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Research Paper

Multi drug resistance in strong biofilm forming clinical isolates of *Staphylococcus epidermidis*

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Abstract

Staphylococcus epidermidis which exists in healthy human skin as a commensal inhabitant is also an important pathogen forming biofilms on many surfaces and recently, increased resistance traits were suggested to be acquired in biofilm environments. In this study; clinical Prevalences, antibiotic resistances and biofilm formations of *S. epidermidis* strains were determined and comparison of all these findings with each other was carried out in order to take precautions against them and figure out if high biofilm forming *S. epidermidis* strains display multi drug resistance. According to our results; samples of wound and blood were the most *S. epidermidis* isolated clinical materials (40%; 35%) and cardiothoracic surgery was the most *S. epidermidis* observed service unit. All of these strains were sensitive to vancomycin, however 65% of them showed resistance to all β-lactam antibiotics (Penicillin, Oxacillin, Amoxicilin / Clavulonic acid), used in this study and 60% of all *S. epidermidis* strains were found as multi drug resistant. When the results of strong biofilm forming *S. epidermidis* strains are examined; they were isolated from sample of blood and service unit of cardiovascular surgery in highest frequency and 80% of them were β-lactam resistant whereas 100% of them were multi drug resistant. One of these multi drug resistant strains which was resistant to maximum amount of different antimicrobial classes, was also observed as maximum biofilm forming strain among all the other *S. epidermidis* isolates. Multi drug resistance in strong biofilm forming strains shows that; biofilms play a role in antimicrobial resistance traits of *S. epidermidis*.

Key words: *Staphylococcus epidermidis*, biofilm formation, β-lactam resistance, multi drug resistance, indwelling medical device related infections.

Introduction

S. epidermidis which is known as a natural colonizer of healthy human skin and mucosa, is also a common nosocomial pathogen along with other Coagulase Negative Staphylococci (CNS) (Otto, 2009) and among them; *S. epidermidis* is the most important pathogen, responsible from many indwelling medical device related infections such as; catather, prosthetic joint, vascular graft, surgical site, central nervous system shunt and cardiac device related ones. Therefore, *S. epidermidis* strains especially emerge as life-threatening pathogens triggering septicaemia, meningitis and other serious conditions in medical device using and immunocomprised patients (Goldman *et al.*, 2001; Guiot *et al.*, 1994; Poutsiaka *et al.*, 2007; Ziebuhr *et al.*, 2006). One of the most important mechanisms making these commensal inhabitants dangerous for medical device using and immunocomprised patients is known as biofilm formation. By means of having ability to adhere to various surfaces and form slimy layer known as biofilm on them; *S. epidermidis* strains and some other biofilm forming microorganisms can encase themselves (Costerton *et al.*, 1995; Costerton, 1999; Shiro *et al.*, 1994; Tunney *et al.*, 1998). Thus; neither immune defense nor antimicrobial agents are able to eradicate biofilm-associated infections and in order to terminate them, the device must be removed or some surgical operations must be applied (Xu and Siedlecki, 2012). Moreover, since *S. epidermidis* strains are natural colonizers of healthy human skin and can easily grow on medical devices because of having strong attachment ability, they

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can easily be transmitted to a medical device and colonize on the surface of it during an implantation process (Kaplan *et al.*, 2004). That is, a natural colonizer of healthy human skin may easily turn into a nosocomial pathogen if it has strong attachment and high biofilm forming abilities. Nowadays, increased antibiotic resistance in some clinical strains is thought to be related with their biofilm forming capabilities. Because, acquirement of some resistance traits via gene transfer is possibble within biofilms (Subramanian *et al.*, 2012). The aim of this study is to determine the clinical prevelances, antibiotic resistances and biofilm formations of *S. epidermidis* strains; compare all these findings with each other, reveal the clinical informations of multi drug resistant and high biofilm forming *S. epidermidis* strains in order to take precautions against them and figure out if high biofilm forming *S. epidermidis* strains display multi drug resistance.

