ORIGINAL ARTICLE

Plasmid Profile and Transformation Efficiency in *Staphylococcus aureus* isolated from different clinical specimens

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ABSTRACT

Staphylococcus aureus a common cause of many diseases related to human health. It colonizes the skin and mucosal surfaces, and the most consistent site from which this bacterium can be cultured in the anterior nares. A total of 261 patients' samples taken were received from the different hospitals in Erbil city from July 2019 to January2020. Using various methods for suspected *S. aureus* identifications. The antimicrobial susceptibility test results against 16 antimicrobials showed variable susceptibility patterns. The transformation efficiency was calculated for ten more resist isolates of *S. aureus*. Based on morphological, cultural, and biochemical tests, 93 isolates of *S. aureus* were isolated and identified from 261 samples (35.63%). Likewise, in molecular identification, PCR was used based on the 16S rRNA and *nuc* genes, and all 93 isolates were reidentified. The antimicrobial susceptibility test results against 16 antimicrobials showed variable susceptibility patterns. The highest resistance was recorded against oxacillin 98.96%, and the lowest was 0% for each of amikacin, gentamicin, and trimethoprim-sulfamethoxazole. To determine the location of responsible genes for antibiotics resistance in *S. aureus* isolate**s**, the genetic transformation process of laboratory *Escherichia coli*DH5α strain with purified plasmid DNA from *S. aureus* isolate**s**, was conducted successfully. Colonies of this process appear to be resistant to ampicillin, nalidixic acid, and penicillin, which indicates that the genes encoding these antibiotics resistance were located on plasmid DNA. The transformation efficiency was calculated for ten more resist isolates of *S. aureus,* and the results were ranged from more efficient ($1.0185*10^4$) in SA59 isolate to the low efficiency of transformation ($0.525*10^3$) for isolate SA69. On the other hand, the transformation frequency was revealed the various range and the transformation frequency for isolate SA59 was shown the highest frequency among all ten isolates ($2.7*10^{-4}$). In contrast, the lowest frequency was $1.4*10^{-5}$ for isolate SA69.
Keywords: *Staphylococcus aureus*, clinical specimens, plasmid profile.

INTRODUCTION

Staphylococcus aureus is an important pathogen both in the hospital and in the community. About 35-50% of normal adults carry it in the anterior nares; it is skin normal flora, and other sites of colonization include the perineum and axillae. It is still one of the five most common causes of nosocomial infections and is often the cause of post-surgical wound infections(Obajuluwa et al., 2015).*S. aureus* is considered a major pathogen of hospital infections for a long time(Tong et al., 2015, Asadpour and Ghazanfari, 2019). This bacterium is one of the most commonly diagnosed causes of infections of the skin and soft tissue, as well as systemic infections. The antibiotic resistance of *S. aureus* strains has become a global warning problem, failing to treat bacterial infections(dos Santos et al., 2018), which threatens the life of the patient and increases treatment costs(Leila et al., 2018, Alinaghi et al., 2011).

Mobile genetic elements (MGEs) play a central role in microbial evolution, serving as a mechanism by which genetic material can be transferred, disseminated, and rearranged, allowing for rapid adaptation to new and changing environments. Nowhere is this more apparent than in the global dissemination of genes encoding mechanisms of antimicrobial resistance and virulence in populations of clinically significant bacteria(Shintani et al., 2015, Hasan et al., 2017).

A plasmid, usually circular but sometimes linear, is a small double-stranded DNA unit, which is chromosome independent and is capable of self-replication. Each plasmid carries only a few genes, the plasmid's size ranges from 1 to more than 1000 kbp. Genes required for organism survival and those that are generally beneficial to the host organism, such as antibiotic resistance, are often found in plasmids(Al Doghaither and Gull, 2019).In plasmids, replication occurs at a specific site known as the origin of vegetative replication (*oriV*). Well-known replication systems of circular plasmids include theta-type replication, rolling-circle replication, and strand displacement-type replication (Shintani et al., 2015).

Gram-positive bacteria transfer plasmids by two methods, although the detailed mechanisms are not well understood. First, a single strand of plasmid DNA is transported via a T4SS, which seems to be widely used as a means for transferring plasmids in Gram-positive bacteria(Goessweiner-Mohr et al., 2013, Ghods et al., 2017). Plasmids perform numerous functions. For instance, the enhancement of organism survival may be found in genes containing genes responsible for killing other organisms or having defense mechanisms for the host through the production of toxins. Also, some plasmids may enable the bacterial replication process. The small size of plasmids limits the genes that can be found, which tend to have a particular function (in contrast to an abundant amount of noncoding DNA). A single cell may have multiple coexisting plasmids, each with various functions (Al Doghaither and Gull, 2019).

Plasmids are essential for the epidemiology and evolution of antimicrobial resistance in *S. aureus*. However, to study their distribution and relationship to the host cells,

a classification system is essential, and several classification systems have been established, divided plasmids into three classes: (i) small rolling-circle, (ii) multi-resistance, and (iii) conjugative. Since then, a fourth class (pSK639 family) has been proposed. Another system, based on incompatibility groups, identified 15 groups within the *Staphylococcus* genus(Lozano et al., 2012).

Moreover, a classification system that uses restriction fragment length polymorphism (RFLP) patterns were suggested for large plasmids. Recently, plasmid classification based on PCR amplification of conserved areas of the replication initiating genes (rep) from 103 plasmids belonging to different Gram-positive bacteria was described (Jensen et al., 2010). Plasmids carry a diverse range of antimicrobial and biocide resistance genes and can carry toxin genes(McCarthy and Lindsay, 2012, Zendehdel and Roham, 2019).

