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Comparison Study of Accuracy of phenotypic Methods for Detecting Biofilm Producer *Staphylococcus aureus* Isolates

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ABSTRACT

Microorganisms growing in a biofilm are correlating with chronic human infections and are highly resistant to immune system, antimicrobial agents and difficult to remove from the infected host. There are different methods to determine biofilm production such as Microtitre Plate (MtP) method , Congo Red Agar method (CRA) and Tube Method (TA) method .This study was carried out from December 2015 to september 2016, 580 specimens were obtained from patients at different hospitals in Erbil city/ Iraq. *Staphylococcus aureus* clinical isolates were identified phenotypically by different biochemical and molecular tests. One hundred isolates were found to be *Staphylococcus aureus*. MtP, CRA and TA tested Biofilm determination. This study was aimed to compare three methods of biofilm production. The results found that the accuracy and specificity of MtP was 100%, while it was 87%, 75% and 91% ,81.25% for CRA and TA methods respectively . The MtP method was considered to be superior to CRA and TA methods, and this method was the better screening for biofilm formation than CRA and TA methods, so this method can be a reliable quantitative tool for detection of biofilm formation in clinical isolates of *S. aureus*.

Introduction

Staphylococcus aureus is one of the most harmful species of staphylococci, the species is identified by a variety of conventional physiological or biochemical characters (1). In addition, *S. aureus* can be determined by PCR methods based on *16S rRNA* gene which is reported to be a taxonomic marker molecules (2). Bacteria can support biofilm formation on a number of various surfaces, such as natural environments, living tissues, medical devices, industrial or drinkable water piping systems (3). Biofilms are sets of microorganisms in which cells affix to each other on a surface. A biofilm, sometimes referred to as slime, a polymeric mixture generally composed of proteins, extracellular DNA, and polysaccharides. Bacterial polysaccharides are a main component of matrix of biofilms and intermediate most of the cell-to-cell as well as cell-to-surface interactions, which required for biofilm formation and stabilization (4).

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Biofilms develop in numerous stages to form extremely ordered multicellular communities. At first the bacteria ought adhere to a surface or host tissue (primary attachment phase) prior they reproduce to form multicellular aggregates (accumulation phase). At the maturation stage, channels and mushroom-shaped structures were formed to allow nutrients to permeate the deeper layers of the biofilm. Lastly, at the dispersal stage, bacteria separate from the biofilm and spread to new sites (5). Bacteria in biofilms show distinct features of their free-living planktonic counterparts such as different physiology , resistant to immune system (6), resistance to antimicrobial agents and difficult to remove from the infected host, as they can show susceptibilities towards antimicrobials less of 10-1000 times (7). Staphylococcal biofilms are a source of chronic and continual infections (8). They are contributed in a wide variety of microbial infections such as gastrointestinal tract, formation of dental plaque, urinary tract infections, cystic fibrosis infections, airway/lung tissue, also found in infections of permanent indwelling devices such as heart valves, joint prostheses, and for chronic administration (9).Various methods are actually used in medical fields for the detection of biofilm production.

However, qualitative methods, such the tube adherence (TA) assay and the Congo red agar (CRA) way and quantitative method (Microtitre plate (MtP) test**)** are utilized in routine laboratories (10).The objective of the current study was to examine the biofilm production in *S. aureus* strains isolated from clinical specimens of patients from different hospitals in Erbil, by using three phenotypic methods and comparing these above mentioned methods.

2. Materials and Methods

2.1.Bacterial isolates

From December 2015 to April 2016, 580 specimens were obtained from patients at different hospitals in Erbil city/ Iraq. The Specimens that were included ; 120 from urinary tract infections , 65 from nose infection, 127 from wound infections, 78 from burns, 85 from tonsillitis, 45 from vaginitis and 60 from ear infection . All samples were taken by disposable cotton swabs or sterile containers .The specimens were plated on mannitol salt agar (MSA) media, (Oxoid, England), and incubated overnight at 37C◦ for 24 hours. Single, well-isolated colonies with the typical appearance of *S. aureus* were subcultured and confirmed by Phenotypic and molecular identification.

