Review Article

Fungal Biofilm & Medical Device Associated Infection: It's Formation, Diagnosis & Future Trends: A Review

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Keywords: *Fungal biofilm; Biomedical device; Quorum sensing; IBIS T 5000; Electrochemical sensor*

ABSTRACT

Fungal biofilm has been associated with a wide range of persistent infections, which responds poorly to antifungal drugs. In modern day critical care; use of life saving medical devices encourages biofilm formation. Formation of biofilm on devices and prolonged hospital stay cause resistance of microorganisms to antifungal drugs. Development of resistance is due to genetic and biochemical changes in fungal cells and production of exopolysaccharide matrix. Fungal biofilm is a heterogeneous structure. There are three stages of biofilm formation, such as, microbial adhesion, maturation and disposal of biofilm. Besides, quorum-sensing play an important role in maturation and dispersion of biofilm cells. Biofilm can be detected by culture – based methods, microscopy and genotypic methods. In future, research on innovation of medical devices, newer and early diagnostic methods, new drug molecules, synthetic peptides, electrochemical sensors, atmospheric pressure and non – thermal plasma have scope to deal with biofilm – associated infection.

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Introduction

The modern definition of a biofilm was very well presented by Donlan and Costerton (2002). They reported that biofilm is a community of sessile microbial cells, irreversibly attached to an interface or to each other and surrounded by an extracellular polymeric matrix produced by them. Their growth rate and gene transcription are altered when trapped in biofilm. [1,2]

Importance of biofilm detection in medical science

Organ transplantation, immunosuppressive drug therapy, extensive use of indwelling catheters and prolonged stay in critical care units promote the prevalence of biofilm associated fungal infections. Formation of fungal biofilms on medical devices is a very serious clinical problem. This can cause dysfunction of medical device and manifestation of chronic and systemic infections. This also causes resistance to antifungal drugs. [3] Biofilm producing Candida albicans (C. albicans) are highly resistant to some of the antifungal drugs, such as, nystatin, Chlorhexidine, fluconazole and amphotericin B. The biofilm phenotypes show increased resistance to antimicrobials and treatment failure. [4] Biofilm associated infections may be polymicrobial and sometimes consist of bacterial and fungal infections together. There, they may act synergistically, resulting an increased resistance to antimicrobials. Adams et al reported a biofilm-associated infection due to Candida albicans and Staphylococcus epidermidis causing reduced susceptibility to both fluconazole and vancomycin. [5] There are several factors regarding biofilm based antimicrobial drug resistance, such as; physical barrier to phagocytosis, opsonization, stress and diffusion of antimicrobials through gel like substances of the biofilm. Further, this exopolysaccharide can electrostatically interact inhibiting action of ionic-based antimicrobials, e.g., aminoglycoside. Besides, this enclosed matrix provides an excellent medium for the exchange of genetic information via plasmids, such as, multidrug resistance. It is still not clear about the exact mechanism of drug resistance in fungal biofilm. It is either due to genetic and biochemical alteration of fungal cells or due to production of extracellular material. Some researchers proposed that antifungal resistance of biofilm producing fungus was due to metabolic quiescence of fungal cells. Another group of workers explained that might not be possible as biofilm enclosed cells actively metabolize certain substrates, such as, XTT and Fun – 1. [3, 6, 7]