Ninety percent of *S. aureus* isolates carry at least one plasmid, and ~80% hold a plasmid >20 kb. Staphylococcal plasmids can be grouped by size and replication mechanism, ranging from small (<5 kb) and often cryptic rolling-circle-replication (RCR) plasmids to more abundant (8–50 kb) theta-replicating plasmids. Self-transmissible conjugative plasmids only represent around 5% of all sequenced *S. aureus* plasmids. Despite the low abundance of conjugative plasmids, phylogenomic comparisons indicate that plasmid exchange between diverse *S. aureus* lineages occurs (Kwong et al., 2017).

The most common mechanism of MGE in *S. aureus* is transduction because there is little evidence that transformation occurs, and conjugative plasmids or transposons are not widespread in *S. aureus*. Either many transduction experiments have been conducted intending to test the transduction ability of staphylococcal phages or prove the mobility of variable genetic elements with genes encoding antibiotic resistance or toxins (Malachowa and DeLeo, 2010).

Staphylococcal plasmids can be classified into one of the three following groups: (1) small multicopy plasmids that are cryptic or carry a single resistance determinant; (2) larger (15–30 kb) soft copy (4–6/cell) plasmids, which usually carry several resistance determinants; and (3) conjugative multiresistance plasmids (Lindsay, 2010). Larger plasmids undergo theta replication (a DNA replication mechanism that resembles the Greek letter theta), whereas small plasmids usually replicate by the rolling-circle mechanism (Khan, 2005).

HGT or lateral gene transfer (LGT) is the movement of genetic material between unicellular organisms and multicellular organisms other than by the vertical transmission of DNA from parent to offspring(Julie, 2011). HGT is an essential factor in the evolution of many microorganisms(Gyles and Boerlin, 2014). MGE is the primary mechanism for the spread of antibiotic resistance in bacteria. It plays a vital role in the evolution of bacteria that can degrade novel compounds such as human-created pesticides and in the evolution, maintenance, and transmission of virulence(Keen, 2012). Genes responsible for antibiotic resistance in one species of bacteria can be transferred to another species of bacteria through various mechanisms of HGT, such as transformation, transduction, and conjugation, subsequently arming the antibiotic-resistant genes' recipient against antibiotics. The rapid spread of antibiotic resistance genes in this manner is becoming medically challenging to deal with(Cairns et al., 2018, Soltany et al., 2020).

Bacterial transformation is a process of HGT by which some bacteria take up foreign genetic material (naked DNA) from the environment. The process of gene transfer by transformation does not require a living donor cell but only requires the presence of persistent DNA in the environment. The prerequisite for bacteria to transform is its ability to take up free, extracellular genetic material. Such bacteria are termed as competent cells. The factors that regulate natural competence vary between various genera. Once the transforming factor (DNA) enters the cytoplasm, it may be degraded by nucleases if it is different from the bacterial DNA. If the exogenous genetic material is similar to bacterial DNA, it may integrate into the chromosome. Sometimes the exogenous genetic material may coexist as a plasmid with chromosomal DNA. The phenomenon of natural transformation has enabled bacterial populations to overcome significant fluctuations in population dynamics and overcome the challenge of maintaining the population numbers during harsh and extreme environmental changes. During such conditions, some bacterial genera spontaneously release DNA from the cells into the environment free to be taken up by the competent cells. The competent cells also respond to the changes in the environment and control the level of gene acquisition through natural transformation process (Green and Sambrook, 2012).

The natural state for many bacteria is not growth in liquid culture but rather, living as a community attached to a surface. These bacterial communities, termed biofilms, exist in the natural world as well as in the human host. The Centers for Disease Control and the National Institutes of Health have estimated that approximately 65-80% of human infections are biofilm-related. A recent burgeoning area of research has examined the role of plasmids in biofilms, including the effect of conjugative plasmid transfer on biofilm formation as well as the role of biofilms in plasmid dissemination (Cook and Dunny, 2014).

The role of plasmids in biofilm formation and stabilization, the typical plasmid genome can be divided into backbone and accessory regions. The traits encoded in the backbone include replication, partition, stability, and mobilization functions and can be thought of as the essentials of a functionally stable minimal plasmid entity. Genes that encode functions, which enhance the fitness of the plasmid's host under a given selective pressure, are typically described as accessory genes(Smillie et al., 2010). Examples of functions of accessory genes include resistance towards antibiotics, metals, bacteriocins, metabolic functions, and attachment to specific surfaces, to name a few (Norman et al., 2009). Biofilm-associated factors (BAFs) can be encoded both by genes in the backbone and the accessory regions of plasmids. This indicates that biofilm formation may be of importance for some plasmids. This is especially true for the BAFs encoded by the backbone genes (e.g., conjugal pili), as such genes are well-integrated parts of the plasmid's biology.Although only a few BAFs of plasmids have been

studied in detail, we will give a few examples to illustrate the variety of factors that can be involved. As the understanding of the interconnectedness between biofilms and plasmids is further explored, many more examples will, no doubt, be uncovered (Madsen et al., 2012).

This study was aimed at determining the plasmid profile of *S. aureus* isolates from different clinical specimens and their relationship to biofilm formation.