Materials and Methods

Bacterial strains

All *S. epidermidis* strains were obtained from a hospital in Ankara, Turkey and were isolated from clinical materials of blood, wound, nose, abscess, synovial fluid and from the services of physical rehabilitation, cardiothoracic surgery, dermatology, intensive care, neurology, general surgery, emergency, thoracic medicine and paediatry. Isolated *S. epidermidis* strains were inoculated in to the Brain Heart Infusion Broth media including 10% glycerol and stored at -20 °C.

Isolates were identified by standard phenotypical methods (Holt *et al.*, 1994) and identification was further confirmed by using Vitek-32 system (BioMérieux, France).

Antibacterial susceptibility testing

The susceptibilities of *S. epidermidis* strains to 11 different antibacterial agents of Amoxicilin/Clavulonic acid (20/10 μg), Erythromycin (15 μg), Oxacillin (1 μg), Ciprofloxacin (5 μg), Trimethoprim/Sulfamethoxazole (1.25 μg), Vancomycin (30 μg) Gentamicin (10 μg), Tetracycline (30 μg), Clindamycin (2 μg), Penicillin (10 Units), Nitrofurantoin (300 μg) were assessed by Disc-Diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) and the strains were classified as Resistant (R), Intermediate (I) or Sensitive (S), according to the zone table, constituted by Clinical and Laboratory Standards Institute (CLSI).

Biofilm formation

The determination of biofilm formation in *S. epidermidis* strains was performed by Crystal Violet Binding Assay described by O'Toole with some modifications (O'Toole, 2011). Briefly, bacterial cells corresponding to a 2.0 McFarland optical density standard were inoculated into Brain Heart Infusion Broth medium and then they were incubated at 37 °C overnight. The overnight culture was 1:100 diluted into a fresh BHI medium and the wells of a polysytrene plate were filled with 1 mL of the diluted inoculum. Then, the plates were incubated for 48 h at 37 °C. Following this, the medium was gently removed and the wells were washed. After allowing wells to dry, each of them were stained with 1% crystal violet for 45 min at room temperature. Afterwards, the unbound crystal violet stain was removed and microscobic observation was done. Finally, bound crystal violet in each well was solubilized by adding 1 mL of Ethanol-Acetic Acid (90:10) solution and solubilized crystal violet for each well was read by a spectrofotometer at 540 nm. According to their biofilm formations, *S. epidermidis* strains were classified into four categories as follows:

$OD = 0$, Non Biofilm Former (NBF)

$0 < OD < 0.4$, Weak Biofilm Former (WBF)

$0.4 \leq OD < 0.8$, Intermediate Biofilm Former (IBF)

$OD \geq 0.8$, Strong Biofilm Former (SBF)

The experiment was performed in triplicate.

Results

S. epidermidis strains were obtained from 5 different clinical materials including; blood, wound, nose, abscess and synovial fluid. Among them; samples of wound and blood were the most *S. epidermidis* isolated clinical materials (40%; 35%) and among all the service units which include patients infected with *S. epidermidis*; cardiothoracic surgery was observed in highest frequency (35%) (Figures 1, 2).

After the application of Crystal Violet Binding Method for biofilm screening; 25% of all *S. epidermidis* strains were determined as strong, 15% as intermediate, 55% as weak and 5% as non-biofilm formers. When the biofilm formations of *S. epidermidis* isolates in different clinical materials are examined, strong biofilm forming strains were isolated from samples of blood, wound and synovial fluid and within these; sample of blood was seen in highest frequency (Figure 1). When the biofilm formations of *S. epidermidis* strains in different service units are examined, cardiothoracic surgery was the most frequent unit for strong biofilm forming *S. epidermidis* isolation and additionally, the other units including patients infected with strong biofilm forming *S. epidermidis* strains were observed as; thoracic medicine, neurology and dermatology (Figure 2).

When the antibiogram tests of *S. epidermidis* strains against 11 different antibiotics are examined; all of them were found as sensitive to vancomycin (Figure 3). However, 65% of them and 80% of strong biofilm formers showed resistance to all β-lactam antibiotics (P, OX, AMC) used in this study (Table 1).

