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Exploration of biofilm formation and its association with the clinical and molecular characteristics of *Staphylococcus aureus*

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Abstract

Bio film is the complex community of different microorganisms that associated or attached to the surfaces. These microbial communities are composed of multiple species in which different organisms interact with each other and with the environment. In these organisms, *Staphylococcus aureus* are the most important which have a vital role in bio film formation. By developing this complex community, *Staphylococcus aureus* become more resistant towards antimicrobial agents. Methiciline resistant *Staphylococcus aurues* have resistant against methicilline by developing the complex community with many other organisms. New methodologies and experimental work have been done to explore the phylogenetic grouping, metabolic interaction, and competition among other members of the bio film. For the mere advancement, we reviewed here the bio film ecology, model organisms which have been investigated using different molecular techniques and identify the gene required for the development of bio film. Recently, bio film formation has led to amazing progress for studying advanced ecological significance of bacteria and provide the new approaches in the molecular genetics of bio film formation.

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Introduction

Staphylococcus aureu sis the pathogens that are medically very important because it has a number of virulence factors, one of which is bio film formation, which is divided in three steps attachment, maturation and dispersal. This bio film formation on an inert surface depends upon different factors as the physical and chemical nature of an object and surface components of bacteria. In *Staphylococcus aureus*, the surface protein G and C along with bio film associated proteins (BAP), fibronectin, fibrinogens and clumping factor B are individually associated with bio film. BAP is involved in intercellular adhesion and complicated cell cluster formation as well as in attachment of bacteria to biotic or a biotic surfaces. Bio film formation is achieved when bacteria attach to the surface and recognize the adhesive matrix molecule, they unite and produce extra polymeric substances which interact with components derived from the host as platelets and produce bio film (Speziale *et al*, 2014).

Bio film formed on mammalian cells is linked to exopolysaccharides production and rough colony formation. The bacteria which are truly encapsulated by exopolysaccharides become more resistant to phagocytes because they inhibit the interaction between receptors present on the phagocyte and bound opsonins, they need anti capsular antibodies for opsonization while in bacteria having microcapsule antibodies concentrating against bacterial capsule play role in opsonization. The chronic infections are mostly due to the involvement of excessive exopolysaccharides (Baselga *et al*, 1994).

Bio film formed by *Staphylococcus aureus* is in response to decreased pH when cytoplasmic proteins associate to the cell surface in reversible manner that are released from stationary phase cells and they contribute in exopolysaccharides production, cytoplasmic proteins along with exopolysaccharides help in bio film formation. *Staphylococcus aureus* seems to recycle these cytoplasmic proteins (Fouls ton *et al*, 2014). Regulated autolysis in cell release extra cellular DNA that makes the structure of biofilm more stable.

The exopolysaccharides enclose non-pigmented and non-hemolytic small colony variants which are formed by host derived stress and external environmental factors in specific Staphylococcal strains, both of these make the bacteria exceedingly resistant to antimicrobials (Bui *et al*, 2015).

Exploration of Bio film Formation

Bio film formation is three step process, in the first step the plank tonic bacteria near a surface experience Vander wall forces as well as net negative electrostatic repulsive force, if it is acted upon by foreign kinetic energy that will increase the chance of attachment to the surface by overcoming repulsive force then it will produce a exopolysaccharides matrix for irreversible attachment to the surface, these bacterial cells multiply and form bulk mass which finally start releasing some bacterial cells from periphery again become plank tonic (Shivakumar and Chakravortty, 2014). In a bio film when irreversible adhesion takes place rate of cell division becomes equal to cell death, which is carried out by some kind of enzymes released by bacteria itself that release some bacteria to colonize another attachment site. During this process operons are up regulated for motility while prions are down regulated to complete genetic cycle. This whole process is carried out by cell to cell communication called as Quorum sensing (Gautam *et al*, 2013).