MATERIALS AND METHODS

Clinical specimens of *S. aureus*: Ninety-threeisolates of*S. aureus* were identified based on biochemicals, VITEK II system compact and molecular approach among 261different clinical specimens collected over seven months from patients submitted to the various hospitals in Erbil city, and this was also done after transferring and cultivating daily all samples in the brain heart infusion broth (BHI), which was then incubated for 24-48 hrs. with shaking at 350×*g*. After that, positive growthwas cultured and identified by biochemical tests, which included catalase, tube coagulase, DNase, lecithinase, protease, oxidase, urease, tellurite reduction, and the identity of the isolates has been confirmed through a PCR based on identification 16SrRNA and*nuc*genes. The sequence of the primers with the PCR setup can be found in Table 1.

Table 1.The oligonucleotide sequencesused for PCR amplification of both 16S rRNAand *nuc* genes.

Genes	Primers detail			References
	Primer Sequence (5´ – 3´) (Oligonucleotide)	Amplicon size (bp)	Cycling program	
16S rRNA	CACCTTCCGATACGGCTACC GTTGACTGCCGGTGACAAAC	372	95°C–30 s; 59°C–45 s; 72°C–1 min; 35 cycles	(Hamasalih and Abdulrahman, 2019)
nuc	AGCCAAGCCTTGACGAACTAAAGC GCGATTGATGGTGATACGGTT	279	94°C–30 s; 53°C–30 s; 72°C–1 min; 35 cycles	

Antimicrobial susceptibility screening: Antimicrobial sensitivity testing was performed by disk diffusion method according to the CLSI (CLSI, 2017), against the following antimicrobials; Amikacin AK 30 µg, Azithromycin AZM 15 µg, Ciprofloxacin CIP 5 µg, Clindamycin CD 2 µg, Erythromycin E 15 µg, Gentamicin G 10 µg, Levofloxacin LEV 5 µg, Netilmicin NET 30 µg, Nitrofurantoin NIT 300 µg, Norfloxacin NOR 10 µg, Oxacillin OX 1 µg, Penicillin P 10 U, Tetracycline TE 30 µg, Tobramycin TOB 10 µg, Trimethoprim+Sulfamethoxazole SXT 1.25+23.75 µg, and Vancomycin VA 30 µg (Bioanalyse, Turkey). A lawn of test *S. aureus* was prepared by evenly spreading 100 µL inoculums was adjusted to ($5*10^5$ CFU/ml) at $OD_{600\ nm}$(Yasir et al., 2019).

Genomic DNA Extraction: Genomic DNA was extracted from pure cultures using the Presto™ Mini gDNA Bacteria Kitfollowing the manufacturer's instructions; 100 µL elution buffer was used for extracting. Extracted genomics was stored at –20°C before running PCR. The NanoDrop 1000 spectrophotometer was used to evaluate the concentration and purity of DNA in which one µL of the DNA genome was used to define the concentration and purity of DNA samples at 260nm.

Amplification of 16S rRNA**and** *nuc***genes:** PCR amplification was carried out to detect the genes with a final volume of one µL of elution buffer in 25 µL. After the addition of 12.5 µL of 2× Master mix, then one µL of each primer added into the tube, and the volume completed with free nuclease water at 25 µL. DNA fragments were analyzed by gel electrophoresis after PCR amplification. Ten µL of the amplification products were subjected to electrophoresis using 1.2 percent agarose gel with a safe GelRed DNA stain and 100 bp DNA ladder used as the standard size.

Transformation profile of plasmid DNA of *S. aureus* isolates

Experimental protocol for plasmid extraction: AccuPrep® Plasmid Mini Extraction kit was used for plasmid DNA extraction from more ten resist isolates of *S. aureus*.

Bacterial cell preparation: A single colony from a fresh cultured LB agar plate was picked up and inoculated the cell into the 1-5 mL of new LB liquid media at 37℃with shaking for 12-16 hrs.The bacterialcells were collected by centrifugation at >8000 ×*g* for 2 min. Also, altogether removed from the media by pipetting.

Cleared lysate preparation: The pellet completely was resuspended by adding 250 µL of Buffer ① by vortexing.250 µL of Buffer ② was added, mixed by inverting 3-4 times gently.350 µL of Buffer ③was added and immediately mixed by inverting the tube 3-4 times, kindly.The tube was centrifuged at 16000 ×*g*, 4℃ for 10 min. in a microcentrifuge.

Plasmid DNA purification: The cleared lysate was transferred to the DNA binding column tube and centrifuged at 13000 ×*g* for 1 min. The flow-through was poured off and re-assembled the DNA binding filter column with the 2.0 ml collection tube.Seven hundred µL of Buffer ④was added to the DNA binding column tube and centrifuged at 13000 ×*g* for 1 min. The flow-through was poured off and re-assembled the DNA binding filter column with the 2.0 mL collection tube. The DNA binding filter column was dried by additional centrifugation at 13000×*g* for 1 min. to remove the residual ethanol.The DNA binding filter column was transferred to the new 1.5 ml microcentrifuge tube.One hundred µL of Buffer ⑤was added to the DNA binding filter column and wait for at least 1 min. for elution.The plasmid DNA was eluted by centrifugation at 13000 ×*g* for 1 min(Macnamara, 2008).

Genetic transformation: To determine the location of the antibiotic resistance genes in *S. aureus*, the purified plasmid from *S. aureus* was transformed into another sensitive strain of *E. coli* DH5α (*E. coli* DH5α was a bacterial strain that does not contain plasmid used in this study).

