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A STUDY ON THE BIOFILM FORMATION BY MRSA ON INDWELLING CATHETERS AND IT'S PHYTO-PROPHYLACTICS

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ABSTRACT

Microbial biofilms are extremely complex, consisting of microorganisms attached to a surface and embedded in an organic polymer matrix of microbial origin. The self- produced matrix of extracellular polymeric substances which is also referred to slime, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms. Extracellular polymeric substances (EPS) are responsible for the adhesion of microbes and among various EPS producing organisms methicillin resistant strains of *Staphylococcus aureus* was isolated and identified from on or within indwelling catheters. For the assay of biofilm formation Congo red agar method, Tissue culture plate method and Tube method were performed and obtain results as adherent Staphylococcal cells formed biofilms on all side walls and were uniformly stained with crystal violet in TCP method .The antibacterial activity of various plant leaf extracts like tulsi (*Ocimum sanctum*), papaya plant, neem and guava(*Psidium guajava*) were studied and among them neem and tulsi has antimicrobial activity at various concentration. Different materials were selected for the study of biofilm formation on different surfaces and materials like copper (7 x 10^5CFU/ml), plastic (15 x 10^5CFU/ml), poly acrylic(15 x 10^5 CFU/ml) wood and stainless steel(30 x 10^5CFU/ml) were selected and studied using Tryptone water broth and TSB glu(1%) BHI suc (2%) medium.

KEYWORDS: Biofilms, Methicillin Resistance *Staphylococcus aureus*, EPS, antimicrobial activity.

INTRODUCTION

In the seventeenth century, Anton Van Leuwenhoek first observed animalcules swarming on living and dead matter. He discovered these animalcules in the tartar on his teeth and even after meticulous cleansing; the remaining opaque deposits contained a mat of various forms of animalcules that we now know were the bacteria dental plaque. It is reasonable to suggest that this early study of dental plaque was the first documented evidence of the existence of microbial biofilm. Today, we generally define this biofilm as microbial communities adhered to a substratum and encased within an extracellular polymeric substance (EPS) produced by the microbial cells themselves (Jones *et al*., 1969; Characklis 1973. The first true analysis of biofilm was not recognized until 1978 (Costerton *et al* 1978).

Properties and stages in the formation of biofilms

Biofilms are aggregated, sessile communities, formed from groups of microorganisms that persist environment and in chronic infections. Biofilms can form on the surfaces of the implanted devices-catheters, cardiac valves, intrauterine devices and contact lens- or even on teeth as dental plaque, but also in the mucus of cystic fibrosis patients and in the wound bed of chronic wounds. Aggregates of microorganisms begin to form by sticking to materials (eg. metals and plastics). Or each other within dead human tissues or other host components using weak vanderwaals interactions. However, stronger, more permanent anchors are put in the place if the initial colonies are not removed quickly enough (sometimes hours). Ultimately, this can lead to the harboring biofilms severe infections that are difficult to treat. (Costerton and Geesey, 1979).

The vast majority of microbes grow as biofilms can benign or pathogenic, releasing harmful products and toxins, which becomes encased with in the biofilm matrix. Biofilm formation is a phenomenon that occurs is natural and man-made environments under diverse condition, occurring on most surface, plant root and nearly every living animals. Biofilm may exist as beneficial epithilic communities in rivers and streams, waste water treatment plant trickling beds or in elementary canals of mammals given a prevalence of biofilm (Percival *et al*., 2007).

Aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS) adhere to each other and/or to a surface. Biofilms are not easily defined as they vary greatly in structure and composition from one environmental niche to another. Microbial biofilms

are extremely complex, consisting of microorganisms attached to a surface and embedded in an organic polymeric matrix of microbial origin (Wilson. M, 2001). Usually biofilms found on solid substrates submerged in or exposed to an aqueous solution although they can form as floating mats on liquid surfaces and also on the surfaces of leaves, particularly in high humidity climates. A biofilm will grow quickly to be a macroscopic when sufficient resource for growth given.

