



GENOTYPIC INVESTIGATION OF INTERCELLULAR ADHESION LOCI (*ICA*) IN *STAPHYLOCOCCUS AUREUS* ISOLATES RESPONSIBLE FOR RECURRENT SKIN INFECTIONS IN HILLA CITY, IRAQ

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ABSTRACT

Staphylococcus aureus one of the most frequent bacterial causes of infections including endocarditis, osteomyelitis and abscesses. In addition, *S. aureus* has the capacity to adhere to catheters and other indwelling medical devices and form biofilm. The antimicrobial susceptibility patterns by using disk diffusion test. The antibiotic susceptibility test revealed that the presences of multi-resistant strains towards the 15 antibiotics, the resistance rate were: oxacillin (25%), cefoxitin and cefipime (100%), erythromycin (50%), gentamycin (6.2%), tetracycline (56%), doxycycline (53%), clindamycin (3%), trimethoprim (3%) All the strains were susceptible to meropenem, imipenem, norfloxacin, chloramphenicol, rifampicin and vancomycin. Phenotypic detection of biofilm by all isolates was assessed. Among the 32 clinical *S. aureus* isolates, 23 strains (74.3%) were biofilm-producers and the remaining 9 strains were non-producers. Molecular detection of *ica* operon was performed by polymerase chain reaction technique. Twenty three *S. aureus* isolates were carrying *icaA* and *icaD*, giving a 188-bp band for the *icaA* gene and a 198-bp band for the *icaD* gene. All of them phenotypically were biofilm positive. In conclusion, the large amounts of *S. aureus* isolates responsible for skin infection cause severe damage to the patient. Our results indicate that the determination of antibiotic susceptibility and biofilm production are a great importance in epidemiological control of spread of multi-resistant isolates.

KEY WORDS: Biofilm, *icaA*, *icaD*

INTRODUCTION

Staphylococcus aureus one of the most frequent bacterial causes of infections including endocarditis, osteomyelitis and abscesses. The National Nosocomial Infections Surveillance System has identified *Staphylococcus* and coagulase-negative staphylococci as the major cause of skin infections^[1]. *Staphylococcus aureus* has the ability to produce several exoenzymes that contribute to its virulence^[2]. In addition, *S. aureus* has the capacity to adhere to catheters and other indwelling medical devices and form biofilm. Slime is a factor that enhances adhesion to plastic or metallic surfaces. Strategies to prevent and control the spread of methicillin resistant *S. aureus* (MRSA) are of major importance in hospital hygiene worldwide^[3]. The most important strategies to control MRSA include early identification of MRSA strains, informing healthcare workers about the MRSA problem and strict hand hygiene between patients to prevent transmission^[4]. Recently *icaA* and *icaD* genes encoding for polysaccharide intercellular adhesion (PIA) were identified in *S. aureus*^[5,6]. Production of PIA is currently responsible for staphylococcal biofilm development^[7,8]. It has been reported that the majority of clinical *S. aureus* isolates contain the *ica* operon^[9]. Many investigators reported that the slime factor increased resistance to antibiotics^[10]. The aim of this study was to determine the antibiotic susceptibility of clinical isolates of *S. aureus*, biofilm production and presence or absence of *icaA*, *icaD* gene.

MATERIALS AND METHODS

Sample collection

A total of 150 swabs were collected from patient's with skin infections who admitted to Al-Hilla General Teaching Hospital and Merjan Hospital, Babylon-Iraq. All samples were collected through a period from April to June 2013. Pus was directly plated on mannitol salt agar and blood agar plates. Identification was based on the colonial morphology and coagulase production. All suspected colonies were subjected to standard bacteriological procedures to confirm identification.

Antimicrobial susceptibility testing:

The antimicrobial susceptibility patterns of isolates to different antimicrobial agents were determined and interpreted according to^[11] recommendations by using disk diffusion test. Fifteen antibiotics were tested: oxacillin, imipenem, meropenem, cefoxitin, cefipime, gentamicin, tetracycline, doxycycline, erythromycin, rifampicin, chloramphenicol, clindamycin, trimethoprim, norfloxacin and vancomycin.

