Original Article

Detection of biofilm-forming genes in *Staphylococcus aureus* clinical isolates by PCR assay

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Abstract

Biofilms are assemblages of bacteria encased in an extracellular matrix, as well as proteins, exopolysaccharides and macromolecules such as DNA. Bacterial biofilms are thought to play a substantial influence in over 80% of bacterial illnesses. Biofilm buildup on medical implants is responsible for about 60% of hospital-acquired infections. In this study, 150 clinical samples (wound, urine and sputum) were collected from patients admitted to surgical wards of Azadi teaching hospital/Kirkuk during the period from February to October 2018. Of these, 98 (65.3%) were females and 52 (34.7%) were males. Congo Red Agar was used to test the potential of *S. aureus* isolates to produce biofilms. The relationship between *S. aureus* biofilm producer isolates and ica ABCD operon genes was studied by using PCR. Out of all samples, *S. aureus* isolates were identified in 29 (19.3%) of clinical samples, and most isolates were from wound infections (n=19, 65.5%) followed by urine (n=10, 43.5%). Whereas, no *S. aureus* isolate was detected in sputum samples. Moreover, *S. aureus* biofilm producing isolates were detected in 9 (24.1%) samples and were represented as black colonies on Congo Red Agar. The results of this study indicated the icaA, icaB, icaC and icaD genes harbored in 4 (44.4%) of *S. aureus* biofilm forming isolates while icaA, icaC, and icaD genes were detected in 6 (66.7%) of isolates. On the other hand, all *S. aureus* isolates were negative for both clfB and fnbB genes.

Keywords: *Staphylococcus aureus*, biofilm formation, ica operon genes, PCR.

Introduction

A biofilm is a community of microorganisms adhered to a surface that provides a protection level of homeostasis and resilience in a changing environment.[1,2] When compared to their planktonic cells, bacterial cells in biofilms are more vulnerable to environmental conditions and antimicrobials.[3] They may also facilitate mutual metabolic functions and promote horizontal transfer of genes.[4]

In medicine, biofilms have a profound impact and their role in human illness has received a great deal of attention. Generally, biofilms associated with moist surfaces such as medical interiors equipment, tubing of medical equipment, and that formed on medical devices such as catheters, implants or contact lenses, serve as a reservoir for bacteria that can be transmitted into the body, resulting in acute and chronic infections. It is suspected that difficult-to-treat chronic infections arise from these biofilms.[5]

Staphylococcus aureus (S. aureus) is recognized as the most common cause of human skin and mucosal surface infections, septicemia and life-threatening diseases with fatality rates which are higher than those associated with AIDS, TB and chronic viral hepatitis.[6-8] It can also result in inducing infections such as wound and endovascular infections. Such infections can result in persistent chronic infection from an acute inflammatory infection. The Small Colony Variants" (SCV) and the invasion of bacterial host cell are associated with the chronic infections caused by S. aureus.[9,10] Such bacteria may survive in a dehydrated state for long time and can develop biofilms on clinically dry surfaces as well.[11] Staphylococci are the bacteria most likely to

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infect medical equipment that penetrates particular areas, such as those implanted after the operation.[12] Often staphylococcal infections lead to acute disease .[13] Studies have shown increased recovery rate of Methicillin-Resistant *S. aureus* (MRSA)in the patients suffering from acute and chronic maxillary sinusitis.[14] Hence, to understand the recovery process, it is important to detect biofilm-forming genes in *S. aureus*.