Structures of biofilm

Most of the studies on fungal biofilms were done on C. albicans and other Candida species. There are wide varieties of fungi, which can form biofilm; such as, Cryptococcus neoformans, Cryptococcus gattii, Rhodotorula species, Aspergillus fumigatus, Malassezia pachydermatis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Pneumocystis species, Fusarium species, Saccharomyces cerevisiae (S. cerevisiae), Trichosporon asahii, Coccidioides immetis, Mucorales and Blastoschizomyces. [8] Structure of Biofilm varies between individual species. This is a spatially heterogeneous structure and regularly varies in form. Millions of microbial cells form pillar and mushroom shaped ultra structures invaginating into surrounding medium. [9] Biofilm matrix contains 97% of water. Out of total organic carbon present in biofilm, 75 – 90% is extracellular polymeric substances, 10 – 25% microbial cells and 1 – 2% proteins, polysaccharides, peptidoglycans, lipids, phospholipids, DNA and RNA. [10,11,12] The exopolysaccharide of biofilm in mucoid strains of microorganisms is mostly made up of alginic acid, whereas, biofilm of non - mucoid strains carry little alginate. Structural integrity of biofilm mainly depends on the binding force of the ionic interactions. [13,14,15] Besides, it has hydrogen bond also, which play a little role in holding the extracellular polymeric network. [1, 2] After sometimes, microbes become dormant due to depletion of nutrients and oxygen within biofilm and accumulation of metabolic waste products. These microbial cells are known as “persister cells” and are resistant to host immune mechanisms and antimicrobials. Persister cells become activated again when nutrition and oxygen supply to them is restored. [16,17] In case of biofilm formed by filamentous fungi, the collection of hyphae and spores are enclosed in a matrix. This leads to some prominent developmental phases guided by complex molecular events. Existence of persister cells in C.albicans biofilm has not been observed directly. Carol A et al found that Biofilm produced by C. albicans on solid surfaces were three-dimensional structures and highly resistant to antifungals. This observation was important in patient care because of increasing use of medical devices in modern health care practice. [18] Biofilms produced by C. albicans is a heterogeneous structure. [19] This consists of cellular and non-cellular components. Certain genes differently regulate planktonic and biofilm grown cells. On the other hand, some workers found that S.cerevisiae adhered to biomedical devices but failed to form mature biofilm. [3]

Pathogenesis of biofilm infection in medical devices

Colonization of microorganisms on medical devices depends on interaction of three factors, namely, device, microorganisms and host factors. There are several stages of biofilm formation, maturation and disposal.

a. **Microbial adhesion** – Initial attachment of microorganisms to inert surfaces is reversible. This is

due to electrostatic, thermodynamic and hydrophobic interactions. This is followed by irreversible attachment and micro colonies formation. [20] After that, host's extracellular fluid forms conditioning film. [21] Presence of bacterial capsule, molecular components of cell wall and cell membrane influence adhesion of microorganisms and subsequent biofilm formation. P – fimbriae and functional flagella also increases the adhesion property of microbial cells. There are also some surface bound protein adhesins, which involve in secondary microbial adhesion. [22] Other factors, such as, area and type of the surface of the device, porosity, surface hydrophobicity and charge of the surface also influence biofilm formation. Prasanna SS et al observed that rough and porous surface of the device was more favorable for microbial biofilm formation. Cataions caused cross-linking of biofilm matrix. [23]

Like bacteria fungi can also form biofilm on medical implants. Hawser and Douglas found that C. albicans could form biofilm on a wide variety of abiotic surfaces. They observed that biofilm formation by C.albicans occurred best on latex (polyvinyl chloride) or silicone elastomer and less on polyurethane or pure latex. [22] The expressions of agglutinin – like (ALS) genes cause attachment to the host cell surface. Sometimes, formation of biofilm depends on mutations, which can reduce its development. These types of mutations can interfere in adherence to surfaces. One of the many examples of this is EFGI gene. This gene is a major regulator of hyphal development and can be affected by mutation. Carol A et al also found that ACE2 gene encodes a transcription factor that regulates expression of chitinase and cell wall proteins. ACE2 deficient mutants show reduced adherence to polystyrene and thereby, less biofilm formation. [18]

b. **Maturation of biofilm** – After accumulation of microbes on biomaterials, multiplication and production of extracellular polymers occur. This creates multiple layers of microorganisms. Maturing biofilm shows increased synthesis of extracellular polymers and more exchange of genetic information between one cell to other. Biofilm organisms also exhibit increase in resistance pattern to antimicrobials, ultra violet light and secondary metabolic products. [18,22]