The process of biofilm formation is complex, but generally recognized as consisting of five stages (Palmer and White, 1997). 1. Development of a surface conditioning film 2. Movement of microorganisms into close proximity with the surface. 3, Adhesion (reversible and irreversible adhesion of microorganisms to the conditioned surface) 4.Growth and division of the organisms with the colonization of the surface, micro colony formation and biofilm formation; phenotype and genotype changes 5. Biofilm cell detachment/dispersal.

Adhesion

Adhesion of the microbes takes place when surface conditioning and transport of microbes into an area close to the substratum surface. Adhesion first proposed in 1943(Zobell 1943) as consisting of a two-step sequence involving reversible and irreversible processes. The surface of a microbial cell has a major impact on adhesion to a substratum. Cell surface hydrophobicity, the presence of fimbriae and flagella and particularly the extent and composition of generated EPS, influence both the rate and extent of microbial adhesion. A possible role of proteins for the bacterial adhesion has been proposed with the treatment of adsorbed cells by proteolytic enzymes found to cause a marked detachment of bacteria (Basshan and Levanony 1988; Danielsson *et al*., 1977). The EPS of biofilms may account for 50-90% of the total organic carbon of biofilms (Flamming *et al*., 2000).EPS is highly hydrated and can be both hydrophobic and hydrophilic with varying degrees of solubility (Brown *et al*., 1977).

Clinical importance's of biofilms

The biofilm growth forms are responsible for infection in humans and animals. Native valve endocarditis(NVE)is a condition that results from the interaction of bacteria, the vascular endothelium and pulmonic valves of the heart and frequently associated with Streptococci, Staphylococci, Gram Negative bacteria and also fungi(Braunwald, 1997).These microbes gain access to the blood and the heart via the oropharynx, gastrointestinal and urinary tract. Once the intact endothelium is damaged, microbes adhere to it and non-bacterial thrombotic endocarditis (NBTE) develops at the injury. At the point of injury, the thrombus develops, which is an accumulation of plates, fibrin and red blood cells (Donlan and Costerton 2002). Treatment is less effective due to a combination of mass transfer limitations and inherent resistance of biofilms (Fletcher *et al*, 1982).

Biofilm resistance depends on aggregation of bacteria into multicellular communities. Therefore antimicrobial strategy might be to develop therapies to disrupt the multicellular structure of the biofilm. *Simarouba glauca* have been used for the treatment of gastrointestinal disorders. Medicinal plant-derived compounds have been reported to be able to prevent the formation of biofilm in some pathogens like *Listeria monocytogenes, Pseudomonas aeroginosa, Streptococcus pyogenes, Streptococcous mutants* and *S aureus*. Most studies have focused on the observation of anti-biofilm activity of herbs taken as a single unit but not in combnation, such as herbal recipes. No attention has been paid to the antibacterial or anti biofilm activity of traditionally used herbal recipes (Patel R., 2005). The present study is to isolate the methicillin resistant *Staphylococcus aureus* forming biofilm indwelling catheters and its phyto prophylactics on biofilm formation.

Inhibiting bacteria from binding to a device can be done by creating a surface that the bacteria cannot physically bind to or by coating the surfaces with antimicrobial agents. In order to effectively kill the bacteria in a biofilm, many times a combination of different antibiotics along with biofilms matrix- degrading substances must be used (Francolin *et al*., 2010). Degradation of the biofilm matrix is important in order to expose all of the sessile microbial cells to both the host immune defenses as well as antibiotics. Dispersin B is an important molecule that can degrade the EPS of many bacteria including S epidermidis. Dispersin B can also be expressed by modified bacteriophages, which is useful for dissolving the biofilm matrix as well as killing the bacterial cells that form the biofilm (Mah *et al* ., 2001; Talsma *et al*., 2007).

