Original Article

Biofilm Forming Ability in Multidrug-Resistant Staphylococcus aureus Strains Isolated from Local Clinical Sources

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Abstract

The improper use of antibiotics is a global issue that leads to the emergence of multidrug-resistant microbes that cause a wide range of chronic and acute infections. Among these microbes, the bacteria *Staphylococcus aureus* represents a dangerous challenge to public health as it is responsible for life-threatening infections ranging from soft tissue infections to severe diseases. These diseases include endocarditis, pneumonia, septicemia, and catheter-related infections. Also, immunosuppression or a lengthy hospitalization can result in the progression of the invasive, opportunistic *Staph. aureus.* The ability of this bacterium to produce biofilm enables it to resist antibiotics. For this reason, one hundred and thirty clinical samples were collected from various clinical sources and healthcare workers in Baghdad City hospitals (Al Numan Teaching Hospital and Central Teaching Hospital Pediatric). All the isolates were identified by conventional methods (cultural, microscopic, and biochemical tests) in addition to identification by the VITEK® 2 Compact system that included several biochemical tests specific to each bacterial species. Therefore, it can give information at the species level. Thirty isolates were tested against ten antibiotics that belonged to distinct classes (Gentamicin, Clindamycin, Azithromycin, Erythromycin, Cefoxitin, Rifampin, Ciprofloxacin, Penicillin, Ceftazidime, and Tetracycline.) The antibiotic susceptibility profiles for thirty *Staphylococcus aureus* isolates from various clinical samples were tested to choose the multidrug resistance isolates for further detection of the biofilm formation using the microtiter plate method. Then, the powerful biofilm producer in nine isolates was tested for the presence of biofilm genes (*ica B* and *ica C*) using a polymerase chain reaction (PCR) technique. And only eight isolates were identified to have both genes (*ica B* and *ica C*), a result that shows the differences between phenotypic and molecular detection methods.

Keywords: Biofilm Genes (*ica B and ica C*), *Staphylococcus Aureus*, Microtiter Plate, Multi-drug Resistance, Virulence Genes.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a gram-positive bacteria that cause a significant risk to public health. *S.aureus* was found to colonize in around (25-30) of healthy people and global concern for treating infections caused by these bacteria was emerged (Kwok, Chow et al. 2003). *S. aureus* has been a prominent cause of infections in the community and healthcare settings for humans and animals during the past few decades. Due to their capacity to acquire antibiotic-resistant genes, *S. aureus* strains frequently exhibit antibiotic resistance. Dispersal of methicillin-resistant *S. aureus* (MRSA) is a widespread concern in hospitals and communities. Vancomycin-resistant *S. aureus* (VRSA) infections have recently been reported. The *S. aurues* can cause a wide range of diseases in both humans and animals. The ability of this bacteria to adhere to different tissues through biofilm formation enables it to evade and resist applied therapies, as it provides a protective means for the survival of the bacteria and adapts to their environment (Tam and Torres 2019, Freiberg, Le Breton et al., 2020). The *S.aureus* characterized by their ability to produce a variety of virulence factors that are responsible for their pathogencity and among these: surface proteins, biofilm, exoenzymes, exotoxins and exfoliative toxins (Hall-Stoodley, Costerton et al. 2004 and O'Neill, Pozzi et al. 2007). *S. aureus* can cause wide range of diseases from soft tissue infections to severe diseases such as endocarditis, pneumonia, septicemia and catheter related infections (Farah, Abdeljelil et al. 2020); the probability of these disease can be higher especially with diabetic persons, health care workers, patients with weak immunity,

