Investigation of Virulence Factors of *Staphylococcus.Aureus* That Isolated from Different Clinical Infections in Baladroz City (Iraq)

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Abstract

One hundred *Staphylococcus* isolates were isolated form different clinical samples which were collected from hospital at Baladruz city (Diyala-Iraq). The specimens included wound, burn, ear and nasal swabs . The highest rate for the isolation of these bacteria from burn swabs 35% , wound swabs was 30%, ear was 25% ,nasal swabs 10% . All isolates produce DNase ,and lipase ,while 70% of them produce protease . An antibiotic sensitivity test was done for the bacterial isolates. All isolates resist to penicillin and Ampicillin with the ratio of 100% , 95% ,and the results showed that there was multiple resistant antibiotics. The pattern of minimum inhibitory concentration of *S. aureus* isolates to vancomycin , erythromycin was determined using MIC method, the MIC of them between (4-256 µg\ml). The results showed that 17 isolate of *S.aureus* with ratio (85%) were vancomycin resistant while in this study found that 3 isolates (15%) have intermediate resistance ,So (75%) of isolates that be found resistance to erythromycin antibiotic. Biofilm detection was tested by Tube method (TM), the method of Congo Red Agar (CRA), and the method Tissue culture plate (TCP) .Out of 20 isolates, method of tube detected 80% of isolates showed strong biofilm ,while by CRA method detected 77.8% as high biofilm producer. In the same time 30% of isolates gave the OD values ranged from 0.430 to 0.542. This isolates were detected for their bacteriocin production that found 35% were as bacteriocin Producers by well diffusion method, (15%) by the stab-over lay.

Keywords:*Staphylococcus aureus*,Infection,Antibiotics,Resistance ,virulence factors**.**

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التحري عن عوامل الضراوة في بكتريا *Staphylococcus.aureus* المعزولة من اخماج سريرية مختلفة في قضاء بلدروز (العراق)

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الخلاصة

تم عزل 100 عزلة من بكتريا المكورات العنقودية الذهبية من مصادر سريرية مختلفة في مستشفى بلدروز العام (ديالى – العراق) تضمنت هذه المصادر مسحات الجروح، الحروق ، الاذن ، الانف . بينت عملية العزل اعلى نسبة لمسحات الحروق 35% ،الجروح 30% ، الاذن 25 % ، الانف 10% . كانت جميع العزلات منتجة للدنيز واللايبيز بنسبة 100% بينما 70% منها منتجة للبروتيز ، اجري فحص الحساسية للمضادات الحيوية وكانت جميع العزلات مقاومة لمضادي البنسلين والامبيسلين وبنسبة 100% ومقاومة لمضاد المثيسلين بنسبة95% ، ايضا بينت النتائج ان هناك مقاومة متعددة للمضادات الحياتية . وتم تحديد التركيز المثبط الادنى لعزلات المكورات العنقودية الذهبية للمضادي الفانكومايسين والاريثرومايسين وكانت قيم التركيز المثبط الادنى تتراوح بين (4-256 مايكروغرام/مل) ، 17 عزلة (85%) مقاومة للفانكومايسين و3 عزلات (15%) كانت ذات مقاومة متوسطة ، بينما 75% منها كانت مقاومة لمضاد الاريثرومايسين . فحصت قابلية المستعمرات على انتاج البايوفيلم ، اذ استخدمت 20 عزلة من بكتريا *S.aureus* . فحصت قابلية العزلات على انتاج البايوفيلم بثلاث طرق هي طريقة الانابيب،طريقة احمر الكونغو ،صفيحة اختبار جهاز الاليزا لمعرفة قدرتها على انتاج البايوفيلم ، اذ كانت 80% من العزلات منتجة للبايوفيلم بطريقة الانابيب ، بينما 77.8% من العزلات انتجت طبقة مخاطية قوية بطريقة احمر الكونغو وبنفس الوقت 30% من العزلات ذات كثافة ضوئية تراوحت بين 0.430 – 0.542نانومتر.اختبرت قابلية العزلات على انتاج البكتريوسين اذ وجد 35% من العزلات منتجة للبكتريوسين بطريقة الانتشار في الحفر ، بينما 15% منها انتجت البكتريوسين بالطريقة الاخرى.

الكلمات المفتاحية : بكتريا المكورات العنقودية الذهبية ، المضادات الحيوية ، مقاومة المضادات ، عوامل الضراوة .