2.2.The conventional identification of *Staphylococcus aureus.*

Conventional identification was performed through microscopic morphology, coagulase, DNase, motility, oxidase, catalase, urease, voges-proskauer, hemolysis and mannitol fermentation tests (11).

2.3. Isolation of DNA from bacterial cell

The method that used for isolation of DNA from bacterial cells was completed by using Presto Mini™ gDNA bacterial kit (Geneaid,Taiwan). Which inclusive following steps: sample preparation, lysis, DNA binding, washing and elution.

2.4. Molecular identification by PCR amplification of 16S rRNA gene

To confirm the obtained results of phenotypic methods for obtained isolates of *S. aureus* , the monoplex PCR by using specific primer pairs of *16S rRNA* which designed for this study by using primers program on the NCBI website and supplied by Bioneer(Korea) (Table 1)These primers amplify 1487bp region of *16S rRNA* gene fragment of *S aureus*, PCR reaction kit (AccuPower PCR PreMix)was purchased from Bioneer Company. The PCR reaction was carried out in 20 µl as shown in Table (2) , after that lyophilized blue pellet dissolved and the applied PCR program was illustrated in Table (3) by using thermal cycler (Eppendorf, Germany) in addition to negative control which containing water in state extracted DNA in the amplification reaction; After the reaction, 10 µL of the PCR product was resolved into amplified fragments by electrophoresis in 1.5% agarose gel (containing ethidium bromide dye with final concentration 0.1 µg/ml) with 45 V for 15 minutes and completed with 100 V for 1 hour which was used to separate DNA molecules according to size. To evaluate the molecular weights of fragments a 100-bp molecular weight ladder was used and the amplicons observed under UV radiation.

Table (1): Primers sequences and their product size.

Primer name	Primer sequences	Product size bp	Annealing Temperature
16s rRNA F	CCTGGCTCA GGATGAACG	1478	55
16s rRNA R	AATCATTTGT CCCACCTTCG		

6s rRNA: 16s ribosomal RNA; R: Reverse; F: Forward

Table (2) : Additive mixture reaction of the PCR PreMix container tubes to investigate *16 S rRNA* gene

components	size for single sample(µl)	concentration
Primer Forward	1	10 Pmol/µl
Primer Reverse	1	10 Pmol/µl
DNA template	2	50-100 ng
D.W	16	—
The total volume	20	—

Table (3) :PCR program for amplification of the targets DNA for *16S rRNA* gene.

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1min	35
Annealing	55°C	1min	
Extension	72°C	1:30min	
Final extension	72°C	7min	1

2.5 Biofilm Formation Assay:

2.5.1 Microtitre plate (MtP) Test:

All isolates were tested for biofilm formation by using the quantitative method of Microtitre plate method(MtP) as described by Christensen and his colleagues at 1985 and altered by (12).The tested strains were inoculated in brain heart infusion broth and incubated for 24 hours at 37 °C. After incubation each culture was diluted 1:100 with sterile fresh medium, 200 µL of the samples were added to each well of a 96-well microplate and sterile broth was used as blank. The microplate were incubated at 37 °C for 24 hours. After incubation, content of each well was gently removed. The wells were washed three times with sterile distilled water to take off unbound bacterial cells subsequently the plates were exposed to air-dry and 200 µL of 0.1% w/v crystal violet solution was added to each well and incubated at room temperature for 30 minutes . The plates were washed off with distilled water and kept for air-dry. The bound bacteria were quantified by addition of 200 µL of absolute ethanol to each well and the absorbance of dissolved dye was measured at a wavelength of 570 nm by using 96-flat wells of ELISA (BioTek ,USA) The isolates were classified according to biofilm production relying on the criteria place down by (13).

2.5.2 Tube adherence(TA) method

This method was done via inoculation a loopful of each tested bacteria into sterile test tubes of brain heart infusion broth and incubated for 24 hours at 37 °C . Then the supernatants were discarded while the tube was stained with safranin stain, excess stain was removed and tubes washed by distilled water then it was dried. A positive result was determined by the presence of visible film padded the bottom and wall of the tubes (14).

