*, *Fusarium*, *Cryptococcus neoformans*, *Pneumocystis*, *Blastoschizomyces capitatus*, *Trichosporum asahil*, *Malassezia pachydermatis*, *Rhizopus*, *Rhizomucor* are reported to form biofilm (Kernien, J. F., *et al.,* 2018). This article provides a thorough perspective on fungal biofilm with the main focus on extracellular matrix of *Aspergillus* and *Candida* biofilm including biofilm structure, its composition and formation and role of its components in virulence and antimicrobial resistance.

BIOFILM FORMATION, STRUCTURE AND COMPOSITION

Advanced techniques like fluorescence and confocal scanning laser microscopy (CSLM) utilizing carbohydrate-specific dyes including calcofluor and concanavalin A, have indicated that fungal pathogen displays two forms of development involving planktonic and biofilm. Fungal biofilm is a community of cells that live in harmony and coordinate the structural and physiological complexity of microorganisms. Cells in biofilm have shown greater resistance to existing antimycotics as compared to planktonic cells (Chandra, J., *et al.,* 2001; Flemming, H. C., & Wingender, J. 2010). This is mainly because the fungal cells fabricate themselves within self-producing extracellular matrix which provides all the obligatory micro environmental condition necessary for the survival of the pathogen. Extracellular matrix along with the adhered cells comprises of biofilm. Pathogen form biofilm to ensure their survival in surrounding environment. Biofilm not only protect the pathogen from environmental insults like fluctuation in temperature, pH, nutrient availability, host immune

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agency and noxious agents but also provide nutrition to indwelling cells. Within biofilm pathogens thrive, breed, communicate and disseminate. Biofilm provide a hideaway environment to the pathogen where they live in a surface adhered manner. It plays an important role in establishing a successful infection which involves close contact of conidia to the hydrophobic surfaces.

Biofilm architecture is highly affected by the substrate surface, for instance when biofilm is formed on a smooth, hydrophobic surface, it develops as a distinct biphasic structure made up of an adherent blastospore layer covered by hyphal elements embedded within a layer of ECM, whereas when biofilm is formed on a rough, irregular surface, it initially appears as dense tracks of cells growing along the raised, rough edges of the surfaces, with an overgrowth of cells and ECM in mature biofilms. The formation of mature biofilm, is a result of growth and accumulation microorganism (pathogen), which can be divided into three phases, early phase (~0 to 11 h), intermediate phase (~12 to 30 h) and maturation phase (~38 to 72 h) (Chandra, J., *et al.,* 2001). During the early phase, cells appear as blastophore which are surface adhered cells and form the basal layer. As the time proceeds the communities start to appear as a thick clump of fungal growth with the multiplication and propagation of the cells. It is in this interphase the non-cellular material starts to appear. As the cells multiply, they start to produce glue like sticky, non-crystalline material together known as extracellular matrix (ECM) in which the fungal cells are embedded or encased (Reichhardt, C., *et al.,* 2016). With maturation phase the ECM material increase and cells completely encase themselves in this pool (Di Bonaventura, G., *et al.,* 2007).

Extracellular matrix composition of fungal pathogen

Extracellular matrix (ECM) is one of the most intriguing aspects of fungal biofilm. It is a highly hydrated pool of non-cellular components like lipids, exopolysaccharides, carbohydrates, proteins and nucleic acids. In addition to providing a constant source of nutrients and preserving their capacity to adapt to environmental changes, exopolysaccharides in ECM shield microbe from antimicrobials, desiccation, severe temperatures, rival microorganisms, and host immune systems (Rendueles, O., & Ghigo, J. M. 2012). Biofilm formation and extracellular matrix composition are modulated by polysaccharides present in the cell wall. It has been reported that azole resistant strains showed higher cell wall polysaccharide content and contribute to overall less hydrophobicity when compared to azole susceptible strain of *C. glabrata* (Vitali, A., *et al.,* 2017).

Biofilm of *Aspergillus fumigatus* is characterized by hyphal cells or mycelia embedded in ECM commonly termed as mycetoma (Loussert, C., *et al.,* 2010). Reports suggests that ECM of *A. fumigatus* is rich in polysaccharides including galactosaminogalactan (GAG), β-(1,5)-galacto-α-(1,2)-/α-(1,6)-mannan (GM) and β- (1,3)-glucans. Galactosaminogalactan (GAG) is associated with protection of cells against antifungals in similar way other extracellular components are associated in the protection of fungal cells (Beauvais, A., *et al.,* 2007). In *A. fumigatus* GAG mask β-1, 3 glucan and limits their recognition by host immune cells. The biofilm of *A. fumigatus* contains small amount of protein (2%), secreted antigens and hydrophobins. It has been observed that about 98% of the matrix protein is of host origin. These proteins include hemoglobin, albumin, alpha-globulins, amylase, fibrinogen, keratin, fibronectin and vitronectin. Extracellular DNA (eDNA) is a vital component of the ECM and serves a variety of functions, such as genetic information transfer, cellular nourishment, cell dispersal, and ECM integrity maintenance which contribute to the overall stability and integrity of biofilms and drug resistance. In *Aspergillus,* it is observed that autolysis