Determination of biofilm production

Biofilm formation was studied by semi-quantitative measurements of biofilm formation by using tissue culture-treated, 96-well polystyrene plates and culturing the isolates on brain heart infusion broth supplemented with 1% glucose. The isolates were cultured on polystyrene plate and incubated at 37 °C for 24 h under aerobic conditions. Results were interpreted as biofilm producer isolates if the absorbance is 0.17 and considered

to be positive, while isolates that have a value less than 0.17 were classified as non-biofilm producing, as described by [12].

Detection of *icaA* and *icaD* loci:

The presence of *icaA* and *icaD* genes was detected by PCR using forward and reverse primers for *icaA* and *icaD*. The sequences of primers were taken from [13]. For *icaA*, the forward primer had the following sequence: 5'-ACACTTGCTGGCGCAGTCAA-3'; and the reverse primer had the following sequence: 5'-TCTGGAACCAACATCCAACA-3' giving a 188-bp. The primer sequences for *icaD* were: forward, 5'-ATGGTCAAGCCCAGACAGAG-3; and reverse, 5'-AGTATTTCAATGTTTAAAGCAA-3' giving a 198-bp. PCR was performed in a final volume of 20 µl contained 10 pmol forward and reverse primers, dNTP mix (100 mM each of dATP, dCTP, dGTP and dTTP), 1 U of *Taq* DNA polymerase (Bioneer/ Korea), 5 µl master mix and DNA template (5 µl). PCR conditions included initial denaturation (94 °C for 5 min), followed by 30

cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 45 s), followed at the end of cycling with final extension (72 °C for 10 min). PCR products (5 µl) were analysed on 1.5% (wt/v) agarose gel stained with ethidium bromide (0.5 mg/µl), and visualised under ultraviolet transilluminator

RESULTS

Among 150 swabs gathered from patients with skin infections only 32 (21.3%) were positive for coagulase positive *S. aureus* isolates (Figure 1). Wenzel and Perl, (1995) [14] found that, among healthy adults, carrier rates of 11-32% were detected in the general population, and a prevalence of 25% was detected in hospital personnel. Using pulsed-field gel electrophoresis (PFGE) for molecular typing, Von Eiff *et al.* (2001) [15] found that, in most patients with *S. aureus* bacteremia, the isolate from the patient's blood is identical to that found in the anterior nares. Persistent nasal carriage depends on host genetic determinants [16].

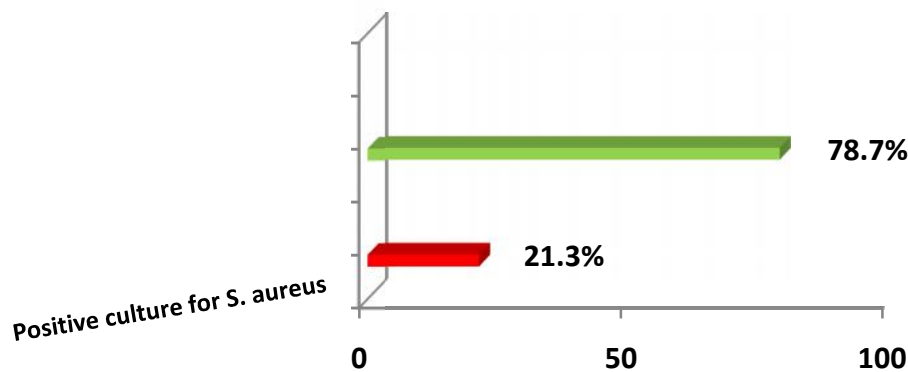


FIGURE 1: The percentage of isolation of *S. aureus*.

