



DETECTION OF BIOFILM –ASSOCIATED GENES IN CLINICAL *STAPHYLOCOCCUS AUREUS* ISOLATES FROM IRAQI PATIENT

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ABSTRACT

Clinical sampling carried out between December 2013 to April 2014. One hundred *Staphylococcus aureus* isolates were obtained from 220 clinical specimens. The sample included of patients in referring various hospital in Baghdad (nose, wound, burn, ear swabs and sputum) .The production of viscous layer in *S. aureus* was investigating by used congo red agar (CRA), showed that 56% of *S. aureus* produce viscous layer and also been screening the ability of isolates to biofilm formation using the microtiter plate, the result appear 57% of isolates produce biofilm. The extraction of DNA was subjected to polymerase chain reaction (PCR) technique in a monplex and multiplex to amplify the virulence factor (*clfA*, *fnbA* and *can*)gene .The result of this investigation showed that (56% ,56% ,81%) respectively .

KEYWORDS: *Staphylococcus aureus*, adhesion gene, *clfA*, *fnbA* and *can* gene.

INTRODUCTION

Staphylococcus aureus represents one of the most serious gram-positive bacterial infections in nosocomial and community settings^[1]. The pathogenicity of *S. aureus* is caused by the expression of an arsenal of virulence factors, which can lead to superficial skin lesions, or to more serious infections, such as pneumonia, mastitis, urinary tract infections, osteomyelitis, endocarditis, and even sepsis. In very rare cases, *S. aureus* causes meningitis^[2]. The first step in *S. aureus* infections is attachment to various surfaces and colonization of host tissues. For this purpose, *S. aureus* carries several surface adhesions (microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)^[2]. Many MSCRAMM proteins interact with various human or animal tissues, serum proteins, and polypeptides of the extracellular matrix, these interactions allow Staphylococci to adhere to a variety of cell lines and promote the invasion and apoptotic death of infected epithelial cells. For example: protein A, clumping factors ClfA and ClfB, and fibronectin binding proteins; FnbA and FnbB^[3]. The ability of *S. aureus* to adhere to plasma and extracellular matrix (ECM) proteins deposited on biomaterials is a significant factor in the pathogenesis of device-associated infections. Several specific adhesions are expressed on the surface of *S. aureus*, which interact with a number of host proteins, such as fibronectin, fibrinogen, collagen, vitronectin and laminin^[4], and have been designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules)^[5].The biological importance of MSCRAMMs and their roles as virulence determinants are still being elucidated. To be classed as a MSCRAMM, the molecule of interest must be localized to the bacteria cell surface, and must recognise a macromolecule ligand found within the host's ECM. These ligands include molecules such as collagen and laminin, which are found exclusively in the ECM, and others such as fibrinogen and fibronectin,

that are part-time ECM molecules but are also found in soluble forms such as blood plasma^[5].

MATERIALS & METHODS

Samples collection

Two hundred -Twenty clinical samples were collected during the period from December 2013 to April 2014. The clinical samples were collected from different patients attending the three hospitals in Baghdad city. The specimens included: nasal swab, wound swab, burn swab, abscess and ear swab, urine and blood culture. Sterile gloves were worn while collecting samples to prevent skin bacterial contamination. Samples from nose (both anterior nares), and ear-swab were collected using sterile swabs, placed into a transport medium and transported to the laboratory immediately.

Bacterial isolates and phenotype identification

One hundred *S.aureus* strain were isolated from patients and used in this study. Isolates were identified morphologically and biochemically by^[6].The coagulase and DNase test were performed for discrimination of *S. aureus* from coagulase negative staphylococcus negative staphylococci. After conventional methods, VITEK2 were employed for the result confirmation.

Slime forming colony

This test was used to evaluate slim formation in *S. aureus* strain on Congo-red agar (CRA), as previously described^[7] pink colonies were recorded as nonbiofilm producers while the black colonies were recognized as biofilm producers .

Biofilm assay

The overnight culture (*S. aureus*), grown in Tryptic Soya broth (TSB) at 37°C, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200 µl of cell suspensions was transferred in a U-bottomed 96-well microtiter plate. Wells with sterile TSB alone were served as negatives controls. The plates were incubated aerobically at 37°C for 24h. The cultures were removed and the microtiter wells were washed twice with

phosphate-buffered saline to remove non adherent cells. Adherent bacteria were fixed with 95% ethanol and stained with 1% crystal violet for 5 min. The wells were washed three times with sterile distilled water and air-dried; the experiment was repeated three times separately for each strain. The optical density of each well was measured at 570 nm (OD570) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy). Biofilm formation was interpreted as followed: highly positive (OD570 > 1), low grade positive (0.1 < OD570 < 1), or negative (OD570 < 0.1)^[8].

