

# Use Ethidium Bromide as curing to plasmid in *Staphylococcus aureus* (MRSA) isolated from Patients Iraqi and Screening for Virulence Factors

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## Abstract

The study was conducted on samples of the ear patients and after the tests biochemical found to be a type of *Staphylococcus aureus*. The most important virulence factors were detected for these bacteria, including the production of bacteria for hemolysin, protease and biofilm.

The result was positive for the production of hemolysin, protease and biofilm. The results of the effect of etidium bromide on the resistance of the isolates under study for antibiotics, which made these isolates sensitive to these antibiotics after treatment with the dye of etidium bromide, and after the rocess of extraction of DNA and conduct the process of Electrophoresis shows the disappearance of the band DNA.

**Keywords:** Ethidium Bromide ,Curing ,Biofilm formation ,Protease , Haemolysin

## INTRODUCTION:

Although *Staphylococcus aureus* this organism is a natural reservoir in humans, it is a pathogen in the world and a disease that causes it severe soft tissue infections such as carbuncles, abscesses, pneumonia, endocarditis, food poisoning, toxic shock syndrome (TSS) throughout the body and as well as scalded skin syndrome in humans, osteomyelitis, pneumonia, arthritis, scalded skin syndrome, endocarditis, myocarditis, pericarditis, and bacteremia [1-3]. *Staphylococcus* are non-motile, non-spore-forming [4]. Optimum growth temperature is in between 30-37°C and the colonies on solid media are round, smooth [5]. *Staphylococcus aureus* bacteria have the ability to produce the virulence factors and this is one of the causes of its pathogenicity and these factors are thermo stable nuclease, enterotoxins serotypes A, toxic shock syndrome toxin-1 (TSST-1), cytolytic toxins ( $\alpha$  and  $\beta$  hemolysins), exfoliative toxins, Panton-Valentine leukocidin (PVL), protein A, several enzymes, Capsule and Surface proteins [6]. MRSA has the ability to resist  $\beta$ -lactam antibiotics either by production  $\beta$ -lactamase enzyme which specifically binds to  $\beta$ -lactam ring rendering them inactivate or resistance could be due to modification target site represent by penicillin binding proteins (PBP2a) causes decreasing their affinity for binding with the antibiotics [7, 8]. Plasmids let the motion of genetic material, including genes of antimicrobial resistance between bacterial species and genera [9].

## MATERIALS AND METHODS

### Microorganism :

The clinical isolates primary identify as *Staphylococcus* species were obtained from hospitalized patients. These patients were suffering from Ear infections. The isolates were primary cultivated on Blood agar and MacConky agar then incubated at 37°C for 18-24 hours. All Pure isolates were Cultured on Mannitol salt agar then incubated at 37°C for 24 hrs. And the biochemical tests identified by system API Staph kit. The isolates are culture on brain heart infusion (BHI) broth and incubated, prepare bacterial suspension about  $10^8$  cell / ml for further use.

### Screening for protease activity :

Skim milk agar was used to screen for protease activity. According to [10] all isolates are cultured on skim milk agar to screen protease production

### Haemolysin production :

Blood agar according to (11) : was used for detection of haemolysin production of all the isolates. All isolates are culture on blood agar to screen haemolysin production.

### Biofilm production

Congo red agar (CRA) method According to (12) was used for detection of biofilm production of all the isolates. All isolates are culture on congo red agar (CRA) to screen biofilm production (13).

