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Susceptibility of planktonic and biofilm forming isolates of *Staphylococcus epidermidis* to antimicrobial agents

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ABSTRACT

The coagulase-negative *Staphylococcus epidermidis* is now well established as a major nosocomial pathogen associated with infections of indwelling medical devices. One hundered and nine (109) clinical isolates of *S. epidermidis* were isolated and identified from the following infection, native valve endocarditis, prosthetic joint infection, and intravascular catheter infection. Screening of isolates for biofilm formation qualitatively and quantitatively was assessed. The isolates were associated with biofilm formation, which may lead to an inability to eradicate the infection due to the intrinsic nature of biofilms to resist high levels of antibiotics. In this study, the sensitivity pattern to 11 different antimicrobial disks were determined.The data revealed that, all isolates were resistant to oxacillin and Amoxicilin-clavulinic acid except one, On the contrary, all isolates were sensitive to vancomycin. Furthermore, Minimum Inhibitory Concentrations (MICs) and Minimum bactericidal Concentrations (MBCs) for 29 isolates of *S. epidermidis* of strong biofilm producing and sensitive isolates were measured against three antimicrobial agents (vancomycin, chloramphenicol and clindamycin) in planktonic form and in biofilm forming. Planktonic isolates were susceptible to all agents studied,but the isolates in biofilms were resistant to high concentrations of most of these agents. The data revealed that, the MICs and MBCs of most isolates in biofilm form were increased 512 to 2048 times or more. These data provide insights into the response of *S. epidermidis* isolates when challenged with various levels of antimicrobial agents in clinical use.

Key Words: *Staphylococcus epidermidis*, adhesion, biofilm, antibiotic resistance

INTRODUCTION

It is well known that implant-related infection is difficult to treat, and curative therapy is still not available. Although healing with retention of an implant is ideal, the implant is often removed after various treatments. This procedure requires much time and places a large burden on patients and the medical staff psychologically and economically. Moreover, when replacement is planned, further burden results. Thus, development of an implantation method, particularly a method for healing from infection with retention of the prosthesis, is needed.

The reason that infections become intractable despite the use of antimicrobial agents with effective spectra is still unclear. Gristaina and Costerton (1985) reported involvement of biofilms as the cause of intractable infection associated with implants. Planktonic bacteria that enter the body adhere to the implant surface and produce a hydrate matrix of extracellular polysaccharides (glycocalyx) and protein, forming a slimy layer defined as biofilm. Some bacteria are released from the biofilms and form other biofilms at different sites. These biofilms provide a barrier that reduces penetration of antimicrobial agents.

Das *et al.* (1998) reported that biofilm plays a significant role in chronic infection associated with implanted medical devices. Ceri *et al.* (1999) reported that biofilm bacteria exhibit dramatically increased resistance to

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killing by antimicrobial agents compared to planktonic bacteria.

Gristina and Costerton (1985) also reported that susceptibility tests with biofilm bacteria have shown the survival of these bacteria after treatment with antimicrobial agents at concentrations hundreds or even a thousand of times the minimum inhibitory concentration (MIC) of planktonic bacteria. However, the susceptibility of biofilm bacteria isolated from the medical devices to any antimicrobial agents is not known well.

Factors which explain the high antimicrobial resistance of biofilms include decreased diffusion of antibiotics through the biofilm matrix, decreased oxygen and nutrient, decreased growth rates and metabolism, quorum sensing and induction of a biofilm specific phenotype and formation of persister cells which contribute to tolerance (Anderson and O'Toole, 2008).

In this study, the (MICs) of various antimicrobial agents against *S. epidermidies* in free (planktonic) or in biofilm forms were determined by microbroth dilution method using microtiter plates.

MATERIALS and METHODS

A total of 320 specimens were collected during the period from May 2011 to November 2011 from patients with medical prosthetic devices admitted to the hospitals of Zagazig University.

Isolation and identification of *S. epidermidis* were processed according to Koneman *et al.* (1997) as follow :

- Microscopical examination: Gram positive cocci arranged in bunches or clusters.
- Biochemical tests:

catalase test is positive, coagulase is negative, *S.epidermidis* grow as white colonies on nutrient agar, mannitol non-fermenter on mannitol salt agar and non haemolytic (gamma) on blood agar.