Bacterial cells in their plank tonic state have flagella and cilia the help them to move and find out a place for attachment and form sessile bio film after which they begin to produce exopolysaccharides and multiply, they have free access of nutrition at that place after maturation of bio film they can release the cells from sessile to plank tonic state (Encarnacion *et al*, 2014). Bio film matrix enables the bacteria to with stand harsh environmental conditions, phagocytosis, and starvation makes them resistant to the effect of antimicrobials; bacteria manage more tolerant phenotype by turning on their stress response genes. Bio film is a three dimensional structure which contain a variety of phenotypes, antibiotic susceptibility is decreased from top to bottom of bio film where oxygen and glucose are completely

consumed rendering it metabolically inactive bacteria have an irregular dissemination in the same micro-environment (Fux *et al*, 2005).

Bacteria after developing bio film become more resistant to the immune system as well as for the physical and chemical agents. In bio film state, bacteria have less nutrient availability so become per sister cells leading to increased resistance to antimicrobial drugs (Jacques *et al*, 2010). Bio film is resistant to antimicrobials, therapeutic failure in most cases causes the mastitis to reoccur, failure of first antimicrobial treatment of mastitis causes increased probability of recurrence of mastitis in the same quarters (Melchior *et al*, 2006). Detection of bio film formed is done by various methods as tube culture method, cover slip method (Fiadh, 2011) Micro titter plate or Congo red method (Melo *et al*, 2013). However, some of Staphylococcus bacteria do not form bio film they have different morphological characters.

Bacterial Bio film and Its Importance in Animal Disease

Bacteria are unicellular microorganisms that were considered to live in the plank tonic mode of growth. A study was conducted to know about the bacterial mode of living and their bio film formation to cause diseases. Bacteria live and modify their growth conditions they can either live as a single cells or in the form of a community called as bio film. This bio film formed by bacteria is believed to support them in causation of diseases and spread of infection. It was studied that bacteria at site of infection detach from their bio film and invade the blood stream and became resistant to phagocytosis, the endotoxins released by bacteria were also considered as part of their bio film. The bacteria as *Staphylococcus* spp, *Streptococcus*, Pseudomonas and Mycoplasma spp. form bio film and cause major infection in animal species. Bio film formation also makes these bacteria resistant towards antimicrobials. The development of pneumonia, dermatitis, arthritis, otitis, abortion, conjunctivitis and mastitis were studied as the major consequences of bacterial bio films.

The initial infections were found to be chronic because of colonization and the formation of bio film by bacteria (Romero *et al*, 2010).

Bio film is a complex structure formed by bacteria, the bio film release extra polymeric substances and embedded in it to form a complex structure. The study was conducted to investigate and understand the insight of bio film association with *Staphylococcus aureus* because this bio film formation is a survival strategy of this bacteria. The formation of bio film by bacterial species as *Staphylococcus aureus* and *Mycoplasma bovis* makes them resistant towards antimicrobials. Bacteria become more resistant when they undergo genomic changes by up and down regulation of their genetic structure, they also modify their phenotypic expressions by forming bio film. These bio film formation by normal commensals help them to colonize and cause chronic infections. The pathogens in a undergo size and phase variation to evade the immune system. The antimicrobials do not penetrate in bio film, so bacteria succeed in establishing chronic infections. In this study a bio film based vaccine was suggested against bacterial adhesions (Prakash *et al*, 2003).

Role of Staphylococcus Aureus in Bio film Formation

Staphylococcus aureus is a normal commensal of human and animal skin and is the cause of a number of infections in both animal and human. In this study the formation of bio film and its dispersal was studied, bio film formation ability make *Staphylococcus aureus* a pathogen of economic importance. As *Staphylococcus aureus* is normal commensal of skin so it's easy for this bacteria to invade and cause infection. The bio film formed by *Staphylococcus aureus* was observed to cause more severe and chronic infections. During the dispersal of cells from a bio film they were found to colonize some other places and enhance the severity of infection. The dispersal of bio film was under the enzymatic action, the Exo-enzymes as nuclease, serine protease and cysteine proteases played an important role in the degradation and dispersal of bio film. It was also established that D-amino acids may also their play a part in bio film dispersal, the bio film matrix and

enzymatic dispersal was found to be varied according to the strain type of *Staphylococcus aureus* (Lister and Horswill, 2014).