### Antimicrobial susceptibility test

The disk diffusion method was used for antimicrobial susceptibility testing [14]. We used (7) antimicrobial disk (HiMedia): Oxacillin OX, Ceftriaxone (CRO), Ampicillin (AM), Nitrofurantion (F), Levofloxacin (LEV), Vancomycin (VN), Erythromycin E (10  $\mu$ g), Tetracyclin TE (30  $\mu$ g). Bacterial culture for 24 h was compared with the standard turbidly solution (McFarland 0.5), this approximately equals to  $(1.5 \times 10^8)$  cfu/ml. A 0.1 ml of the culture was spreaded on the surface of Mueller-Hinton agar (HiMedia) plates, left to dry for 15 minutes at room temperature. The discs were placed on the medium and incubated at  $35 \pm 2$  °C / 24 h. To determine the sensitivity or resistance of isolates to antibiotics based on measurement of diameters of inhibitory zones [20]. *E. coli* ATCC 25922 was used for quality control.

### Plasmid curing experiment:

Prepare tubes from the nutritious broth supplemented with 4 mg / ml of ethidium bromide and inoculated the tubes with isolates of *Staphylococcus aureus* and Incubated for 24 hours / 37 °C. Take a loopful of growth and cultured on Mueller Hinton Agar (MHA) plates and then put the antimicrobial disk on (MHA). Absence of zone of inhibition on Mueller Hinton agar was indicative of plasmids-mediated resistance (plasmid cured), while presence of zone of inhibition on Mueller Hinton agar was indicative of chromosome-mediated (plasmid not cured).

### Plasmid Isolation

A colony of test organism, cultured on fresh agar plates, was picked with the aid of a sterile wire loop and inoculated into sterile test tubes containing 8 ml of fresh nutrient broth and then incubated at 37 °C for 72 h. The antibiotic resistance plasmids in the cultures were isolated using the alkaline lyses protocol [17,18].

### Preparation of Agarose gel:-

Take 0.8 g of agarose powder and dissolve in 100 ml Tris-Borate-EDTA (TBE) buffer concentration (0.5X). The solution was cooled at 45-50 °C. Added 10  $\mu$ l of ethidium bromide to agarose solution [19].

### Agar Gel Electrophoresis

Twenty  $\mu$ l of the plasmid DNA samples mixing with 2  $\mu$ l of Dye loading (bromophenol blue) and loaded into the Agarose gel wells and DNA marker. The gel was thereafter electrophoresed at a constant voltage of 70V for about 1 hour and a half. Plasmid DNA bands were observed with UV transilluminator and photographed [19].

**RESULTS AND DISCUSSION**

All isolates of *S.aureus* were cultured on Mannitol salt agar to know the ability of *S.aureus* to tolerate sodium chloride[19]. The pure colony of the isolate was confirmed by several tests (Table-1). Colony and cells shape,gram stain ,motility, mucoid ,smooth and golden colonies. On blood agar medium they were able to produce golden yellow pigment and were surrounded by a zone of clear (beta) hemolysis (Figure1). The color of mannitol salt agar change from red to yellow because fermenting the mannitol and this considered as a positive test and Protease production on Skim milk agar (Figure 2) . After the isolates were cultured on Congo Red Agar (CRA) at 24 h / 37°C. The colonies color will change to black an indication of the productivity of biofilms (Figure 3) . The most important factors of formation biofilms that synthesis of of the polysaccharide intercellular adhesion by the organism .

**Table 1: General properties of local isolate *Staphylococcus aureus* (MRSA)**

Test	Result
Gram stain	+
Colony shape on Mannitol salt agar	large golden colonies
Motility	non-motile
Protease production on Skim milk agar	+
Blood hemolysis	Beta
Biofilm production on congo red agar	+

This antimicrobial susceptibility test performed for all isolates which gave positive phenotypic tests for methecillin resistance against 7 different antibiotic Oxacillin(OX), Ceftriaxone(CRO),Tetracycline(T),Ampicillin(AM),Nitrofurantion(F), Levofloxacin(LEV),Vancomycin(VN),Erythromycin according to the recommendation of[20]. The results of susceptibility test in Table (2) .