The prevalence of MRSA has increased dramatically in the last several decades. The formation of *S. aureus* biofilm begins with attachment to surfaces and is controlled by the regulation of different microbial surface components. When these molecules are recognized, the production of polymeric intercellular adhesion protein is triggered. This protein modulates cell-to-cell attachment, and is encoded by ica Operon (icaABCD). This synthesis is mediated by fibronectin-binding proteins, clumping factors and biofilm-binding proteins. Studies on icaAD and adhesion gene have demonstrated that biofilm development depends heavily on these four factors. Understanding phenotypic and genetic characteristics of biofilms can also help us avoid, control and treat infections in the future.[4, 15-19]

It has been observed that MRSA presence leads to failure of "anti-microbial therapy" and these organisms are able to distribute to other sites of the body as well causing invasion in the host cells leading an acute infection to chronic infection. Thus, current research aimed to identify the biofilm producing capability, existence of icaABCD, fibronectin-binding proteins (Fnb) and clumping factors (Clf) genes in clinical isolates of *S. aureus* by PCR technique. However, the β- lactamase producing MRSA are able to respond to the treatment of β-lactam antibiotics and even continue to protect "penicillin-susceptible pathogens" from penicillins.[20]

Materials and Methods

Bacterial Strains

This study was conducted in the Department of Medical Microbiology/ Faculty of Medicine/ University of Kirkuk. One hundred fifty clinical samples (wound, urine and sputum) were collected from patients admitted to Azadi teaching hospital/Kirkuk during the period from February 2018 to October 2018. The age of these patients varied between 1-75 years. Out of 150, female patients were 98 (65.3%) and male patients were 52 (34.7%). Samples were cultured on blood agar, followed by an incubation period of 24 - 48 hours at 37°C under aerobic conditions. *S. aureus* strains were screened through gram staining, colonies' structure and growth (golden colonies on nutrient medium, beta hemolytic colonies on blood agar and yellow discoloration on mannitol salt agar), standard biochemical tests (positive catalase and coagulase tests) and API-Staph Ident System (bioMerieux, Nürtingen, Germany).

Biofilm production test

Congo Red Agar (CRA) was used to investigate the biofilm-forming ability of *S. aureus* isolates. The isolates were cultured on CRA medium which was prepared by adding 0.8g of Congo red stain and 36g of sucrose to 1 L of BHI. After 24h incubation at 37°C, the isolates were examined with red colonies being considered as non-slime producers while those with black colonies were regarded as slime or biofilm producers.[21]

DNA extraction

Bacteria DNA Preparation Kit (Jena Bioscience GmbH, Germany) was used for extraction of DNA. According to the protocol, the sample was mixed by inverting the tube for 1 minute followed by centrifuging it for 1 minute at15000 g. The supernatant was discarded and, the contents of the tube were drained onto a clean, absorbing piece of paper. Then DNA pellet was washed by adding 500 microliter of buffer and the tube was rotated several times. Later the tube was centrifuged again for 1 minute at 15000 g. The ethanol was discarded carefully and the DNA was then air dried at room temperature for 10-15 min. Later, 50-100 microliter of hydration solution was added to the dried DNA pellet. The DNA was incubated again at 65°C for 1hr and then stored at -20 °C.

PCR Primers and Amplification

The biofilm genes were determined and amplified by using primers (Qiagen, Germany) as listed in Table 1.

Table 1: Details of the primers used for the amplification of biofilm genes

Genes	Nucleotides sequence of primers (5'-3')	Amplicon size (bp)	Reference
icaA	GAC CTC GAA GTC AAT AGA GGT CCC AGT ATA ACG TTG GAT ACC	814	[22]
icaB	AGAATCGTGAAGTATAGAAAATT TCTAATCTTTTTCATGGAATCCGT	880	[23]
icaC	CTTGGGTATTTGCACGCATT GCAATATCATGCCGACACCT	209	[18]
icaD	ACCCAACGCTAAAATCATCG GCGAAAATGCCCATAGTTTC	211	[18]
fnbB	ACGCTCAAGGCGACGGCAAAG ACCTTCTGCATGACCTTCTG-CACCT	197	[18]
clfB	AACTCCAGGGCCGCCGGTTG CCTGAGTCGCTGTCTGAGCCTGAG	159	[18]

Detection of biofilm-producing genes

The ica ABCD gene was amplified through PCR (BIO-RAD T100 Thermal cycler, USA) to identify biofilm producing *S. aureus* isolates. The PCR products were run on 1.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator.