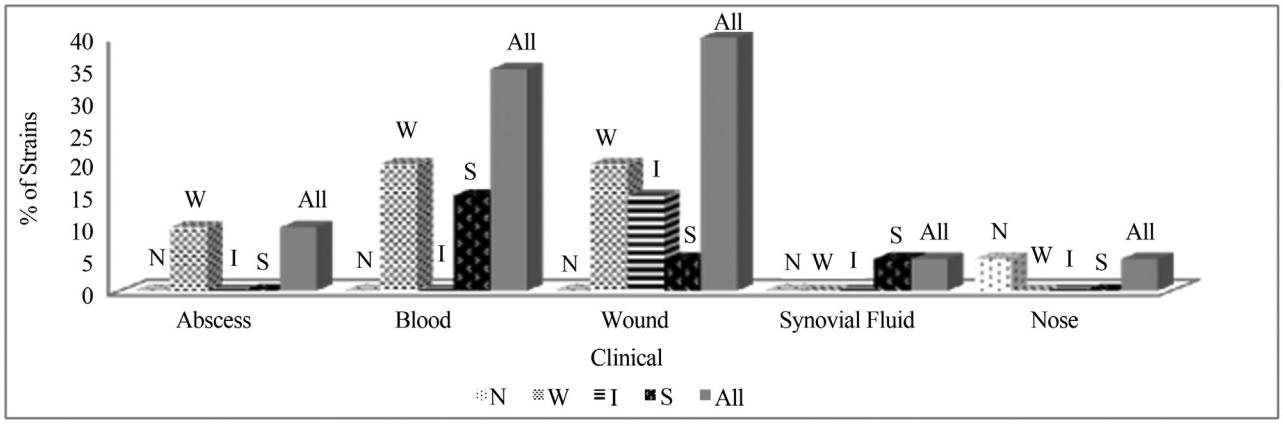

Figure 1 - Percentage of *S. epidermidis* strains in different clinical materials according to their biofilm formation levels. (N: Non Biofilm Formers, W: Weak Biofilm Formers, I: Intermediate Biofilm Formers, S: Strong Biofilm Formers).

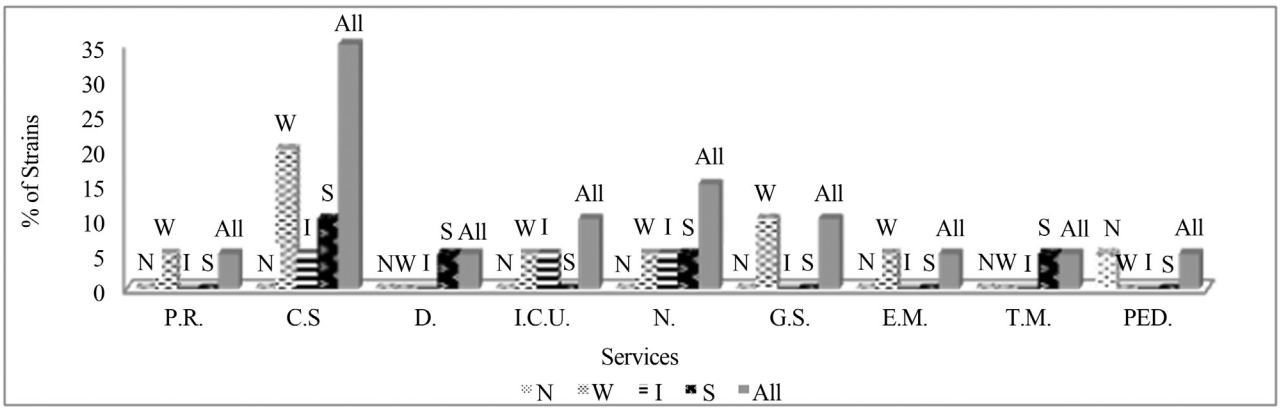

Figure 2 - Percentage of *S. epidermidis* strains in different service units according to their biofilm formation levels. (P.R.: Physical Rehabilitation, C.S.: Cardiothoracic Surgery, D.: Dermatology, I.C.U.: Intensive Care Unit, N: Neurology, G.S.: General Surgery, E.M.: Emergency, T.M.: Thoracic Medicine, PED: Pediatrics, N: Non Biofilm Formers, W: Weak Biofilm Formers, I: Intermediate Biofilm Formers, S: Strong Biofilm Formers).

