



Study the effect of Lysostaphin, on methicillin resistant *Staphylococcus aureus*(MRSA) biofilm formation

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Abstract

This study included collection of 100 specimens from patients in AL-Kindy Teaching Hospital and teaching laboratories of Medical City Hospitals in Baghdad during the period from August to December 2012, these specimens differed in their sources which included 19 nasal swab, 16 wound swab, 27 burn swab, 7 pus, 15 sputum, 10 corneal swab and 6 urine. Only 38 (38%) isolates was identified as *Staphylococcus*. In this study, 29 isolates (76.3%) were coagulase-positive (COPS), while only 9 isolates (23.6%) were coagulase negative (CONS), from total 38 isolates of *Staphylococci*.

The distribution of Methicillin resistance among *Staphylococcus* spp. was investigated by disc diffusion method. In this study, 21 isolates (55.26%) showed resistant to the Methicillin while 17 isolates (44.73%) were sensitive.

The ability to produce slime layer by MRSA isolates was also investigated and the results showed that all isolates of MRSA was produced slime layer when tested by tube method, but the amounts of adherent materials were differ among the isolates. However, the results by Congo red agar method showed that 57% of MRSA isolates produced strong slime layer and 43% of MRSA obtained negative result. Similarly the ability of MRSA to produce biofilm by tissue culture plate (TCP) was investigated and the results indicated that MRSA isolates showed highly and strong biofilm formation, and the OD value of biofilm formation ranged between 0.262 - 0.311. Additionally the OD value of biofilm formation significantly increased in addition of 1% glucose to the media.

Statistical analysis showed slightly effect of lysostaphin under (5.625µg/ml MIC) on biofilm formation ability of (MRSA S3) optical density was reduced to (0.312 nm) in comparable with control group (0.389nm).

Key word : lysostaphin, *S.aureus*, biofilm, MRSA.

دراسة تأثير الاليسوستافين على قابلية المكورات العنقودية الذهبية المقاومة للمثسلين على تكوين البايوفلم (MRSA)

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الخلاصة

شملت هذه الدراسة جمع 100 عينة سريريّة من المرضى الراقدين في مستشفى الكندي التعليمي و المختبرات التعليمية لمدينة الطب في بغداد للفترة من حزيران ولغاية كانون الاول 2012. هذه العينات توزعت بين مسحات (للأنف) (19)، للجروح (16)، الحروق (27)، التقيحات (7)، القشع (15)، القرنية (10) والادار (6). وتم تحديد 38 عزلة (38%) تعود الى جنس *S.aureus*. وجد ان 29 عزلة (76.3%) منها

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كانت موجبة لفحص الأنترايم المخثر للبلازما [(COPS) Coagulase-Positive Staphylococci] في حين اظهرت 9(23.6%) نتيجة سالبة اتجاه هذا الفحص [Coagulase-Negative Staphylococci (CONS)].

تم التحري عن مقاومة العزلات للمضادات الحيوية بواسطة طريقة الانتشار القرص على الاكار الصلب وقد اظهرت الدراسة ان 55.26(%) من العزلات كانت مقاومة للمثليين في حين ان 44.73(%) من العزلات كانت حساسة تجاه هذا المضاد.

وقد تم التحري عن قابلية العزلات MRSA على إنتاج الطبقة اللزجة وأظهرت النتائج أن جميع العزلات MRSA لها القابلية على إنتاج الطبقة اللزجة عند اختباره بواسطة طريقة الأنايب باختلاف كمية المواد المتصقة بين العزلات . ومع ذلك، أظهرت النتائج وفقا لطريقة اكار الاحمر الكونغو أن 57% من MRSA كانت لها قابلية قوية على تكوين الطبقة اللزجة و 43% من MRSA اعطت نتيجة سالبة . وبالمثل تم التحري عن قابلية MRSA على إنتاج البايوفلم بواسطة الطبق الزرع النسيجي (TCP) وأشارت النتائج إلى أن العزلات MRSA لها القابلية العالية والقوية على تكوين البايوفلم وقيمة OD من تشكيل بايوفلم تراوحت بين (0.262-0.311). بالإضافة إلى ذلك اظهرت زيادة كبيرة في قيمة OD لتكوين بايوفلم عند إضافة 1% من الكلوكوزالى الوسط.

