Plasmid transformation and curing of nalidixic acid gene in *Staphylococcus aureus* isolated from buffaloes mastitis and worker's hands

D.A. Khaleel, R.M. Othman* and B.Y. Khudaier

Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq
*Corresponding author e-mail: rashamunther2014@yahoo.com

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Abstract

The present study was designed to detect resistant site of nalidixic acid through transformation and plasmid curing of *S. aureus* strains isolated from buffalo milk with subclinical mastitis and workers' hands. A total of 37 *S. aureus* isolates including 17 isolates recovered from buffalo milk infected with subclinical mastitis, in addition to 20 isolates recovered from workers' hands. All 37 isolates were investigated by detection of the 23S rRNA gene and various other species specific genes including *coa*, *nuc* and *clf*A. The antibiotic resistance of *S. aureus* isolates was performed by the discs diffusion method using 19 antibiotics. Plasmid transformation method was carried out by transferring the plasmid isolated from *S. aureus* into competent *Escherichia coli* HB 101 in order to detection the resistant site of nalidixic acid. Plasmid curing was accomplished by preparing different concentrations of nalidixic acid (100, 150, 200, 250 and 300 µg/ml) and cultured transformed *E.coli* on LB agar supported with each of the aforementioned concentrations. The molecular results showed that six isolates (five isolates from milk samples and one from workers' hands) were identified as *S. aureus* by *coa*, *nuc*, and *clf*A species specific primers. The six *S. aureus* isolates were found to be resistant to at least 5 antibiotics which included the nalidixic acid. The results of plasmid transformation revealed that *E. coli* was able to grow on LB agar supported with 100µg/ml, 150 µg/ml, 200 µg/ml and 250 µg/ml-of nalidixic acid and failed to grow on 300 µg/ml concentration.

Keywords: *Staphylococcus aureus*, Buffaloes mastitis, nalidixic acid, *coa* gene, *nuc* gene, *clfA* gene
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التحول والتحييد البلازميدي لجين Nalidixic Acid في المكورات العنقودية الذهبية المعزولة من التهاب الضرع في الجاموس وايدي العاملين

دعاء عبد الرزاق خليل، رشا منذر عثمان و بسام ياسين خضير

فرع الاحياء المجهرية، كلية الطب البيطري، جامعة البصرة، البصرة، العراق

الخلاصة

تهدف هذه الدراسة عن الكشف عن موقع المقاومة لجين nalidixic acid من خلال التحويل وعلاج البلازميد للمكورات العنقودية الذهبية المعزولة من حليب الجاموس المصاب بالتهاب الضرع تحت السريري وأيدي العاملين. من مجموع ٣٧عزلة من المكورات العنقودية المذهبة منها ١٧ عزلة تم الحصول عليها من حليب الجاموس المصاب بالتهاب الضرع التحت السريري بالإضافة الى ٢٠ عزلة من ايادي العاملين. واختبرت ٣٧ عزلة بواسطة تشخيص جين 23S rRNA الخاص ببكتريا المكورة العنقودية الذهبية وبمختلف عوامل الضراوة التي تتضمن جينات *coa*,nuc, وclfA. وتم اختبار المقاومة الحيوية للمكورات العنقودية الذهبية بواسطة طريقة Kirby-Bauer disc diffusion تجاه 19 نوع من المضادات. كما أجريت عملية التحويل بواسطة نقل البلازميد المعزول من المكورات العنقودية الذهبية الى بكتريا *E. coli* HB 101 المختصة من اجل الكشف عن موقع مقاومة المضاد الحيوي nalidixic acid. تم انجاز علاج البلازميد من خلال إعداد عدة تراكيز للمضاد nalidixic acid (١٠٠، ١٥٠، ٢٠٠، ٢٥٠ و ٣٠٠ مايكروغرام) وتم زراعتها على وسط LB المدعوم بالتراكيز أعلاه. وقد بينت

نتائج الاختبار الجزيئي بوجود ٦ عزلات (٥ من حليب الجاموس و١ من ايدي العاملين) من المكورات العنقودية الذهبية بواسطة استخدام بادئات خاصة بالنوع لجينات *coa*, nuc, وclfA . حيث وجد ان ٦ من عزلات المكورات العنقودية مقاومة ٥ انواع على الاقل من المضادات الحيوية ومن ضمنها المضاد الحيوي nalidixic acid. كما وجد ان بكتريا *E. coli* المتحولة لها القابلية على النمو في وسط LB المدعوم بالمضاد الحيوي nalidixic acid وبالتراكيز (١٠٠، ١٥٠، ٢٠٠ و ٢٥٠ مايكروغرام) إلا أنها فقدت قدرتها على النمو بالتركيز ٣٠٠ مايكروغرام.