The antibiotic susceptibility test results as shown in figure (2) revealed that the presence of multi-resistant strains towards the 15 previously cited antibiotics: the resistance rates were oxacillin (25%), cefoxitin and cefipime (100%), erythromycin (50%), gentamycin (6.2%), tetracycline (56%), doxycycline (53%), clindamycin (3%), trimethoprim (3%). All the isolates were susceptible to meropenem, imipenem, chloramphenicol, rifampicin and vancomycin. These results were in accordance with results obtained by [17,18], who found that all *S. aureus* isolates were resistant to oxacillin and susceptible to non-lactam antibiotics including norfloxacin, chloramphenicol, rifampicin and vancomycin. The results of this study were in accordance with the results obtained by Abdullah *et al.* (2006) [19] who found that 61.8% of skin infection caused by -lactam resistant *S. aureus*. Staphylococcal resistance to either oxacillin or methicillin occurs when the organism including an altered PBP (PBP2A) that is encoded by the *mecA* gene [20]. Oxacillin inhibition zone of resistant strains in DD test were determined at 10 mm (11). This result was less than the result obtained by Al-Fu'adi, (2010) [21] who found that the percentage of oxacillin resistance was 32.2% and more than the result obtained by Al-khudheiri, (2008) [22] which was 22.5% in Najaf / Iraq.

Cephamicin, cefoxitin and cefepime (4th generation cephalosporin) showed that percentages of *S. aureus* isolates resistant were 100% for each (Figure 2). This result was in accordance with the results obtained by Al-Hassnawi *et al.* (2010) [23]. Clindamycin showed resistance at rate 3%. This result was less than the results (35%) reported by Sattler *et al.* (2002) [24]. On the other hand, all bacterial isolates exhibited high sensitivity (100%) to carbapenems (imipenem and meropenem) (Figure- 2). This result was in accordance with the results obtained by [23]. Regarding the percent of resistance to erythromycin (50%) which less than that obtained by [22, 25], (81.5%, 93%) respectively, who isolated erythromycin resistance *S. aureus* from hospital infections in Najaf and European countries. In the present study, *S. aureus* isolates showed high resistance rate (56%) to tetracycline. On the other hand, results recovered by this study indicated that doxycycline resistance rate was (53%). This result was in disagreement with results obtained by [21] who found resistance rate to tetracycline was (32.2%). As shown in Figure (2), the percentage of resistance for gentamicin was 6.20%, This result was less than the result obtained by [23] while in other study [26], found higher resistance rate (19%).