Screening for biofilm formation was assessed qualitatively and quantitatively as follow:

Qualitative assessment of biofilm by tube method

The isolates were tested for their ability to form biofilm according to Stepanovic *et al.* (2007) with some modifications. Tested isolates were grown on Tryptone Soya Broth with 1% glucose (TSB_{glu}) in Falcon tubes overnight, then the turbidity was adjusted to match that of a 0.5 McFarland standard. Five ml of inoculated broth were transferred to new sterile Falcon tubes for qualitative assessment. Uninoculated TSB_{glu} tubes were used as negative controls. Tubes were incubated overnight at 37°C. The content of each tube was carefully aspirated with a pipette, and the tubes were immediately stained with 5 ml of 0.25% safranin for one minute. The tubes were decanted and inverted without washing. After overnight, standing at room temperature, the tubes were examined for biofilm formation. The test was considered positive when there was a stained film or adherent layer on the inner surface of the tube. The biofilm formation was estimated as negative (0), weak (+), moderate (++), or strong (+++).

Quantitative assessment of biofilm by spectrophotometric method

Quantification of biofilm production was assessed according to Stepanovic *et al.* (2007). Five milliliter quantities of inoculated TSB_{glu} with optical density matching that of a 0.5 McFarland standard were distributed in sterile Falcon tubes. Uninoculated TSB_{glu} tubes were used as negative controls. The tubes were incubated for 24 hours at 37°C; the content of each tube was aspirated, and washed three times with sterile saline to remove any non-adherent bacteria. For fixation of adherent bacteria, 99% methanol was

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added for 15 minutes. Then, the tubes were decanted, left to dry, and stained with 2% Hucker crystal violet for 5 minutes. Excess stain was rinsed off by running tap water. After the tubes were air dried, the dye bound to the adherent cells was resolubilized with 33% (v/v) glacial acetic acid. The Optical Density (OD) was measured at 570 nm using spectrophotometer (UV-1800 Shimadzu, Japan). Based on the measurement of ODs, the bacterial biofilms were classified into four categories, non-biofilm forming (if OD ≤ ODc), weak biofilm forming (if OD > ODc, but ≤ 2x ODc), moderate biofilm-forming (if OD > 2x ODc, but ≤ 4x ODc), and strong biofilm forming (if OD > 4x ODc). The cutoff OD (ODc) was defined as equivalent to three times standard deviations above the mean OD of the negative control. The test was made in triplicates and repeated three times, and the data were expressed as average.

Susceptibility by disk diffusion

Clinical isolates of *Staphylococcus epidermidis* were tested for their susceptibility to different antimicrobial agents by disk diffusion method according to CLSI (2006) criteria.

The following antibacterial disks were obtained from Oxoid, hamphsire, England: Methacillin (ME, 5 µg), Amoxicillin-Clav (AMC) (20, 10 µg), Vancomycin (VA, 30 µg), Gentamycin (GN, 10 µg), Azithromycin (AZM, 15 µg), Erythromycin (E, 15 µg), Doxycycline (Do, 30 µg), Ciprofloxacin (Cip, 5 µg), Clindamycin (DA, 2 µg), Sulfamethoxazole-trimethoprime (SXT, 1.25/23.75 µg) and Chloramphenicol (30 µg)

Four separate colonies of each isolate were transferred to a sterile tube containing 5 ml of Mueller Hinton Broth (MHB).

The broth was incubated overnight at 37°C, and the turbidity was adjusted to approximately equivalent to 0.5 McFarland turbidity standard. Broth cultures were further diluted to 1:200 in broth to obtain inoculum density ranged between 10^5 and 10^6 CFU/ml.

A sterile cotton swab was dipped into the bacterial suspension (within 15 minutes of adjusting the density of inoculums) and the excess was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The surface of a dried Mueller Hinton agar plate was streaked with inoculating swab in different directions.