c. **Disposal of biofilm –** After certain period in a biofilm, microbes suffer from lack of nutrition. Due to lack of nutrients and accumulation of metabolic products microbial populations within a mature biofilm suffer. Therefore, to maintain their populations biofilm cells are genetically encoded to seek fresh surface for colonization. The process of disposal of biofilm from a surface are sloughing, abrasion, erosion and human interference. Sloughing or shedding is a more random process than either erosion or abrasion. Dispersion of a biofilm is a complex process and includes multiple effectors, signal transduction pathways and environmental signals. It has three phases – detachment from existing biofilm, translocation to other area and adherence to a new surface. Dispersion of biofilm increases the infection of medical device that is already exists. It also causes rapid spread of infection to other parts. During dispersion of a part of biofilm, some biofilm cells undergo genotypic changes and revert back to planktonic phenotype. One of the examples is up regulation of genes associated with flagella and down regulation of genes coding for exopolysaccharide synthesis and accumulation. Besides, productions of proteases also initiate breaking down of the outer part of the biofilm. Glycosidase, proteases and deoxyribonucleases are few examples of enzymes among many that involve in breaking down of biofilm. Other than that, there are certain surfactants molecules produced by microorganisms, which contribute in dispersion of biofilm. These surfactants act by decreasing cell surface and cell to cell binding force. [24,25,26,27] Hall – Stoodley et al described three stages of dispersal of biofilm – detachment, translocation and attachment to a new surface. [28]

d. **Quorum sensing –** The formation, maturation and dispersion of biofilm cells occur through gene expression. This gene expression is regulated between single and multiple cells via production, release, detection and response to cell-to-cell signaling molecules. These signaling molecules are called auto inducers. The entire communicative system is called quorum - sensing. Quorum- sensing allows the microbial populations to act together as a community. This helps in growth, survival and colonization of microorganisms in a biofilm. Atkinson et al described that quorum-sensing molecule should accumulate in extracellular matrix at a required stage of growth under specific physiological conditions. It also should have the ability to recognize a specific cell receptor present in the biofilm. Besides, it should initiate a cellular response more complex than the process required to metabolize the molecule. [29] Biofilm formation is not dependent upon one specific quorum – sensing pathway. There are two quorum-sensing molecules – farnesol and tyrosol. These two molecules regulate "germ tube" formation. They also influence conversion of yeast to filamentous form and play a major role in pathogenicity and formation of biofilm. [30]

In case of C.albicans biofilm, increased expression of drug resistance genes may be responsible for antifungal drug resistance. Some of these genes are CDR1, CDR2 and MDR1. [31]

Sites of biofilm formation

In modern day medical practice in a hospital, there are increasing uses of life saving devices. Examples include, needle less connectors, central venous catheters, urinary catheters, endotracheal tubes, prosthetic joints, mechanical heart valves, intrauterine devices etc. All these devices are potential sites for biofilm formation. Biofilm may form on solid surfaces having optimum moisture, soft tissue surfaces of living organisms and at liquid – air junctions.[32]

Collection of samples for biofilm detection

Samples can be collected by removal of biofilm by scrapping exposed surface or removal of part of the system carrying biofilm. Biofilm can be loosened up from the system by treating a segment of it with Ultrasonication. Catheter tips can be directly rolled over blood agar plates and incubated for culture of biofilm producing organisms[33]

Culture based methods for detection of biofilm

There are three common medical device associated infections by biofilm producing organisms, such as; catheter associated urinary tract infection (CA UTI), catheter related blood stream infection (CRBSI) and ventilator associated pneumonia (VAP). For CA UTI, urine is collected from sampling port of the catheter with a sterile syringe and needle. The sample is cultured and tested for biofilm production. In case of CRBSI, infection site may show inflammation at the site of insertion. These patients may also have bacteremia. CRBSI can be diagnosed by direct inoculation of catheter tip by rolling and pressing it on culture plate. Fluid from the catheter can also be cultured. Besides, to dislodge the biofilm accumulated inside and outside the catheter, it can be sonicated and the material thus collected can be cultured. For detection of VAP, bronchoalveolar lavage (BAL) through endotracheal intubation or tracheal aspirate is collected. All the samples thus collected are inoculated in blood agar and MacConkey's agar and incubated for 24 hours.