Introduction

In dairy animals, *S. aureus* still remains one of the most significant worldwide investigated organisms associated with clinical and subclinical mastitis (1). Mastitis is the most economically important disease of dairy industries around the world. The major cause of bovine mastitis is the infection with pathogenic bacteria (2,3). The emergence of antibiotic-resistance *S. aureus* strains resulted in significant treatment difficulties which imposed burden on health care systems and simultaneously intensifying the need for new antibiotics (4). In most clinical laboratories, identification methods depend on microbial culture of milk. The cultural method examination is however less sensitive method and time consuming (5). Contrariwise, molecular-based diagnostic technique such as polymerase chain reaction (PCR) are considered rapid, sensitive and more reliable to identify various pathogenic bacteria isolated from bovine mastitis. (6,7).

Bacteria can acquire new genetic information by three means: (conjugation, transduction and transformation). During conjugation, DNA is transferred directly from one organism to another, whereas in transduction, the DNA is carried by bacteriophage. Transformation involves the acquisition of naked DNA from the extracellular environment, and genetic competence is the ability to undergo transformation. Early experiments on transformation showed that DNA is the genetic material (8). The transfer of DNA is from one organism to another, often using a carrier called a vector (such as a plasmid, virus, or another form of mobile DNA) (9). There are two basic methods to transform *E. coli*: chemical transformation (heat shock) and electroporation methods. Chemical transformation is more convenient while the electroporation is more efficient (10). The heat shock method is regarded as the basic technique for the transformation of the plasmid DNA into *E. coli* and includes inserting a foreign plasmid or ligation product into bacteria (11). The present study was designed to investigate the plasmid curing in *S. aureus* bacteria isolated from buffalo milk and workers' hands and to determine the site of antimicrobial resistance genes by transformation its plasmid into the genetically engineered *E. coli* HB101.

Materials and methods

Bacterial isolates

A total of 37 *S. aureus* isolates were obtained from previous study (12) includes, 17 isolates recovered from buffalo's milk infected with subclinical mastitis and 20 isolates recovered from workers' hands.

Antibiotics susceptibility test (AST)

The AST was achieved by the discs diffusion method as described by (13) and results interpretation were completed according to the CLSI guidelines (14).

Molecular identification of isolates

The whole genomic DNA was extracted using QIAamp® DNA Mini and Blood Mini -kit according to the manufactures instructions (Qiagen, USA). The primers used for amplification of 23S rRNA, *coa*, *nuc* and *clfA* genes, summarized in table (1). For 23S rRNA, *coa* and *clfA* genes, the PCR amplifications were performed in 25 µl reaction mixtures with 0.7µl (10 pmol/µl) for forward primer and reverse primer, 12.5µl ready to use green master mix (Promega/USA) and 9.1 µl nuclease-free water. Finally, 2 µl DNA template was added to each reaction tube. For *nuc* gene, the PCR amplifications were performed in 25 µl reaction mixtures with 1.0 µl (10 pmol/µl of each primer, 12.5 µl ready to use green master mix and 5.5 µl of nuclease-free water and finally 5 µl of DNA template and (15).

For 23S DNA amplification the following PCR program was used: one step of 5 min at 94ºC; 37 cycles, with 1 cycle consisting of 40s at 94ºC, 60 s at 64 °C, and 75 s at 72ºC; and one step of 10 min at 72ºC. For amplification of *coa* gene the following PCR program was used: one step of 5 min at 94ºC; 30 cycles, with 1 cycle consisting of 40s at 94ºC, 60s at 58°C, and 60 s at 72ºC; and one step of 10 min at 72ºC. For amplification of *clfA* gene the following PCR program was used: one step of 5 min at 94ºC; 35 cycles, with 1 cycle consisting of 60s at 94ºC, 60 s at 57 °C, and 60 s at 72ºC; and one step of 10 min at 72ºC. For amplification of *nuc* gene the following PCR program was used (16): one step of 5 min at 94ºC; 30 cycles, with 1 cycle consisting of 30s at 94ºC, 30 s at 55 °C, and 60 s at 72ºC; and one step of 10 min at 72ºC. The PCRs were performed with prime full size (Techne, UK).