Table 2: Detection of biofilm-producing genes by using PCR.

Genes	Initial denaturation (° C/ min)	Denaturation (° C / sec)	Annealing (° C / sec)	Extension (° C / sec)	Final extension (° C/ min)	Cycles
icaA	94 / 3	94 / 60	54 / 39	72 / 60	72 / 7	30
icaB	94 / 5	94 / 60	52 / 30	72 / 90	72 / 10	
icaC	95 / 15	94 / 30	60 / 90	72 / 90	72 / 10	35
icaD	95 /15	94 / 30	60 / 90	72 / 90	72 / 10	
fnbB	95 / 15	94 / 30	60 / 90	72 / 90	72 / 10	
clfB	95 / 15	94 / 30	60 / 90	72 / 90	72 / 10	

Every biofilm-producing gene has its own capability of colonizing the human body that is why PCR reaction for each strain was different. . It was observed that the strain with more number of biofilm-producing genes was able to highly colonize the human body. In addition, they were able to enhance the resistance to antibiotics and pathogenesis.

Results

From February 2018 to October 2018, 150 clinical samples were collected from patients admitted to surgical wards of Azadi teaching hospital/Kirkuk. Their ages ranged from 1 - 75 years. Amongst these, 65.3% (n=98) were females and 34.7% (n=52) were males. Of all the patients, *S. aureus* isolates were identified in 29 (19.3%) samples. Most of the isolates belonged to wound infections (n=19, 65.5%), followed by 10 urine samples (43.5%). Whereas, no *S. aureus* isolates were identified in sputum samples as shown in Table 3. Regarding the biofilm producing capability, 9 (24.1%) isolates out of 29 samples produced black colonies on CRA and considered as slim producers.

Table 3: Percentage and number of S. aureus according to the source of infection.

Source of isolate	Frequency	Percentage (%)
Wound	19	65.5
Sputum	0	0
Urine	10	34.5
Total	29	100

Detection of icaA, icaC, icaD, clfB and fnbB genes

PCR was used to study the biofilm related genes icaC and icaD (intercellular adhesion gene C and D), clfB (clumping factors B) and fnbB (fibronectin binding proteins B). Results showed that the icaA gene amplicon was present in 7 (77.8%) S. aureus isolates. The size of amplified DNA is 814 bp as shown in figure 1.

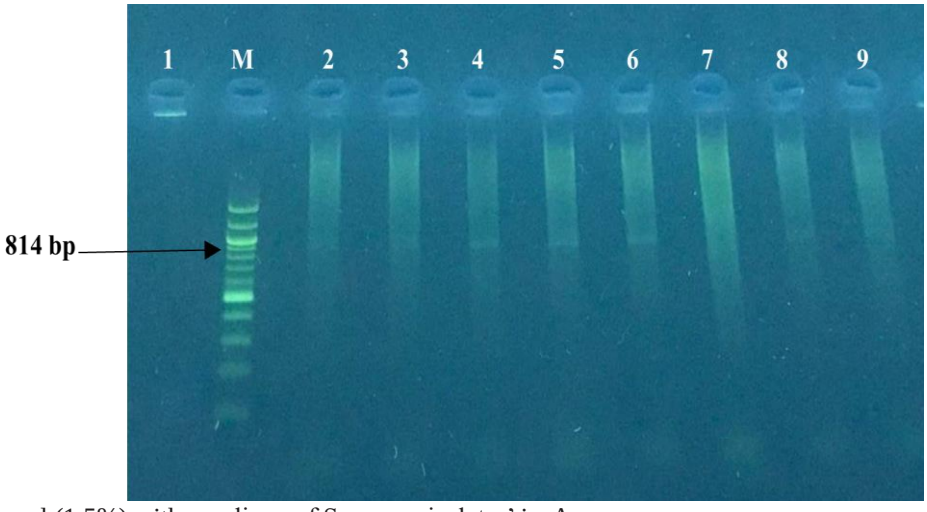

Figure 1: Agarose gel (1.5%) with amplicon of S. aureus isolates' icaA gene.

