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Microscopic Study on the Development of Biofilm by *Staphylococcus aureus* on Prosthetic Device (Catheter)

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Staphylococcus aureus (S. aureus) is a common cause of biofilm-mediated prosthetic device-related infection. Bacteria that adhere to implanted medical devices or damaged tissue can encase themselves in an extracellular polymeric substance (EPS) and form a slimy layer known as a biofilm. Formation of these sessile communities can become the cause of persistent and chronic bacterial infections. The purpose of this study was to investigate the morphology of in vitro biofilm formation for duration of time using live cell imaging and scanning electron microscopy. S. aureus ATCC 12600 was cultivated in Luria Bertani (LB) broth and diluted with freshly prepared LB broth for overnight (16-18 hours) to achieve standardized 10^8 CFU/ml cell suspensions. Cell suspension of S. aureus was inoculated into glass bottom petri dish and incubated under live cell imaging for 10 hours. At various times of incubation (day 1, 9, 15 and 17), the catheters that were incubated with S. aureus were collected and then processed for morphology analysis using scanning electron microscopy. Microscopy study of S. aureus biofilm formation in vitro suggests that the pattern of development involves initial attachment to a solid surface, the formation of microcolonies and finally differentiation of microcolonies into exopolysaccharide-encased as a matured biofilm. In conclusion, the microscopic study of S. aureus ATCC 12600 biofilm may be useful for morphological identifiers in classifying bacteria biofilms.

Keywords: *Staphylococcus aureus*, biofilm, scanning electron microscopy, live cell imaging system

INTRODUCTION

Modern medical science has designed numerous types of prosthetic devices for implantation into patients. The use of surgically implanted devices has increased as a result of their beneficial effect on quality of life, and in some circumstances, on patient survival rates. However, microbial adhesion and biofilm formation on medical implants is a common occurrence and represents a serious medical problem. By one estimate, 80% of biofilm infections have been found to be involved in a wide variety of microbial infections in the body such as catheter infections, urinary tract infections, formation of dental plaque, coating contact lenses and also orthopaedic devices infections [1]. These infections are often

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difficult to resolve because of their increased resistance to host defense mechanisms and antimicrobial therapy. Once the infection is established, the patient must endure a lengthy regimen of antimicrobial therapy. Furthermore, the biofilm can act as a nidus for recurrence of infection when antimicrobial therapy stops. Biofilm infections usually persist until colonised surface is surgically removed from the body [2]. Staphylococci are among the most common pathogens associated with device-related infections [3].

Biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface, embedded within a self-secreted extracellular protective and adhesive matrix of a polymeric substance (EPS) [4]. Structural thick biofilms comprise mushroom-shaped microcolonies of bacteria separated by fluid-filled channel [5].

Three steps are involved in the development of biofilm. The formation of biofilm begins with the adhesion of planktonic bacteria to surfaces. Subsequent to cell surface interaction, these organisms enter the "accumulative phase" of biofilm formation. This involves cell-cell interaction and the formation of cell aggregates on the surface. The second step is the proliferation of the primary colonizers and the maturation of the biofilm. The biofilm grows through a combination of cell division and recruitment of bacteria. At the same time, most biofilm bacteria produce EPS or exopolysaccharide that hold the cell aggregates together, protects cells within it and facilitates communication among them through biochemical signals. In addition it also stabilizes the biofilm architecture. In the final step, previously sessile members of the mature biofilm detach and may lead to spread and cause formation of new colonies at different sites [6].

The present study investigated the morphology of *in vitro* biofilm formation according for a duration of time by employing two different microscopy techniques.

MATERIALS AND METHODS

Bacterial Strains

S. aureus ATCC 12600 was cultured in Luria Bertani (LB) broth and incubated at 37°C overnight (16-18 hours). Following incubation, the cultured broth was diluted with freshly prepared LB broth to achieve standardized 10^8 CFU/ml cell suspension.

Catheter-associated Biofilm

Biofilm of *S. aureus* was developed on a 14-gauge Teflon intravenous catheter. Briefly, the catheter was cut into 1 cm segments and each piece was sterilized with 70% ethanol and air dried. The biofilm was developed on the catheter by placing individual segment into each tube containing 2 ml of cell suspension (in exponential phase of growth) as described above.