Preparation of competent cells: A single colony of *E. coli* DH5α (16-20 hrs. old) was picked up and transferred the colony into 10 ml SOB medium. The culture was incubated for 3 hrs. at 37°C with vigorous agitation, monitoring the

growth of the culture. As a guideline, 1 OD_{600nm} of culture contains approximately $1*10^9$ bacterial cells/mL.The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml tubes. The cultures were cooled to 0°C by storing the tubes on ice for 10 minutes. The cells were recovered by centrifugation at 7500 ×*g* for 10 min. at 4°C.The medium was decanted from the cell pellets. The tubes were stand in an inverted position on a pad of paper towels for 1 min. to allow the last traces of media to drain away.Each pellet was resuspended by swirling or gentle vortexing in 30 mL of ice-cold $MgCl_2$-$CaCl_2$ solution.The cells were recovered by centrifugation at 7500 ×*g* for 10 min. at 4°C.The medium was decanted from the cell pellets. The pellet was resuspended by swirling or gentle vortexing in 2 mL of ice-cold 0.1 M $CaCl_2$ for each 50 mL of the original culture.At this point, either use the cells directly for transformation as described below or dispense into aliquots and freeze at -70°C. Include all of the appropriate positive and negative controls (Green and Sambrook, 2012).

DNA uptake: To transform the $CaCl_2$-treated cells directly, 200 µL of each suspension of competent cells was transferred to a sterile, chilled tube using a chilled micropipette tip. DNA (no more than 50 ng/10 µL) was added to each tube. The contents of the tubes were mixed by swirling gently. The tubes were stored on ice for 30 min.The tubes were transferred to a rack placed in a preheated 42°C water bath and kept the tubes in the stand for exactly 90 sec. Do not shake the tubes.The tubes transferred rapidly to an ice bath. The cells were allowed to chill for 1-2 min.Eight hundred µL of SOC medium was added to each tube. The cultures were incubated for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.Two hundred µl of transformed competent cells were transferred onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.The plates were inverted and incubated at 37°C. Transformed colonies should appear in 12-16 hrs.(Green and Sambrook, 2012).

The genetic transformation frequency and efficiency were calculated according to the Equation (3.1 and 3.2) described by Flynn et al. (2002) and Equation (3.3 and 3.4) byTu et al. (2005):

$$\text{Transformation frequency} = (\text{No. of transformants}) \times (1\text{ml} \div \text{plated volume}) \times (1 \div \text{viable cells}) \ldots\ldots\ldots\ldots\ldots 3.1$$

$$\text{Viable cells} = (\text{No. of colonies}) \times (1\text{ml} \div \text{plated volume}) \times (\text{dilution factor}) \ldots\ldots\ldots\ldots\ldots 3.2$$

$$\text{Transformation efficiency} = \frac{\text{Transformant CFU}}{\text{plasmid DNA (µg)}} \ldots\ldots\ldots\ldots\ldots\ldots 3.3$$

$$\text{Transformant cfu} = \frac{\text{No. of bacterial colonies} \times \text{dilution ratio} \times \text{original transformation volume}}{\text{plated volume}} \ldots\ldots\ldots\ldots 3.4$$

Assessment of biofilm formation using polystyrene microtiter plate assay: Biofilm presence and activity were assessed with colorimetric methods based on crystal violet. Briefly, each strain was grown overnight in bottles containing the BHI for bacteria, at 37ºC in an orbital shaker at 150–180 ×*g*and then harvested and centrifuged at 3000×*g* for 5 min at 4ºC. The supernatant was removed, and the pellet was washed twice with Phosphate Buffered Saline (PBS). The washed cells were then resuspended in a TSB (Tryptic Soy Broth) to obtain a final density of $1.0*10^6$CFU/mL, by adjusting the density after spectrophotometrical readings at OD_{600nm} and the calculation with the regression equation of the species-specific curves. One hundred µL of this standardized cell suspensions were seeded in each selected well of a 96-well microtiter plate; one unseeded well-acted as the negative background control for the subsequent steps. Three different replicates for each strain were set into each plate. These microtiter plates were then closed, sealed, and incubated for two hrs. at 37ºC. After biofilm surface priming, the medium in each well was removed carefully with a multichannel pipette, taking care not to disrupt the biofilm; each well was subsequently washed three times with PBS. At this stage, it was not possible to spectrophotometrically evaluate the biofilm formation because its optical density was under the detection limit of the plate reader. After these washing steps, 100 µL of the appropriate medium was added to the wells. Each plate was then closed, sealed again and incubated for 24 h at 37ºC to permit the biofilm development. The plates were subsequently recovered, and the same washing procedure described above was applied. The plates were stained with 1% solution of crystal violet (100 µL each well) for 15 min; they were then washed three times with water and dried at room temperature, and the absorbance of adherent biofilm cells was measured with an ELISA plate reader (BioTek ELx800, USA) at 570 nm. Each strain was tested for biofilm production in triplicates, and the assay was repeated three times (Corte et al., 2019). Based on the absorbance of crystal violet stain linked to the adhered cells, biofilms formed by various strains have been analyzed and categorized (**Table 2**)(Hamasalih and Abdulrahman, 2019).

Table 2.Classification of biofilm formation abilities by Microtiter plate method

Cut-off value calculation	Mean of OD_{570} values results	Biofilm formation abilities
ODnc > 4×ODC	OD > 0.557	Strong
2×ODC < ODnc ≤4×ODC	0.278< OD ≤ 0.557	Moderate
ODC< ODnc ≤ 2×ODC	0.139< OD ≤ 0.278	Weak
OD ≤ 0.139	OD ≤ 0.139	None

RESULTS AND DISCUSSION

Isolation and Characterization of S. aureus: Based on morphological features, cultural characteristics, biochemical tests, and molecular approach isolates from 261different clinical specimens, 93 isolates (35.63%) were identified as *S. aureus*. The strains were from samples belonging to urine, wound, blood, and burn, and the distribution of each source was clarified in **Table (3)**.