The inoculated plates were left on a flat level surface undisturbed for 3-5 minutes; the antibacterial disks were placed on the inoculated plates by using fine pointed sterile forceps and lightly pressed into the agar with the forceps. The disks were arranged at 15 mm from edge of the Petri dish and 30 mm from each other. This arrangement reduces zones overlapping each other. The plates were incubated inverted at 37°C for 18 hours. Plates were examined and diameters of the complete inhibition zones were measured in millimeters, and interpreted according to CLSI (2006).

Determination of MICs and MBCs for planktonic bacteria

The strong biofilm forming and sensitive isolates to the antimicrobials namely, vancomycin, chloramphenicol and clindamycin were included in this part of investigation.

The MICs and MBCs of the antimicrobial agents were determined by micro broth dilution method according to CLSI (2006). Suspension of *S. epidermidis* were prepared by resuspending one colony of an overnight culture from Tryptone Soya agar in Tryptone Soya broth. The turbidity was adjusted to approximately 0.5 McFarland turbidity standard. Broth cultures were further diluted to 1:200 in broth to obtain inoculum density ranged

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between 10^5 and 10^6 CFU/ml. Each well of polystyrene microtiter plate containing 100 µl of the antimicrobial agent at two fold serial dilution was inoculated with 100 µl of the bacterial suspension and incubated at 37°C for 24 hours. Then, the wells were visually inspected for growth.

The MIC was defined as the lowest concentration that did not show growth. Positive and negative controls were included.

Following the MIC assay, MBCs were determined by plating 10 µl of each clear wells onto TSB agar plates. The MBC was defined as the lowest concentration yielding no growth following incubation at 37°C for 24 hours.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

The MBICs of the three antimicrobials were determined according to Cernohorska and Votava (2008). The experiments were done in 96-wells polystyrene microtiter plates with round bottoms. An overnight culture adjusted with TSB to achieve a turbidity equivalent to that of a 0.5 McFarland standard, then 75 µl aliquots of the inoculated media were added to the wells of microtiter plates. The plates were incubated for 24 hours at 37°C. The wells were washed three times with phosphate-buffered saline (PBS) under aseptic conditions to remove unattached bacteria and dried in an inverted position. Volumes of 100 µl of appropriate two-fold dilutions of the respective antimicrobial agents or the potential antibiofilm agents in Mueller-Hinton broth were transferred into the dried wells with established biofilms. The microtiter plates were incubated for 18-20 hours at 37°C, and Minimum Biofilm inhibitory Concentration (MBIC) was determined, which corresponds to the lowest concentration of antimicrobial which inhibits the growth of biofilm's cells as indicated by absence of visible growth in the wells. A positive control and a negative control were included in all experiments. The experiment was repeated three times.

RESULTS

As figure (1) revealed, about 109 isolates of *S. epidermidis* were isolated from 320 clinical specimens; 93 from different medical prosthetic devices and 16 from blood after bacteremic infection due to implanted devices.

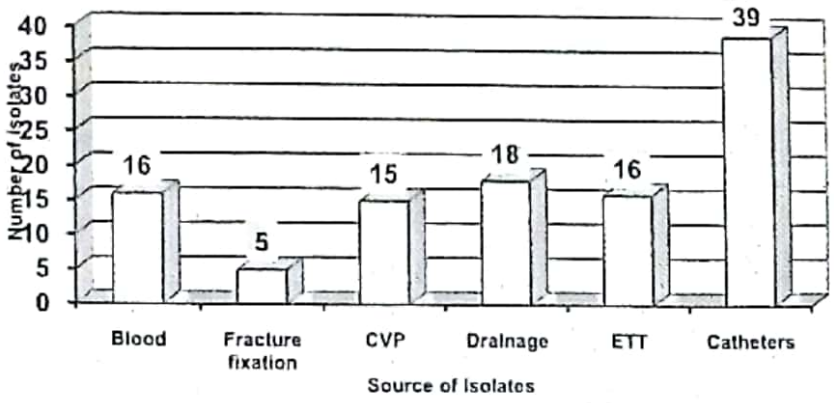

Figure 1. Number of isolates from different medical prosthetic devices
CVP= Central venous pressure ETT= endotracheal intubation

Screening for biofilm formation is done qualitatively and quantitatively and the results were summarized in figure (2) which shows that 31 isolates were non-biofilm producer, 30 isolates were weak, 19 were moderate and 29 were strong biofilm producer.