In case of CA UTI, if there are more than or equal to 10^5 colony forming units (CFU)/ ml, it is considered as significant. For BAL, 10^3 CFU/ml and for tracheal aspirates, 10^5 CFU/ml are significant. [34,35,36,37,38] The culture isolates are identified by conventional methods and tested for biofilm production. There are several culture-based techniques for detection of biofilm, such as; Congo red agar (CRA) method, tube method (TM) and tissue culture plate (TCP) method.

In CRA method, concentrated aqueous solution of Congo red stain is prepared and sterilized by autoclave. This stain such prepared is added to autoclaved brain heart infusion agar. Then, sucrose is added to it at 55^0 C and dispensed in petri plates. The organism to be tested is inoculated in CRA plate and incubated at 37^0 for 24 to 48 hours. Biofilm producing microorganisms show dry, crystalline, black coloured colonies on CRA plate. [39,40]

In TM, a tube with 10 ml of tryptic soy broth is taken and 1% glucose is added to it. This broth is inoculated with test organism and incubated at 37^0 C for 24 to 48 hours. After incubation, supernatant is discarded and the tube is gently washed with phosphate buffer saline. Then the tube is air-dried and the adherent deposit (due to biofilm production) at the bottom and wall of the tube is stained with 0.1% crystal violet. After washing with de ionized water the tube is dried in an inverted position. [39,40]

In TCP method, the wells of the plate are inoculated with fungal suspension along with positive and negative controls. The TCP is incubated for 24 to 48 hours. Non-adherent cells are removed by washing it with phosphate buffered saline. Biofilm such produced are fixed with 2 % sodium acetate and stained with 0.1% crystal violet. After washing with deionized water the plate is dried and optical density of stained biofilm is procured by spectrophotometry.

Mathur T et al and Bose S et al found that TCP was a better test for detection of biofilm producing organisms than CRA and TM. [39,40]

Biofilms can also be grown on polymethylmethacrylate strips or on silicone elastomer disks of 1.5cm^2 diameter. In both the methods standard inoculum of fungus, i.e., 1x 10^7 cells from overnight culture are used to form biofilm. Fungal suspension is added to polymethylmethacrylate strips and incubated in RPMI 1640 medium. In case of silicone elastomer, the disks were placed in a 12 well tissue culture plate. Fetal bovine serum is added to it and the plate is incubated at 37^0 C for 24 hours on a rocker. Then the disks were washed with phosphate buffered saline (PBS) and dipped in 3 ml of standardized cell suspension (1x 10^7 cells). The plate is incubated at 37^0 C for 90 minutes. After that, wells were washed with PBS and disks were immersed in YNB medium with 50 mM glucose. The plate is again incubated at 37^0 C on a rocker. Chandra J et al reported that tetrazolium XTT reduction assay and dry weight measurement quantitate biofilm formed on both polymethylmethacrylate and silicone elastomer disks. Disks without fungal cells act as control. Assay is done in 4 replicates. [3]

Microscopy

Light Microscopy – Layer of biofilm is scrapped off from the medical device and inoculated into a culture medium. This suspension is then poured into a flat-bottomed plate. A coverslip is placed over the top of the plate, so that upper most surface of the liquid reaches the centre of the coverslip. The plate is incubated at 37^0 C for 18 hours. After that, the coverslip is taken out and dipped in 0.1% crystal violet. With the help of a light microscope, the stained cells attached to the coverslip can be visualized. [41]