Table 3. The types and numbers of different clinical specimens used in the analysis (*n* = 261)

Sources of specimens	Total no. (%) of samples	Positive no. (%) of samples
Urine	32 (12.26)	14 (15.05)
Wound	96 (36.78)	23 (24.73)
Blood	21 (8.05)	9 (9.68)
Burn	112 (42.91)	47 (50.54)
Total	261 (100)	93 (35.63)

To endorse the identification of *S. aureus* isolates by a conventional method, the VITEK II system was conducted,andthis system reidentified all strains as *S. aureus* (**Table 4**).The present results of the VITEK II system show that all isolates were identified as *conventional methods confirmed S. aureus*. To further confirm the identity of the *S. aureus* isolates, all *S. aureus* were examined for the presence of the 16S rRNA and *nuc* genes to characterize and validate the staphylococcal species. All of the strains were confirmed as *S. aureus* by the occurrence of 16S rRNA and *nuc* genes (**Figure 1**).

Table 4. Cultural, biochemical characteristics, VITEK II system, and molecular technique of *S. aureus* identification results in different clinical specimens.

Identification aspects	Positive results	
	n.	(%)
Coagulase	93	100
Catalase	93	100
Oxidase	0	0
DNase	93	100
Tellurite reduction (Black colonies)	93	100
Shiny colonies	93	100
Lipase activity (Clear zone)	64	68.81
Lecithinase production (Opaque of precipitation)	74	79.56
VITEK II System	93	100
16S rRNA gene	93	100
nuc gene	93	100

Figure1A. Agarose gel electrophoresis of PCR amplification products for the 16S rRNA gene of *S. aureus*. M: The DNA marker (100 bp ladder), lanes 1-16positive amplification of 372 bp for 16S rRNA gene.

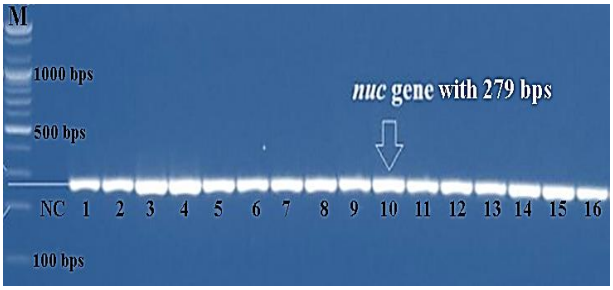

Figure1B. Agarose gel electrophoresis of PCR amplification products for the *nuc* gene of *S. aureus*. M: The DNA marker (100 bp ladder), lane NC: *negative control*, lanes (1-16) positive amplification of 279 bp for *nuc* gene.

The mostcomprehensiveroutinetest is the coagulase test, which is performed by TCT. The essential component of plasma known as coagulase reacting factor (CRF), which binds to the coagulase in *Staphylococcus* (SC) and forms SC–CRF complex, and the result represents the basis for a test of coagulase which converts the fibrinogen to fibrin and leads to clot formation. Rabbit plasma has been considered as the first fitting plasma for coagulase testing with a CRF ideal concentration (Manukumar and Umesha, 2017). All 93isolates of *S. aureus*were branded as Gram-positive; other cultural characteristics and biochemical test results are stated in Table 3. Subramanian et al. (2017)reported that among the 281 isolates of *S. aureus* that were positive for *femA* gene (*femA* gene was used as a marker gene test for identification of *S. aureus*), 268 isolates (95.37%) and 277 isolates (98.58%) revealed clot creation in the TCT using HP and RP, respectively.

Another famous test used for *S. aureus* identification is the DNase test. In the current study, all *S. aureus* isolates were positive for DNase activity. Similar results have been reported previously with no further clarification of the causes for these findings that require further investigation identical to the current study. Also, there might be technical difficulties with regards to interpretation in the case of frail positive reactions. In this way, for diagnosis of *S. aureus*, the DNase test incapable of being used as an independent test, which was evident with low concordance rate with PCR, among other individual tests. Furthermore, in the presentstudy, 74 and 64% of *S. aureus*isolates secrete lecithinase and lipase enzymes, respectively. Our finding agrees with results presented by Karmakar et al. (2016) in which 81%, 51%, and 48% of *S. aureus* strains were reported to have a lecithinase, lipase, and nonwhite pigmented colonies, respectively.

All isolates of *S. aureus* were undergone PCR using universally preserved 16S rRNA and *nuc* genes, which were explicitly designed to discriminate against *S. aureus* isolates. The PCR successfully amplified the part–size DNA fragments of 16S rRNA and *nuc* genes. In the results of Karmakar et al. (2016), among 100 isolates of *S. aureus* were subjected to detection, the *nuc* and *mecA* genes found that all isolates confirmed by PCR. Khan et al. (2007)identified 17 isolates of *S. aureus* by morphological and biochemical characteristics; also, these strains were further characterized by amplification of the *nuc* gene.

Susceptibility Pattern of *S. aureus* against Different antimicrobials: The antimicrobial sensitivity test results for

all *S. aureus* have been demonstrated against 16 antimicrobials in **Table 5**. The highest percentage of resistance was against oxacillin 95.7%), followed by penicillin (86.02%). However, each of gentamycin and netilmicin was recorded the lowest resistant at8.6 percent.