In the last part of this study; multi drug resistant strains were determined and their biofilm formations were examined. According to results; 60% of all *S. epidermidis* strains and each of strong biofilm forming *S. epidermidis* strains were found as resistant to 3 or more antimicrobial classes and defined as multi drug resistant (Table 1). In addition; maximum biofilm forming isolate was found as resistant to 7 different (Aminoglycoside, β-lactams, Macrolide, Tetracycline, Fluoroquinolone, Lincosamide and Sulfonamide) antimicrobial classes.

Discussion

Technological developments have provided several benefits for human life, both in industry and medical fields. Eradication of pathogen microorganisms by antimicrobial agents and usage of many indwelling medical devices in order to increase the function of any defective organ, are some significant advances which can be given as examples to these benefits (Aybar *et al.*, 2012). Inspite of all these beneficial things, increase in antimicrobial resistant microorganisms rises every passing day and as a consequence; persistent nosocomial infections in hospitals, mortality and huge economic damages come true. Therefore, investigation of mechanisms playing a role in drug resistance and fighting against them, have great place in preventing these casualties. One of these mechanisms playing a role in persistent infections is known as biofilm formation (Djeribi *et al.*, 2012). Therefore, this study is carried out to reveal the occurrences of *S. epidermidis* strains in different clinical materials and service units, determine antibiotic resistances of them and evaluate all these results according to *S. epidermidis* strains' biofilm formation performances.

When the occurrence of *S. epidermidis* strains according to clinical information of patients from which they were isolated are examined, it is seen that our findings are in agreement with a recent study, suggesting; samples of blood and wound are the most *S. epidermidis* isolated clinical materials (Sheikh and Mehdinejad, 2012). In addition, according to some other studies; similar to our results, *S. epidermidis* is indicated as the most important pathogen playing a role in cardiovascular infections (Michelim *et al.*,