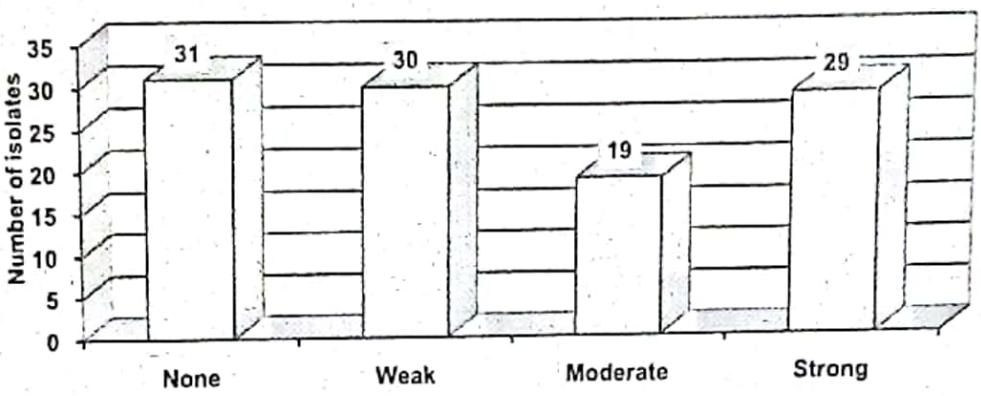

Figure 2. Number and degree of biofilm production and adhesion of *Staphylococcus epidermidis* isolates.

Susceptibility patterns

The sensitivity of isolates to 11 antimicrobial agents was done by disk diffusion method. The data shown in figure (3) revealed that, all isolates were resistant to oxacillin and AMC except one. On contrary, all isolates were sensitive to vancomycin. In addition, 87.1% and 80.7% of isolates were sensitive to chloramphenicol and clindamycin, respectively.

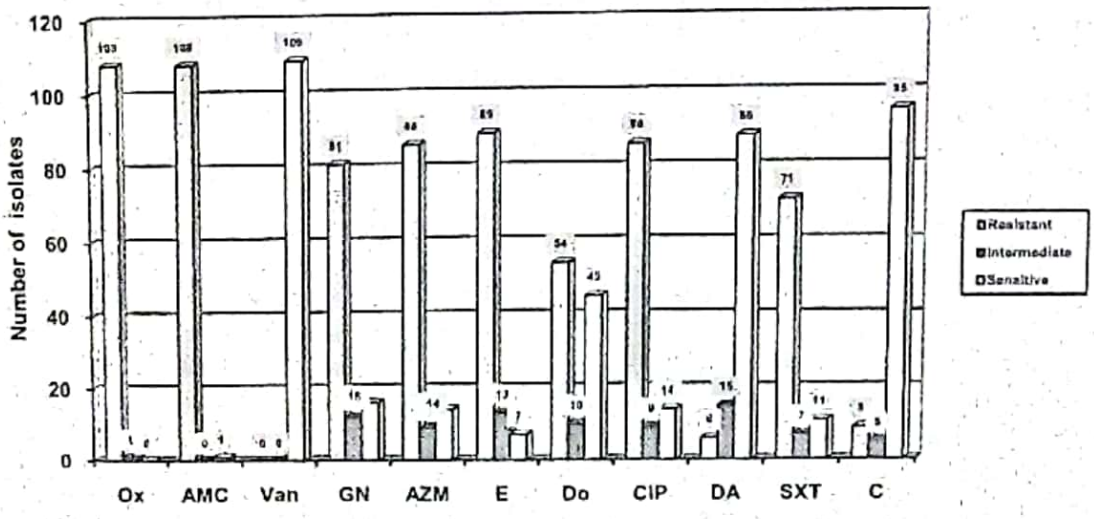

Figure 3. Susceptibility patterns of *S. epidermidis* isolates to different antimicrobial disks.