Introduction

Staphylococcus aureus is one of among S*taphylococci* belonging to the *Micrococaceae* family, that can cause under appropriate conditions, minor skin infections such as (pimples, cellulites, toxic shock syndrome, impetigo and sores) in addition to life threatening diseases (pneumonia, endocarditis, meningitis, and septicemia); it is also responsible for severe morbidity and mortality worldwide (Al-Gosha'ah *et al*.,2014**)**

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Staphylococcus aureus can be considered as a commensal on the skin and nasal flora , and cause native, severe, and invasive infections, such as bacteremia or pneumonia (Türk *et al*.,2015) . The most of staphylococcal infections can be treated with antibiotics; however, in the latest years *Staphylococcus* found its way to resist the commonly used and active antibiotics; these antibiotics include group of macrolides, streptogramin, tetracycline, gentamicin, and beta-lactams especially methicillin (Delorme *et al*.,2009 **) (**Juayang *et al*.,2014) **.** The various range of *S. aureus* disease has been credited to its ability to produce of virulence factors, the expression of which is firmly regulated by global regulatory systems in response to cell density, energy availability, tension of oxygen, and other environmental signals (Maria *et al*.,2016). *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 environments. The example of changing of genotypic is the acquirement of the β-lactamase gene conferring penicillin resistance. The formation of biofilm is an example of phenotypic change. Biofilm Formation is the hallmark characteristic of *S. aureus* infection which consists of several layers of bacteria encased within an exopolysaccharide glycocalyx. Presence of glycocalyx may protects the enclosed bacteria from host defenses and inhibits delivery of antibiotics**.** (Al-Sheikh and Yosif**,** 2014**).** A biofilm can be defined as a sessile community, surface-associated microorganism characterized by cells that are irreversibly attached to a living or nonliving substratum to form a multilayered cell clusters that embedded in a matrix of extracellular polysaccharide (slime), that they have produced, which facilitates the adherence of these microorganisms to the surfaces and protect them from host immune system and antimicrobial therapy (Mathur *et al*.,2006) **,**and other virulence factor is bacteriocin. Bacteriocins are antibacterial proteins produced by bacteria that may be kill or inhibit the growth of other bacteria (Cleveland *et al.,* 2001) **.**and there are more virulence factor such as beta lactamase. Beta-lactam antimicrobial agents show the most private treatment for bacterial infections and continue to be the prominent cause of β-lactam antibiotics resistance to among Gram-negative bacteria worldwide.(Pitout and Laupland, 2008). Beta-lactam antibiotics produce a bactericidal effect by binding penicillin protein PBP

to β-lactam inhibiting the membrane-bound enzymes responsible for catalyzing vigorous stages in the biosynthesis of the cell wall. In contrast to the PBPs in methicillin-susceptible strains, which have high empathy for most β-lactam antimicrobials**.** (Chambers, 2003**).** In certified instances of life threatening infections such as bacterial endocarditis, or infections caused by highly or multiple resistant organisms, the physician may require a quantitative measurement of microorganism sensitivity rather than the qualitative report of resistance, intermediate, sensitive ,Therefor use minimum inhibitory concentration method

Amis of study

The aims of present study were to determine the antimicrobial susceptibility, virulence factors such as bacteriocin ,Beta-lactamase and biofilm formation among hospital isolates of *Staphylococcus aureus* with MIC detection.

Materials and methods

Isolation and Identification

Collection of specimen and isolation and identification of hundred specimens were collected from different sources including wounds, burns, ears ,urine in Baladroz city (Iraq) from 1/3/2016 to 1/8/2016. *S.aureus*, was identified, according to the morphology, cultural characteristics and biochemical tests (Baron and Feingeld, 1990). All specimens were streaked on mannitol salt agar for detecting on mannitol fermentation and Blood Agar for detecting hemolysis type. Thereafter, all plates were incubated aerobically for 24 h at 37°C. To isolate and identify the bacteria used appropriate media and identification of this bacteria depending on the basis of morphological and biochemical characterization as according to Bergey`s manual of systematic bacteriology. (Layer *et al*.,2006)..Finally the Analytical Profile Index API Staph system was used for precise identification of the isolates at generic and typing of *S.aureus* .

Antibiotic Sensitivity test

Antibiotic Sensitivity tests were used, and detected by the Kirby-Bauer disk diffusion method suggested by the Clinical and Laboratory Standards Institute (CLSI , 2012). The antibiotic susceptibility pattern of all isolated *S. aureus* was tested against 13 antibiotics (Bioanalysis Turkey), included Penicillin (10)µg/ml Ampicillin (1) µg/ml , Cloxacillin(1) µg/ml, Oxacillin(10) µg/ml, Clindamycin(2) µg/ml, Ciprofloxacin(5) µg/ml, Amoxicillin(25) µg/ml, Erythromycin(15) µg/ml, Gentamicin(10) µg/ml, Rifampicin(5) µg/ml, Vancomycin(30) µg/ml , Methicillin(5) µg/ml and Imipenim(10) µg/ml.