Table 1: Oligonucleotide primer sequences for PCR amplified of 23S rRNA, *coa*, *nuc* and *clfA* genes

Primer name	Sequence (5'-3')	Size of PCR product (bp)	Reference
23S rRNA F	ACGGAGTTACAAAGGACGAC	1250	14
23S rRNA R	AGCTCAGCCTTAACGAGTAC		
coa gene F	ATAGAGATGCTGGTACAGG	Polymorphism size	14
coa gene R	GCTTCCGATTGTTCGATGC		
nuc gene F	GCGATTGATGGTGATACGGTT	280	15
nuc gene R	ACGCAAGCCTTGACGAACTAAAGC		
clfA gene F	GGCTTCAGTGCTTGTAGG	1024	14
clfA gene R	TTTTCAGGGTCAATATAAGC		

F= forward primer; R = reverse primer.

The PCR products (7µl) were run on a 2% agarose gel containing 0.5 µl/25 ml ethidium bromide with the addition of loading buffer and a DNA molecular size marker as standard in electrophoresis which run at 80V for 1 h. The products were visualized by UV transilluminator (Vilber Lourmater UV light EEC /France).

Plasmid extraction

The plasmid was extracted by using plasmid extraction kit using according to the manufactures instructions (Promega, USA). The results were detected by electrophoresis on 1% agarose gel and exposed to UV transilluminator (Vilber Lourmater UV light EEC/France).The DNA plasmid appeared as compact bands.

Transformation of *E. coli* competent cells

The transformed *E. coli* HB 101cells were demonstrated by their culturing on the LB agar selectable medium supported with 100µg/ml nalidixic acid, this method was done according to the protocol provided along with the genetic modified competent *E. coli* HB101 (Promega®): The competent cells were removed from frozen -70ºC, and placed on ice for 5 minutes or until just thawed. The thawing cells were pipetted quickly to 10 ml sterile screw culture tubes using chilled 4ºC pipette tips to prevent the cells from warming above 4ºC and mixed gently by flicking the tube, then 100µl of the thawed competent cells were transferred to each chilled culture tube. One µl of the plasmid was added to 100 µl of competent cells. The tube was flicked quickly several times and returned immediately to ice for 10 minutes. The cells were heat-shocked without shaking for 50 seconds in a water bath at exactly 42ºC then placed directly on ice for 2 minutes. Each transformation reaction was added to 900µl of cold 4ºC LB broth medium, and incubation for 60 minutes at 37ºC with shaking (approximately 225 rpm). For each transformation reaction, the cells were diluted 1:10, 1:100 and 1;1000. Then 100 µl of each dilution cells were plated on nalidixic acid antibiotic plates, the plates were incubated at 37ºC for overnight. To confirm the results of the transformation step, the random choice of single positive transformed colonies growing on nalidixic acid LB medium were spread on LB agar supported with ampicillin (final concentration 100µg/ml).

The efficiency of transformation

Transformation efficiency is defined as the number of cfu produced by one µg of plasmid DNA (17). To determine the efficiency of transformation, several dilutions of transformed *E. coli* HB101 were cultured and grown on LB agar with nalidixic acid. The transformation efficiency was found to be sufficiently (16×10^5 cfu/g plasmid DNA) on LB / NA plates at 100µg/ml concentration, as shown below.

$$\frac{\text{cfu on transformant plate}}{\text{ng of competent cells DNA plated}} \times \frac{1\times 10^5 \text{ng}}{\mu\text{g}}$$

Plasmid curing

Curing trials were conducted by using different concentrations of nalidixic acid (100, 150, 200, 250 and 300 µg/ml). Each concentration was added to LB medium and cultured transformed *E. coli* HB101 on their plates and incubated overnight at 37ºC to be observed growth of transformed *E. coli* HB101.