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occurred by increased activity of chitinase enzyme which ultimately leads to the cell death and thus releasing DNA into the extracellular matrix (Shopova, I., *et al.,* 2013). In *C. albicans* eDNA contribute to about 5% of total dry weight of ECM (Zarnowski, R., *et al.,* 2014). The eDNA of *Candida* enters extracellular matrix probably by the process of autolysis and cell to cell gene transfer occurs by horizontal gene transfer mechanism (Rajendran, R., *et al.,* 2013). Matrix composition varies among species of *Candida*, *C. tropicalis* comprises of 27% higher hexosamine content as compared to *C. albicans*. Hexosamine content are not disrupted by DNase treatment (Costa-Orlandi, C. B., *et al.,* 2017; Desai, J. V., *et al.,* 2009). Similarly, environment conditions and media condition play an important role for their contribution to matrix composition, *C. albican*s biofilm cultured in RPMI (Roswell Park Memorial Institute) media showed 1000x fold higher matrix eDNA concentration as compared when cultivated in Nitrogen base Media (Mitchell, K. F., *et al.,* 2016).

Prokaryotes and eukaryotes live in a combination with symbiotic relationship where they prosper, grow and protect each other against antifungal and antibacterial agents giving rise to polymicrobial biofilm (Harriott, M. M., *et al.,* 2009). This multispecies interaction could be prokaryotic-prokaryotic, eukaryotic-eukaryotic or prokaryotic-eukaryotic. Multispecies interaction is observed in many species like *candida-aspergillus* along with *Psaeudomonas aeruginosa* in lung infected patients suffering from cystic fibrosis (Bakare, N., *et al.,* 2003). Most microorganisms live in a close relationship with each other and give rise to the concept of polymicrobial biofilm. Polymicrobial biofilms are most frequently observed in lungs, urogenital cavity, inner ear, oral cavity, on the site of wounds and frequently seen on abiotic surfaces. Biofilm at these sites can give rise to potential life-threatening infections (Costa-Orlandi, C. B., *et al.,* 2017). It has been observed that multispecies interaction and biofilm formation facilitates and protects the cells against antifungals and antibacterial agents. Harrott M.M and Noverr MC in 2009 shown that when *S. aureus* biofilm when grown with *C. albicans* biofilm improves resistance to vancomycin (Manavathu, E. K., *et al.,* 2014; Harriott, M. M., & Noverr, M. C. 2009).

ECM associated antimicrobial resistance mechanism

Microbe biofilm holds an extraordinary feature which is its extracellular matrix which not only provides protection against environmental insult including host defenses and antimicrobial therapy but also provides a suitable environment to the indweller cell community (Desai, J. V., *et al.,* 2014). It provides nutrition to the developing microbial community, improves cellular communication, provide the microenvironment for the exchange of genetic material, adhered the cells with each other and to the surface and protect the developing and propagating pathogen from radiation and dematerialization (Flemming, H. C., & Wingender, J. 2010). Cells in biofilm shows 1000-fold higher withstanding capacity against antifungals as compared to planktonic cells (Mathé, L., & Van Dijck, P. 2013; Taff, H. T., *et al.,* 2013; Bink, A., *et al.,* 2012). Fungal matrix associated resistance against several anti-mycotic are interlinked; this was discovered in Douglas Laboratory where they co-related matrix abundance with biofilm tolerance against two antifungal drugs, amphotericin B and Fluconazole (Al-Fattani, M. A., & Douglas, L. J. 2006; Baillie, G. S., & Douglas, L. J. 1998). β-1, 3 glucan is a fungal matrix polysaccharide which is associated with the biofilm resistance mechanism against multiple drugs. Studies have shown that when *C. albicans* and *A. fumigatus* biofilm are treated with DNase enzymes, reduces the resistance to antifungals, thus modulating biofilm related drug resistance to certain level (Martins, M., *et al.,* 2012; Krappmann, S., & Ramage,

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G. 2013; Taff, H. T., *et al.,* 2013).