individuals with long hospital stays, recipients with previous methicillin-resistant *S. aureus* (MRSA) infection and people with skin infections. This bacteria is implicated in both community-acquired and nosocomial infections with considerable morbidity (Yılmaz and Aslantaş 2017). The *S. aureus* that are found in the mucous membranes and skin can be progress to invasive opportunistic *S. aureus* and become more virulent and able to produce respiratory, skin diseases or bacteremia (Alsaimary 2020). The ability of this bacteria to adhere to different tissue through biofilm formation enabling them to evade and resist applied therapies as it represent a protective way for the survival of the bacteria and adapt to their environment (Freiberg, Le Breton et al. 2020). The biofilm is an extracellular polymer matrix that surrounds the population of microbial cells. It enhances their attachment to the surfaces that provide a perfect barrier against applied antibiotics and helps the bacteria to evade the immune system (Nguyen et al., 2019). The ability of this bacteria to adhere to different tissue through biofilm formation enabling them to evade and resist applied therapies as it represent a protective way for the survival of the bacteria and adapt to their environment (Freiberg, Le Breton et al. 2020). The bacteria that produce the biofilm defend the host mechanisms during their growth and protect themselves from opsonophagocytosis. They tolerate all traditional antimicrobials designated to eliminate free-floating, single-cell (planktonic) bacteria, especially those associated with the inhibition of the biosynthesis of the cell wall, making them a concern in nosocomial infection and a global health risk (AL-hadeith, Jasim et al., 2022). According to the latest studies, Biofilm is associated with several genes. The intracellular adhesion (*ica*) genes encode for proteins mediating the synthesis of polysaccharide intracellular adhesion (PIA), the N-acetylglucosaminyltransferase encoded by the *icaA* gene. Others include the de-acetylation of mature PIA and the trans-membrane protein encoded by the *icaC* (transporter of PIA) (Ghasemian, Najar-Peerayeh et al., 2015). This study's goal is to detect the presence of *icaB* and *icaC* genes in the *S. aureus* isolates obtained from different clinical sources and their relation to the ability of the bacteria to resist different types of antibiotics.

MATERIALS AND METHODS

Bacterial isolates

Thirty *S. aureus* strains were isolated from patients and health care workers from hospitals in Baghdad and identified by conventional culture and biochemical methods. The *S. aureus* isolates were obtained from various clinical specimens such as sputum (29.6%), nasal swabs (22.3%), urine (18.5%), double lumen (14.8%), and wound swabs (14.8%). The results appear in Table 1. After identification and characterization, the *S. aureus* was maintained in brain heart infusion broth (BHI), to which 15% glycerol was added and stored at -20 C.

Table 1. The biochemical test for the staphylococcus aureus isolates

Test	Results
Gram stain	Gram positive
catalase	positive
coagulase	positive
Mannitol fermentation	positive with yellow colonies
oxidase	negative
Colony morphology	grape like cluster

Antibiotic susceptibility test

The antibiotic susceptibility profile for the positive *S. aureus* isolates against ten types of antibiotics (Gentamicin, Clindamycin, Azithromycin, Erythromycin, Cefoxitin, Rifampin, Ciprofloxacin, Penicillin, Ceftazidime, and Tetracycline) that inhibit the bacteria growth through various mechanisms were tested. Swabs from an overnight bacterial suspension (turbidity is equal to McFarland No. 0.5) were taken and inoculated on Muller Hinton agar plates to examine their antibiotic resistance according to the Kirby-Bauer disk-diffusion technique. The antibiotic discs were placed in agar with sterile swabs and pressed to ensure contact with the agar. Subsequently, the plates were incubated for 24 hours at 37 °C, according to Clinical Laboratory Standards Institute (CLSI, 2021). The inhibition zone was measured in diameter (mm) of each antimicrobial agent, compared with the standard inhibition zone, and interpreted as susceptible, intermediate, or resistant to particular antibiotics.

Biofilm assay

The tissue culture plate method was used for biofilm detection described by (Christensen, Simpson, et al. 1985). The *S. aureus* was isolated from fresh agar plates and inoculated in brain heart infusion broth (BHI) at 37 C for 24 h. Then, the grown colonies were suspended in sterile physiological saline with 0.5 McFarland for turbidity adjustment. The microdilution plates (cell and tissue culture plates, flat well bottom) have 96 wells, each well filled with 180 µL BHI, 1% glucose, and 20 µL of bacteria suspension added. One well was inoculated with sterile (BHI) and acted as the negative control. The plates were inoculated at 37 °C for 24 h. after incubation, the broth of each well was carefully removed and washed three times with sterile phosphate-buffered saline (pH 7.2). This cleaning removed free-floating bacteria and the biofilm produced by these bacteria adherent to the wells, and then the plates were dried at room temperature. The next step was staining the bacteria with crystal violet (0.1%) for 15 min. Then the wells were washed with sterile PBS three times to remove the excess crystal violet. The crystal violets bound to the biofilm of the

bacteria and were extracted by 200ml of 33% glacial acetic acid. Finally, the optical density (OD) of the stained biofilm was determined using a Microelisa Autoreader. An OD of 595 nm > 0.12 is a positive biofilm sample. In Table 2, the classification of *Staphylococcus* spp. is based on the OD value obtained for each isolate (Stepanović, Vuković et al., 2007).