Results

Antibiotic susceptibility test

All 37 *S. aureus* isolates (100%) were susceptible to norfloxacin, clindamycin, tobramycin, ciprofloxacin, gentamycin, kanamycin and rifambin, resistant (100%) to ampicillin, amoxillin, methicillin and nalidixic acid and intermediate (100%) for erythromycin. Most of them (83.3%) were susceptible to vancomycin, (66.7%) to oxacillin, (50%) to neomycin and less than (16.7%) to tetracycline and chloramphenicol, whereas (66.7%) showed resistance to chloramphenicol, (50%) to tetracycline and (33.3%) to ceftriaxone and oxacillin, but (16.7%) resistance to vancomycin. Intermediate susceptibility to ciprofloxacin

was observed in (66.7%) of the *S. aureus* isolates, and (50%) intermediated for neomycin and (33.3%), (16.7%) for tetracycline and chloramphenicol, respectively. The results of antibiotic susceptibility test were shown in table (2).

Table 2: Antibiotic Susceptibility for *S. aureus*

Type of antibiotic	Sensitive (%)	Intermediate (%)	Resistance (%)
Ampicillin	0 %	0 %	100 %
Amoxillin/clavulanic acid	0 %	0 %	100 %
Ceftriaxone	0 %	66.7 %	33.3 %
Chloramphenicol	16.7 %	16.7%	66.7%
Ciprofloxacin	100 %	0 %	0 %
Clindamycin	100 %	0 %	0 %
Erythromycin	0 %	100 %	0 %
Gentamycin	100 %	0 %	0 %
Kanamycin	100 %	0 %	0 %
Oxacillin	66.7 %	0 %	33.3 %
Rifampicin	100 %	0 %	0 %
Methacillin	0 %	0 %	100 %
Norfloxacin	100 %	0 %	0 %
Neomycin	50%	50%	0%
Naldixic Acid	0 %	0 %	100%
Tetracycline	16.7 %	33.3 %	50 %
Tobramycin	100 %	0 %	0 %
Streptomycin	100%	0%	0%
Vancomycin	83.3 %	0 %	16.7 %

Molecular identification of *S. aureus*

The 23S rRNA gene was detected in all 37 *S. aureus* isolates (n= 17 milk isolates and 20 hand isolates) (figure 1). Meanwhile, genes *coa*, *nuc* and *clf*A were amplified in five isolates of *S. aureus* of milk samples and one isolates of *S. aureus* from human hand swab origin yielding PCR products of 910 bp, 710bp and 627 bp (figure 2), 280bp (figure 3) and 1024 bp (figure 4), respectively.

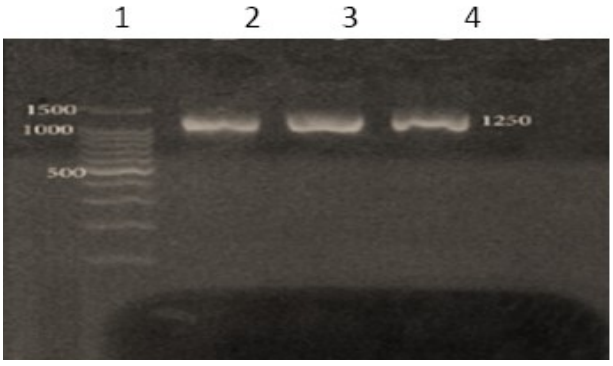

Figure 1: Amplification of 23S rRNA gene (1250 bp). Lane 1: DNA Ladder; Lanes 2, 3 and 4: *S. aureus* isolates.

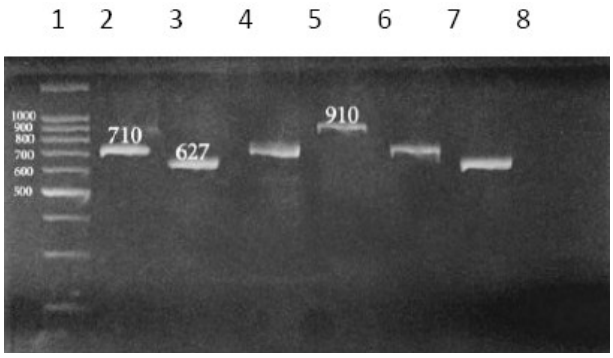

Figure 2: Amplification of S. aureus coa gene Lane 1: DNA Ladder; Lanes 2, 4 and 6: 710 bp; Lanes 3 and 7: 627 bp; Lane 5: 910 bp.