Bacterial DNA extraction

Bacterial genomic DNA was extracted from isolates using DNA kit (Geneaid Kit- Korea) following the manufacturer’s protocol. The resultant DNA extract with a final elution volume of 100 µl was frozen at - 20°C until use.

PCR and agarose gel electrophoresis

The purpose of using simplex and multiplex PCR was to determine the distribution of 3 biofilm genes in 100 different clones of *S. aureus* isolates. Total chromosomal DNA was extracted from the isolates using a Geneaid Kit-

Korea). All the 3 genes were amplified by simplex PCR. The distribution of biofilm genes in different clones of *S. aureus* isolates was examined by multiplex PCR method. All primer sets for adhesion and biofilm genes were described in the table (1). The reaction mixture of PCR was 25 µL in total volume containing 12.5 µL of master mix, 1.0 µL of primers (forward 0.5 µL and reverse 0.5 µL), 2 µL genomic DNA, and 9.5 µL of distilled water (dH2O). PCR was carried out with an initial denaturation step of 5 min at 94 C, followed by 25 cycles of denaturation (1 min at 94 C), annealing (1 min at 55 C), and extension (1 min at 72 C); the reactions were finalized by polymerization for 10 min at 72 C. Multiplex PCR was carried out using the Geneaid Multiplex PCR kit, the method as described program reaction, mixtures containing 1 µg chromosomal DNA, 25 µL of master mix with 3 mM MgCl₂, 5 µL of primer mix (2 µM in TE buffer for each primer), and RNase-free water in a final volume of 50 µL was prepared. The PCR amplicons were visualized using UV light after electrophoresis in a 2% (w/v) agarose gel.

TABLE 1: PCR target genes and primer used in this work

Primer name	Primer sequence	Size of product (bp)
<i>clfA</i>	F/5-CCGGATCCGTAGCTGCAGATGCACC-3	1000
	R/5-GCTCTAGATCACTCATCAGGTTGTTTCAGG-3	
<i>fnbA</i>	F/5-GATACAAACCCAGGTGGTGG-3	191
	R/5-TGTGCTTGACCATGCTCTTC-3	
<i>cna</i>	F/5-GTCAAGCAGTTATTAACACCAGAC-3	423
	R/5-AATCAGTAATTGCACTTTGTCCTG-3	

RESULT & DISCUSSION

Staphylococcus aureus identification depended of Microscopic examination (isolates demonstrated gram-positive purple colour spherical cells or cocci arranged in irregular grape-like clusters), cultural examination (slightly yellow, flat, large circular in Nutrient agar and opaque, while the colonies on Blood agar plates appeared large, round, golden-yellow to creamy/buff colored colonies, producing complete clear zones of α -hemolysis. Then, the isolates were cultured on mannitol salt agar (MSA), after incubation at 37°C for 24 hours; the colonies appeared golden-yellow along the lines of streak. The colonies were large, convex, elevated, shining, opaque and circular in appearance and Biochemical tests (Catalase test was performed for all isolates which gave positive reaction as suspected for all *Staphylococcus* species; the positive reaction indicated by the formation of gas bubble after mixing hydrogen peroxide solution with little amount of bacterial growth, coagulase test was also performed for all *Staphylococcus* isolates, the results marked the formation of clot when the tube method was applied while the presence of agglutination within 10 seconds indicated positive reaction when slide method was applied, and all *S. aureus* isolates were positive in both methods. One hundred isolates gave a positive result as coagulase positive, mannitol fermenters and DNase positive were diagnosed as *S. aureus*; The result of VITEK2 were employed for the result confirmed the primary identification of *S. aureus* isolates collected throughout the study. The ability of *S. aureus* strains to adhere and form multilayered biofilms on host tissue and other surfaces is

one of the important mechanisms by which they are able to persist in the diseases^[9]. The results from workers showed a high percentage of up to 77 isolates of 100 *S. aureus* isolated produce slime positive in Congo red agar. This result agreed with the study by^[10] who found that 77.8% of *S. aureus* isolates showed strong slime layer on the Congo red agar.

The Microtiter Plate Method result showed 57 of 100(57%) *S. aureus* were biofilm-positive, that 14(14%) strains were highly biofilm-positive (OD570 > 1), 43(43%) were low-grade biofilm and 43(43%) were biofilm-negative. This result agree with the study of (Mathur *et al.*, 2006), 57.8% of staphylococcal clinical isolates displayed a biofilm-positive phenotype and 14.47 and 39.4% exhibited high and moderate biofilm formation, respectively; while 46% were weak isolates with no biofilm detected.