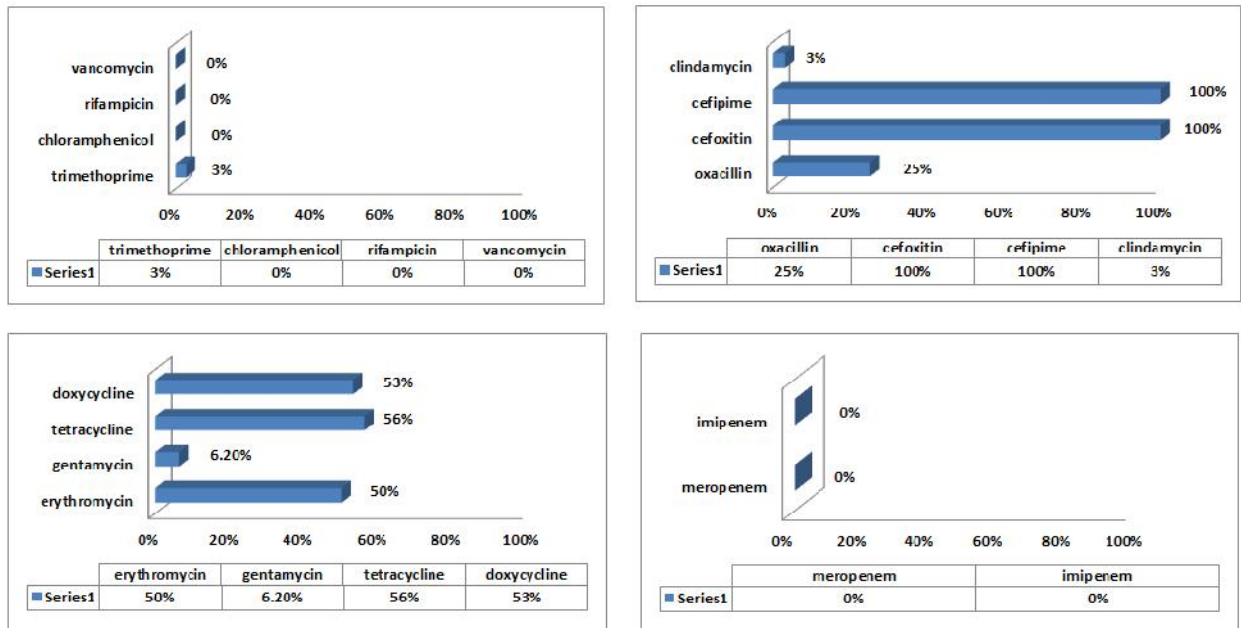


FIGURE 2: The Percentage of antibiotic resistance of *S. aureus*.

In relation to the resistance rate of trimethoprim 3%, this result was in less than the results obtained by [22, 23], who found resistance rates 13.6% and 42%, respectively. Concern phenotypic detection of biofilm, the results displayed that among the 32 clinical *S. aureus* isolates, 23 (71.9%) were biofilm-producers and the remaining 9

(28.1%) isolates were non-producers Figure (3), this result was more than the result obtained by [23], who found 61% of isolates were biofilm producers, but in accordance with the results of [27], who found that 74.3% of MRSA isolates were biofilm producers.

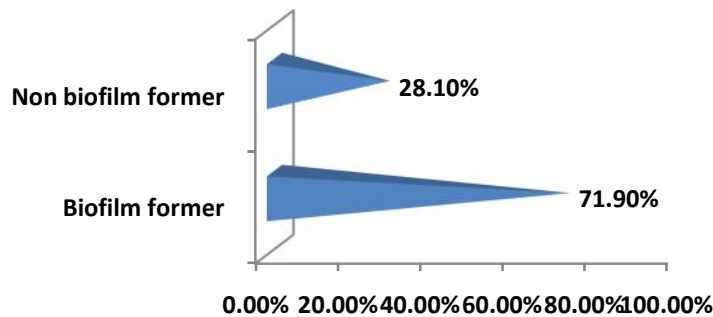


FIGURE 3: the percentage of biofilm formation of *S. aureus*.

Detection of *icaA* and *icaD* genes:

Twenty three *S. aureus* isolates were carrying *icaA* and *icaD*, giving a 188-bp band for the *icaA* gene and a 198-bp band for the *icaD* gene (Figure 4 and 5). All of

them phenotypically were biofilm positive. These findings were in accordance with the result carried by [27], who found that among the 35 *icaA/icaD* positive isolates, 26 were biofilm positive and nine were biofilm negative.

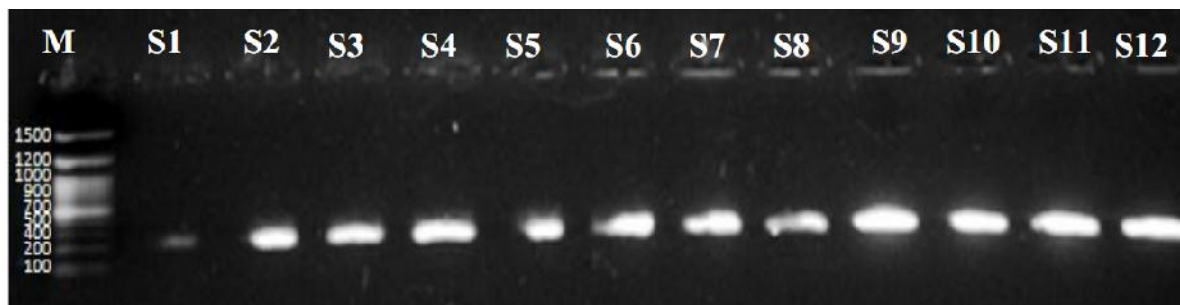


FIGURE 4: Agarose gel electrophoresis of PCR amplification of *icaD* genes. Lane M, 100 pb DNA molecular size marker; lanes, S (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) PCR amplification of *icaD* gene (198bp).