Ox = Oxacillin, AMC, Amox-Clavulanic acid, Van = Vancomycin, GN = Gentamicin, Azm = Azithromycin, E = Erythromycin, Do = Doxycycline, CIP = Ciprofloxacin, DA = Clindamycin, SXT = Trimethoprime-sulfamethyazoline, C = Chloramphenicol

The MICs and MBCs were determined for 29 isolates in planktonic and biofilm forming; selected on bases of: they were strong biofilm producers and were sensitive to 3 antimicrobial agents namely, vancomycin, chloramphenicol and clindamycin, In addition to the standered strain of *S. aureus* ATCC 6538 (strong biofilm producer).The data in table (1) revealed that, non of the isolates under investigation in planktonic forms were resistant to the 3 antimicrobial agents. These results were interpreted according to the CLSI (2006).

Table (1). The MICs and MBCs of selected planktonic isolates of *S. epidermidis*

Isolate number	Vancomycin		Chloramphenicol		Clindamycin	
	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
1	0.5	2	0.5	2	0.25	2
2	0.5	1	2	4	0.25	1
3	0.5	2	1	4	0.5	2
4	0.5	2	2	8	0.5	2
5	1	4	0.5	4	0.5	4
6	0.5	2	0.5	2	1	4
7	0.5	2	1	4	0.25	2
8	0.5	2	0.5	2	0.5	4
9	0.5	2	4	16	0.5	4
10	0.5	2	2	4	0.5	4
11	0.5	1	0.5	8	0.5	4
12	0.5	2	0.5	2	0.5	4
13	1	2	0.5	4	0.5	2
14	0.5	2	0.5	4	0.5	2
15	0.5	2	1	4	0.25	2
16	0.5	2	1	4	0.25	2
17	0.5	2	1	4	0.25	1
18	1	2	1	4	0.5	1
19	1	2	1	2	0.5	1
20	1	4	1	2	0.5	4
21	1	4	0.5	8	0.5	4
22	1	4	0.5	4	0.5	2
23	0.5	1	0.5	4	0.5	2
24	0.5	2	0.5	4	0.25	1
25	0.5	2	2	8	0.25	1
26	0.5	2	2	8	0.25	2
27	1	2	2	2	0.25	2
28	1	4	2	4	0.25	2
29	0.5	2	0.5	4	0.5	4
ATCC6538	0.5	1	1	2	0.5	4

As shown in Table (2), The susceptibility of 29 isolates and standard strain of *S. aureus* ATCC 6538(strong biofilm producer) in biofilms against vancomycin, chloramphenicol and clindamycin were presented in table (2).The data shown in table (2) revealed that,the MICs and MBCs of most isolates were increased 512 to 2048 times or more.

Table (2). MICs and MBCs of isolates in biofilms.

Isolate number	Vancomycin		Chloramphenicol		Clindamycin	
	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
1	512	2048	256	1024	128	512
2	512	2048	256	1024	256	1024
3	512	1024	512	2048	256	512
4	1024	> 2048	256	1024	256	1024
5	1024	> 2048	1024	> 2048	128	512
6	512	2048	512	2048	512	2048
7	2048	> 2048	512	2048	512	1024
8	512	> 2048	512	2048	256	1024
9	512	> 2048	512	2048	256	512
1	512	> 2048	1024	> 2048	256	512
0	512	2048	256	1024	256	1024
11	512	> 2048	512	2048	512	1024
12	512	> 2048	256	1024	512	2048
13	512	1024	256	1024	128	512
14	1024	2048	256	1024	256	1024
15	1024	2048	256	1024	256	512
16	1024	2048	512	2048	256	1024
17	1024	2048	512	2048	128	512
18	512	1024	256	1024	128	512
19	512	> 2048	256	1024	256	1024
20	512	> 2048	256	1024	256	1024
21	1024	2048	1024	> 2048	256	512
23	1024	> 2048	1024	> 2048	256	512
24	2048	> 2048	1024	> 2048	256	1024
25	512	2048	512	2048	256	1024
26	2048	> 2048	512	2048	256	1024
27	512	> 2048	1024	2048	256	1024
28	512	1024	512	2048	128	512
29	512	1024	512	1024	128	512
ATCC6538	512	1024	512	2048	512	512