أظهر التحليل الإحصائي تأثير قليل للإيسوستافين تحت تركيز (5.625 مايكروغرام/مل) على قابلية (S3 - MRSA) على تكوين البايوفلم حيث انخفضت قراءة (OD) إلى (0.312 نانومتر) مقارنة بمجموعة السيطرة (0.389 نانومتر).

INTRODUCTION

Staphylococcus aureus is a causative agent of many types of diseases throughout the world. *Staphylococcal* infections are of particular concern because of the causative bacteria offering resistance to a wide range of commonly used antibiotics [1]. *S. aureus* is consistently one of the top four causes of nosocomial infections [2]. Patients hospitalized for long periods of time are usually predisposed to infection by methicillin resistant *S. aureus* [3]. At present approximately 40% of *Staphylococcus aureus* are resistant to methicillin and the incidence of methicillin resistance increases year by year [2]. MRSA poses increasingly serious health care problem in many parts of the world. Several studies have reported increased morbidity and mortality associated with MRSA compared to methicillin sensitive *Staphylococcus aureus* infections [4]. Methicillin resistant strains of *S. aureus* are more difficult to treat because multidrug resistance is more common in these isolates as compared to the MSSA isolates. Vancomycin is considered as the treatment of choice for MRSA cases but there are reports of emergence of vancomycin resistance in *S. aureus* [5].

Staphylococcus aureus is an adaptable, pathogenic organism. In the presence of environmental challenges, *S. aureus* can alter its genotype and/or phenotype to adapt to its surroundings. An example of genotypic change is the acquisition of the β -lactamase gene conferring penicillin resistance. The formation of biofilm is an example of phenotypic change. Formation of a biofilm is the hallmark characteristic of *S. aureus* infection which consists of multiple layers of bacteria encased within an exopolysaccharide glycocalyx. Presence of glycocalyx protects the enclosed bacteria from host defences and impedes delivery of antibiotics [6]. Infact biofilms can resist antibiotic concentration 10-10,000 fold higher than those required to inhibit the growth of free floating bacteria [7]. Biofilm formation in *S. aureus* is regulated by expression of Polysaccharide Intracellular Adhesion (PIA) which mediates cell to cell adhesion PIA synthesis depends on the expression of the *icaADBC* operon, which encodes three membrane proteins (IcaA, IcaD, and IcaC) with enzymatic activity and one extracellular protein (IcaB), and is the gene product of Ica (IcaA, IcaB, and IcaC and IcaD) [8].

Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle ear infections, formation of dental plaque, gingivitis, coating contact lenses and less common but more lethal processes such as infective endocarditis, cystic fibrosis and infections of permanent indwelling devices such as joint prosthesis and heart valves [9,10]. A serious problem especially of chronic infections is formation by microorganisms of biofilm, which is difficult to eradicate with standard antibiotic therapy. Thus, researchers are still looking for alternative options to eliminate the biofilm-forming microorganisms

[11,12].this study aimed to Investigating the effect of lysostaphin on biofilm of the multi-drugs resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

***Staphylococcus aureus* isolation and identification**

Specimen's collection

From August to December 2012 One hundred specimens were collected from patients in AL-Kindy Teaching Hospital and Medical City Teaching Hospitals, the specimens were included nasal swab, wound swab, burn swab, corneal scraping swab, pus, sputum and urine culture.

***Staphylococcus aureus* isolation**

The collected specimens were inoculated on the blood agar incubated at 37°C for 24 hours. The isolates were examined for their shape, size, colour, pigments, and haemolytic activity. Then transferred and streaked on mannitol salt agar which considered as selective and differential medium for the isolation, purification and identification of *Staphylococci*, and for detecting the ability of each isolate to ferment mannitol. All plates were incubated at 37°C for 24 hours then a single pure isolated colony was transferred to Nutrient agar medium for the preservation and to carry out other biochemical tests that confirmed the identification of isolates.

Identification of *Staphylococcus aureus*

A-Microscopic examination:The isolates were stained by Gram stain to detect their response to stain, shapes and their arrangement[13].

B-Cultural characteristics: The colonies were grown on blood agar and tested about their shape, size, color and blood hemolysis[14].

I) Growth on mannitol salt agar:The plates were streaked with a pure colony of tested bacteria, then incubated at 37°C for 24 hours. This medium was used for selective isolation and cultivation of bacteria from clinical and nonclinical specimens[15].

II) detection of hemolysis on human blood agar:Human blood agar was inoculated with an overnight bacterial culture and incubated at 37°C for 24 hours. A clear zone around the colonies was considered as beta hemolysis behavior[14].