Use of fluorescent dyes, such as; 4,6 diamidino – 2 phenylindone (DAPI) or acridine orange (AO) to visualize biofilm cells; enhance the function of light microscopy. These dyes can be used directly on catheter surfaces. They can stain nucleic acid of both live and dead microbial cells. Propidium iodide can stain cells with damaged cytoplasmic membrane and 5 – cyano – 2,3 – ditolyltetrazolium chloride can stain viable cells. Live cells reduce the dye into 5 – cyano – 2,3 – ditolyltetrazolium chloride formazan. This can be appreciated by visualizing evolution of a red fluorescent precipitate. Therefore, these dyes can be used to detect effectiveness of the treatment. But Donlan et al observed that thick biofilm cells could not be detected by this method due to scattering of light by thick layers of biofilm. [2,42,43]

Scanning Electron Microscopy (SEM) – By this method, catheter tip as well as culture of biofilm producing microbes both can be stained. The sample is sputter coated with a gold or palladium film. This coating allows visualizing the surface attached cells and extent of growth of biofilm. But during the preparation of the sample for electron microscopy, cells of biofilm may shrink due to complete dehydration. This excessive shrinkage can be avoided by treating the sample with ruthenium red prior to dehydration.

A modified SEM method, environmental scanning electron microscopy (ESEM) has been developed, but its magnification is less than the conventional SEM. Taj et al and Schaule et al also observed similar findings in their research. [41,43]

Genotypic Method

These molecular techniques are very sensitive and time saver but costlier and need appropriate training. This can also detect viable but non - culturable (VBNC) as well as fastidious microorganisms within biofilm. Therefore, molecular techniques help to a great extent in identification and treatment processes of a patient. Now a day, molecular method has been adopted globally. [44,45]

Polymerase chain reaction (PCR)

PCR amplifies gene sequences of a target organism causing infection. There are three steps in a PCR –

1. Nucleic acid extraction
2. Amplification of the extracted nucleic acid
3. Detection of amplified product by gel electrophoresis

There is a chance of false positive result during amplification of bacterial DNA. This can be rectified by detection of messenger RNA (m RNA) and ribosomal RNA (r RNA) for detection of biofilm. Only viable cells produce m RNA, therefore, detection of m RNA in a biofilm confirms cell viability. Disadvantage of detection of m RNA in a biofilm is its rapid degradation after cell death. It can be detected only in active infection. Patrick FB et al found that low copy number and absence of universal target sequences for all bacterial cells were other limitations of detection of messenger RNA in biofilm. Ribosomal RNA is present in abundance in viable bacterial cells and disappears rapidly after cell death. A segment of r RNA is unique for a particular species of microorganism. [45,46,47] While working on clinical cases of fungal keratitis, Embong et al demonstrated that rRNA PCR technique was 90.9% sensitive and 94.7% specific. [47]

Fluorescent in situ hybridization (FISH)

In FISH, fluorescent labeled probes identifies DNA sequences of denatured DNA samples. In this method, detection of culture- negative microorganisms can also be possible in approximately six hours. But one of the disadvantage of this technique is fading of fluorescent marker and therefore, not suitable for all types of laboratories as samples cannot be stored. On the other hand this is a quick and reliable method and very useful in rapid diagnosis and treatment of biofilm-associated infections. [48,49] Goncalves AV et al (2006) experimented with FISH using universal rRNA probe EUK516 labeled with the red CY3. After that, they stain the biofilm with calcoflour white MR_2 fluorescent dye, which stained fungal cell wall blue. They observed that, by using CW, it took less than one hour while by FISH, five hours to demonstrate filamentous fungi in water biofilm. [50]