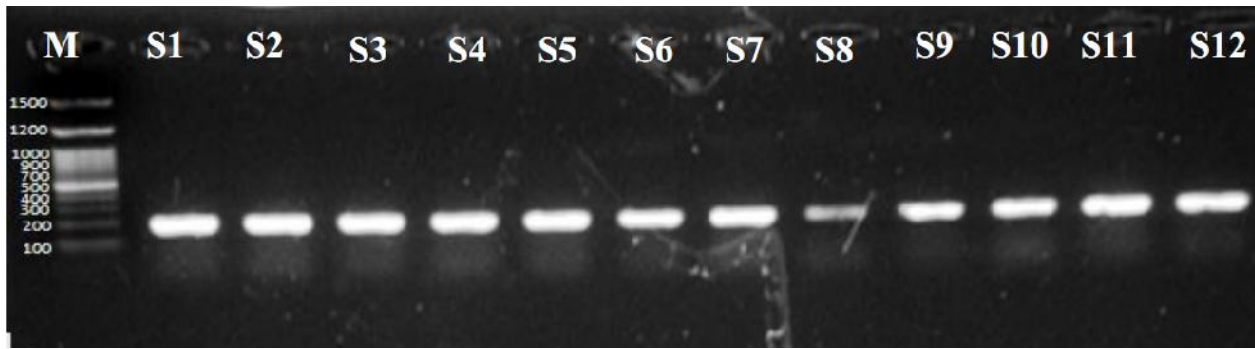


FIGURE 5: Agarose gel electrophoresis of PCR amplification of *icaA* gene. Lane M, 100 pb DNA molecular size marker; lanes, S (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) PCR amplification of *icaA* gene (188bp).

DISCUSSION

The postulated sequence of events that leads to infection is initiated with carriage of the organism. The organism is then disseminated via hand carriage to body sites where infection may occur (either through overt breaks in dermal surfaces, such as vascular catheterization or operative incisions, or through less evident breakdown in barrier function, such as eczema or shaving-associated micro trauma^[28]).

The hallmark of staphylococcal infection is the abscess, which consists of a fibrin wall surrounded by inflamed tissues enclosing a central core of pus containing organisms and leukocytes. Persistent deep-seated infections have now been linked to small-colony variants of the organism. This population is more resistant to antibiotics and grows slowly. These organisms have been described in patients with cystic fibrosis and may contribute to the persistence of *S aureus* in these patients^[29].

The percentage of antibiotic resistance is flexible and may be accredited to drug overuse or misuse.

Such high percentage may be due to frequently use β -lactam antibiotics by patients. In the present study patients infected with recurrent skin infections, that increase the chance of antibiotic resistance. This can be explained by the fact that all staphylococcal isolates produce β -lactamase which destroys the β -lactam ring resulting in inactive products^[30]. Resistance to cephalosporins mediated by cephalosporinase production^[31]. Furthermore; β -lactamase produced by staphylococci excreted into the surrounding environment by which the hyper production of β -lactamase will give longer validity and surviving to this bacterium, because the hydrolysis of β -lactams takes place before the drug can bind to PBPs in the cell membrane^[31]. The relatively high percentage of resistance to these antibiotics was not attributed only to production of β -lactamase enzyme, but could be due to the decreased affinity of the target PBPs or decreased permeability of the drug into the cell^[32]. These organisms not only survive penicillin therapy but can also protect penicillin-susceptible bacteria from penicillin by releasing the free enzyme into the infected tissue or pus^[33]. Imipenem and meropenem are broad-spectrum carbapenems antibiotics. β -lactam rings of these antibiotics are resistant to hydrolysis by most β -lactamases^[34]. Imipenem inhibits bacterial cell wall synthesis by binding to and inactivating PBPs, and the activity of meropenem against most clinical isolates was comparable with imipenem^[35]. For the variations in clindamycin resistance rate, this is due to

differences conditions of tests used and type of techniques, these factors may lead to differences in resistance levels^[36].

