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# Molecular Identification of 16S rRNA gene in Staphylococcus aureus Isolated from Wounds and Burns by PCR Technique and Study Resistance of Fusidic acid

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## Abstract:

Thirty six samples were collected from patient suffering from wounds and burns in Baghdad Hospitals .The samples were analyzed for the presence ofStaphylococcus aureus. Ten positive samples screened by phenotypic microbiological and Biochemical tests and genotypic by DNA extracted from all isolates and the PCR carried out using 16S rRNA gene for S. aureus. In this method for identifying and confirmed all the staphylococcal isolates as S. aureus.The present study was carried out in an attempt to detect the distribution of antibiotic-resistant and we have tried to cover the ever increasing problems facing the treatment and containment of bacterial skin infections.Therefore we are studySusceptibility of Staphylococcus aureus to antibiotics .The results showed relatively high resistance to MethcillinME ,Amoxicillin-Clavilonicacid, Azithromycin with frequencies of 100%, 100% and 70% respectively and moderate resistance to Vancomycin VA, Amikacin AK with frequencies of 60% and 62% respectively, low resistance to Fusidic acid with frequencies of 30% because that we determined the Minimum Inhibitory Concentration for Fusidic acid ,the isolates incorporated in this test were those have intermediate resistant to Fusidic acid.

***Keyword:*** *wounds and burns,Staphylococcus aureus, 16S rRNA gene, PCR.Fusidicacid .*

## Introduction:

*Staphylococcus aureus* is a Gram positive coccus (Steinberg et al., 1996) with circular chromosome located on those pathogenesis and antibiotic-resistant genes (Novick, 1990). It is one of the most important bacteria in Micrococcaceae family, which is also responsible for a wide variety of community- and hospital-acquired infections (Abed EI-Jalilet al., 2008). This bacterium is one of the most versatile organisms. It is found world wide and is a leading cause of disease. Even though it is not classified as a true pathogen (an organism that is expected to always cause disease in humans),(Abed EI-Jalilet al., 2008).but an opportunistic pathogen, it has a diverse repertoire of possible infections. Normally, it is a transient colonizer of the skin and body entry portals (ears, eyes, nasal passages, etc.), and an estimated 20% of humans are carriers (asymptomatic permanent colonization). However, any break in the skin, or colonization of individuals with compromised immune systems can provide an opportunity for this bacteria to cause infection.

The disease process can be mediated via two possible mechanisms (Carboneauet al ;2010) Staphylococcus aureus from the Staphylococcus genus of Micrococcaceae family is a leading cause of community-acquired infections in humans and a cause of mastitis and skin diseases in milk producing animals (EbrahimRahimiet al., 2013) . S. aureus is commonly resident on skin and mucous membranes of cattle (Kluytmans, J. A 2010). Despite its generally benign nature, in changing circumstances, as response to damage and exposure of structures below the epithelial or mucosal surface, S. aureus can behave as a pathogen (EbrahimRahimiet al., 2013; Kluytmans, J. A 2010 ). Illness through S. aureus range from minor skin infection such as pimples, boils, cellulites, toxic shock syndrome, impetigo, and abscesses to life threatening disease such as pneumonia, meningitis, endocarditisand septicemia (Adwan, G ;2005). The majority of skin and

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soft tissue infections are abscesses or cellulitis, but up to one quarter are superficial infections such as impetigo (Naimiet al., 2001).

Fusidic acid belongs to the fusidanes, which have molecular structures similar to corticosteroids without the steroid-like effects(Wilkinson JD.,1998). It is able to achieve a high penetration and concentration at the site of infection, and is highly effective against S. aureus. Many guidelines suggest fusidic acid as first line in the treatment of superficial skin infections and infected eczema, as the main bacterial culprit is S. aureus. (Abeck D.,1998) .Topical fusidic acid and mupirocin appear to be equally effective in cases of primary cutaneous infections (Morley P.,1988). Both ointments appear to be effective against Gram-positive, Gram-negative or a combination of these organisms. The only adverse effect was that of greasiness, which was higher in the mupirocin group ( Perera. G and Hay. R;2005).