Recent studies highlight that *Candida* matrix have low level of β-1,3 glucan level and high level of α-mannans and β-1,6 glucan. These both interact and contribute for the formation of mannan-glucan complex (MGCx), the complex provide immense protection against antifungal treatment (Zarnowski, R., *et al.,* 2014). In some papers it has been reported that β-1, 3glucan in the extracellular matrix can interconnect with several antifungal like amphotericin B, echinocandins, flucytosine and block their further entry into the matrix and to the cell, thus contributing to biofilm related drug resistance. Exopolysaccharide degradation within extracellular matrix by an enzyme alginate lyase enhances the activity of certain antifungals like polyenes (Amphotericin B) (Bugli, F., *et al.,* 2013). Exopolysaccharide play an important role in cell-to-cell communication via quorum sensing (QS) molecules. These are the low molecular weight signaling molecules which triggers series of signaling cascade by which cellular communication occurs. Exopolysaccharide may act as (QS) molecules like in the case of *B. subtilis*, it governs its own production (Elsholz, A. K., *et al.,* 2014). For the better understanding of drug resistance to biofilm, Samaranayake in 2005 measured drug absorption by the filtered grown biofilm of several *candida* specie, they found that absorption of antifungals tested including fluconazole, amphotericin B and flucytosin were inhibited by *C. albicans* biofilm; *C. parapsilosis* and *C. krusei* inhibited amphotericin B absorption but the absorption of flucytosine and fluconazole were higher as compared to *C. albicans* biofilm. This suggested that specie specific biofilm contribute for the drug resistance by limiting drug diffusion within the extracellular matrix of biofilm (Samaranayake, Y. H., *et al.,* 2005).

Drug sequestration is also one of the mechanisms of drug resistance to biofilm matrix of *candida.* In a paper, Nett in 2007, their group isolated *candida* biofilm matrix and performed planktonic minimum growth inhibition assay, they found that extracellular matrix is able to provide antifungal resistance to non-biofilm cells and isolable fluconazole was traced through biofilm to test whether matrix interact with antifungals (Nett, J., *et al.,* 2005). They concluded that vast majority of antifungals were associated with matrix supporting the phenomenon of drug sequestration. When biofilm matrix is exposed to enzymes targeting matrix components, it was found that β-1,3 glucan is a key component of extracellular matrix of *C. albicans* and with their degradation impairment associated with biofilm resistance against antifungals occurred (Nett, J., *et al.,* 2005). Most specie of *candida* including *C. parapsilosis*, *C. glabrata*, *C. tropicalis* produce matrix β-1, 3 glucan that help in drug sequestration (Taff, H. T., *et al.,* 2013). β-1, 3 glucan binds to amphotericin B and flucytocin and sequesters these drugs, thus contributing to biofilm associated drug resistance (Nett, J. E., *et al.,* 2010; Vediyappan, G., *et al.,* 2010).

Another mechanism of biofilm-based resistance is the upregulation of genes responsible for the activation of efflux pumps. Hyperactivity of efflux pumps is required for the outflow of drugs that have been accumulated within the matrix-cell. This mechanism has crucial role in resisting early biofilms against fluconazole but no significant role is seen with mature biofilm (Mukherjee, P. K., *et al.,* 2003). For *Candida* and *Aspergillus* species it is observed that efflux pump activity is increased during the growth of biofilm (Mukherjee, P. K., *et al.,* 2003). Increased expression of efflux pump is responsible for azole resistance. Accumulation of persister cells due to the production of cell surface superoxidase dismutase is another mechanism of biofilm-based drug resistance (Bink, A., *et al.,* 2012; Høiby, N., *et al.,* 2010; Robbins, N., *et al.,* 2010).

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As it is mentioned in previous section that eDNA helps in maintaining integrity of the extracellular matrix, they also help in biofilm associated drug resistance. When *candida* biofilm is treated with DNase enzyme, this decreases the biomass of the biofilm and increases the susceptibility of the cells to Amphotericin B but when treated with fluconazole and caspofungin no significant change in their activity was observed. This study shows that eDNA might interact with amphotericin B and establish an eDNA-amphotericin B interaction and block its access to the biofilm and thus making biofilm resistance to amphotericin B (Rajendran, R., *et al.,* 2013*;* Shopova, I., *et al.,* 2013). In another study conducted by Bugli in 2013 shown that when *A. fumigatus* biofilm are subjected to digestion by alginate lyase enzyme, this increases the invitro susceptibility to amphotericin B. Alginate lyase weakens the extracellular matrix by digesting carbohydrate content of the uronic acid (Bugli, F., *et al.,* 2013).

Physiological state and nutrient limiting condition modulates drug resistance mechanism of the pathogenic fungi. *C. albicans* when grown under iron and glucose limiting condition were reported to be highly resistance against amphotericin B (Baillie, G. S., & Douglas, L. J. 1998). Cell density is another important factor responsible for biofilm related drug resistance; in 2007 it was observed that the cell density is directly proportional to resistance against antifungals mainly azoles (Perumal, P., *et al.* 2007).