C- Biochemical tests: Catalase test ,Oxidase test ,Coagulase test ,Acetoin production test DNase production test,Mannitol fermentation Gelatin liquefaction, Protease production,Nitrate reduction test, Urease test and methicillin susceptibility test [16]and Autoanalyzer staph system (vitak II system).

Detection of biofilm formation was done by the following methods:

Tissue culture plate method : The tissue culture plate assay described by Christensen *et al.* is the most widely used and was considered as standard test for the detection of biofilm formation. This method was applied on five isolates of Methicillin resistance *Staphylococci* MRSA (S3,S1,S3,S4,S5) .These isolates selected according to the previous tests (Congo-red agar and Tube Method) as the isolates that produce highest amount of slime layer and also selected according to the multi-drug resistance pattern. The influence of media composition on biofilm formation were also investigated, therefore two media were used to evaluate biofilm formation; brain heart infusion broth (BHI), and BHI with 1% glucose.

A suspension of bacterial isolate that equivalent to the McFarland No. 0.5 turbidity standard were inoculated in BHI and incubated for 18 hours at 37°C in stationary condition then diluted 1:100 with fresh BHI and with BHI supplemented 1% glucose. Individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plate were filled with 0.2ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media.

The tissue culture plates were incubated for 24 hours at 37°C. The contents of each well were gently removed by tapping the plates, the wells were washed three times with phosphate-buffered saline (pH 7.2) to remove free-floating "planktonic" bacteria. Adherent organisms were fixed by air drying and stained with 0.1% safranin. Excess stain was rinsed off by distilled water or tap water and plates were kept for drying. The optical density (OD) of stained adherent bacterial films were determined with a Micro ELISA autoreader at wavelength of 490nm. Adherences measurements were performed in sex triplicate and repeated at least three times the values were then averaged.

The mean of (OD) value obtained from media control well was deducted from all the test OD values. Classification (Table 1) based on OD values obtained for individual strains of *Staphylococcus* spp. were used for the purpose of data simplification and calculation [17].

Table 1: Classification of bacterial adherence by tissue culture plate method [17]

Mean OD values	Adherence	Biofilm formation
< 0.120	Non	Non/ Weak
0.120 – 0.240	Moderately	Moderate
> 0.240	Strong	High

Tube method: This is a qualitative assessment of slime production a standard glass culture tubes were used in this method as the following: Tryptone soya broth (10ml) were inoculated with a suspension of bacterial isolate that turbidity is approximately equivalent to the McFarland No. 0.5 turbidity standard. The tubes were incubated overnight (18-24) hours at 37°C. The cultured tubes were then emptied of their contents and stained by adding 10ml of safranin stain solution (2-2-2-6). Each tube was then gently rotated to ensure uniform staining of any adherent material on the inner surface and the contents gently decanted. The tubes were then placed upside down to drain. A positive result was indicated by the presence of an adherent layer of stained material on the inner surface of the tube or visible film lined the walls of the tube. Ring formation at the liquid-air interface was not considered indicative of slime production [17,18].

Congo red agar method: The Congo-red agar medium was inoculated with a suspension of bacterial isolate that turbidity is approximately equivalent to the McFarland tube No. 0.5 turbidity standard. The plates were incubated aerobically for 24-48 hours at 37°C. A positive result was indicated by black colonies with a dry crystalline consistency. Non- slime produces usually remained pink. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology [17,18].

Effect of Lysostaphin, on MRSA biofilm

Preparation of lysostaphin stock solution: Different concentrations of lysostaphin (from Bio near company) were prepared by dissolving 4.5mg of lysostaphin in 50 ml of sterilized D.W.to prepare 90µg/ml as a stock solution then serial concentrations were prepared (90,45,22.5,11.25 and 5.62 µg/ml). Each concentration was filtered with Millipore filter unit (0.2 µm) for their sterility.

Different concentrations of,lysostaphin were performed to detect their effect on biofilm formation depending on the method that was applied by [19] with simple modification as following steps:A- suspension of bacterial isolate that equivalent to the McFarland tube No.0.5 were inoculated in tryptone soya broth and incubated for 6 hours at 37°C. B- The broth culture was diluted with the same volume of antibiotics solution in combination by adding 1ml of the broth culture to each tube that content antibiotic solution in combination to obtain the final concentration .C- bacterial-drug solution (0.2ml) from each tube was placed into the plastic tissue culture plate wells in triplicate for each tube, and allowed to adhere up to an additional 6 hours. The control was performed in the absence of antibiotics as negative control. After incubation the contents of each well was removed and washed three times with phosphate-buffer saline (pH 7.2) then the adherent bacteria were fixed by air drying and stained with 0.1% safranin. Excess stain was removed by washing with water. After drying the OD of each well was measured by Micro ELISA autoreader at wavelength of 490nm. and compared with the negative control.