All isolates showed significantly different resistance patterns to 16 different antimicrobials. Antimicrobial susceptibility carried out for 16 antimicrobials through disc diffusion methods. All of the isolates showed resistance to OX (*n=89*), while the remaining isolates showed a range of susceptibility patterns. Reported that 26.44% of 121 *S. aureus* isolates resist against each of tobramycin, kanamycin, amikacin, and gentamycin, 47.93%, 20.65%, and 3.3% of *S. aureus*isolates were resistant to tetracycline, doxycycline, and minocycline, respectively. Based on the antimicrobial susceptibility reports byBoada et al. (2018) among the 765 assessed *S. aureus* isolates, the maximum resistance rates were observed to penicillin, followed by azithromycin, erythromycin, and clindamycin, with resistance percentages of 87.1%, 11.6%, 11.2%, and 9.7%, respectively. A total of 1.3% of strains were resistant to oxacillin; 83 isolates were not resistant to the tested antibiotics (10.8%). The basis of the resistance mechanism to oxacillin and methicillin is a gene encoding a PBP2 homolog called PBP2a or PBP2' which is resisting to drug action. This is due to the active TP of the PBP2a site of serine that is not accessible to β–lactams. In this way, the enzymes can take over peptidoglycan biosynthesis ifthe housekeeping PBP2 TP site is inactivated. Despite the fact, the activity of PBP2 transglycosylase is vital for the biosynthesis of peptidoglycan because the moiety of PBP2a is not working. Thus, the biosynthesis of peptidoglycan is, therefore, a collaborative effort between the two proteins in the inactivation of the PBP2 TP site(Foster, 2017).

Table 5. Resistant percent of *S. aureus*isolates toward antimicrobials.

Antimicrobials	Resistance *n.* (%)	Intermediate *n.* (%)	Sensitivity *n.* (%)
AK*	9 (9.68)	3 (3.23)	81 (87.10)
AZM	52 (55.91)	9 (9.68)	32 (34.41)
CIP	17 (18.28)	3 (3.23)	73 (78.49)
CD	39 (41.94)	19 (20.43)	35 (37.63)
E	69 (74.19)	6 (6.45)	18 (19.35)
G	8 (8.60)	2 (2.15)	83 (89.25)
LEV	19 (20.43)	4 (4.30)	70 (75.27)
NET	8 (8.60)	11 (11.83)	74 (79.57)
NIT	37 (39.78)	11 (11.83)	45 (48.39)
NOR	31 (33.33)	20 (21.51)	42 (45.16)
OX	89 (95.70)	1 (1.08)	3 (3.23)
P	80 (86.02)	3 (3.23)	10 (10.75)
TE	39 (41.94)	13 (13.98)	41 (44.09)
TOB	42 (45.16)	9 (9.68)	42 (45.16)
SXT	22 (23.66)	8 (8.60)	63 (67.74)
VA	20 (21.51)	11 (11.83)	62 (66.67)

*: AK: Amikacin, AZM: Azithromycin, CIP: Ciprofloxacin, CD: Clindamycin, E: Erythromycin, G: Gentamicin, LEV: Levofloxacin, NET: Netilmicin, NIT: Nitrofurantoin, NOR: Norfloxacin, OX: Oxacillin, P: Penicillin, TE: Tetracycline, TOB: Tobramycin, SXT: Trimethoprim+Sulfamethoxazole, and VA: Vancomycin.

Genetic Transformation in *S. aureus:* A genetic transformation experiment was performed to determine the site of genes that responsible for antibiotic resistance in *S. aureus*. The method of Tu et al. (2005) was followed by extract plasmid DNA from *S. aureus* presenting more resistance to antibiotics understudy, as shown in **Table (6)**. The plasmid DNA content of *S. aureus* was plated on nutrient agar to ensure either it is contaminated with *S. aureus* or not, after incubation time, there was no growth obtained, this means that the extracted plasmid DNA did not contaminate during the extraction of plasmid DNA.

Table 6.No. of transformation colonies, transformation frequencies value, the transformation efficiency of more resistant isolates of *S. aureus*.

Isolates	Biofilm status through MTP assay	Number of bacterial colonies	No. of transformant colonies tested	No. of transformant colonies on nutrient agar with different antibiotics (µg/mL)			Transformation frequency	Transformation efficiency (Transformants/µg plasmid DNA)
				Amp	NA	P		
SA38	Strong	157	50	S	34	42	$2.75*10^{-4}$	$1.6485*10^{4}$
SA40	Strong	113	50	27	37	33	$8.69*10^{-4}$	$1.1865*10^{4}$
SA55	Moderate	122	50	S	4	S	$5.545*10^{-4}$	$1.281*10^{4}$
SA59	Strong	197	50	S	5	34	$2*10^{-4}$	$2.0685*10^{4}$
SA64	Strong	161	50	20	37	3	$2.639*10^{-4}$	$1.6905*10^{4}$
SA69	Strong	57	50	42	S	15	$1.14*10^{-3}$	$0.5985*10^{4}$
SA70	Moderate	53	50	30	32	S	$1.514*10^{-4}$	$0.5565*10^{4}$
SA73	Strong	67	50	29	9	S	$8.815*10^{-5}$	$0.7035*10^{4}$
SA92	Strong	91	50	S	31	42	$1*10^{-3}$	$0.9555*10^{4}$
SA94	Strong	111	50	30	36	42	$1*10^{-3}$	$1.1655*10^{4}$

Figure (2) shows the plasmid profile of transformant cells of *E. coli* DH5α strain after transformation with purified plasmid DNA from the ten isolates of *S. aureus* (38, 40, 55, 59, 64, 69, 70, 73, 92, and 94) and appeared that these plasmids approximately the same molecular weight identically that the antibiotic-resistant genes of *S. aureus* had been transferred successfully to *E. coli* DH5α strain.