Table 2. The classification of the bacteria adherence according to the tissue culture plate method

Mean OD values	Adherence biofilm formation
<0.120	Weakly or non-adherent
0.12-0.24	Moderately adherent
>0.24	Strongly adherent

Detection of icaB and icaC genes by PCR

After identifying the isolates and recording them as *S. aureus*, their selection depends on their resistance profile and ability to produce biofilm in microtiter tubes for molecular screening of *icaB* and *icaC* genes using the polymerase chain reaction (PCR) method. The method achieved by using specific primers for each gene appears in Table 3.

Table 3. Oligonucleotide primers that used for amplification of icaB and icaC genes (Nourbakhsh, Namvar et al. 2016).

Primer name	Sequence (5-3)	Annealing temperature (°C)	Product size (bp)
icaB-F	5-ATACCGGCGACTGGGTTTAT-3	60	140
icaB-R	5-TTGCAAATCGTGGGTATGTGT-3		
icaC-F	5-CTTGGGTATTTGCACGCATT-3	60	209
icaC-R	5-GCAATATCATGCCGACACCT-3		

The lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100pmol/µL as a stock solution. It was prepared by adding (10 µL) of primer stock solution to (90 µL) of nuclease-free water to obtain the working primer solution and stored in a freezer at -20 C. A single colony of the bacterial isolate was taken from nutrient agar and cultivated in 1 ml of brain heart broth for 24 hr at 37 °C. The bacterial genomic DNA was extracted with an ABIOpureTM Total DNA (ABIOpure, USA), as recommended by the manufacturer. The total volume of the PCR mixture should be 20 µL, as in Table 4.

Table 4. The mixture of PCR reaction

Component	Reaction volume(µl)
master mix	10
Forward primer	1
Reverse primer	1
DNA template	Single colony
Nuclease free water	8
Final volume	20

The (0.2 ml) Eppendorf tube contained primers, nuclease-free water, and a master mix. The PCR tubes were then transferred to a thermal cycler to initiate the amplification according to the program in Table 5.

Table 5. The PCR amplification program of icaB and icaC genes.

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	35
Annealing	60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Results and Discussion

One hundred thirty-five clinical samples were collected from clinical sources and healthcare workers in Baghdad hospitals. Thirty isolates (22%) were characterized as *S. aureus* using the conventional cultural, biochemical, and microscopic examination methods in addition to the VITEK® 2 Compact system. The rest of the clinical samples, which represent (88%), were related to different genera of pathogenic bacteria. The antibiotic susceptibility profile results for the *S. aureus* isolates against applied antibiotics appear in Table 6 with the published data update by the CLSI (2021).

In our study, thirty isolates of *S. aureus* presented different degrees of resistance against diverse types of antibiotics. Ceftazidime and tetracycline were the least active antibiotics. The tested isolates had a high degree of resistance, as recorded in Table 6. The ratios were 76.7% and 66.7%, respectively. On the other hand, the highest sensitivity rate was for *S. aureus* isolates against Ciprofloxacin and penicillin (86.7%). Also, Gentamicin and Rifampin were highly active as we recorded a sensitive rate of 76.7% for both. The sensitivity rate of *S. aureus* against Azithromycin was 73.3%, Clindamycin 66.7%, Erythromycin 56.6%, and Cefoxitin 50%. As a final result, nine isolates were recorded as Multidrug Resistant (MDR)

since they resist five or more antibiotics that belonged to different classes.

Table 6. Percentage of susceptibility profile for each tested antibiotic compared with the standard published inhibition zone by (CLSI 2021).

Antibiotic	R%	I%	S%
CN	(7) 23.3%	0	(23) 76.7%
DA	(7) 23.3%	(3) 10%	(20) 66.7%
AZM	(6) 20%	(2) 6.7%	(22) 73.3%
E	(11) 36.7%	(2) 6.7%	(17) 56.6%
CX	(10) 33.4%	(5) 16.6%	(15) 50%
RA	(5) 16.6%	(2) 6.7%	(23) 76.7%
CIP	(3) 10%	(1) 3.3%	(26) 86.7%
P-10	(4) 13.3%	0	(26) 86.7%
CAZ	(23) 76.7%	0	(7) 23.3%
TE	(20) 66.7%	(3) 10%	(7) 23.3%

The isolates were tested for biofilm formation using the microtiter plate method. Biofilm formation was observed in 28 (93.3%) of them after measuring the optical density (OD) of the stained biofilm at 595 nm. A strong biofilm formation was detected in 9 (30%) of the tested isolates, while 11 (36.7%) of them were able to form moderate biofilm. On the other hand, 8 (26.7%) were weak biofilm producers. Two isolates (6.6%) were non-producers of biofilm, as shown in Table 7. We selected nine biofilm-producing isolates that showed MDR, as they are more pathogenic.