Statistical analysis.

The Statistical Analysis System- SAS (2010) was used to effect of treatments in study parameters. The LSD test the comparative between means and Chi-square test to comparative between percentage in this study[20].

Results and Discussion

Microscopically Characteristics : Microscopic examination was applied to all 38 isolates after staining by Gram stain and the cells appeared as Gram-positive cocci irregular clustered in large number (in grape- like irregular clusters).

Biochemical Tests

The results of *S. aureus* are summarized in table (2).

Table 2- The biochemical tests and their results for *S. aureus*

Test	38 Isolates
Gram stain	100% Gram positive
Acetoin production	100% Positive
Catalase	100% Positive
Oxidase	100% Negative
Coagulase	76.3% Positive
DNase	100% Positive
Mannitol fermentation	100% positive
Gelatin liquefaction	100% Positive
Protease production	100% Positive
nitrate reduction	100% Positive
Urease	100% Positive
Hemolysis behavior	86% β -hemolysis
Gram stain	100% Gram positive

Methicillin susceptibility test: From the collected clinical samples, 21(55.26%) out of 38 were MRSA and the rest were MSSA 17(44.73%), present results were agree with [21] observed that only (51.4%) of isolates were methicillin resistant and (48.6%) were sensitive. Also, previous study by ALalem showed that the ratio of MRSA strain was (56%), while MSSA strains was (44%) in Turkish hospitals besides, the prevalence of MRSA strain was (59%), and MSSA strain was (41%) in Libyan hospitals [22].

Resistance to methicillin may due to prevalence of *mecA* gene among *S. aureus* isolates, which coding for penicillin- binding proteins with very low affinity to β -lactam antibiotics including Methicillin [11,23]. On the other hand, current results did not agree with the results of a local study by ALmaliki who showed that the percent of Methicillin resistance *S. aureus* (MRSA) to the Methicillin sensitive *S. aureus* were (80.3% -16.4%) respectively, while the intermediate resistance to the Methicillin in the *S. aureus* was 3.3% [24]. As well as, other study reported that the ratio of Methicillin resistance *S. aureus* (MRSA) was(83.70%) [25].

Detection of Slime layer and Biofilm formation by Methicillin Resistance Staphylococci

Congo-red agar method (CRA): The results showed that 57% of MRSA isolates produced strong slime layer indicated by formation of black colonies with dry crystalline consistency, while 43% of MRSA isolates were non-slime producer indicated by formation of pink colonies with no change in the color of the medium. This result agreed with other study found that 50% of *S. aureus* isolates produced strong slime layer on the Congo red agar and 40% was indeterminate producer and only 10% showed negative result (pink colonies) [26].

Tube Method: The results showed that 100% of MRSA tested isolates were produced slime layer on this method but the amount of adherent material was differ among the isolates (ranged from weak to strong) .

Biofilm assay by Tissue Culture Plates Method (TCP): the test was performed by using two types of media, the first one was Brain heart infusion broth (BHI) and the second medium was BHI supplemented with 1% glucose (BHI_{glu}). the twenty –one isolates in the BHI gave the OD values ranged from 0.262 to 0.311 .These values indicated highly biofilm formation and strong adherence according to the classification of (17,18).Currently, the results showed that the use of second medium (BHI_{glu}) significantly increased bacterial growth, the OD values of 12 isolates were (S-10, S-15,S-3,S-43,S-57,S-22,S-35,S-72,S-86,S-18,S-11 and S-37. However, the increasing in the OD value of (S-79, S-66,S-91,S-56,S-40,S-35,S-30,S-26, S-64 and S-50) was considered non-significant .This results agreed with previous studies mentioned that the presence of sugar was played an important role in the stimulation of biofilm formation in *Staphylococcus* spp figure (1). The impact of glucose in the induction of biofilm formation in *S. aureus* and *S. epidermidis* also reflected by the fact that most of the biofilm adherence assays included high concentration of either glucose or sucrose[27,28] . Fitzpatrick *et al.* demonstrated that biofilm formation was increased four- to eight fold in all MRSA isolates when grown in brain heart infusion (BHI) medium supplemented with glucose compared to BHI alone [29] .