After incubation for 2 to 3 hours at 37°C, colonized catheters were rinsed once with LB broth to remove unbound bacteria. The catheters were incubated further in fresh LB broth at 37°C for 1, 5, 9, 13, 15 and 17 days. During incubation, the medium was replaced every 8 to 12 hours with fresh LB broth. Control catheters were prepared as above but without the bacterial inocula. The catheter-associated biofilms were used for microscopy studies (Modified method from Kadurugamuwa *et al.,* 2003 [7]).

Microscopic Techniques

For visualization by live cell imaging system (Inverted Motorized Microscope TI-E, Nikon, Japan), a total of 2 ml of 10^8 CFU/ml cell suspensions in LB broth was incubated in the glass-bottom chamber (FluroDish™, USA) for 10 hours. Visible biofilms were documented with an attached digital camera.

Sample preparation for scanning electron microscopy (SEM) was performed. Catheter-associated biofilms were fixed in 4% glutaraldehyde and kept at 4°C for 24 hours. Then, they were washed with 0.1M sodium cacodylate buffer three times at 10 minutes each washing. Then, post-fixation was carried

out in 1 % of osmium tetraoxide for about 2 hours in 4°C. Dehydration was performed by sequential immersion in serial diluted ethanol solutions of 35, 50, 60, 70, 80, 90, and 100%. The samples were kept in absolute alcohol and critical point dried using CO_2 (BAL-TEC CPD 300, Germany). The samples were then sputtered with palladium gold for SEM analysis (JOEL 6400, Japan).

RESULT

Microscopic Evaluation of S. aureus Biofilm

The development of *S. aureus* biofilm was viewed under live cell imaging system during 10 hours incubation. Observation of *S. aureus* revealed that the organisms swam along the surface before attached to the bottom of petri dish.

As illustrated in *Fig. 1* within 6 hours or less, planktonic bacteria form a monolayer on the bottom of glass petri dish. It appears that, once bacteria initiate surface contact, they come to rest on the surface. However, time-lapse microscopy revealed that the bacteria continue to move. This movement occured only on the surface and used twitching motility. By 7 hours, this monolayer covered the surface punctuated by microcolonies. These microcolonies were dispersed and/or moved as a unit across a surface and completely covered the surface by 8 hours. After 9 hours of biofilm formation, the microcolonies of cells form as the consequence of individual cells twitching across the surface towards each other and as a result a thicker biofilm was formed. The biofilm was easily visualized during 9 to 10 hours post-inoculation.

The morphology of *S. aureus* biofilm on the surface of catheters was viewed under SEM.