The role of *Staphylococcus aureus* in bio film formation is very critical it's because of colonization or adhesion and bio film formation of bacteria in mammary tissue. The bio film formed by the *Staphylococcus aureus* increase its pathogenicity, this study was conducted to know about the components of *Staphylococcus aureus* involved in bio film formation. The animals at the sub clinical stage of mastitis were tested and a total of 102 strains were isolated of which about fifty percent were found to produce bio film by the Congo red agar method. The genes involved in adhesion of bacteria to intracellular surface were studied by polymerase chain reaction, both ice A and ice Dgenes were investigated in bio film. It was established that both ice A and ice D play roles in bio film formation. The use of PCR was established as a novel method for the detection of genes involved in intracellular adhesion and in bio film formation (Dhanawade *et al*, 2010).

Staphylococcus family is increasingly involved in bio film formation and methicillin resistant *Staphaureus*, which is becoming a frequent human pathogen was investigated in a study conducted in Belgium. In that study one hundred and eighteen herds were monitored and a total of one hundred and eighteen strains of *Staph. Aureus* was examined for the presence of mec A genes. The use of disk diffusion, multiloccus sequence technique, somatic cell mec typing and spa typing were carried out in that study. The herd prevalence was found 0-7.4% of methicillin resistant strains while mec A genes were detected in 9.3% of Belgian cows.

The antibiotic sensitivity tests also revealed that MRSA positive strains of *Staph.aureus* were resistant to tetracycline and macrolides. The study suggested that livestock associated multiresistant strains of *Staph aureus* are becoming future threat in biofilm formation and its treatments (Vanderhaeghen *et al*., 2009).

*Staphylococcus aureus*is a major pathogen causing high economic losses and remain untreated in most of the cases, persist even during antibiotic therapy due to its ability to form biofilm that helps to evade the host immune system along with other bacterial pathogen of Staph.

Family increasingly involved in mastitis during the last decade is *Staphylococcus epidermidis*. The work was done to establish new techniques as fluorescent in situ hybridization for the observance of biofilm directly in milk samples. In this study it was established that *Staphylococcus epidermidis* was 81% in isolated strains indicating its prevalence and colonization. Some other strains of Staphylococcus species were also found that were coagulase negative, the colonization and biofilm formation by CNS was equally high irrespective of its place of isolation (Oliveira *et al*., 2006).

Role of Biofilm in Bacterial Diseases

In a study conducted on mastitis prevalence it was found to be most problematic disease of dairy cattle and mastitis caused by *Staphylococcus aureus* a biofilm producing bacteria was found to be resistant to antibiotic therapy and leads to chronic mastitis, biofilm forming property of bacteria help to colonize and survive them in hostile conditions and establish infection, it was suggested to give proper consideration to the infection caused by bacteria that produce biofilm. The intra mammary infusions were considered as less effective in treating chronic infections produced due to biofilm so development of the operative vaccine contrary to biofilm producing bacteria was suggested as a tool to control the infections (Raza *et al*., 2013).

The colonization of *Staphylococcus aureus* is supported by its virulence factors, by many factors biofilm formation is an important factor that makes *Staphylococcus aureus* more resistant to antimicrobials. In this study the formation of biofilm by different strains of methicillin resistant *Staphylococcus aureus* and their response to antimicrobials was studied under in vitro conditions.

A total of eighty six isolates were evaluated of which forty four produced biofilm containing eight isolates that produced tough biofilm. The studies showed the bacteria in planktonic state were sensitive to almost all antibiotics, but when they form a biofilm they became resistant to antimicrobial therapy. In this study the strong biofilm producing strains were subjected to six novel antimicrobials and they were resistant to high concentrations of antibiotics except gentamicin and tigecycline that showed effective response in bactericidal action (Cha *et al*., 2011).

Staphylococcus aureus is major mastitis pathogens, in a study of clinical mastitis cases hundred samples were collected and preceded for bacterial isolation. Ninety bacteria were isolated of which thirty three were *Staphylococcus* spp and fifty seven were miscellaneous bacterial species. Twenty three were positive for *Staphylococcus aureus* and ten were positive for *Staphylococcus epidermidis*.