Recently many PCR based molecular methods were developed as an alternative ways for accurate identification (Emelet al; 2010). Amplification of 16S rRNA gene sequences (228) bp) is the most commonly used method for identifying and classifying bacteria, including staphylococci (Mohammad et al;2007).

The aim of present study was to Presence of S. aureus inpatients wounds and burns ,can be detected by simple molecular analysis of 16S rRNA gene. Based on the culture methods and PCR technique and study the resistance forfusidic acid due to the most commontopical treatment for wounds and burns.

## Materials and Methods:

**Bacterial Isolation:**

Thirty six samples were obtained from patient suffering from wounds and burns in Baghdad during November 2015 obtaining 10 from isolate as Staphylococcus aureus. Swaps of wounds and burns samples were collected in sterilized screw-cap containers, then the samples were inoculated on culture media (Mannitol agar and blood agar) and incubated aerobically at 37°C for 24hr.Biochemical tests were performed by using API 20 E system which provides a typical identification for all isolates.

**Antibiotic Susceptibility Testing**

Stock solution of the antibiotics Fusidic acid was prepared at final concentration 10 mg/ml according to Clinical Laboratory Standard Institute (CLSI) recommendations using distilled water. Sterilized by filtration and stored at -20°C in small container . Susceptibility testing was conducted by disc diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013) .Antibiotic discs (Becton Dickinson, USA) were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each zone was measured in millimeters. The following antibiotic discs were used:8 Antimicrobial disks tested included Methicillin ME(10µg),Vancomycin VA(10µg), Azithromycin Azi(15 µg) , (30 µg ), Fusidic acid; FA(10µg) , Amikacin AK (10 µg), Amoxicillin + glavulanic acid(30 µg).

**Determination of the Minimum Inhibitory Concentration (MIC):**

Stock solution of the antibiotics Fusidic acid was prepared at final concentration 10 mg/ml according to Clinical Laboratory Standard Institute (CLSI) recommendations using distilled water. MIC was determined for all isolates according to the CLSI (2013) criteria by a standard agar dilution method as follows: Serial two fold dilution of each antibiotic being tested ranged from 0. 5 to 256 µg /ml were prepared of (Fusidic acid).Mueller-Hinton agar medium was prepared, sterilized by autoclave and cooled to 45°C, then the antibiotics were added in appropriate amount from their stock solution , mixed well and poured into the plates. Few colonies (2-4) from overnight culture were transferred to 2ml of normal saline in order to prepare the bacterial suspension and were adjusted to 0.5 McFarland turbidity equal to 1.5×108 CFU/ml .Five microliter of each inoculum was spotted on the agar surface by micropipette. The plates were left to dry for 5 minutes and then incubated at 37°C for 18-24 hr. Mueller-Hinton agar medium without any antibiotic was used as a positive control .MIC results were read after 18-24 hr. The lowest concentration of antibiotic inhibiting the bacterial growth was recorded as the MIC.

**Genomic DNA extraction**

Total DNA was extracted from S. aureus grown on mannitol salt agar plates by using the boiling approach as described previously (Zhang et al.,2004). In brief, for rapid DNA extraction, one to five colonies of each freshly subcultured strain were suspended in 50 µl sterile distilled water and heated at 99°C for 10 min.After centrifugation at 30,000 xg for 1 min, the supernatant was used as a DNA template and stored at -20ºC until PCR was performed.

**PCR amplification procedure**

The sequence of oligonucleotide primers that were used in PCR to detect the presence of 16s rRNA gene were synthesized in Alpha DNA (Canada).Table (1) shows primer sequence and product.

**Table (1): Sequence and molecular size of PCR products of 16s rRNA**

| Gene | Primer Sequence | Size of product (bp) | Cof |
|---|---|---|---|
| 16srDNA | F : GTAGGTGGCAAGCGTTACC<br>R : CGCACATCAGCGTCAG | 228 | Mastafa Al-musawi*et al*., (2014) |

Attend each solution separately primer concentration of (10) Pmol/µl by dissolving 10µl of stock solution for each primer and add to 90µl distilled water unionic mix well and keeping in the ice until use .the stock solutions of the primers were store in -20 ,then mixing by vortex to homogenized before uses.