DISCUSSION

The use of medical implants has increased immensely over the last few decades. This ranges from the simple use of catheters to draw blood to life-saving devices such as the total artificial heart. A major barrier to the long use of medical device is development of infection. *Staphylococcus epidermidis* is one of the most common bacterial isolates from these infections. *S.epidermidis* is one of most etiological agents of several nosocomial infections (e.g.bacteremia, endocarditis, osteomyelitis, peritonitis, postoperative cardiac infection and urinary tract infections) which is associated with biofilm and colonization on the catheters and implants (Boyce, 1999). Currently, antibiotics are used as the main type of therapy; however, with the emergence of staphylococcal resistance, this kind of therapy is fast becoming ineffective.

In this study, 109 isolates of *Staphylococcus epidermidis* were isolated from 320 clinical specimens (33.4%) from bacteremic or patients implanted with prosthetic devices.

Among these 109 isolates, 78 isolates (71.5%) were biofilm producers. The rate of biofilm production ranged from weak (38.4%),

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moderate (24.3%), to strong (37.7%). The above data revealed a strong correlation between the use of medical devices and ability of *S. epidermidis* to adhere and colonize the prosthetic devices in biofilm forms.

In this investigation, out of 109 isolates, 108 isolates were resistant to oxacillin and AMC ,74.3 % were resistant to gentamycin, 81.7% were resistant to erythromycin, 65.1% were resistant to SXT, 78.9% were resistant to ciprofloxacin .The study revealed a good clinical sign that, all isolates were sensitive to vancomycin, this was in agreement with that reported by Michelim *et al.* (2005). They stated that of the 98 clinical isolates, 82.6% were resistant to gentamycin, 79.6% to erythromycin, 76.5% to sulphamethoxazole/trimethoprim, and 71.4% to ciprofloxacin. All the isolates were susceptible to vancomycin.

In this sense, various studies have shown that staphylococci resistant to oxacillin were resistant to all penicillins (including semi-synthetic penicillins congruent resistant to beta-lactamases) as well as penens, carbapenens and cephalosporins (Chambers, 1997). Staphylococcal resistance to these chemotherapeutic agents having been attributed to the production of the Pencillin Binding Protein (PBR-2′) protein which links to the antibiotics (Boyce, 1999). An elevated frequency of oxacillin resistance has been described by De Giusti *et al.* (1999).

In this study, The MICs and MBCs of the three antimicrobials namely, vancomycin, chloramphenicol and clindamycin against the planktonic and biofilm forming isolates were determined. The results showed that all planktonic cells were susceptible to tested antimicrobials according to CLSI (2006). On the other hand, the cells in biofilm showed high resistance to test antibiotics, and this could be estimated from MBIC/MIC ratio.

The MBIC/MIC ratio in vancomycin is 1024. This means that the MIC of vancomycin against the isolates in biofilm forms increased by 1024 folds. This was in agreement with that reported by Nishimura *et al.* (2006). They found that even though the antimicrobial agents were effective against planktonic bacteria, the MBC values for biofilm bacteria of all antimicrobial agents were high, demonstrating that treatment with antimicrobial agents is generally ineffective after a biofilm has formed. In clinical practice, although inflammatory findings disappear after administration of antimicrobial agents and the infection is thought to have resolved, the infection may relapse after a while such as with the excretion of exudates. In such cases, only planktonic bacteria may be eliminated during the early phase symptom disappearance. Although the symptoms transiently disappear, biofilms may remain and release bacteria, resulting in infection. To attain a complete healing, a strategy for preventing the presence of biofilm bacteria behind the planktonic bacteria must taken into consideration.

The persistence of bacteria in biofilm was explained by Stewart and Costerton (2001) in spite of antimicrobials therapy. They suggested three main hypotheses. The first hypothesis was the possibility of slow or incomplete penetration of antibiotic into biofilm. The second hypothesis is an altered chemical microenvironment within the biofilm. For example, acidic waste products in biofilm might lead to pH differences greater than one between the bulk fluid and the biofilm interior, which could directly antagonize the action of the antibiotic.