2.5.3. Congo red agar (CRA)method

Tested bacteria were inoculated onto the surface of the Congo red agar medium which was solid medium -brain heart infusion broth supplemented with 5% sucrose and Congo red. The medium was composed of BHI (37 gm/L), sucrose (50 gm/L), agar (18 gm/L) and congo red stain (0.8 gm/L), and incubated for 24 hours at 37 °C. Biofilm producers form black colonies on congo red agar medium while non-producers form red colonies (15) .

3. Results and Discussion

In this study, 100 (17.24%) *S. aureus* strains were isolated from all specimens, wound infection (31%), burns (12%), UTI (15%), tonsillitis (8%), vaginitis(3%) and ear infection(13%) and nose infection (18%), All isolates were identified as *S. aureus* by conventional phenotypic tests and there was 100% similarity between the conventional identification results and the amplification of the 1478 bp fragment of the species-specific gene 16S rRNA (Fig1).

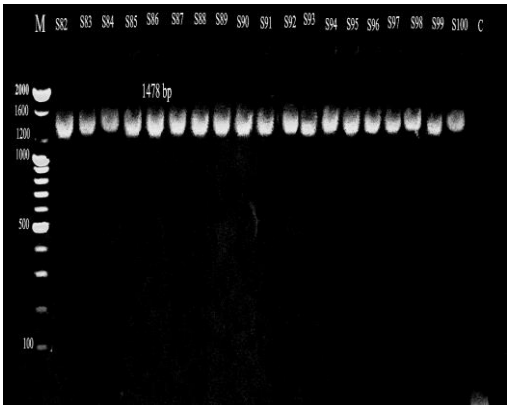


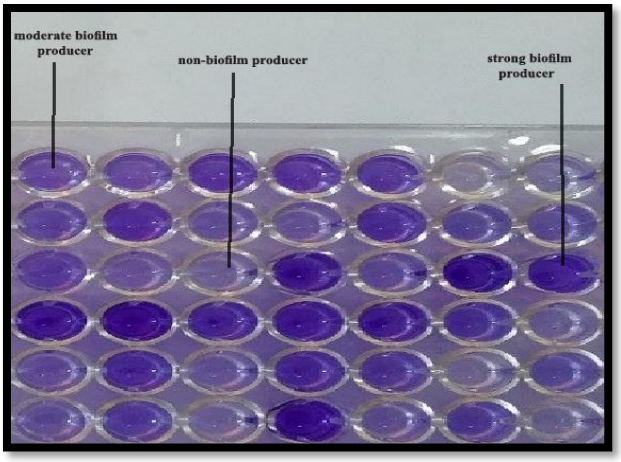
Figure (1): Polymerase chain reaction products on gel electrophoresis (1.5%) for *16S rRNA* gene. M: DNA ladder (100 bp). Lane S82 to S100: Amplified PCR product of 16S rRNA gene (1478 bp) for *S.aureus* isolates. C: negative control.

These results were in agreement with (16) who found that *S. aureus* clinical isolates identified phenotypically by different biochemical tests correspondence the molecular identification was done by PCR using species specific *16S rRNA* primer pairs .In this study we found 100 isolates organized to be positive as *S. aureus*. This result also consistent with the study conducted in Baghdad and Al-Anbar (17, 18) governorate, with 43 (20.7%), 15 (14%) respectively and less than in Turkey and other study in Baghdad ,49(46.7%) (19) and 64 (57.14) (20) respectively. *S.*

aureus isolates obtained from this study differed in their efficiency in the formation of the biofilm under stable conditions. Three methods were used for this purpose (Microtitre Plate method , Tube adherence method and Congo red agar method). The results were shown in Table (4) and Figure (2) with the Microtitre Plate method, 61% were biofilm producer isolates while 39% were non-biofilm producer isolates. These results were in agreement with (21) in Which they that indicated 64.89% of *S. aureus* isolates were found to be biofilm producers and 35.11% strains where non-biofilm producers. Whereas (22) found that 69% of the isolates were biofilm producers and 31% of the isolates were non-biofilm producers by using MtP method. While (23) stated that in MtP method biofilm formation was observed in 54.19% isolates and non-biofilm producers were 45.81%. The second method was the tube adherence method which biofilm was detected in 52%, while the proportion of *S. aureus* isolates that make biofilm by using the third method (congo red agar) was 48%.