Primers obtained from Alpha DNA (USA) using the conditions in table 2 using Conventional PCR

**Table 2: The conditions used for the amplification of 16s rRNA geneby Conventional PCR.**

| Initial denaturation | Denaturation in each cycle | Annealing | Primers extension | Final extension |
|---|---|---|---|---|
| 94 °C, 5 min | 94°C, 1 min | 51.5°C,30s | 72°C, 1min | 72 °C,10 min |
| | 30 cycles | | | |

Amplification products then separated by electrophoresis on agarose gel 1% stained with ethidium bromide (0.5 µg/ml) in Tris - borate - EDTA TBE buffer. The bands were detected using UV transillumination.

**Gradient PCR amplification procedure**

The reaction was performed using PCR thermal cycler apparatus.The following program was adopted:-PCR consisted of a preheating at 94ºC after this initial denaturation step, the mixture was subjected to 30 amplification cycles as follows:-

**Table 3: The Program of gradient PCR for 16s rRNA gene by Conventional PCR.**

| Initial denaturation | Denaturation in each cycle | Annealing gradient | Primers extension | Final extension |
|---|---|---|---|---|
| 94 °C, 5 min | 95°C, 1 min | (55ºC Gradient Δ10ºC)1 min | 72°C, 1min | 72 °C,10 min |
| | 30 cycles | | | |

## Result and Discussion:

**Isolating and Diagnosing of Staphylococcus aureus**

In this study, Thirty six specimens were collected from burn and wound patients from Baghdad Hospitals. Swaps of wounds and burns samples were collected and asabsorved by other report Rittenhouse et al.(2006) that wounds are common infections caused by S. aureus . Then the samples were inoculated on culture media (Mannitol agar and blood agar) ,as shown in Figure1,they were initially diagnosed on blood agar some of the isolates produce a clear translucent area surrounding the colonies as result for ß-haemolysin production.Hemolysis by this bacteria considered the most important features for diagnosis and identification the pathogenic isolates of S.aureus from other Staphyloccusspp(Atlas et al .,1995).

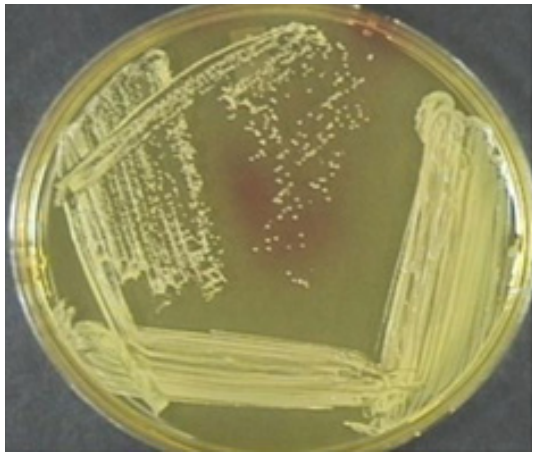

**Figure 1: *S.aureus* on Mannitol Salt agar.**

Microscopic examination was applied to the all isolates after staining by Gram stain and the cells appeared as Gram-positive cocci arranged in pairs or grape-like irregular clusters, as represent the phenotypic characteristics for this genus (Baron et al .,1994). Mannitol salt agar is selective differential growth medium. It encourages the growth of Staphylococcus isolates, while inhibiting the growth of others. It contains a high concentration 7.5-10.0 % of NaCl salt, making it selective for Gram-positive bacterium Staphylococcus and Micrococcus.

S.aureus produces yellow colonies, whereas other Staphylococci produces small pink or red colonies with no color change to the medium. If an organism can ferment mannitol, an acid is formed that will cause the phenol red in the agar to turn yellow (Anderson and Cindy, 2013).

**Susceptibility of Staphylococcus aureus to antibiotics:**

According to the recommendation of (CLSI, 2013), ten isolates of S.aureus isolated from burn and wound patients were tested for antibiotic sensitivity by disc diffusion method (Kirby-Bauer test).The results showed relatively high resistance to MethcillinME ,Amoxicillin-Clavilonicacid AMC, Azithromycin AZM with frequencies of 100%, 100% and 70% respectively. Kader et al. (2011) reported that 88.24% of isolates were resistant to methicillin and Oxacillin discs, while Odonkora and Addob(2011) reported that methicillin disc diffusion test detected 54 MRSA ( 21.6%) , whilerelative moderate resistance to Vancomycin VA, Amikacin AK with frequencies of 60% and 62% respectivelyhas been observed in our study. Alebachewet al. (2012) found a resistance of S.aureus isolates towards Vancomycin and Amikacin 9.7% and 10.0%, respectively.