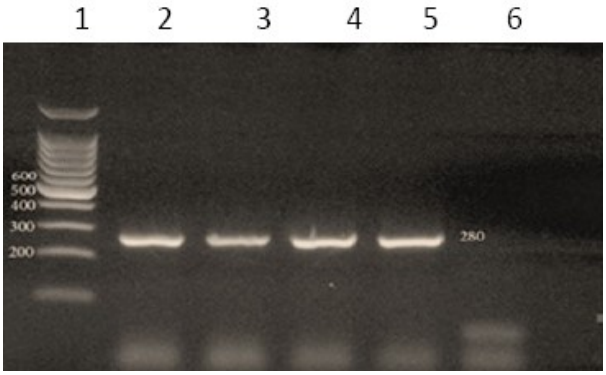

Figure 3: Amplification of *S. aureus* nuc gene (280 bp). Lane 1: DNA Ladder; Lanes 2, 3, 4 and 5: S. aureus isolates; Lane 6: negative sample.

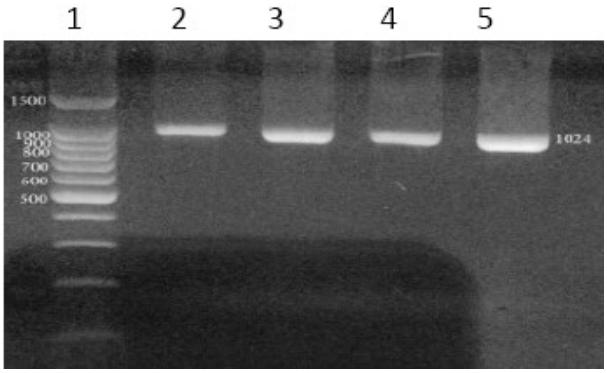

Figure 4: Amplification of clfA gene (1024 bp) . Lane 1: DNA Ladder; Lanes 2, 3, 4 and 5: *S. aureus* isolates.

Transformation of *E. coli* competent cells and the efficiency of transformation

The results of plasmid DNA appeared as compact bands shown in figure (5). The agarose gel electrophoresis showed clearly that plasmid DNA does not undergo any degradation during extraction. On the other hand the results of transformation of *E. coli* HB101 competent cells were observed and given in figure (6). Figure (7) displayed the result of transformed *E. coli* HB101 In this figure, only 16 single colonies were counted, and the transformation efficiency was found to be sufficiently (16×10^5 cfu/g plasmid DNA) on LB / NA plates at 100µg/ml concentration.

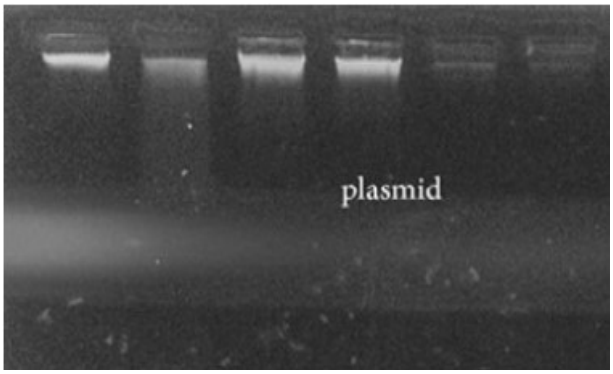

Figure 5: Plasmid DNA extraction from *S. aureus* isolates using 1% agarose gel electrophoresis.

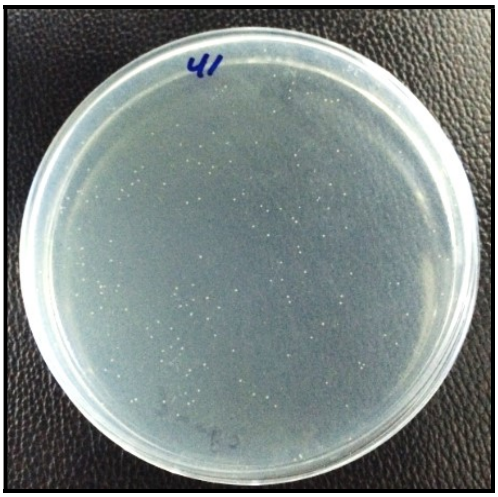

Figure 6: Transformed E. coli HB 101 competent cells on LB selectable medium.