The biochemical tests were performed for *Staphylococcus aureus* and *Staphylococcus epidermidis*, beta hemolysis, mannitol fermentation and deoxyribonuclease were positive for *Staphylococcus aureus* but were negative for *Staphylococcus epidermidis*. Both bacteria of the staph family were resistant towards antimicrobials, but percentage was more for *Staphylococcus aureus*than *Staphylococcus epidermidis* (Arshad *et al*, 2006).

Biofilm forming ability of *Staphylococcus aureus* was tested in which milk samples of forty five herds were analyzed and two hundred and twenty one *Staph.aureus*were isolated from one hundred and seventeen milk samples, thirty four from the milking machine equipment's and seventy from the skin of the teats. Pulse field gel electrophoresis was used to sort out biofilm forming ability of these bacteria. It was established that the *Staph.aureus* isolated from milk samples were more proficient to form biofilm than the other isolates.

The biofilm formation by *Staph.aureus* from milk samples was because of their attachment to the mucosal surface of the udder that helps them to colonize and form biofilm, that biofilm then provide a setback for non-biofilm forming bacteria to colonize and cause infection in the udder. In that study it was proposed that pre milking teat washing and cleaning of the milking machine reduced the chance of biofilm formation by those Staph aureusisolates so a concept of strong and weak biofilms was presented and in tramammary infection was established by *Staphylococcus aureus* with other bacterial species (Fox *et al*., 2005).

Biofilm Formation in Negative Regulatory System

In a study the biofilm formation was observed in absence of Two component system, *Staphylococcus aureus* possess a positive and negative regulatory system. The biofilm formation was also affected by environmental factors. The gene involved in biofilm formation was targeted in many of the procedures. A chemically defined medium was used in biofilm formation and negative regulators were identified by disrupting the two component system and by using transposon mutagenesis.

The studies showed that in biofilm formation ar lRS was a repressor. It was found involved in attachment to a surface and it enhances the accumulation of poly-N-acetyl glucosamine (PANG) but act as repressing agent in both steady and flow state. The absence of ica ADBC operon did not affect the biofilm formation by ar lRS that indicated independence of biofilm formation from PANG. In that study the sar A and atl removal was reported not to affect the biofilm formation while removal of agr was found to reinforce the biofilm formation by ar lRS mutation (Arana *et al*., 2005).

Intramammary infections and colonization by *Staphylococcus aureus*in mastitis is a burning issue for the dairy industry. A study was conducted to reveal the pathogenesis and biofilm forming ability of *Staphylococcus aureus*. The chronic infections caused by *Staphylococcus aureus* were associated with its biofilm formation. In study the animals were divided into three sets based on the presence and absence of bap and *ica* genes.

One set was positive for both of the genes other was negative for both while the third group was negative for bap and was positive for *ica*genes. It was observed that the isolates which were negative for both of the genes associated with biofilm formation had a greater somatic cell count than others. The isolates with presence of bap genes were more resistant to antibiotic therapy and the establishment of chronic infections. The presence of *ica* genes were not found to play a significant role in mastitis and in chronic infections (Cucarella *et al.*, 2004).

The pathogenesis of *Staphylococcus aureus* is associated with its adherence factors which are varied as biotic or abiotic surface, biochemical components. In this review it was studied that *Staphylococcus aureus* adopt various ways to colonize and evade the immune system in which biofilm formation is the major factor. *Staphylococcus aureus* adheres to epithelial cells of the mammary glands and secretes extracellular components and undergoes association with other bacterial pathogens. The extracellular components help the bacteria to evade phagocytosis and the physiochemical interactions make them able to colonize other cellular components as well. The antibiotic resistance was also found to associate the secretion of extra polymeric substances. The cellular adhesion by *Staphylococcus aureus* was associated with receptors; α-Toxin and β-toxin was involved in increased adhesion of *Staphylococcus aureus* to mammary epithelial cells. The derangement of plasma membrane and redirection of other cellular components also play their role in bacterial adhesion, evasion and its pathogenesis in mastitis (Dego *et al.*, 2002).