S.aureus showed relatively low resistance to Fusidic acid with frequencies of 30% has been detected with other study of Masonet al.(2003) who reported low value for Fusidic acid. as shown in Figure 2.

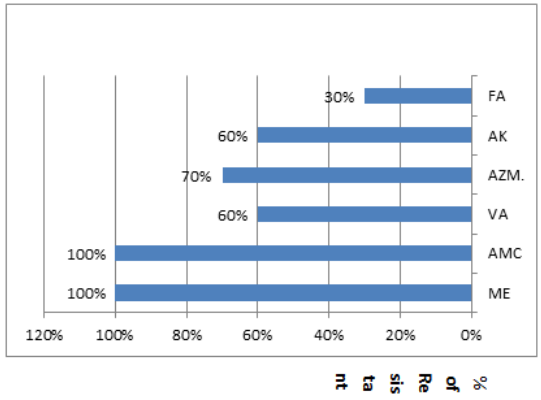


**Figure 2:Percentage of Resistance antimicrobial agents forStaphylococcus aureus**

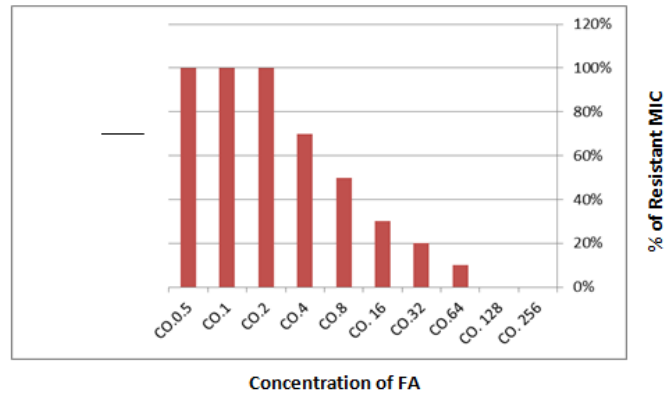


**Figure 3: Percentage of Resistance Minimum inhibitor concentration agar diffusion method for Fusidic acid (co; concentration 0.5-256) for *Staphylococcus aureus.***

**Determination of MIC for Fusidic acid antibiotic of S.aureus:.**

Determination of MICfor Fusidic acid resistant isolates of S. aureusshown intermediate resistant to Fusidic acid CLSI(2013). The results show MIC range from 8-256 µg /ml. The resistance was presented in Figure 3which summarized the result of MIC experiment. . Perera.G and Hay. R (2005) highlighted this concern overgrowing fucidin resistance. The West Yorkshire study found that50% of fusidic acid-resistant strains were from dermatology patientsexposed to topical fucidin in the 6 months prior . The lowest rate of resistance to Fusidic acid was 10% in the concentration 64 µg /ml the MIC values.Mason (2003) indicted that the resistance level to fucidin is low at present, most likely due to its unique molecular structure and therefore is less likely to share resistance mechanisms with other antibiotics. Prolonged treatment with fucidin ointment should be avoided, even in the community setting.

**4:Genetic Detection of S. aureus by 16s-rDNA**

The total DNA (chromosomal) will be served as a template since they willreleased out side the bacterial cell . The present research findings pertain to the isolation of S. aureus ,samples were confirmed as S. aureus on the basis of morphological, biochemical and molecular characterization .After gradient PCRhas been performed(55ºC Gradient Δ10ºC) ,bands has been obtained as presents in (Fig-4). Showing that the best annealing a temperature to give16s-rDNA was(51.5°C,30s) depending on results of gradient PCR amplification procedure