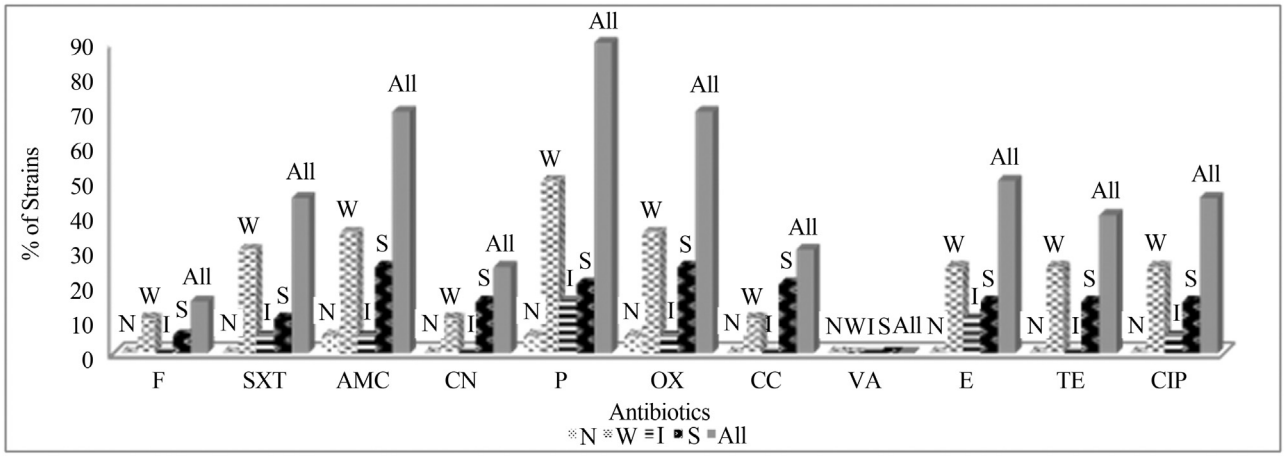

Figure 3 - Percentage of *S. epidermidis* strains displaying resistance to 11 different antibiotics according to their biofilm formation levels. (F: Nitrofurantoin, SXT: Trimethoprim /Sulfamethoxazole, AMC: Amoxicilin /Clavulonic acid, CN: Gentamicin, P: Penicillin, OX: Oxacillin, CC: Clindamycin; VA: Vancomycin, E: Eritromycin, TE: Tetracyclin, CIP: Ciprofloxacin, N: Non Biofilm Formers, W: Weak Biofilm Formers, I: Intermediate Biofilm Formers, S: Strong Biofilm Formers).

2005; Villari *et al.*, 2000; Vuong and Otto, 2002). Furthermore, *Staphylococci* are stated as the most commonly diagnosed microorganisms in microbial infections on blood-contacting devices (Xu and Siedlecki, 2012). Existence of high proportion of *S. epidermidis* strains in cardiovascular infections and in sample of blood confirms that, *S. epidermidis* is one of the most important pathogens causing bloodstream infections and the reason of this is suggested to be associated with their biofilm formation abilities on implanted medical devices like prosthetic heart valves (Xu and Siedlecki, 2012).

According to their biofilm formation performances, *S. epidermidis* strains were classified in different groups. Within these, percentage of strong biofilm forming strains were found as 25%. Studies, which have been applied for several years indicate that; usage of intravascular devices in different types, is the reason of most nosocomial blood stream infections (Fux *et al.*, 2003) and the increased usage of these indwelling medical devices such as; cardiac devices, vascular grafts and intravascular catheters, is observed to enhance frequency of biofilm related *S. epidermidis* infections (Arciola *et al.*, 2012). Therefore, it is concluded that; the existence of strong biofilm forming strains in different cardiothoracic surgery unit patients and in sample of blood may be related with enhanced usage of some indwelling medical devices.

When the antibiotic test results of *S. epidermidis* strains are examined; our findings are in agreement with those of other studies suggesting; vancomycin is the most efficient antibiotic for *S. epidermidis* inhibition (Al *et al.*, 2005; Chaieb *et al.*, 2005; McCann *et al.*, 2008). However; deficient inhibition effect of vancomycin in biomaterial re-

Table 1 - Percentage of Strong Biofilm Forming and all *S. epidermidis* strains displaying resistance to different antimicrobial classes.

Resistance to antimicrobial classes	% of SBF *S. epidermidis* strains	% of All *S. epidermidis* strains
Fully Sensitive	-	5
Resistance to 1 Antimicrobial Class	-	15
Resistance to 2 Antimicrobial Classes	-	20
Resistance to 3 Antimicrobial Classes	20	10
Resistance to 4 Antimicrobial Classes	20	15
Resistance to 5 Antimicrobial Classes	40	20
Resistance to 6 Antimicrobial Classes	-	5
Resistance to 7 Antimicrobial Classes	20	10
Resistance to 8 Antimicrobial Classes	-	-
Resistance to 9 Antimicrobial Classes	-	-
β-lactam (OX, AMC, P) Resistance	80	65
*Multi Drug Resistance	100	60

(*Each of Strong Biofilm Forming strains were multi drug resistant).
(Antimicrobial Classes: Aminoglycoside, β-lactam, Macrolide, Tetracycline, Fluoroquinolone, Lincosamide, Furan, Sulfonamide, Glycopeptide).