IBIS T 5000 Technology

This technique combines broad range PCR (16s RNA PCR) and high performance mass spectrometry. By 16s RNA PCR, genes of microorganisms in biofilm are amplified. [51] Then base composition signatures are obtained by mass spectrometry. This is a highly sensitive technique and can detect culture negative or difficult to culture microorganisms also. IBIS T 5000 technology is useful for detection of microorganisms up to species level and genes

responsible for drug resistance. [52] The advantage of broad range PCR over traditional PCR is that, it can produce products from a number of microorganisms at the same time. In conventional PCR a specific primer is required for detection of a specific microorganism. On the other hand, IBIS T5000 technology uses mass spectrometry derived base units of pathogens obtained from amplified PCR products of the sample.

Mass spectrophotometer analyzes base composition data of PCR products rapidly. Suci PA et al described that this method could be used for large-scale analysis of samples. IBIS T 5000 technology is a fully automated system. Therefore, no specialized expertise is required to run this machine. [53]

Future trends - A multidisciplinary approach is required for prevention and treatment of biofilm and medical device associated infections. Multipronged actions like patient care and cleanliness, innovative devices and methods, newer drug molecules, synthetic antimicrobial peptides, use of acoustic waves, atmospheric pressure and non – thermal plasma should be adopted to minimize device – associated infection. New designs of biomaterials with less chance of biofilm formation look promising. [54] Newer diagnostic methods that can detect early formation of biofilm are need of the hour. Otto and Silhavy (2002) observed that disruption of quorum sensing between microbial cells might play an important role in reducing cell reproduction and biofilm formation. [55] Bellin et al (2014) showed that square wave voltammetry could be used for identification and quantification of several distinct redox active metabolites from microorganism. Development of specific electro chemical sensors for species identification and biofilm formation might be useful. [56] Biomedical devices impregnated with antimicrobials are increasingly being used to prevent colonization and biofilm formation.8 Resistance to antimicrobials are becoming more problematic with the increased use of life saving devices and prolonged hospital stay. The potential cytotoxicity and stability of the surface coating of the medical devices are to be considered while working for development of antimicrobial medical device. [57] Silver containing medical devices is widely used in practice. Silver has a broad spectrum anti bacterial, antifungal and protozoal activity. [58] But there are also some reports on silver resistant genes found in microorganisms. [59] Martinez et al (2010) reported anticandidial biofilm activity of chitosan. They observed that, chitosan inhibited Candidal biofilm in vivo. They also found that chitosan reduced metabolic activity of biofilms and the cell viability of C.albicans and C. parapsilosis biofilm in vitro. By using SEM, these researchers demonstrated that Candidal biofilm was invaded and damaged by chitosan treatment. Chitosan is a polymer extracted from exoskeleton of crustaceans. Chitosan interferes with synthesis of messenger RNA and proteins of microorganisms. Interaction between negatively charged microbial cell membrane and positively charged chitosan causes leakage of intracellular constituents leading to cell death. This observation may allow further study of invention of chitosan- coated device to prevent biofilm-associated infections. [60] There is increased use of infection resistant biomaterials to prevent biofilm related infections. Most of such materials only act actively for first few days after application. Therefore, more researches are required to improve longevity of their action and to reduce microbial resistance. [61, 62]

Conclusion

The adherence of microorganisms to medical devices leads to colonization and subsequently formation of biofilm. The use of life saving indwelling medical devices causes an ongoing challenge to clinicians. To deal with this problem, we should update our understanding of human immune response to the implantation of devices as well as microbial pathogenicity cycle and biofilm formation. This approach may reveal new biomarkers with their use for diagnostic purpose and new biofilm- related targets for chemotherapeutic challenge. There is a lot of scope for future research for early detection of biofilm formation, design of new types of biomedical devices and newer drug delivery methods to combat with biofilm associated infection.

Acknowledgement

Authors are grateful to the Pravara Institute of Medical Sciences for the encouragement and support throughout the study.

Funding

Nil

Competing Interests

None declared

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