Biochemical tests and enzymes

This study include detection some enzyme and tests such as Catalase test ,Oxidase test ,Coagulase test , DNase production test ,Mannitol fermentation, Protease production, Lipase production .(Forbes *et al*.,2007)

Detection of Bacteriocin

1-Well diffusion method

The bacteriocin production was determined using the well diffusion method. The supernatant from a culture of *S.aureus* was purify sterilized by passage through a 0.45 um pore size membrane filter. sterile supernatant were placed in 4-mm-diameter wells that had been cut in Mueller-Hinton agar plates formerly inoculate with the indicator bacteria. After 12-18 h of incubation, inhibition zones of growth were measured. (Naz *et al*.,2013)

2-Stab-overlay method

Brain heart infusion agar plate was stabbed with the *S. aureus* and incubated at 37°C for 24h. Next day plate was exposed to chloroform vapours to kill the producing strain (Keeping the plates inverted and 9cm piece of Whatman No.1 filter paper was introduced into the lid and impregnated with 1mL of chloroform for(20-30 min). Plate was then overlaid with 3mL soft agar containing 0.1mL of indicator/sensitive organism. Plate was again incubated at 37°C for overnight and observed for clear zone around the producer culture **(**Naz *et al*.,2013**).**

Biofilim formation

This include three methods the first Tissue culture plate method (TCP) .Biofilm assays were executed in 96-well polystyrene micro plates, by using the medium of trypticase soy broth (TSB) with 1% (w/v) glucose (TSB-1% Glc). isolates of *S.aureus* in 5 mL were incubated in a shaker with 250 rpm at 37˚C for 18 hours. Isolates were diluted 1:100 in TSB-1% Glc and 200 mL were inoculated into well. The micro plate was incubated at 37˚C for 20 h. removed Supernatants from each well and washed biofilms gently twice with PBS, then dried and fixed at 65˚C for 1 h. Finally, use of crystal violet 1% to stain plates and gently washed twice with PBS. The absorbance at 492 nanometers (nm) was calculated in a spectophometer. (Souza *et al*.,2014)**.**

The second method Congo red agar (CRA). The medium was prepared by using brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red stain 0.8 g/L. Congo Red stain was prepared as a determined aqueous suspension and autoclaved distinctly from the other medium components. This stain added to the autoclaved brain heart infusion agar with sucrose. Inoculate CRA plates with test organisms and incubate at 37oC for 24 h aerobically. Biofilm production indicated by the presence of black colonies. (freeman *et al*.,1989) (Rewatkar *et al*.,2013).

And the third procedure Tube Method (TM) . Aqualitative method for biofilm detection. using loopful of test organisms inoculated in 10 ml in the medium of trypticase soy broth with 1% glucose in test tubes. the tubes were incubated at 37C for 24 h. After incubation, tubes were emptied and washed with phosphate buffer saline (pH 7.3) and dried. then staining tubes with crystal violet (0.1%). Extra stain washed with distilled water. Tubes were dried in upset position. The positive result confirmed by visible thick film covered the wall and the lower surface of the tube . (Mathur *et al.*, 2006) .

Detection of β-lactamase Production by Iodometric Methods

This test include three procedure the first Agar Method in which Pure colonies of *S.aureus* isolates were inoculated on 0.2% starch in nutrient agar. After overnight period of incubation at 37ºC, Penicillin solution was added to the *s.aureus* full-grown culture plate. The excess

Penicillin solution was flowing after 15 min. Iodine solution in 1:5 dilution was added above the growth and later inverting the plate to remove the excess solution. Interpretation was taken after 30 minutes at room temperature. The isolate 0f *S,aureus* that producing β-lactamase would produce characteristic decolonization around the β-lactamase producing colonies. (Devapriya *et al*.,2013) . The second Chromogenic method in this method the test organism isolates were applied directly over a sterile, distilled, water-moistened nitrocefin disc.the positive result can be detected by change in color from light yellow to deep red color within 15 seconds to 5 minutes and refer to hydrolysis of *β* lactam antibiotic by induced *β* lactamase enzyme, while negative result can be noticed without change in color within 5 minutes. (Bidya *et al*.,2014) . Finally acidimetric method by suspending the isolates in penicillin-phenol red test reagent dispensed in 100 µl volume in microtiter wells .the Change in color from purple pink to yellow within 15 minutes may be considered positive result while the negative result there is no change within 15 minutes for the production of *β* lactamase.(Thornsberry and Kirven .,1974).

Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) is the lowest concentration that inhibits the visible growth of bacteria (Islam *et al*.,2008) . The value of minimum inhibitory concentration (MIC) for Vancomycin and erythromycin included the two-fold dilution susceptibility. Susceptibility test results were assessed after incubation for 24 h at 37°C**.** (Morello *et al*.,2006) **.** Minimum inhibitory concentrations of Vancomycin and erythromycin to 20 *S aureus* isolates were determined. and compare the results with the (CLSI ,2012).

Results and Discussion

Isolation and identification from the 100 sample collected from different sources only 20 isolates found to be *S.aureus* .All the isolates of *S.aureus* were gram-positive, the test of positive catalase- cocci **.** After incubation on mannitol salt agar in which *S. aureus* ferments mannitol, result converting of the colour the medium from pink to yellow.The higher numbers of isolates were distributed among burns specimens with the ratio (35%) wounds (30%) ear

(25%) and the lower isolate was distributed though nasal specimens (10%) (table 1) figure 1. All isolates 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. The isolates appear Gram positive cocci mostly arranged in grape-like irregular clusters.

Table 1 Number and percentage of bacterial isolates from different sources

Source of samples	No. of isolates	Percentage %
Wounds	6	30%
Burns	7	35%
Ear	5	25%
nasal	2	10%

Antimicrobial susceptibility test

Antimicrobial susceptibility test against 13 antibiotics to investigate the multidrug resistance pattern . All isolates were resistant to ampicillin with ratio (100%), penicillin G (100%), methicillin (95%) , Gentamicin (90%) Erythromycin (75%),Vancomycin (60%),Cloxacillin ,Oxacillin(55%) , Clindamycin(35%), Ciprofloxacin(20%), while all isolates sensitive to Amoxicillin, , Rifampicin, , Imipenem and Chloramphenicol with the ratio 100% sensitive (figure 2). This resistance of Penicillin by *Staphylococci* strains can be explained according to the production of β-lactamase enzyme that destroyed the β-lactam ring.while in this study high resistance of these isolates against methicillin, gentamycin, erythromycin approximately agrees with other previous studies (Ounnga and Awhowho, 2012).

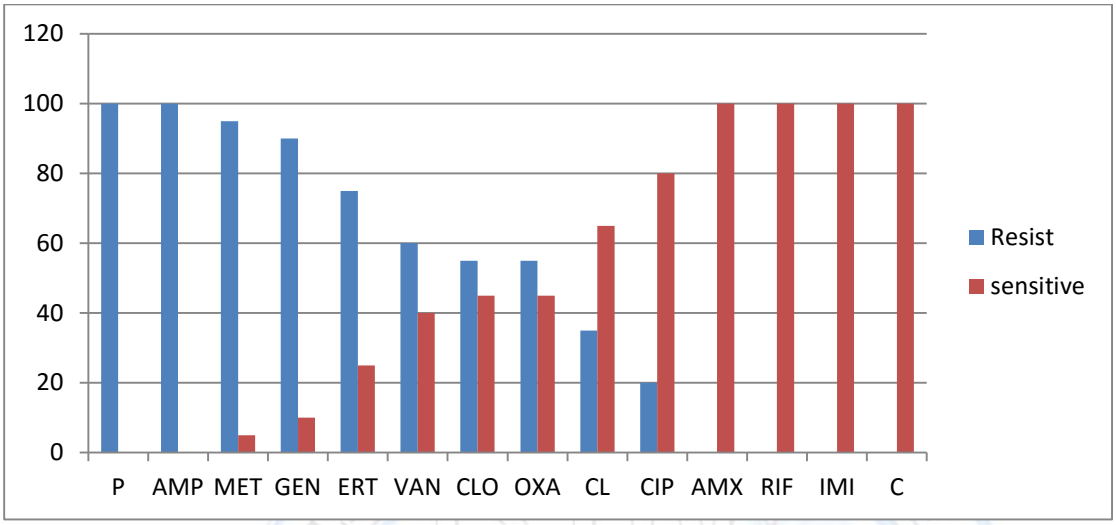

Figure (2) the Percentage of Antibiotic-resistance of *S. aureus* isolates from different sources

Production enzymes and Biochemical tests Results

The results of production of lipase 100% that partially agreed with Al-jundi (2005) who found that 94.6%o of *S.aureus* isolates produce lipase . The lipase play important role in colonization and persistence of resident organisms on the skin .in this study also found that the production of DNase (100%) .the function of DNase is damage host DNA and lead to increase in pathogenicity of Staphylococci, while 70% of isolates produce protease. Recent studies found that proteases play important role in the alteration of *S. aureus* cells from an adhesive to an invasive phenotype by destroying bacterial cell surface proteins.