Antimicrobials of plant origin ie tulsi (*Ocimum sanctum*), papaya plant, neem and guava(*Psidium guajava*) are effective in the treatment of infectious diseases and simultaneously mitigating many side effects often associated with synthetic antimicrobial agents have been discovered(Whittaker *et al*., 1996; Bryers J., 2008; Rittman BE, 1989).Total extracts of these medicinal plants effective against killing or inhibiting the growth food borne bacterium *Staphylococcus aureus, E coli, Salnonella enteritidis* and *Bacillus sereus* which can cause food borne illness and spoilage(Arciola *et al*., 2012;Boles *et al*., 2008).

MATERIALS AND METHODS

Isolation and Identification of Microorganisms

Specimen collection

specimens from indwelling catheters were collected by using sterile swabs from hospitals. Sample collected and inoculated into sterile Brain Heart Infusion Broth for 2-3hrs and after 3hrs of incubation the broth was serially diluted up to 10^{-7} and spread plate technique was carried out on Nutrient agar plates and incubated at 37^0C for 24hrs. Nutrient agar plates were then observed after incubation period and isolated colonies were selected for

the further studies. The organisms were identified by using various tests including Grams staining and biochemical tests. Isolated gram positive coccus was inoculated onto various solid media like blood agar plates, MacConkey agar and Mannitol salt agar. Plates were incubated at 37^0C for 24hrs.

Screening for Methicillin Resistant *Staphylococcus aureus* (MRSA)

Methicillin resistance was confirmed by disk diffusion method as described in CLSI guidelines (NCCLS 2000. Performance standards for antimicrobial disk susceptibility tests, 8th edition) Muller Hinton agar plates were prepared and a suitable dilution (McFerland standard 3) of broth suspension was spreaded on the surface of plates using sterile swabs. After drying the plates, antibiotic disks (Cefoxitin-CX30) were placed using sterile forceps and incubated at 37^0C for overnight. The degree of sensitivity is determined by measuring the zones of inhibition of growth around the discs.

Biofilm formation Assay for *S aureus*

1. Congo Red Agar Method (CRA)

Freeman *et al*., 1989 has described a method for screening the biofilm forming *S aureus* which requires specially prepared solid media- Brain Heart Infusion broth supplemented with5% sucrose and congo red. Congo red prepared separately and autoclaved at 121^0C for 15mins and then added to the media when the agar had cooled to 55^0C. Plates incubated aerobically for 24-48hrs at 37^0C.

Congo red agar (pH-7.2)

BHI	- 37g
Sucrose	- 50g
Agar	- 10g
Congo red stain	- 0.8g
Distilled water	- 1000ml

2. Tissue Culture Plate Method (TCP)

In present study, we screened the isolates for their ability to form biofilm by TCP method as described by Christensen *et al* with a modification in duration of incubation which was extended to 24hrs.Previous reports have indicated the influence of the media composition on biofilm production, therefore we had evaluated biofilm production in three different media, Tryptone soy broth(TSB Himedia), TSB with 1%glucose(TSB glu) and Brain Heart Infusion(BHI Hi media) with 2% sucrose(BHI suc).

Isolates from fresh agar plates were inoculated in respective media and incubated for 18hrs at 37^0C. Individual wells of sterile, polystyrene, 96 well flat bottom tissue culture plates (Tarson, India) and wells were filled with 0.2ml aliquots of the diluted cultures and only broth served as control. Tissue culture plates were incubated for 18-24hrs at 37^0C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2ml of Phosphate buffer saline (PBS pH-7.2) to remove free floating planktonic bacteria. Biofilms formed by adherent sessile organisms in plates were fixed with sodium acetate (2%) and stained with crystal violet (0.1%w/v). excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent Staphylococcal cells usually formed biofilm on all sides of well and were uniformly stained with crystal violet.

3. Tube Method (TM)

TSB glu (10ml) was inoculated with organisms from overnight culture plates and incubated for 24hrs at 37^0C. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet(0.1%). excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying in inverted position and observed for biofilm formation and it considered positive when a visible film lined on the wall and at the bottom of tube. Ring formation at the liquid interface was not indicative of biofilm formation.