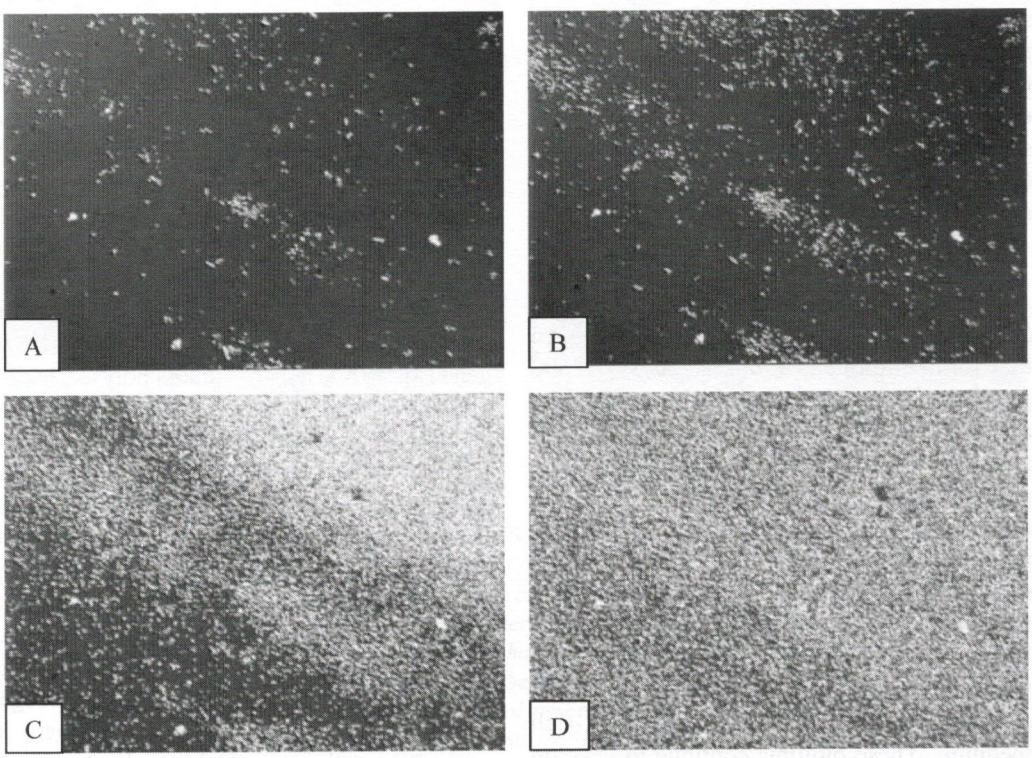

Fig. 1: Phase contrast micrographs of live biofilm formation by S. aureus at (A) 6 hours post inoculation, (B) 7 hours post-inoculation, (C) 8 hours post-inoculation and (D) 9 to 10 hours post- inoculation. All micrographs are shown with the same magnification, 40x

Fig. 2 showed the morphology of *S. aureus* biofilm according to the time interval from day 1 to day 17. The first step in biofilm formation involves attachment of the organisms to the catheter surfaces with host protein (*Fig. 2B).* Subsequent to cell attachment on the surface of catheter, the floating organisms accumulated on top of those that adhered to the catheter surface. This second step involved cell to cell interactions and the formation of cell aggregates on the surface. As illustrated in *Fig. 2C* primary colonizers recruited and co-aggregated planktonic members and formed multiple cell layers. The dense aggregates of bacteria were held together by diffuse substances known as either EPS or exopolysaccharide or slime. These 9-day old biofilm partially covered the catheter surface.

By day 15 old biofilm, the surface of catheter was fully covered by cocci shaped *S. aureus* biofilm (*Fig. 2D*) which formed dense layers that cling tightly to the artificial surfaces. However, by day 17 (*Fig. 2E*) the mature *S. aureus* biofilm grew with thickened mushroom-like structure, multilayered, stacked more densely and formed a three-dimensional structure encased in EPS. A complex "slime" (EPS or exopolysaccharide) was clearly seen surrounding the bacteria in *Fig. 2F.*

DISCUSSION

Staphylococcus aureus is an important pathogen that forms biofilms. *S. aureus* ATCC 12600 grows well in Luria Bertani (LB) at 37°C under ambient aeration. LB broth is an excellent medium because it is very efficient at stimulating growth and is suitable for many different organisms. This broth is categorized as a rich medium because it contains all the nutrients such as peptides and peptones, vitamins, and trace elements needed for bacteria to proliferate. Briefly, *S. aureus* undergo a transition from free-living, planktonic cells to sessile, surface-attached cells in response to a nutrient-rich medium. These biofilms continue to develop as long as fresh nutrients are provided, but they detach and slough from the surface and then return to a planktonic mode of growth when they are nutrient deprived [5]. They also may grow more slowly and have restricted mobility. Most probably, this starvation response allows the cells to search for a fresh source of nutrients. In addition to the nutritional content of the medium, other environmental cues that can influence biofilm formation include temperature, osmolarity, pH, iron and oxygen [5].

Microscopy of *S. aureus* ATCC 12600 biofilm formation *in vitro* suggests that the pattern of development involves initial attachment to a solid surface, the formation of microcolonies on the surface and finally differentiation of microcolonies into exopolysaccharide-encased, matured biofilm. These findings was supported by Ganderton and colleagues [8] who examined the presence of bacterial biofilm on the surface of Foley bladder catheters for the period ranging from 3 to 83 days (35 days) using SEM. In addition, the investigation by George and colleagues [9] showed direct observation of *Pseudomonas aeruginosa* biofilm formation on polyvinylchloride (PVC) over 7.5 hours using phase contrast microscopy.