CONCLUSION

Biofilm is an important virulence factor of pathogenic fungi. These accommodate extracellular matrix (ECM) that play an important role in shielding the pathogen against environmental insults and avoid its recognition by host immune surveillance. Biofilm are crucial for establishing a successful infection. Each ECM component has a role in the pathogenicity of the pathogen. Therefore, a clear understanding of production of ECM and its components is needed. Exopolysaccharide in ECM are responsible for integrity and stability of biofilm including β-glucan which is a matrix polysaccharide that mask the cells and avoid their recognition by host immune agency. They help in drug sequestration by interacting with drugs and blocking their passage into the matrix cells. Targeting β-glucan could prove to be a promising drug target for developing new class of antifungals. Therefore, studies should be conducted on genes producing β-glucan, inactivating these genes would lower the β-glucan production which ultimately lowers the content in ECM.

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TABLES

Sr. No	Fungi	Biofilm composition	Biofilm structure	References
1.	*Aspergillus fumigatus*	Galactomannans, GAG, α-1,3 glucan, melanin, hydrophobic protein and eDNA.	Filamentous fungi, hyphal cells, hyphal fragments embedded in ECM.	Rajendran, R., *et al.,* 2012; Desai, J. V., *et al.,* 2014
2.	*Candida*	Polysaccharides, monosaccharide, uronic acid, DNA, hexosamine, phosphorus and carbohydrates, β-1,6 glucan	Basidiospores are settled at the basal layer, pseudohyphae, Hypha, Hyphal fragments.	Al-Fattani, M. A., & Douglas, L. J. (2006).
3.	*C. neoformans*	Higher sugar components is reported, Sugars such as glucose, xylose, mannose and glactoxylomannan are present.	Yeast cells embedded in a matrix material.	Martinez, L. R., &Casadevall, A. (2007).
4.	*Trichosporum* spp.	Mannose and glucose present in cell wall Polysaccharides.	Both yeast and hyphal cells are present and are embedded in ECM.	Di Bonaventura, G., *et al.,* 2007

Table1. Showing biofilm composition and structure of pathogenic fungi.

FIGURES

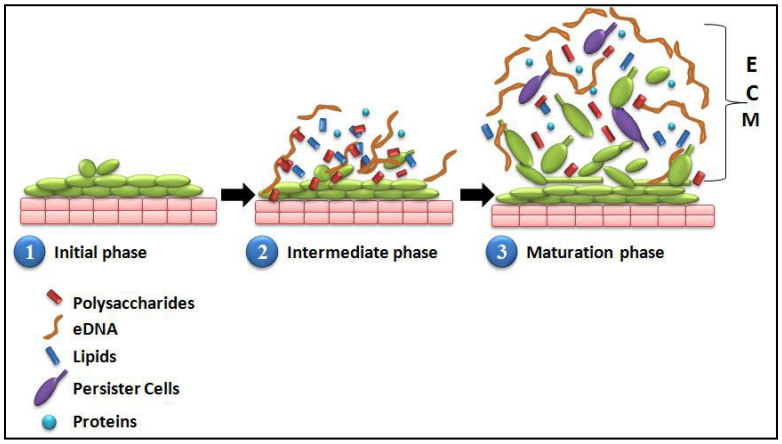

Fig1. Figure showing Biofilm Formation and Components of extracellular matrix (ECM)

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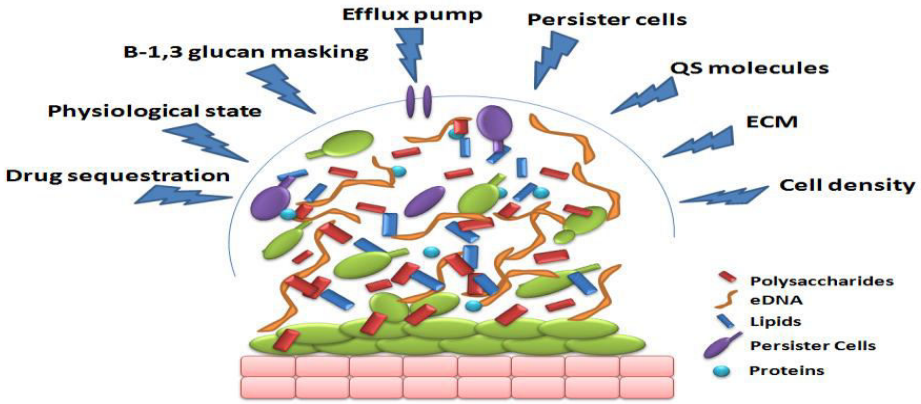

Fig2. Overview of biofilm related resistance mechanism

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