Table 7. The biofilm formation ability of Staphylococcus aureus using microtiter plate method.

Type of biofilm formation	Number(No.)	Percentage (%)
Strong	9	(30%)
Moderate	11	(36.7%)
Weak	8	(26.7%)
Non-producers	2	(6.6%)

The biofilm-producing isolates selected for detecting (icaB, icaC) biofilm genes represented the most pathogenic isolates. Nine of thirty *S. aureus* isolates were recorded as strong biofilm producers and previously recorded as multidrug-resistant. The PCR technique detected the presence of a single band with a molecular weight of 140 bp and 209 bp for the *icaB* and *icaC* genes, respectively. The band that appeared was compared to the DNA marker, as described in the figures. In Figure 1, all the isolates except the (7r) isolate produced a single band with a molecular weight of 140 bp that indicates the presence of the *icaB* gene. The result appears in Figure 2.

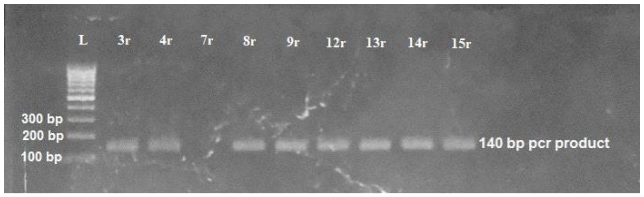

Figure 1. PCR profile for the amplified icaB gene of Staphylococcus aureus samples.

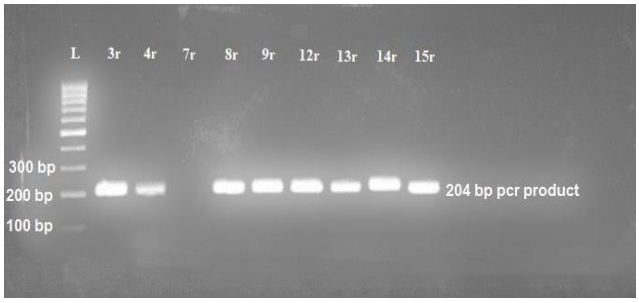

Figure 2. PCR profile for the amplified icaC gene of Staphylococcus aureus samples.

The *S. aureus* is a cause of various diseases in both humans and animals (Arciola, Campoccia et al., 2018). The *ica* genes of *S. aureus* is an important genes for production of biofilm, usually the *S. aureus* isolates that have these genes are more capable to form biofilm (Nourbakhsh, Namvar et al. 2016). The maturation of the biofilm matrix is initiated by polysaccharide intercellular adhesion (PIA). PIA is synthesized by intracellular adhesion *ica* (*icaA, icaB, icaC, icaD*) and a regulatory gene (*icaR*) (Trivedi, Parameswaran, et al. 2014). These genes are encoded for these proteins (*ICAA*, *ICAB*, *ICAC*, and *ICAD*) respectively. Each protein has a role in biofilm synthesis.

Our result shows that all MDR *S. aureus* isolates were strong biofilm producers, and subsequently, only eight (88.9%) of these isolates have *icaB* and *icaC* genes. The current result came from correspondence with Prasanth and Saravanakumari (Prasanth and Saravanakumari 2017), as they represent that *icaA, icaB,* and *icaD* genes were present in all tested isolates. Also, (Gowrishankar, Kamaladevi, et al., 2016) reported that (84%) of the *S. aureus* isolates carry (*icaABCD*) genes. This study found a critical relationship between biofilm formation and antibiotic resistance in locally isolated *S. aureus* strains. Crucial virulence factors form an apparent border between the bacteria and any antibacterial agents, so the bacteria become more pathogenic and harmful and cause chronic and persistent infections.

CONCLUSIONS

This study highlighted the genetic diversity of Staphylococcus aureus isolates from various sources in Iraq and demonstrated the ability of these isolates to produce biofilms.

REFERENCES

AL-hadeithi, Z. S., S. A. Jasim and O. D. Salahdin (2022). "Relation between resistance of Klebsiella pneumoniae to certain antibiotics and ESBL/PBP genes." Biodiversitas Journal of Biological Diversity 23(8).

Alsaimary, I. E. (2020). Comparative Molecular Analysis of Meca, Sea and Seb Genes in Methicillin-Resistant Staphylococcus Aureus (MRSA) Journal of Clinical & Biomedical Research, SRC/JBBR-110.