Biofilm Formation and Antibiotic Resistant Capacity

As a major human pathogen, Biofilm formation and antibiotic resistant capacity had a major role in the success of *Staphylococcus aureus* in both community settings and health care. Independently, Both these virulence factors had no major role in pathogen city but the expressed phenotype by clinical isolates was influenced by the acquisition of *Staphylococcus aureus* that is methicillin resistant due to mec A gene.

The strains of Methicilline *sensitive Staphylococcus aureus* (MSSA) usually produced a dependent biofilm of ica ADBC operon-encoded Polysaccharide Intercellular Adhesin (PIA). The release of cell surface expression, extracellular DNA (eDNA) and the major autolysin had been associated with the phenotype of methicillin resistant *S. aureus* (MRSA) biofilm. The high level methicilline resistant expression in the MSSA resulted in the several consequences such as biofilm production by the repression of PIA, down regulation of regulator (Agr) system of accessory gene and the attenuation of virulence in device infection and murine sepsis models. The ica locus was observed to be the major cause of MRSA biofilm formation. The biofilm formed by MRSA isolates were considerably more likely induced by the supplementation of glucose in the growth medium as compared to other clinical isolates of NaCl induced biofilm by MSSA. MRSA biofilm is more susceptible to proteinase K treatment, but resistant to sodium metaperiodate method in which different adhesin proteins implicating in biofilm phenotype.

Discussion

Biofilms are enclosed masses of bacteria that adhere to the living or non-living surfaces. Diverse range of microorganisms are involved in biofilm formation including bacteria and archaea, it's a key factor for the bacteria to survive in diverse range of environments. This protective mode of living makes it easy for the bacteria to survive in hostile environment and to colonize new niches. Biofilm formation is a basic virulence factor of *Staphylococcus aureus*, which shows unique mechanisms for pathogen city. The bacteria which contain exopolusaccharide develop biofilm formation and became them more resistant. Predominantly, Clinical MRSA strains form biofilm that is dependent on PIA and ica ADBC operon. The stability of biofilm formation by MRSA is associated with down-regulation of gene expression. In human and animal disease as urinary tract infections and mastitis respectively the biofilm formation by *Staph aureus* has much important revelance and key adhesion proteins involved in biofilm formation are members of MSCRAMM family proteins.

The development of an effective vaccine against the infections of Staph aureus remained unsuccessful due to failure in selection of an appropriate antigen. The whole genome sequence of *Staph aureus* and MRSA has been done but still there are confusions regarding the selection of an antigen either a bacterial component from the biofilm or the matrix itself may be used for vaccine development. Currently, PIA and PANG which are components of extracellular metrix have been used for vaccine development. A number of animal diseases have been associated with biofilm formation but still less research is carried out on determining the revelance of biofilm with animal disease such as mastitis and pneumonia. Recent researches have shown the presence of certain biofilm forming proteins in the mammary glands of animals that increases the ability of the bacteria to causes infection than nonbiofilm producing strains of bacteria. The formation of biofilm in the udder increases the antibiotic resistance capacity of the bacteria thus causes persist ant infection.

Many other bacteria also contributed with the *Staphylococcus aureus* to develop biofilm and develops many serious diseases as otitis media, pneumonia, urinary tract infections and wound infection in human. Biolfim is the complex community in which *Staphylococcus aureus* attached with the associated interfaces or surfaces. It is found that *Staphylococcus aureus* in clinical, natural and industrial settings attached with the surfaces and develops its microbial community. In this community, several other bacteria also contributed to develop more complex community. Many experiments and methodologies have been developed to explore the phylogenetic grouping, biofilm formation and metabolic interaction of *Staphylococcus aureus* with other members of the community. Further research is needed to identify the components of biofilm that can be used as antigens to develop vaccines against MSSA and MRSA strains in order to minimize the infections associated with the bacteria. The homologous and heterologous biofilm formation by different bacterial strains may potentiate the disease progression in both human and animals.

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