lated endocarditis infections is also pointed out and the reason of this is indicated to be related with biofilm formation (Juárez-Verdayes *et al.*, 2006). When the microorganisms spread to external surface of a medical device, they begin to grow and multiply to colonize on it and in order to fasten their colonization, they produce extracellular polymeric substances known as biofilms (Kumar and Anand, 1998). As a result of including extracellular polymeric substance, biofilms act as preventive barriers both to antimicrobial agents and immunological responses. For this reason, in order to eradicate biofilm related infections, some surgical operations are required to be applied (Baillie and Douglas, 2000; Xu and Siedlecki, 2012). Apart from this, inside biofilms; huge amount of extracellular DNA exists and too many distinct microorganisms have a chance to come together and stay immobile. As a result, in addition to acting as a preventive barrier; biofilm environments also promote genetic exchange of antimicrobial resistance genes, increasing bacterial virulence and contributing to the development of multi resistant phenotypes (de Araujo *et al.*, 2006; Parsek and Singh, 2003; Subramanian *et al.*, 2012). Confirming these, in our study; high amount of β-lactam resistance was observed in strong biofilm forming *S. epidermidis* strains (80%) and additionally, each of strong biofilm formers were determined as multi drug resistant because of being resistant to 3 or more antimicrobial classes (Magiorakos *et al.*, 2012) (Table 1). Moreover, one of these multi drug resistant strains which was found as resistant to maximum amount of different antimicrobial classes (7) was also observed as maximum biofilm forming strain among all the other *S. epidermidis* strains.

In parallel with our findings, Ponnusamy *et al.* (2012) demonstrate that, strong biofilm producers are more resistant to antimicrobial agents than weak biofilm producing ones. Similar with this; Kwon *et al.* (2008) also suggest that, multi drug resistant clinical isolates of *S. aureus* have a greater capability of developing biofilms on medical devices. In addition, according to an another study carried out on antibiotic resistance of exopolysaccharide forming *S. epidermidis* strains from orthopedic implant infections, similar to our results; up to 80% of all isolates were found as β-lactam resistant (Arciola *et al.*, 2005).

All these findings show that, strong biofilm forming *S. epidermidis* strains display increased antimicrobial resistance and the reason of this is supposed to be related with genetic modifications occurring in the matrix of a biofilm. That is, our findings are in agreement with those of other studies suggesting; biofilm formation plays a huge role in gaining new virulence factors for any kind of microorganism (Donlan, 2001; Parsek and Singh, 2003; Subramanian *et al.*, 2012).

To sum up, according to our results; samples of blood and wound were the most *S. epidermidis* isolated clinical materials and cardiothoracic surgery was the most *S. epidermidis* isolated service unit (Figures 1, 2). Similar to this; strong biofilm forming *S. epidermidis* strains were also isolated from sample of blood among different clinical materials and from service of cardiovascular surgery among different service units in highest frequency (Figures 1, 2). On the other hand, vancomycin was the most efficient antibiotic against all *S. epidermidis* strains whereas efficiency of β-lactam antibiotics all together (P, OX, AMC) was determined as 35% (Table 1). Additionally, each of strong biofilm forming *S. epidermidis* strains were multi drug resistant and high amount of them were resistant to all β-lactam antibiotics (P, OX, AMC) used in this study (Table 1). Furthermore, one of these multi drug resistant strains which was found to display resistance to maximum amount of different antimicrobial classes was also observed as maximum biofilm forming strain among all the other *S. epidermidis* strains.

Conclusion

This study shows that, a commensal skin colonizer may easily turn in to an important pathogen. Therefore, as well as preventing medical device using patients from extrinsic *S. epidermidis* contaminations, taking precautions against patient's own skin during surgical operations is also extremely necessary in order to prevent *S. epidermidis* related nosocomial infections. Otherwise, once *S. epidermidis* spreads to external surface of any blood-contacting medical device during the settlement of it by surgical operation, colonization and biofilm formation of *S. epidermidis* on device's surface may come true and this may be the result of every reoccurring, persistent infections. Additionally, inside the matrix of biofilms, some genetic modifications may occur and turn these commensal microorganisms into multi drug resistant pathogens. As a result, surgeons must strictly take precautions against both extrinsic *S. epidermidis* contaminations and patient's own skin during surgical operations.

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