Table (4) : Determination of biofilm forming isolates of *S. aureus* by using three methods (Microtitre Plate, tube adhesion, Congo red agar) .

Testing method	No.(%) biofilm producers isolates	No.(%)non- biofilm producers isolates
Microtitre Plate	61(61%)	39(39%)
Tube adherence method	52(52%)	48(48%)
Congo red agar method	48(48%)	52(52%)



A- Microtitre Plate method

B- Congo red agar method

C- Tube adherence method

Figure (2): Detection of biofilm production in *S. aureus* by different methods A- Tissue culture plate method, B- Congo red agar method, C- Tube adherence method

The results of the present study showed that the highest percentage of the *s.aureus* isolates forming biofilm in MtP method were 61 isolates attributed to the wounds with 25 (80.65%) followed by12 (75%) isolates obtained from burns while the lowest percentage Was obtained from ear isolates with 4 (30.77%) as shown in Table (5). The high percentage of *S.aureus* infections caused by wounds and burns were due to the fact that the skin is not a good environment for the growth of most microorganisms due to secretion of the sebaceous glands that inhibit the growth of bacteria The salts present in the sweat are high in osmotic pressure, making it difficult for most living organisms to colonize the outer layer of the skin (24). However, when skin damage such as bruises, wounds or other factors occurs, colonization begins with bacterial pathogens; *S. aureus* caused tissue destruction and abscesses (25).

Table (5): Number and percentage of biofilm forming *S.aureus* isolates by using Microtitre Plate (MtP) method and sources of infection .

Type of infections	No. of *S.aureus* isolates	No. of *S.aureus* isolates Produced Biofilm by MtP	Percentage of *S.aureus* isolates Produced Biofilm by MtP
Wound Infections	31	25	80.65%
Burns	12	9	75%
Urinary tract Infections	15	9	60%
Tonsillitis	8	5	62.50%
Nose Infection	18	7	38.89%
Vaginitis	3	2	66.67%
Ear infection	13	4	30.77%
Total Number	100	61	61%

MtP was based on spectrophotometric measurements which supply quantitative information on the capability of bacterial strains to rapidly grow and it was extremely sensitive test (26) .Therefore, the standard method was better compared to the other methods. The comparison was made among the three methods as shown in Table (6). Indeed, 52 isolates in tube adherence method and 48 isolates in congo red agar were gave positive results for biofilm formation , while 9 isolates in tube adherence method and 13 isolates in Congo red agar were non-biofilm formers , but they were produced biofilm in the Microtitre Plate (MtP) method, so they were considered as false negative .The results of this study found that the non-producing biofilm isolates by using Microtitre Plate (MtP) method method also non-produced biofilm in other ways .Therefore 39 isolations were real negative, while no false positive isolates were found .

Table (6) Estimation of the Congo red agar and tube adhesion methods to determine the ability of *S aureus* isolates (N = 100) for formation of biofilm comparing with the Microtitre Plate (MtP) method

MtP method for determine the ability of isolates to form biofilm		Tube adherence method		Congo red agar method	
		Positive	negative	Positive	negative
Positive	61	52	9	13	48
Negative	39	0	39	39	0
Total No.	100	100		100	

The sensitivity, specificity, positive and negative predictive values and the accuracy of each method were calculated using tissue culture method as a standard method and based on the data that found in Table (6) and applying the following laws to obtain the required data. Sensitivity = A / (A + C), specificity = D / (B + D), positive Predictive value (PPV) = A / (A + B),negative Predictive value (NPV) = D / (D + C), and accuracy = (A + D) / (A + B + C + D), A indicated to actual positives of biofilm producers by MtP, TM and CRA , B: false positives were biofilm producers by TM and CRA method but negative by MtP method, C: false negatives of non- biofilm producers by TM and CRA, but biofilm producers by MtP, D: true negatives of non-biofilm producers by all the methods [12].Sensitivity and negative predictive value were 100% for all methods while specificity was 100%, 81.25% and 75% whereas, the actual positive values were 100%, 85.25% and 78.69% ,also The accuracy was 100%, 91% and 87% for the Microtitre Plate (MtP) method, tube adherence method and Congo red agar respectively and as shown in Table (7).