Plasmid transfer among bacteria provides a means for the dissemination of resistance to multiple antibiotics. Resistance to various antibiotics is frequently found with *S. aureus* clinical isolates and is often plasmid-mediated. To transform, bacteria need to develop a specific physiological state called genetic competence. Competence is achieved through the regulated expression of the genes encoding the DNA uptake machinery (Thomas and Nielsen, 2005). In general, Gram-positive DNA uptake machinery is formed by a pseudopilus (*ComG* proteins) that brings extracellular DNA to the cytoplasmic transport machinery, a DNA-binding protein (the receptor *ComEA*) and a channel (*ComEC*). Only a single strand enters the cytosol, while the

complementary strand is degraded by an endonuclease (Cafini et al., 2017).

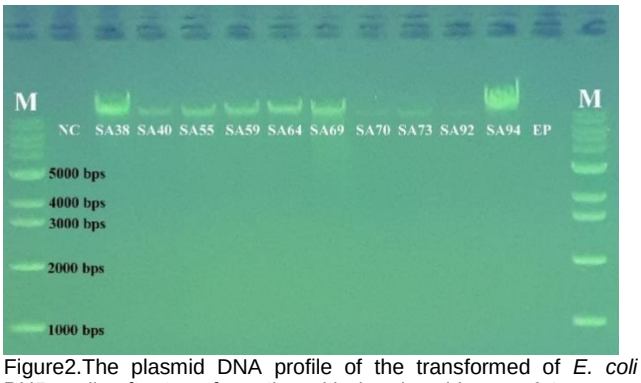

Figure2.The plasmid DNA profile of the transformed of *E. coli* DH5α cells after transformation with the plasmid DNA of *S. aureus* isolates. Lane M: DNA ladder 1000 bps; lane NC: negative control; lanes (SA38, SA40, SA55, SA59, SA64, SA69, SA70, SA73, SA92and SA94); lane EP: *E. coli* DH5α plasmid extraction.

Competence development is a species-specific process that requires particular environmental conditions. These conditions include nutrient access, starvation, altered growth conditions, and cell density(Thomas and Nielsen, 2005). Under these growth conditions, wild-type strains (N315 and it is derivative) show low transformation frequencies ($<10^{-11}$), partly attributed to the subpopulation limited *SigH* expression. Overexpression of *SigH* increases the transformation frequencies up to $\sim10^{-9}$ when purified plasmid DNA is used as donor and to $\sim10^{-7}$ if living *S. aureus* COL cells carrying pT181 are used. Moreover, there seem to be more excellent growth conditions that facilitate transformation. Importantly, antimicrobial agents also affect the transformation efficiencies in the *SigH*-expressing cells(Romero and Morikawa, 2016).

The process of genetic transformation depends on the ability of lab *E. coli* DH5α strain which has known genotype to be transformed from sensitive strain to resist one for some genotypic traits after treating with $CaCl_2$ and exposing to the prepared plasmid DNA from *S. aureus* isolates using heat shock, consequently, gaining of the *E. coli* DH5α strain with new genetic traits primarily exist on plasmid DNA of donor bacteria; thus, they assist us in determining the genetic sites that endow resistance to antibiotics.

For this purpose, 50 ng/10 µL of the prepared plasmid DNA was added to laboratory *E. coli* DH5α. **Table (6)** shows that *E. coli* DH5αcould receive purified plasmid DNA from *S. aureus*, and transformed successfully; also the Table indicates that the number of purified transformant colonies produced by the plasmid DNA were less than those isolated from, where the number of transformant colonies obtained was 157, 113, 122, 197, 161, 57, 53, 67, 91, and 111 colonies for SA38, SA40, SA55, SA59, SA64, SA69, SA70, SA73, SA92, and SA94, respectively.

From **Table (6)**, also we can notice that these *E. coli* DH5α transformant colonies, when grown in the presence of these antibiotics, show variation in their number of survivals. For example, fifty colonies appear after transformation with the plasmid DNA of SA38; the number declined to 34 colonies for nalidixic acid and 42 colonies for penicillin. On the other hand, the transformant colonies produced by the plasmid DNA already purified from all isolates failed to grow on nutrient agar with ampicillin. This finding may be related to that the genes which are responsible for a given resistance to this antibiotic, either located on a chromosome or large plasmid DNA, cannot enter *E. coli* DH5α acting as a recipient(Abdulrahman, 2005).

The value of transformation frequency also exhibits variation among isolates, as shown in **Table (6)**. The value of transformation frequencies of *E. coli* DH5αb by prepared plasmid DNA from *S. aureus* isolates was generally low because the normal values of transformation frequencies are 10^{-3} to 10^{-4}(Al-Saffawi, 2001) but still, transformation occurs successfully.

The transformation frequencies value obtained from our study was within a normal range, and near to results ofAl-Saffawi (2001), that was 10^{-4} for *S. aureus*, while higher when compared with that reported by (Abdulrahman, 2005) in which the value of transformation frequency for *S. aureus* was 10^{-6}.