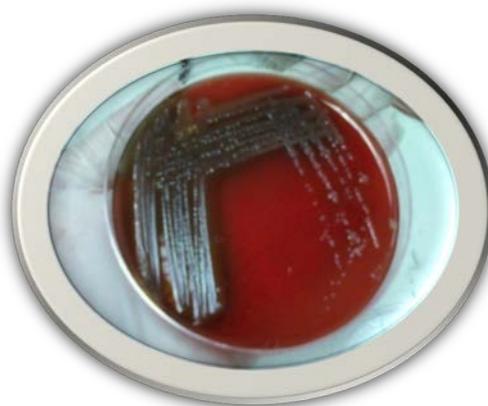
**Table (2): Antibiotic Resistance Pattern of *Staphylococcus aureus* before & after treatment with Ethidium bromide**

Antibiotics Types	Before Treatment with Ethidium bromide	After Treatment with Ethidium bromide
	Diameter Antibiotics of Inhibition Zones (mm)	
Oxacillin (OX)	-	-
Ceftriaxone (CRO)	-	25
Ampicillin(AM)	-	-
Nitrofurantion (F)	-	-
Levofloxacin(LEV)	-	20
Vancomycin(VN)	-	-
Erythromycin (E)	-	-
Tetracyclin (TE)	-	15

All the resistant *Staphylococcus aureus* (MRSA) were cured the plasmid by ethidium bromide in the end the strains lost their plasmids by mutagenic matter (ethidium bromide).The growth of (MRSA) on mannitol salt agar could be attributed to its ability of *S.aureus* to tolerate high concentrations of sodium chloride as contained in the medium [21]. *Staphylococcus aureus* has virulence factors such as coagulase and hemolysin which cause the pathogenicity of these bacteria and production of virulence factors in *Staphylococcus aureus* is in conformity with the reports of [22,23] .The results showed that the addition of Ethidium bromide dye (mcg / ml to the brain heart infusion broth medium and when the incubated of bacterial isolates in the degree in which 37 ° C for 24 hours , then softened samples and spread over nutrient agar plates and then incubated in the degree of 37 ° C for 24 hours. after a period cuddling conducted screening test sensitivity for all the colonies developing on the dish for the

purpose of knowing for neutralization of the plasmids through the loss of sensitivity or no loss of sensitivity to antibiotics, which conducted its examination become sensitive to the antibiotics that were originally resistant to them prior to the neutralization process, were some of the colonies that make them sensitive assay sensitivity for all types of antibiotics which indicates that the prescription of resistance to these antibiotics are all mounted on the plasmid results also showed that the other colonies were sensitive to some antibiotics and resistant to some of the other, and this shows that the resistance genes for anti-mounted chromosome(Figure4).

One of the reasons for the chang in the molecular structure that affect the composition of nucleic acid and leads to the elimination of plasmids is the association of ethidium bromide with DNA, is responsible for loss of the phenotypic characteristic [24],this indicates that antimicrobial resistant genes were plasmid located and this result in agreement with loss of resistance determinants (plasmids) that was investigated as previously reported [25,26].



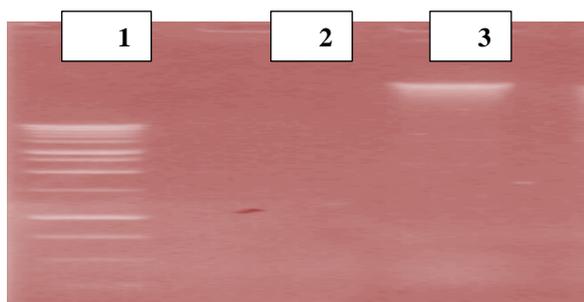
**Fig.(1):- Isolates producing Hemolysis production on blood agar**



**Fig.(2):- Isolates producing protease on Skim milk agar**



**Fig.(3):- Isolates producing biofilm on congo red agar**



**Fig.(4):Plasmid curing profile of *Staphylococcus aureus* isolates– Stained 0.8%agarose gel**

**Lane 1: represents marker DNA**

**Lane 2: represents isolates after curing treatment**

**Lane 3: represents isolates before treatment**

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