Plasmid curing

Transformed *E. coli* HB101 was grown in 100 µg/ml, 150 µg/ml, 200 µg/ml and few single colonies were grown on a concentration of 250 µg/ml but no growth was observed in a concentration of 300 µg/ml. Figure (8) shows the result of plasmid curing where only few single colonies were appeared in LB medium supported by nalidixic acid with a concentration of 250 µg/ml.

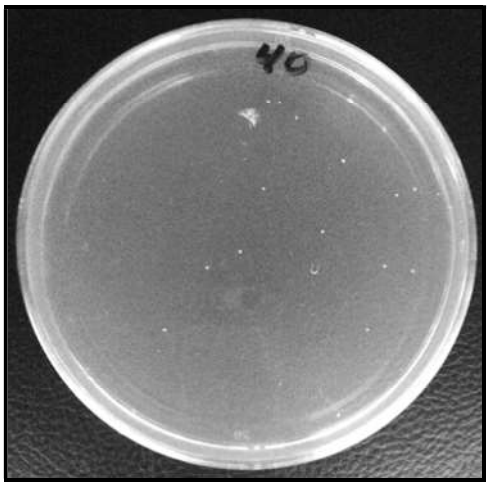

Figure 7: Single colony transformed E. coli HB 101 competent cells on 1: 100 LB selectable medium supported with nalidixic acid for determination the efficiency of transformation.

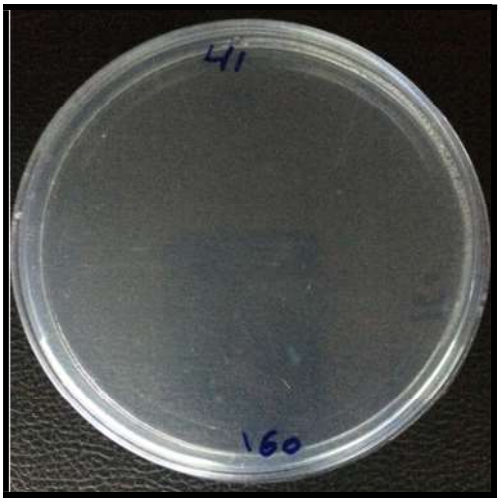

Figure 8: Few single colony was appeared in LB medium supported with nalidixic Acid with concentration 250 µg/ml.

Discussion

The antibiotic-resistance of *S. aureus* strains is another serious concern beside the pathogenicity. Strains of *S. aureus* have been observed to show resistance against

multiple antimicrobials (18,19). The antimicrobial results showed the absolute sensitivity of *S. aureus* isolates to norfloxacin, gentamycin, tobramycin, ciprofloxacin, clidamycin, kanamycin and streptomycin and resistance (100%) to methicillin, ampicillin, amoxillin/clavulanic acid, and nalidixic acid but intermediate (100%) to erythromycin. This result were consisted to the study of (20) concerning gentamicin, rifampin and tobramycin and nalidixic acid, and to (21) with respect to the resistance ampicillin. Also (22) was closely relate to the results of the present study who were reported (86.95%) sensitivity for amoxillin and gentamycin while (82.6%) for ampicillin and ciprofloxacin. Additionally the antimicrobial results of the present study were comparable to the results reported by (23) were (66.7%) sensitive to oxacillin and (33.3%) resistance to same antibiotic, and closely related which was sensitive (79%) to rifampin and vancomycin and (50.6%) resistance to tetracycline.

On the other hand, (24) disagreed with the present study that was resistant to gentamycin and streptomycin. The development of resistance to earlier antibiotics could be due to their indiscriminate usage (25). On the other hand plasmid borne genes associated with antibiotic resistance can easily be transferred from one strain to another or from one organism to another within the same environment this also played important role in the development of resistance to antibiotic (26).

The PCR methods were used accurate and precise techniques for the identification of bacteria (27). In the present study, *S. aureus* was identified by PCR assay using specific primers for housekeeping genes, such as the 23S rRNA region and various virulence factors genes including *coa*, *nuc* and *clf*A. All these target genes allowed a rapid identification of these species with high sensitivity and specificity.

The PCR method for identifying *S. aureus* isolates recovered from various origins were successfully applied other authors (15,20,28,29) using 23S rRNA gene as specific-specific target sequence.