The early attachment in the biofilm development by *S. aureus* ATCC 12600 occurs initially via cell-surface interactions. These interactions may be mediated through a number of factors, including uncharacterized surface proteins, extracellular proteins, polysaccharide intercellular adhesin (PIA) [10] and the cell surface–localized autolysin encoded by the *atlE* gene [5]. PIA is a β -1,6-linked polysaccharide that is strongly associated with the staphylococcal cell surface and mediates cell-to-cell adhesion. *AtlE* is synthesized as a proteolytically cleaved precursor of a ~120-kDa protein. This protein is an adhesin for binding to both biotic and abiotic surfaces and has vitronectin-binding activity [11]. Numerous studies confirmed that polysaccharide intercellular adhesin (PIA) mediates bacterial cell-cell adhesion and is required for biofilm formation, which is thought to increase the virulence of both pathogens in association with prosthetic biomedical implants [10, 12].

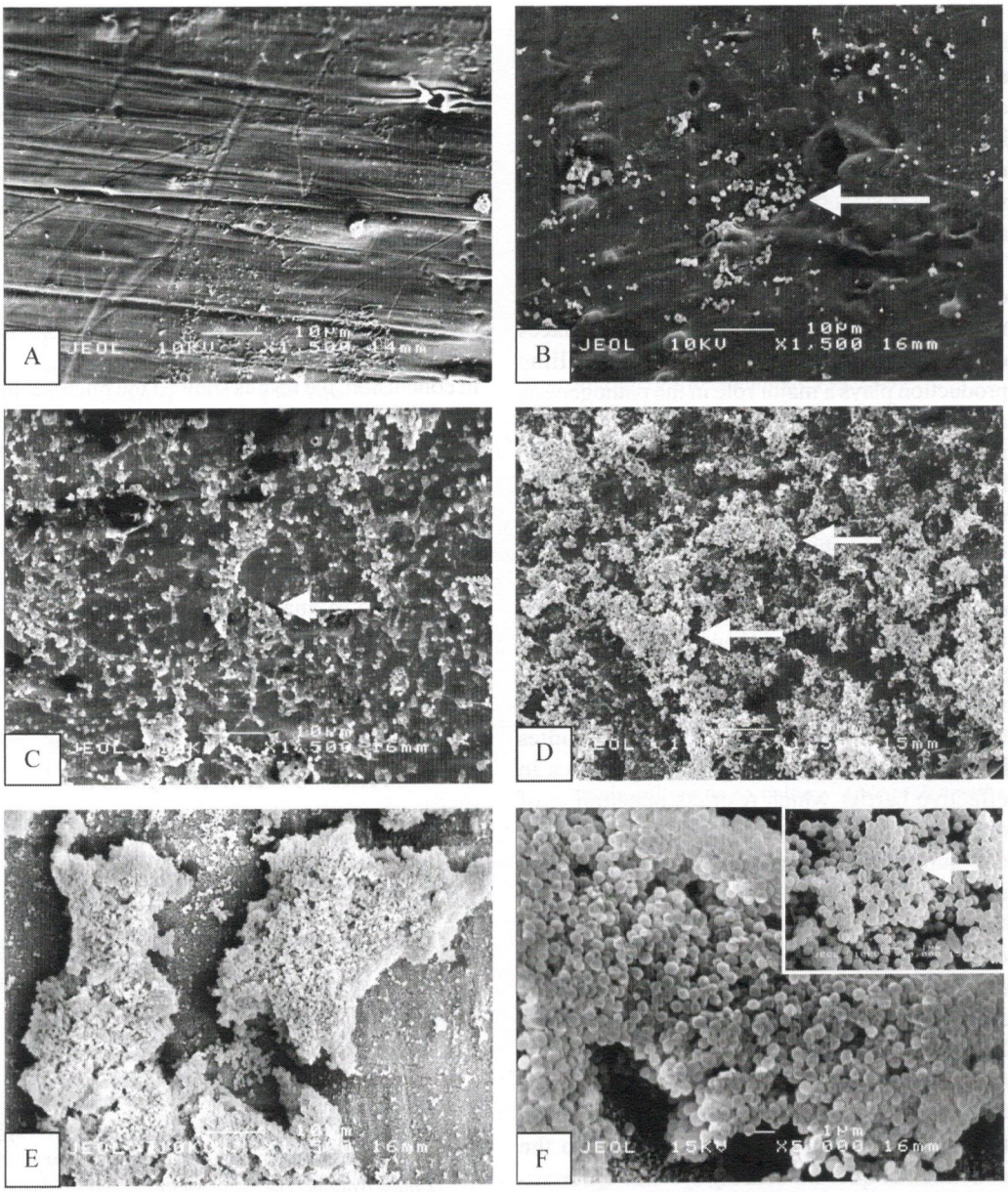

Fig. 2: Scanning electron micrographs of S. aureus *biofilm on the surface of catheters from day 1 to 17 incubation. (A) Surface of catheter without bacteria growth as control (1,500x) (B) Day 1, planktonic of* S. aureus *clumps (arrow) on catheter surface (1,500x) (C) Day 9, the dense aggregates of bacteria held together by diffuse extracellular polymeric substances (EPS) or exopolysaccharide (arrow) (1,500x) (D) Day 15,* S. aureus *biofilm grown thicker and covered fully the surface of catheter (1,500x) (E) Day 17, the mature biofilm form a three-dimensional structure (1,500x) (F) The bacteria tend to grow in clumps (microcolonies) and covered by EPS (5,000x) was clearly seen at high magnification (arrow) (10,000x)*

Scanning electron micrographs from this study showed *S. aureus* form multiple cell layers and mature biofilm on the catheters surface. The cells in these layers are enveloped and protected by a slimy matric (extracellular polysaccharide matrix (EPS) or exopolysaccharide). Slime is an outer structure, formed after growth in special media and placed outside the cell wall. This slime layer composed of extracelullar carbohydrates forming a net where cells can reproduce themselves and adhere to the surfaces of prosthetics and plastic catheters. Slime production plays a major role in the pathogenesis of infections by Gram positive and Gram negative bacterias [13]. The functions of slime layer