Table (7): Comparison of the sensitivity, specificity, PPV, NPVand accuracy for the methods which used to determine the ability of the *Staphylococcus aureus* to form biofilm (n = 100)

Method of testing biofilm formation	Sensitivity (%)	Specificity (%)	Positive Predictive Value (PPV) (%)	Negative Predictive Value (NPV) (%)	Accuracy (%)
Tube adherence	100%	81.25%	85.25%	100%	91%
Congo red agar	100%	75%	78.69%	100%	87%
Microtitre Plate	100%	100%	100%	100%	100%

The tissue culture plate method showed its efficiency in determining the isolates producing biofilm compared with other methods and most sensitive and accurate way to estimate the efficiency of isolates on the formation biofilm, the MtP method was associated with the tube adherence method more

strongly than it did with the Congo red agar method because. Optical density absorption measurements supply quantitative information on the ability of the isolates to form biofilm, whereas the Congo red agar was based on the colony color scale resulting from the Congo red dye interaction with multi-polysaccharides (27). As for The method of tube adhesion was not appropriate because it depended only on the formation of a visible layer adhering to the walls and bottom of the tube thus it could not determine if isolates produced the strong biofilm ,moderate and weak biofilm formation (28) . In this study, the microplate method was associated with the method of tube adhesion more than the association with the method of Congo red agar.This result in agreement with results of (29) but disagreement with (30) They reached the sensitivity and specificity of Congo red agar when compared with Microtitre Plate 90.63% and 90.6%, respectively. Whereas (31) found from studying the sensitivity and specificity of the methods for determining biofilm formation of *S.aureus* that isolated from infected cows in which the percentage of isolates that formed biofilm were 85%, 95.7% and 98.9% by using congo red agar, molecular methods and tissue culture plate method respectively .The sensitive and specificity of MtP method when compared with the molecular methods were 100% and 25%, respectively, whereas the sensitivity and specificity of the CRA were 85% and 100%, respectively.

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دراسة مقارنة لدقة الطرق المظهرية للكشف عن إنتاج الغشاء الحيوي في عزلات المكورات العنقودية الذهبية

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الخلاصة

الكائنات الحية الدقيقة التي تنمو في الغشاء الحيوي تكون مرتبطة مع الالتهابات البشرية المزمنة إذ تقاوم بدرجة عالية الجهاز المناعي، والعوامل المضادة للميكروبات وتكون صعبة الاستئصال من المضيف المصاب. هناك طرق مختلفة لتحديد إنتاج الغشاء الحيوي مثل اطباق المعايرة الدقيقة (MtP) ، طريقة اكار احمر الكونغو (CRA) ، وطريقة الالتصاق بالانابيب (TA) نفذت هذه الدراسة من كانون الاول 2015 إلى أيلول 2017 ، تم الحصول على 580 عينة من المرضى في مستشفيات مختلفة في مدينة أربيل / العراق . العزلات السريرية للمكورات العنقودية الذهبية تم تشخيصها من خلال الاختبارات البيوكيميائية المختلفة والتشخيص الجزيئي ، فوجد مئة عزلة كانت تعود للمكورات العنقودية الذهبية ، وتم تحديد تكوين الغشاء الحيوي بواسطة اطباق المعايرة الدقيقة، طريقة اكار احمر الكونغو ، وطريقة الالتصاق بالانابيب. كان هدف هذه الدراسة هو مقارنة ثلاث طرق لتحديد الأغشية الحيوية، فاظهرت النتائج أن دقة وخصوصية MtP تكون 100٪ ولكن 87٪ ، 75٪ و 91٪ ، 81.25٪ كانت دقة وخصوصية CRA و TA على الترتيب. وجد أن طريقة MtP تكون متفوقة على طريقة CRA وTA وكانت هذه الطريقة هي الافضل لفحص تشكيل الغشاء الحيوي من طريقةCRA و TA ، كذلك وجد أن MtP طريقة كمية موثوق بها للكشف عن تشكيل الغشاء الحيوي في العزلات السريرية للكائنات الحية الدقيقة.