In the present study, PCR products of the *coa* gene for 6 isolates (n=5 milk isolates and n=1 hand isolate) were displayed polymorphism size includes 910 bp; 710 bp, and 627 bp in 1, 2 and 2 isolates, respectively. this result agreed with (29) and (30) whereas, (31) found that the *coa* gene revealed polymorphic coagulase amplicon 480 bp, 640 bp and 840 bp. Analysis of coagulase-encoding *S. aureus* DNA (*coa*) genes has revealed a variable sequences in the 3-end coding region. This region contains polymorphic repeat regions which differ among *S. aureus* isolates and that can be used to identify *S. aureus* isolates (32,33). Also present study revealed that *nuc* gene and *clf*A gene were amplified in all 6 isolates with size of 280 bp and 1024, respectively. This result is complies with (30) in which *clf*A gene was detected in all isolates of *S. aureus* with the size 1024 bp while they found *nuc* gene in 97.29% of isolate with size 279 bp. However, (28) recorded that the amplification of *clf*A gene revealed polymorphisms size approximately 1000 bp and 890 bp while *nuc* gene presented in 279 bp. According to the PCR results, we concluded that all *S.aureus* species have 23s rRNA, but not all of them were identified as pathogenic strains. This finding can be explained by two possibilities. The first explanation suggest that the use of 23s rRNA in the diagnosis allows the discrimination between *S.aureus* strains according to the similarities in many phenotypic characteristic (34). The second explanation revealed that virulence factors investigated by using PCR are more related to the pathogenic *S.aureus* strains and play a role in the pathogenicity of mastitis (35).

In the present study the transformation of *E. coli* competent cells was investigated. The growth of *E. coli* HB101 on LB agar was supported with nalidixic acid as an indicator to their acquisition for plasmid DNA and became resistant to nalidixic acid. This result was considered an evidence that the gene was responsible for resistance of *S. aureus* to nalidixic acid carried on the plasmid. The results of the present study were similar to the results reported by (36) where the genes responsible for resistance to nalidixic acid and 12 other antibiotics were located on the plasmid DNA isolated from *E. coli* O157:H7.

In the present study the transformation efficiency was found to be sufficiently (16×10^5 cfu/µg plasmid DNA) on LB / NA plates at 100µg/ml concentration. This result was less than resulting of Sambrook and (37) who observed that the transformation efficiency was enhanced when using SOC medium. The transformation frequency differs from one type of bacteria to another. These differences may be due to the size and shape of transferred plasmid; small circle plasmids are transferred much more efficiently than large circle plasmids in addition to the fact that the purity of plasmid affects transformation (36). Concerning plasmid curing, the trials were conducted by using different concentrations of Nalidixic acid (100, 150, 200, 250 and 300 µg/ml) to determine the concentration of their antibiotics inhabited or killed transformed *E. coli*. Each concentration was added to LB medium and cultured transformed *E. coli* on their plates and incubation overnight at 37ºC to be observed if transformed *E. coli* was grown or not. The result of the present study demonstrated that transformed *E. coli* was grown in 100 µg/ml, 150 µg/ml, 200 µg/ml and few single colonies were grown on the concentration of 250 µg/ml but did not grow in the concentration of 300 µg/ml, this result was an indicator for unable transformed *E. coli* to grow in this concentration. On the other hand, this concentration had eliminated the plasmid from transformed bacteria and made it sensitive to nalidixic acid in this concentration or may be have killed it.

(38) used two plasmid curing agents sodium dodecyl sulphate and ethidium bromide on Pseudomonal isolates and they observed that no cured cells were obtained for all antibiotics used from treatment with 700 µg/ml ethidium bromide, whereas in the treatment done for three incubation times using SDS at a concentration of 1% (W/V) with different incubation periods as curing agent, the bacterial colonies that lost their antibiotics resistance appeared with different curing rates.

On the other hand (39) used three plasmid curing agents, acridine orange, ethidium bromide and sodium dodecyl sulfate on multi drug resistant *Escherichia coli* with three different concentrations of each curing agents. Consequently, (39) reported that only acridine orange treated cells could eliminate only plasmids of 2.7 MDa and another smaller than 2 MDa: While (40) used plasmid curing agents for proving the presence of gene that encodes resistance to copper in plasmid.

The loss of resistance after curing indicates the presence of the resistance character in the plasmid and such strains were unable to grow on medium supported with this antibiotic became sensitive. From the previous reports, plasmid curing can be used for detecting the presence of gene in plasmid or in chromosome and also for eliminating plasmid from the cells.

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