Specific primers were used to determine the (*clfA*, *can* and *fnbA*) genes in this study; the optimal conditions for identified in Tabl (2). Several experiments have proven the purpose of reaching these conditions. This study was found 81 out of 100 *S. aureus* (81%) have the adhesions *clfA* gene; this result was nearly with another study which found that 91.3% of the tested strains were *clfA* positive (12). While 56 out of 100 strains (56%) have the adhesions *clfA* gene. This result was agreement with another report which found that 56% of the tested strains were *fnbA* positive^[13].

The collagen adhesion plays an important role in the pathogenesis induced by *S. aureus*, therefore In this study, showed 56 out of 100 strains (56%) have the adhesins *cna* gene, Our result was nearly with another

report which found that 52% of the tested strains were *cna* positive [14].

Table 2: The PCR program for the (*clfA*, *fnb* and *can*) genes amplification

No.	Step	Temperature(°C)	Time	No. of Cycles
1.	Initial denaturation	94°C	5 min.	1
2.	Denaturation	94°C	1 min.	
3.	Annealing	55°C	1 min	
4.	Extension	72°C	1 min	25
5.	Final extension	72°C	10 min.	1

In this study was used three genes (*clfA*, *fnb* A and *cna*), firstly we tested the ability of each primer pair to detect a specific target DNA sequence within *clfA*, *fnbA*, *can* genes

respectively (Fig1, 2, 3), then co-amplifications using different combinations of two, and finally all three primers pairs mixed together with samples of DNA extracted.

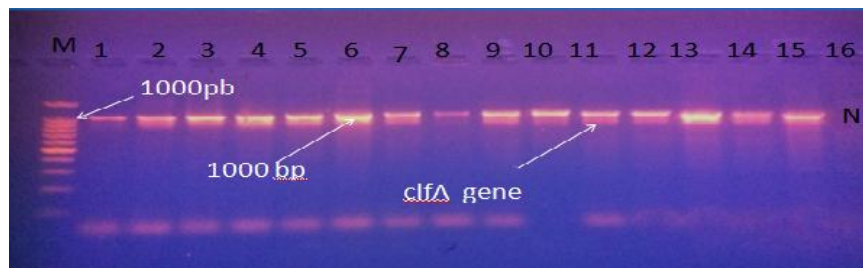


FIGURE 1: Agarose gel electrophoresis of PCR amplification products of *S. aureus*, *clfA* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular weight marker (100 bp ladder); lanes (1-15) positive amplification of 1000 bp for *clfA* gene. N is a PCR product of negative control.

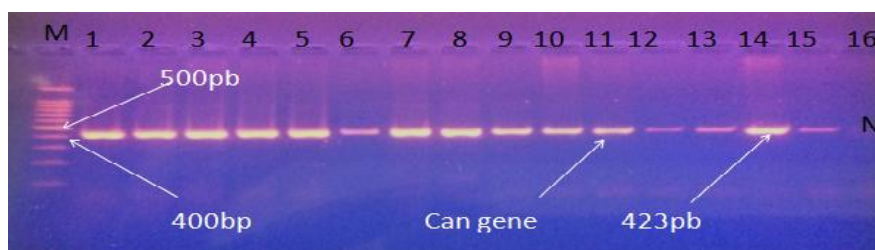


FIGURE 2: Agarose gel electrophoresis of PCR amplification products of *S. aureus*, *cna* gene (2 % agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular weight marker (100 bp ladder), lanes (1-15) positive amplification of 423 bp for *cna* gene. N is a PCR product of negative control.

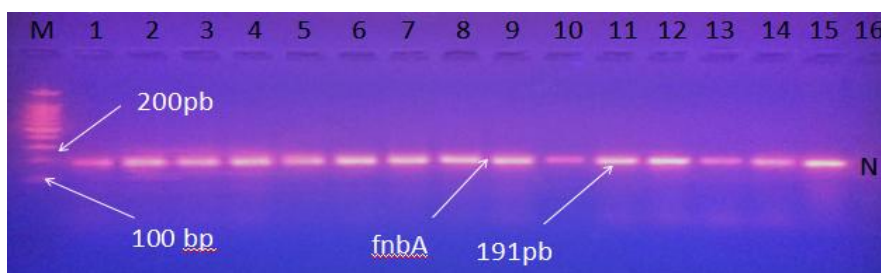


FIGURE 3: Agarose gel electrophoresis of PCR amplification products of *S. aureus*, *fnbA* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular weight marker (100 bp ladder), lanes (1-15) positive amplification of 191 bp for *fnbA* gene. N is a PCR product of negative control.

The results of the present study showed three, *clfA*, *fnbA*, *can* genes band detected at (1000bp, 423bp, 191bp respectively) only 36% isolated strains were produced three gene (1000bp,423bp,191bp) band. All the components were adjusted to give an excellent product for the two genes which were mixed together to be used as a single

step for the more precise test for the detection of our bacterium in different precise samples under study. 10 µl of the reaction mixture was put on an argarose gel of 2 % (wt/vol) for electrophoresis and visualization of the product after staining with ethidium bromide on a UV transilluminator as seen in figure (4).