Biofilim formation

The results in Congo-red agar method showed that 70% of isolates produced slime layer indicated by formation of dark black colonies with dry crystalline, while 30% of isolates were non-slime producer indicated by formation of pink colonies without any change in the medium colour this is agreed with the study of(Turkyilmaz and Kaya ,2006) that showed 77.8% of isolates were slime producer .The capacity of *S.aureus* to produce biofilms is an important virulence mechanism that lead to complicates infections, especially those containing foreign materials like catheters, surgical implants resulting in continuing infections

that are difficult to treat. (El-Gayar *et al*.,2014). Many environmental factors can influence the slime layer production when tested by this method like O_2 , temperature, pH, and other stress conditions that may be gave variable results**.** In Tube method, noticeable thick film was obtained inside the wall of tube and bottom. 80% of isolates were revealed thick film inside the wall of the tube showing strong biofilm production while 20% of isolates do not shown any biofilm formation. And the latest method Tissue culture plate method (TCP) in which method was applied on six isolates 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 multidrug resistance pattern. The results showed that the six isolates 30% 0f isolates gave the OD values ranged from 0.430 to 0.542. These values indicated the high biofilm formation and strong adherence according to the classification of (Mathur *et al.*, 2006)

Bacteriocin production

In this study found that 7 bacterial isolates with 35% produced bacteriocin, identified by wells diffusion method, depending on the size of inhibition zone and the highest sensitive number of the basic indicator isolates. These isolates were used as indicator local in bacteriocin typing **.**the other method is the Stab-overlay method found that 3 bacterial isolates (15%) produce bacteriocin**.** Reason of using two methods was to compare some of the most usually used methods for the activity of bacteriocin detection. our studies it was found to produce bacteriocin by both the methods **.** Bacteriocins may serve as anti-competitor compounds enabling an invasion of a strain or species in an established microbial community; (Desriac *et al.*, 2010). Bacteriocinogenesis and the term `Colicins` were first devised about 90 years ago and in recent years all major groups of bacteria including gram negative and the gram positive have been described to produce these lethal proteins. The bacteriocin producing bacteria found in different places and isolated from a diversity of ecological sites including soil, water, food and human body (Shand & Leyva, 2008**)** .

Beta -lactamase Detection

In this study found that 15 isolates (75%) positive results for B-lactamase detection by the agar Method , followed by 11 isolates (55%) by iodometric method and 8 isolates

(40%) by acidometric method. In this method the β lactamase enzyme performances on penicillin by forming of penicilloic acid. This method lead to detect extra β lactamase producers as compared to Iodometric method with less practical, since pH and buffer controls must be included and required large amount of penicillin G . Iodometric test is an easy to perform, efficient, and relatively recognize rapid method. Iodometric slide test results have been described to be similar to nitrocefin disk test, and thus can be used in detecting staphylococcal *β* lactamase when chromogenic methods are not obtainable (Bidya *et al*.,2014)

Detection of MIC

To extra sensitively detect differences between groups, MIC were compared, as opposed to comparing categorical resistance data. Measuring MICs allows detection of small changes in sensitivity that do not appear around defined resistance breakpoints. This approach identified important differences in susceptibility for medicines where no isolates with MICs above the CLSI resistance breakpoint were identified (Rubin *et al*.,2011) . Determination of MIC antibiotics of bacteria acting a important role for determination of bacterial resistance to antibiotic of the present research work was assumed for determining resistance of *S. aureus* against commonly antibiotics.((Islam *et al*.,2008) .In this study found that 17 isolate (85%) resistant to Vancomycin while 3 isolates (15%) have intermediate resistance.on the other hand 15 of this isolates (75%) resistance to Erythromycin with the 5 isolates (25%) intermediate and. this results partially agree with **(**Flayyih and Abd rabaa**,**2015) who found that 40% of isolates resistance to Vancomycin .

Conclusion

The presence of some virulence factors of *S.aureus* could increase their potential threats especially that most of them have multidrug resistance, rendering it difficult to treat. Further studies have to be conducted in the future targeting these virulence determinants as an additional approach that might aid in the prevention and control of such resistant organism seen in our hospitals.

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