A third hypothesis was that a subpopulation of microorganisms in a biofilm forms a unique, highly protected, phenotypic state; a type of cell differentiation similar to spore formation.

CONCLUSION

The data obtained in the present work confirmed the importance of antibiotic resistance and the necessity of particular care in the antibiotic treatment of nosocomial infections caused by *S. epidermidis* in biofilms. Since the implant-related infection is difficult to eradicate with antimicrobial agents alone. Further investigation must be conducted for the use of co-therapy with antimicrobial agents which could be able to prevent or decrease the adherence of biofilm forming isolates to the prosthetic devices or at least change the hydrophobicity of prosthetic devices.

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حساسية الخلايا العالقة وخلايا البيوفيلم لميكروب ستافيلوكوكس ابيدرميدز للمضادات الميكروبية

أشرف أحمد قدري، منى عبدالمنعم السيد، شيماء إبراهيم نزيه
قسم الميكروبيولوجي والمناعة- كلية الصيدلة-جامعة الزقازيق

من المعروف حاليا ان ميكروب الايستافيلوكوكس ابيدرمدز اصبح ميكروبا اساسيا في العدوي المكتسبة من المستشفيات خاصة الناتجة عن استعمال الاجهزة المنزرعة داخل جسم الانسان. في هذه الدراسة تم جمع ٣٢٠ عينة من مختلف الاجهزة المنزرعة داخل جسم الانسان من (قساطر بولية – قساطر وريدية مركزية- درانق- مسامير وشرائح لتثبيت العظام- الانابيب المنزرعة داخل القصبة الهوائية- الدم الناتج من عدوي بكتيرية نتيجة لاستخدام هذه الاجهزة) وقد تم التعرف علي ١٠٩ عزلة من ميكروب الايستافيلوكوكس ابيدرميدز.

لقد تم معرفة درجة تكوين الغلاف الحيوي (البيوفيلم) في جميع العزلات عن طريق تحليل كيفي (وصفي) بالمشاهدة بالعين وهو مصبوغ علي جدران الأنابيب وأيضا بطريقة أخري عن طريق ملاحظة لون المستعمرات علي وسط CRA وعن طريق تحليل كمي يعتمد علي قياس درجة اللون بجهاز التحليل الطيفي، وقد تم تقسيمها الي درجات بناء علي قدرتها علي تكوين الغلاف الحيوي (البيوفيلم) من قوي (٢٩) عزلة، ومتوسط (١٩) عزلة، وضعيف (٣٠) عزلة، ولايكون البيوفيلم (٣١) عزلة.

تم اختبار جميع العزلات (١٠٩) لاختبار الحساسية ضد ١١ نوع من انواع المضادات البكتيرية المختلفة والتي تنتمي للفئات المختلفة من عائلات المضادات البكتيرية، وقد أظهرت النتائج مقاومة شديدة للبنسلين والبيتالاكتاميز بينما أظهرت حساسية شديدة للفانكوميسن، وقد اظهرت الدراسة نتائج متفاوتة للمقاومة لمضادات الكليندامايسن والكلورامفينيكول والجنتاميسين والسيبروفلوكساسين.

تم اختيار (٢٩) عزلة والتي اوضحت قدرتها القوية علي تكوين الغلاف الحيوي (البيوفيلم) لتحديد اقل تركيز من المضادات البكتيرية (الفانكوميسن، الكلورامفنيكول و الكليندامايسين) لمنع النمو وأقل تركيز لقتل البكتيريا وذلك مرة لتحديد التركيزات للبكتيريا العالقة ومرة اخري لنفس العزلات في الغلاف الحيوي (البيوفيلم). وقد أظهرت النتائج مقاومة شديدة للعزلات في شكل البيوفيلم تتروح في الزيادة من ٥١٢ الي ٢٠٤٨ مرة عن نفس العزلات في الصورة العالقة وهذا يوضح الدور الخطير الذي تلعبه البيوفيلم في زيادة مقاومة البكتريا للمضادات الحيوية .