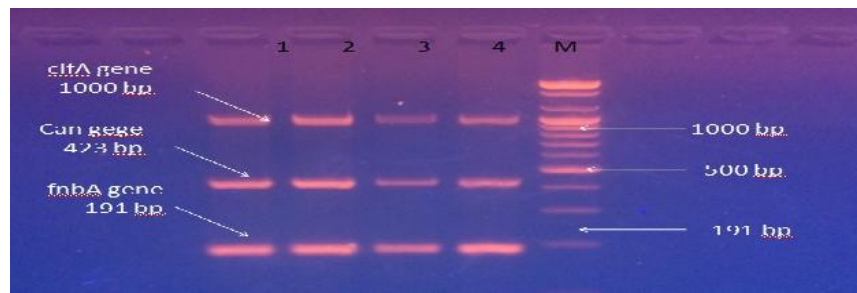


FIGURE 4 : Gel electrophoresis of amplified PCR products of *clfA* (1000bp), *fnbA* (191bp), *can*(423bp) genes of *S. aureus* isolates in multiplex PCR technique a garose (2%), TBE buffer (1X), 5V/Cm for 2 hrs. stained with ethidium bromide. M: The DNA molecular weight marker (100 bp ladder); Lane 1-4 a positive result.

REFERENCES

- [1]. Valaperta, R., Tejada, M.R., Frigerio, M., Moroni, A., Elisa Ciulla, E., Cioffi, S., Paolo Capelli, P. and Costa, E. (2010) *Staphylococcus aureus* nosocomial infections: the role of a rapid and low-cost characterization for the establishment of a surveillance system. *New Microbiologica*. 33:223-232.
- [2]. Al-Hasani, H.M.H. (2011) Comparative study between methicillinresistant coagulase positive and negative staphylococci on some virulence factors (2005). MSc. Thesis college of Science, University of Baghdad, Iraq
- [3]. Cucarella, C., Tormo, M.Á., Knecht, E., Amorena, B., Lasa, Í., Foster T.J. and Penadés, J.R. (2002) Expression of the Biofilm-Associated Protein Interferes with Host Protein Receptors of *Staphylococcus aureus* and Alters the Infective Process. *Infect. Immun.*70 (6):3180-3186.
- [4]. Foster, T.J., McDevitt, D. (1994) Surface-associated proteins of *Staphylococcus aureus*: their possible role in virulence. *FEMS Microbiol Lett* **118**: 199-206.
- [5]. Patti, J. M., Allen, B. L., McGavin, M. J. and Hook, M. (1994) MSCRAMM mediated adherence of microorganisms to host tissue. *Annu. Rev. Microbiol.* 48:585–617.
- [6]. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. (1994) *Bergey, s Manual of Determinative Bacteriology*. 9th.ed. Williams and Wilkins, USA. 532-551.
- [7]. Frank, K. and Patel, R. (2007) Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilm formed by *icaADBC*-positive *staphylococcus lugdunensis* isolates. *infect. immun.* 75(10):4728-42.
- [8]. Bekir, K., Ben Abdallah, F., Ellafi, A. and Bakhrouf, A. (2011) Adherence assays and slime production of *Staphylococcus aureus* strains after their incubation in seawater microcosms. *Ann. Microbiol.*, 61(4): 819-823.
- [9]. Jabra-Rizk, M.A., Meiller, T.F. James, C.E. and Shirliff, M.E. (2006) Effect of Farnesol on *Staphylococcus aureus* Biofilm Formation and Antimicrobial Susceptibility. *Antimicrob. Agents Chemother.* 50(4):1463-1469.
- [10]. Türkyilmaz, S. and Kaya, O. (2006) Determination of some Virulence Factors in *Staphylococcus* Spp. Isolated from Various Clinical Samples. *Turk. J. Vet. Anim. Sci.* 30:127-132.
- [11]. Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. (2006) Detection of Biofilm Formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian. J. Med. Microbiol.* 24: 25-29.
- [12]. Karahan, M., Acik, M. N. and Cetinkaya, B. (2011) Investigation of Virulence Genes by PCR in *Staphylococcus aureus* Isolates Originated from Subclinical Bovine Mastitis in Turkey. *Pak. Vet.J.*(31):249-253.
- [13]. Bekir, K., Haddad, O., Grissa, M., Chaieb, K., Bakhrouf, A., Elgarssdi, S.I. (2012) Molecular detection of adhesins genes and biofilm formation in methicillin resistant *Staphylococcus aureus*. *AFR. J. Microbiol. Res.*6:4908-17.
- [14]. Peacock, S.J., Foster, T.J., Cameron, B. J., and Berendt, A.R. (1999) Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology*145:3477–3486.