This study was revealed high ratio of resistance (56%, 53%) for tetracycline and doxycycline respectively, due to the samples were taken from recurrent skin infections and empirical prescription of these drugs by physicians and overuse by patients. The variation between both antibiotics can be assign to the popular administration of tetracycline without physician's consulting and its availability in the hospitals and pharmacies rather than doxycycline will be elevating. Highly resistant to erythromycin in this study agreed with many studies reported that transposon Tn554^[37], encoding resistance to macrolides (erythromycin) was located in upstream of the *mecI-mecR1-mecA* gene complex that carrying directly on MRSA isolates, while the Plasmid pUB110, encoding resistance for aminoglycosides (gentamicin), was inserted between two insertion sequences IS431 (or IS257) at the left of *mecA* gene^[38]. Trimethoprim was approved for the treatment of SSTIs caused by *S. aureus*^[39]. This antibiotic has moderate therapeutic effect, and could be used for the treatment of infections caused by *S. aureus* that resist to different types of antibiotics. Variations in sensitivity are related to the frequency of usage of the individual antibiotics. The results of vancomycin, chloramphenicol and rifampin resistance agreed to expect, because the resistance is very rarely reported^[11,40]. This pattern is closely related to one pattern of the primary types causing community acquired infections nationwide^[41]. Tissue culture plate method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation by *S. aureus* and has the advantage of being a quantitative model to study the adherence of this bacterium on biomedical devices^[42]. Crystal violet is a basic dye known to bind to negatively charged molecules on the cell surface as well as nucleic acids and polysaccharides, and therefore gives an overall measure of the whole biofilm. It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of Gram positive and Gram-negative bacteria^[43]. *S. aureus* known to form biofilm on various biomaterials^[44]. It can persist and gain increased resistance to antibiotics through biofilm formation that appears to be a bacterial survival strategy^[45]. *S. aureus* is a common pathogen responsible for nosocomial and community infection. It readily colonizes tissues forming microbiotic communities termed biofilms

in which *S. aureus* are protected from killing by antibiotics and body's immune system. For years, one mechanism behind biofilm resistance to attack from the immune system's sentinel leukocytes has been conceptualized as a deficiency in the ability of the leukocytes to penetrate the biofilm^[46]. As an opportunistic pathogen, it can cause infections that vary widely in their susceptibility to antibiotic treatment. Many studies revealed that Fibronectin-binding proteins (Fnbps), besides its role in binding to fibronectin, it could promote biofilm formation in clinical MRSA isolates^[13]. The expression of multiple distinct surface proteins that mediate the binding of *S. aureus* to the cells, a substantially reduced ability to clear the bacteria, and an inability to mount an adequate immune reaction have been proposed to have a role in this relation^[47]. The findings for *icaA*, *icaD* operon could be due to the fact that *ica* expression is subject to environmental conditions [48,49]. Furthermore, the ability of staphylococci to adhere and proliferate may explain their ability to colonize biomaterials in hospital. In addition we found that slime-producing strains were multi-drug resistant. Most of the *S. aureus* isolates analyzed so far contain the entire *ica* gene cluster^[5,9,50], but only a few express the *ica* operon and produce biofilms. The investigation of environmental strains of *Staphylococcus epidermidis* shows no correlation between the qualitative biofilm production and the presence of *ica* genes^[51]. Stimuli such as high osmolarity (3% NaCl), growth in anaerobic conditions, high temperature and sub-inhibitory concentrations of certain antibiotics are known to enhance *ica* transcription and biofilm formation^[48,49].^[5] Demonstrated that the deletion of the *ica* operon in *S. aureus* resulted in biofilm-negative phenotype. Many investigators reported that the slime factor increased resistance to antibiotics by hindering entrance of antibiotics into the bacteria^[10]. In conclusion, the large amount of *S. aureus* isolates responsible for skin infection causes severe damage to the patient. Our results indicate that the determination of antibiotic susceptibility and biofilm production are a great importance in epidemiological control of spread of multi-resistant isolates

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