include the aggregation of bacteria cells, the formation of an active gel-like structure that maintain cell cohesion, stabilizes the biofilm structure and protect microbes against noxious environmental condition [14]. Environmental factors that have been reported to promote slime include high level of oxygen, limited availability of nitrogen, desiccations, low temperature and low pH [15]. Many studies have reported that the slimy matrix provides an effective barrier which restricts penetration of chemically reactive biocides, cationic antibiotics and antimicrobial peptides [16] and also host immune-attack [17].

As biofilms mature, the increase in cell density creates gradient of nutrient and oxygen availability leading to reduction in metabolic activity and growth rate. Bacteria located in the biofilm periphery have better access to nutrients and oxygen than bacteria located deeper in the biofilm community [16]. Furthermore, the increase in cell density also leads to the activation of quorum-sensing system. Quorum-sensing or cell-to-cell signaling controls the expression extracellular virulence factors, the stationary-phase sigma factor *rpoS* (αS) and biofilm differentiation in response to population density [18]. The sigma factor *rpos* is a general stress response regulator that activates expression of a number of genes necessary to maintain cell viability during stationary phase when cell experience nutrient starvation. Thus, conditions found inside biofilm (e.g. nutrient limitation and oxygen limitation) would induce up-regulation of efflux pumps [19] and the expression of the *rpos* alternative sigma factor, resulting in physiological changes that would mediate protection against environmental stress, antimicrobial agents [16] as well as innate and adaptive host response mechanisms [20]. The detachment of mature biofilm from the catheters may result in infection [21].

CONCLUSIONS

In conclusion, the results showed the microscopic study of *S. aureus* ATCC 12600 biofilm may be useful for morphological identifiers for classifying bacteria biofilms. Furthermore, *S. aureus* biofilm could play a significant role in recurrent and chronic infections. With the basic parameter evaluated in this study, it will be possible to analyze the biofilm phenotype and to explore the *S. aureus* biofilm behavior in developing prevention strategies and therapy concepts.

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