Transformation efficiency is critical in molecular cloning experiments and can be affected by many factors. The most important being that the bacterial cells must in their early logarithmic growth period. Bacteria that can take up DNA are called "competent," and competency can be induced by treatment with calcium chloride in the initial log phase of growth. The bacterial cell membrane is permeable to chloride ions but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA; the exact mechanism of this uptake is unknown. A second factor, which can have an impact on the transformation efficiency, is that the competent cells must be maintained in a cold environment, both during storage and in use. Another critical factor is the concentration of $CaCl_2$. Although 50-100 mM calcium chloride can be used, 75 mM $CaCl_2$ in TB solution was found to be the optimum concentration(Tu et al., 2005).

The bacterial culture medium can also affect transformation efficiency(Fierro et al., 2004)and (Maeda et al., 2004)suggested the SOC medium for the growth of bacteria for the preparation of competent cells. SOC is a more vibrant medium than LB medium, which therefore results in faster growth of bacteria; not only can transformants be observed sooner in SOC medium after 12 hrs. as opposed to 24 hrs. in LB medium, but the transformation efficiency is much higher; S.O.C. giving 10-30 times higher efficiency than LB.

Biofilm formation: Thirty-one isolates (33.33%) gave a strong positive result for the biofilm production test (**Figure3**). The strong biofilm formation ability was highly related to*S. aureus*isolates, followed by weak biofilm formation with 23 isolates (24.73%).

Khan et al. (2011) assessed for the biofilm formation among 262 clinical isolates of S. aureus by tissue culture plate method, and they found that 38 (14.51%) isolates were strongly positive for biofilm production, 132 (50.38%) were moderate biofilm producers whereas 92 (35.11%) were negative for biofilm formation. The TCP method was used by

Manandhar et al. (2018) to assess biofilm production using three variations in media. In TCP with TSB only, 21 (5.6%) isolates with 4 (2.5%) *S. aureus* showed strong biofilm production. An addition of 1% glucose to TSB medium increased biofilm detection in 83 (22.1%), comprising 48 (19.8%) *S. aureus*. In BHI, incorporated with 2% sucrose, also increased biofilm detection, including 41 (25.5%) *S. aureus*.

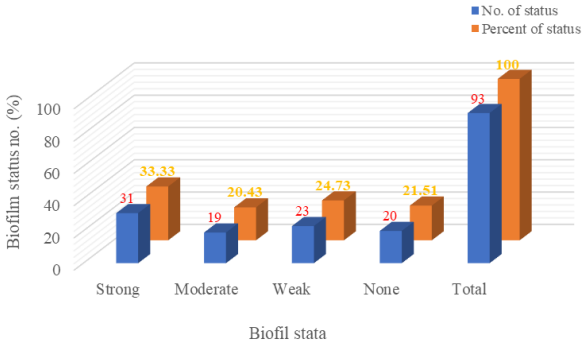

Fig.3. Screening of *S. aureus* isolates from biofilm production by MPM assay

The pool of extracellularDNA found in bacterial biofilms provides a richsubstrate for naturally occurring genetic transformation,which is the only alternative to mobile genetic elementsand bacteriophage induced gene transfer. This observation led to investigations into the role ofDNA donor cells in biofilms, and the conclusion that biofilm cells actively donate DNA to their prokaryotic neighbors. Soon after, experiments with *P. aeruginosa* showedthat very closely linked chromosomal markers could beco-transferred by *P. aeruginosa* extracellular DNA at afrequency of 10^{-7}–10^{-8}. Althoughthese results were obtained under artificial laboratory conditions,these findings implied that transformation througheDNA could also occur in natural environments. ExtracellularDNA active in the natural transformation was shown to bereleased by both Gram-positive and Gram-negative membersof soil bacteria, thereby facilitating naturally occurringgenetic transformation. Natural habitatssuitable for horizontal gene transfer are not limited to the soil.The eDNA accompanies the majority of bacterial populations on earth, andit is known that such eDNA is suitable for horizontal genetransfer (Kaneko and Itaya, 2010). Today, it is well establishedthat gene transfer occurs with enhanced efficiency in biofilms (Okshevsky and Meyer, 2015).

Competence has been closely linked to the release of eDNA and subsequent formation of biofilms on the genetic level. For example, a putative murein hydrolase, choline-binding protein D (CbpD), was identified in one study on *S. pneumoniae* as a key component of competence-induced cell lysis, which, along with DNA release, is strongly attenuated in a *cbpD* mutant (Kausmally et al., 2005).Induction of the competent state of *S. pneumoniae* triggered lysis of a subfraction of the bacterial population, resulting in the release of DNA. DNA is spontaneously released into the medium in a process initiated by competence induction for genetic transformation and which is dependent on the autolysin genes *lytA* and *lytC*(Moscoso and Claverys, 2004). This competence-induced DNA release is an integral part of natural genetic transformation that has evolved to increase the efficiency of gene transfer between pneumococci. In the closely related *S. mutans*, the competence associated genes *comA*, *comB*, *comC*, *comD* and *comE* be necessary for biofilm formation (Li et al., 2002), and a link between competence, eDNA release and biofilm formation has been demonstrated in various other streptococcal species(Petersen et al., 2004). In mixed-species biofilms, DNA release and competence in streptococci are triggered by the interspecies antagonism (Kreth et al., 2008).

CONCLUSION

We believe that to understand how virulence mechanisms such as biofilm formation are established. To achieve the goal, as mentioned earlier, we must gain a better understanding of the molecular mechanisms involved. HGT and MGEs, such as plasmids, are at the very heart of this. In this review, we have argued that the interconnectedness between biofilm formation and plasmid biology may act as a positive loop that promotes both. The perspective extends to an overall interdependence between HGT, MGE, and the social evolution of bacteria.

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