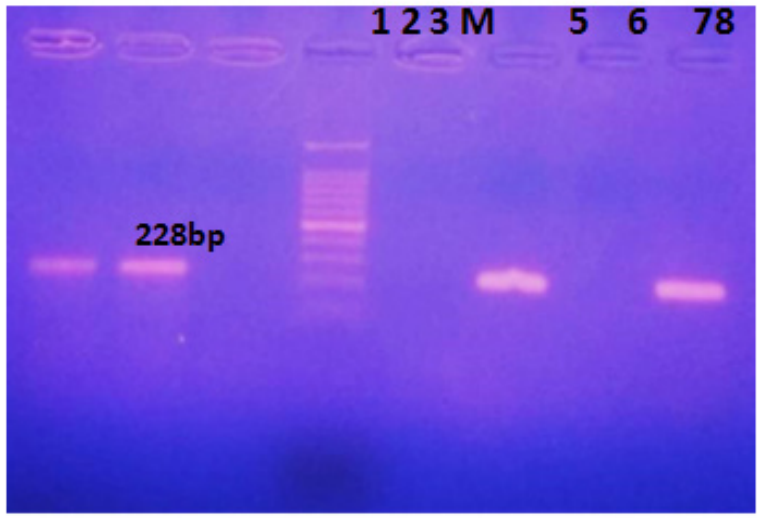


**Figure 4 :Agaros gel electrophoresis(1% agarose, 7 v/cm2 ) and Ethidium bromide staining .Lane M, molecular size DNA ladder (100 bp DNA Ladder); lanes 1-2-6-8, DNAs isolated from S. aureus samples Positive PCR bands were show to be give(band 228bp) on the set suggesting that bands are 16srRNA gene The PCR was carried out in duplicate. The photocomposition of the figure was obtained with Gel documentary camera.**

Several studies have used PCR for the detection of 16S-rDNA by PCR method more rapidly and reliably . The results of current study agree with result of a same study in Iran reported by.( Maliheet al;2011) ) who noticed that amplification of 16s-rDNA confirmed all the 126 staphylococcal isolates as S. aureus.

**Conclusions**

PCR is a rapid, sensitive, specific and inexpensive method; we suggest that it can be replaced to traditionally assays for detecting S. aureus. Although the 16S rDNA procedure differentiated of all tested clinical isolates identified 100%. Therefore, if an unknown organism needs to be identified, 16S rDNA sequencing is the method of choice because of the availability of universal primers. Furthermore, antibiotic susceptibility tests and MIC should be done besides detecting bacterial factors in order to enhance treatments for decreasing infections of human staphylococciand study the resistance for fusidic acid due to the most common topical treatment for wounds and burns

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# التشخيص الجزيئي لجين 16 S rRNA في بكتريا المكورات العنقودية الذهبية المعزولة من الجروح والحروق بواسطة تقنية PCR ودراسة مقاومتها لمضاد الفيوسديك اسد .

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**الخلاصه:**

ستة وثلاثون عينة جمعت من مرضى يعانون من الجروح والحروق من مستشفيات بغداد .وقد تم تحليل العينات عن وجود بكتريا المكورة العنقودية البرتقالية. عشرة عينات إيجابية للفحص من قبل الميكروبيولوجية, المظهرية والاختبارات البايوكيميائية والفحوصات الوراثية بواسطة استخلاص الدنا لكل العزلات واجراء تقنية التضاعف التسلسلي PCR باستخدام جين 16S rRNA للبكتريا المكورة العنقودية البرتقالية استخدمت هذه الطريقة لتحديد وتأكيد جميع والمكورة العنقودية وتأكيدها انها العنقودية الذهبية. وقد أجريت هذه الدراسة في محاولة للكشف عن انتشار مقاومة المضادات الحيوية، وحاولنا لتغطية المشاكل المتزايدة التي تواجه معالجة وتلوث اصابات الجلد بالبكتريا. لذلك درسنا حساسية بكتريا المكورات العنقودية الذهبية للمضادات . اظهرت النتائج مقاومة عالية لمضاد Methcillin بترددات 100%, 100% و 70% على التوالي . مقاومة متوسطة لمضادات -Vancomy ME ,Amoxicillin-Clavilonic acid, Azithromycin cin VA, Amikacin AK بترددات 60% و 62% على التوالي , مقاومة منخفضة لمضاد Fusidic acid بتردد 30% وبسبب ذلك حددنا التركيز المثبط الادنى(MIC) لمضاد Fusidic acid على عزلات بكتريا المكورات العنقودية الذهبية حيث اظهرت مقاومة متوسطة لهذا المضاد .