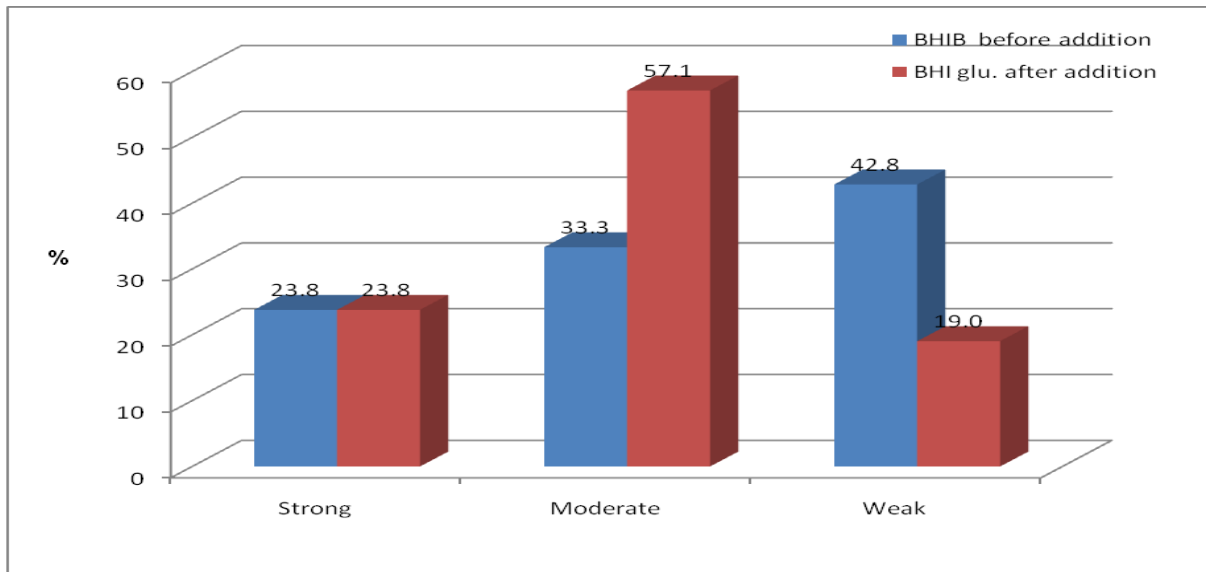


Figure 1- Percentage of biofilm formation using BHI & BHI_{glu}

Activity of lysostaphin to reduced *S.aureus* (MRSA) biofilm formation: this study investigated ability of lysostaphin to inhibit biofilm formation capacity by selected isolate (MRSA S3). Table (3) showed weak decrease in the biofilm forming capacity of the tested isolate S3 detected by lysostaphin alone. Statistical analysis showed slightly effect of lysostaphin under concentration (5.625 μ g/ml), optical density at(490nm)was reduced to (0.312 nm) in comparable with control group (0.389nm). While, other study showed effective role of lysostaphin against biofilm formation as a coating for catheters[30]; Also, in a mouse model, lysostaphin has been used to eradicate *S. aureus* biofilms from a catheterized jugular vein[31].

Table 3- Inhibition of biofilm –forming capacity of the selected isolates at different lysostaphin concentrations

Isolate	lysostaphin (μ g/ml)				
	90	45	22.5	11.25	5.625
<i>S. aureus</i> 3					
OD of control cell	0.389				
OD of turbidty cell	0.183 1	0.214	0.263	0.264	0.312
LSD value	0.113 *	0.084 *	0.069 *	0.078 *	0.089 NS

* (P<0.05).

The difference between current study and previous may due to lysostaphin –resistance of selected strains for present study.Wu *etal.* showed that the disruption of *S. aureus* biofilms was specific for lysostaphin-sensitive *S. aureus*, as biofilms of lysostaphin-resistant *S. aureus* were not affected [32].

A more likely explanation, however, was that the disruption of staphylococcal biofilms by lysostaphin occurs through the rapid lysis of the sessile staphylococci, which may be sufficient to destabilize the entire biofilm matrix in such a manner as to allow detachment from artificial surfaces [33].

The resistant of bacteria growing in biofilms remains an incompletely understood process and is an area of active research. Several factors may explained bacterial resistance in established biofilms, including the multilayer structure of biofilms and/or the unique genetic characteristics bacteria in biofilms compared to those of planktonic cells[34].

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