Arciola, C. R., D. Campoccia and L. J. N. r. m. Montanaro (2018). "Implant infections: adhesion, biofilm formation and immune evasion." 16(7): 397-409.

Christensen, G. D., W. A. Simpson, J. Younger, L. Baddour, F. Barrett, D. Melton and E. J. J. o. c. m. Beachey (1985). "Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices." 22(6): 996-1006.

Craft, K. M., J. M. Nguyen, L. J. Berg and S. D. J. M. Townsend (2019). "Methicillin-resistant Staphylococcus aureus (MRSA): antibiotic-resistance and the biofilm phenotype." 10(8): 1231-1241.

Farah, A., O. B. Abdeljelil, W. Jomaa, K. B. Hamda and F. J. A. o. C. D. S. Maatouk (2020). "Infective endocarditis; Staphylococcus aureus versus other pathogens." 12(1): 98.

Freiberg, J. A., Y. Le Breton, J. M. Harro, D. L. Allison, K. S. McIver and M. E. J. M. Shirtliff (2020). "The Arginine deiminase pathway impacts antibiotic tolerance during biofilm-mediated Streptococcus pyogenes infections." 11(4): e00919-00920.

Ghasemian, A., S. Najar-Peerayeh, B. Bakhshi and M. J. A. o. P. I. D. Mirzaee (2015). "High prevalence of icaABCD genes responsible for biofilm formation in clinical isolates of Staphylococcus aureus from hospitalized children." 3(3).

Gowrishankar, S., A. Kamaladevi, K. Balamurugan and S. K. J. B. r. i. Pandian (2016). "In vitro and in vivo biofilm characterization of methicillin-resistant Staphylococcus aureus from patients associated with pharyngitis infection." 2016.

Hall-Stoodley, L., J. W. Costerton and P. J. N. r. m. Stoodley (2004). "Bacterial biofilms: from the natural environment to infectious diseases." 2(2): 95-108.

Hatem, Z. A., Jasim, S. A., & Mahdi, Z. H. (2021). Phenotypic and Genotypic Characterization of Antibiotic Resistance in Staphylococcus aureus Isolated from Different Sources. Jundishapur Journal of Microbiology, 14(4). DOI: 10.5812/jjm.115221

Kwok, A. Y., A. W. J. I. j. o. s. Chow and e. microbiology (2003). "Phylogenetic study of Staphylococcus and Macrococcus species based on partial hsp60 gene sequences." 53(1): 87-92.

Nourbakhsh, F., A. E. J. G. H. Namvar and i. control (2016). "Detection of genes involved in biofilm formation in Staphylococcus aureus isolates." 11.

O'Neill, E., C. Pozzi, P. Houston, D. Smyth, H. Humphreys, D. A. Robinson and J. P. J. J. o. c. m. O'Gara (2007). "Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections." 45(5): 1379-1388.

Prasanth, P. and P. J. I. J. P. P. S. Saravanakumari (2017). "Detection of intercellular adhesion genes (ica) in Staphylococcus aureus Causing implant associated infections." 9: 76-80.

Saxena, P., Y. Joshi, K. Rawat and R. J. I. j. o. m. Bisht (2019). "Biofilms: architecture, resistance, quorum sensing and control mechanisms." 59(1): 3-12.

Stepanović, S., D. Vuković, V. Hola, G. D. Bonaventura, S. Djukić, I. Ćirković and F. J. A. Ruzicka (2007). "Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci." 115(8): 891-899.

Tam, K. and V. J. J. M. s. Torres (2019). "Staphylococcus aureus secreted toxins and extracellular enzymes." 7(2): 7.2. 16.

Torlak, E., E. Korkut, A. T. Uncu, Y. J. J. o. i. Şener and p. health (2017). "Biofilm formation by Staphylococcus aureus isolates from a dental clinic in Konya, Turkey." 10(6): 809-813.

Trivedi, U., S. Parameswaran, A. Armstrong, D. Burgueno-Vega, J. Griswold, S. Dissanaike and K. P. J. J. o. p. Rumbaugh (2014). "Prevalence of multiple antibiotic resistant infections in diabetic versus nondiabetic wounds." 2014.

Yılmaz, E. Ş. and Ö. J. A. P. j. o. t. m. Aslantaş (2017). "Antimicrobial resistance and underlying mechanisms in Staphylococcus aureus isolates." 10(11): 1059-1064.