Medicinal plants-collection and extraction method

Guava (*Psidium guajava*), Neem (*Azadirachta indica*), Thulasi (*Ocimum tinuiflorum*), Papaya (*Carica papaya*) and Lakshmitaru (*Simarouba gauca*) collected and leaf extractions were prepared within 2hrs of collection. The leaves were grinded and 10g was taken and added to 100ml water to obtain water extract 100mg/ml concentration at RT. And then diluted using double fold serial dilution by adding one part of plant extract with one part of sterile distilled water. This gives the concentration of 50mg/ml. further dilution of the sample by transferring from first tube to the second tube containing distilled water to get the concentration of 25mg/ml.

Determination of Antibacterial activity

Kirby Bauer –disc agar diffusion method was performed using Muller Hinton agar medium. The plates were inoculated with the *S aureus* (1.0OD adjustment with the standard of MacFerland's standard) which was isolated from the clinical isolates forming biofilms on the indwelling medical devices, by means of sterile cotton swab to ensure the growth of microbes. The sterile filter paper disc was placed on the agar plates and each disc were added with different dilutions of leaf extracts. Then the plates were incubated for 24hrs at 37^0C. the antibacterial activity evaluated by measuring zone of inhibition.

Prevention of biofilm formation by medicinal plant extract

To 20µl of overnight culture of *S aureus* was placed in each micro titre wells which was cultivated with 2ml of BHI suc (2%) and incubated at 37^0C for 24hrs and then the wells was washed with PBS solution in order to remove unattached cells and again the micro titre plates incubated with the addition of leaf extracts for 12-18hrs.

RESULTS AND DISCUSSION

From indwelling catheters bacterial isolates were isolated and identified using various staining and biochemical tests and the isolates identified as *S aureus* and shows methicillin resistance in zone of inhibition method. Positive result was indicated by black colonies with a dry crystalline consistency in Congo red agar. Microbial biofilm often develop on or within the indwelling medical devices such as contact lenses, central venous catheters, urinary catheters (Donlon, 2001; Percival and kite, 2007). Different materials like copper (7 x 10^5CFU/ml), plastic (15 x 10^5CFU/ml), poly acrylic(15 x 10^5 CFU/ml) wood and stainless steel(30 x 10^5CFU/ml) was inoculated with 10ml of tryptone water broth and TSB glu(1%) BHI suc medium with culture and incubated for 18-24hrs. the number of organisms adhered to the surface of materials was enumerated by serial dilution and maximum was found in wood(85 x 10^5CFU/ml).

Adherent Staphylococcal cells formed biofilm on all side walls and were uniformly stained with crystal violet in TCP and a visible film lined the wall and bottom of the tube was considered to be the biofilm formation for *Staphylococcus sp* in tube method. *S aureus*. A medically important organism associated with a vast variety of chronic infections through biofilm formation (Costerton *et al*., 1984) .Five different plant leaf extracts has taken at different concentration like 100, 50 and 25mg/ml by disc diffusion method and zone of inhibition was measured. Neem(10, 6 and4mm) and thulsi(8, 6 and 2mm respectively) has antimicrobial activity in all concentration. Guava and papaya doesn't have antimicrobial activity. Lakshmitharu extract gives 2mm zone at 100mg/ml concentration and no zone found at 50 and 25mg/ml.

Biofilm formation on different surfaces like copper, plastic, poly acrylic, wood, steel and aluminum were used for study and wood surface shows maximum ability for the biofilm formation than other materials. The extracts of neem could be useful for the inhibition of growth of carcinogenic bacterium (Marsh PD., 1995). In the ethanolic extract of plants were found to be more effective than the water extract. The phyto constituents like alkaloids, glycosides, flavonoids and saponins are antibiotic principles of the plants. This principle is the defense mechanism of the plants against different pathogens (Fletcher M., 1977).

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