The icaB gene amplicons were present in 5 (55.6%) biofilm producing S. aureus isolates. The size of amplified DNA is 880 bp (figure 2).

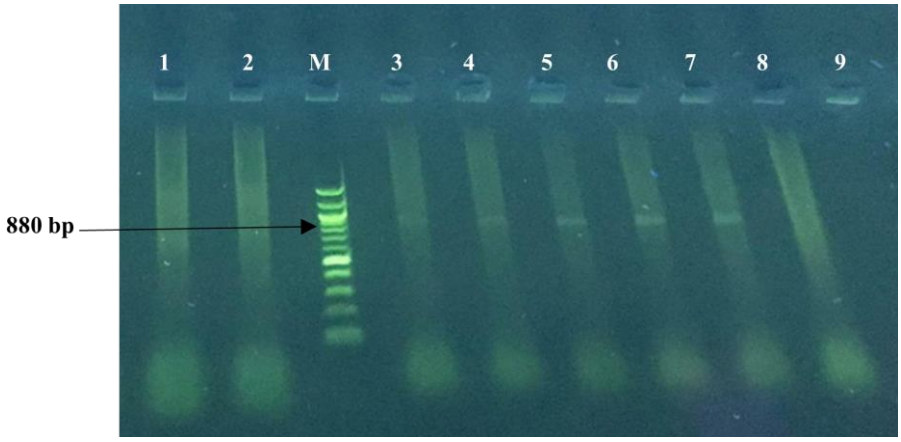

Figure 2: Agarose gel (1.5%) with amplicon of S. aureus isolates' icaB gene.

While 8 (88.9%) biofilm producing *S. aureus* isolates showed positive results for icaC. The size of amplified DNA is 209 bp (figure 3).

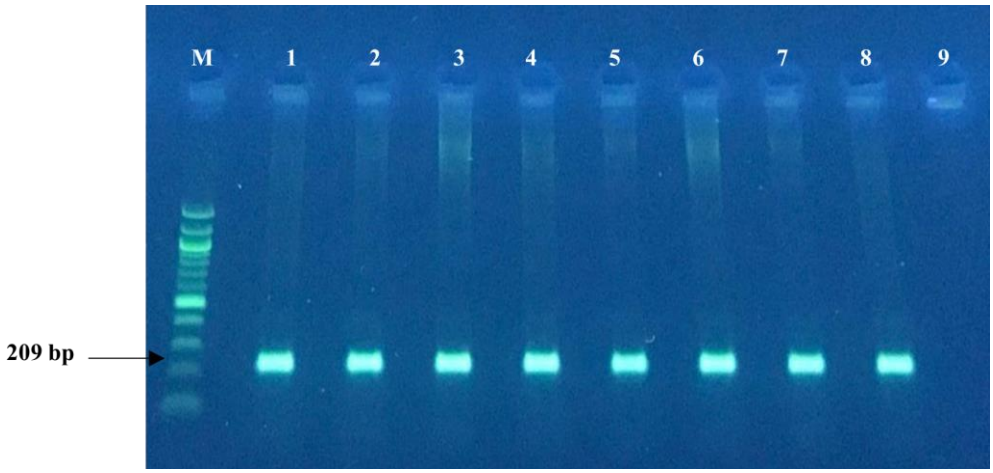

Figure 3: Agarose gel (1.5%) with amplicon of S. aureus isolates' icaC gene.

Similar results were also shown by icaD gene as shown in figure 4. The size of amplified DNA is 211 bp

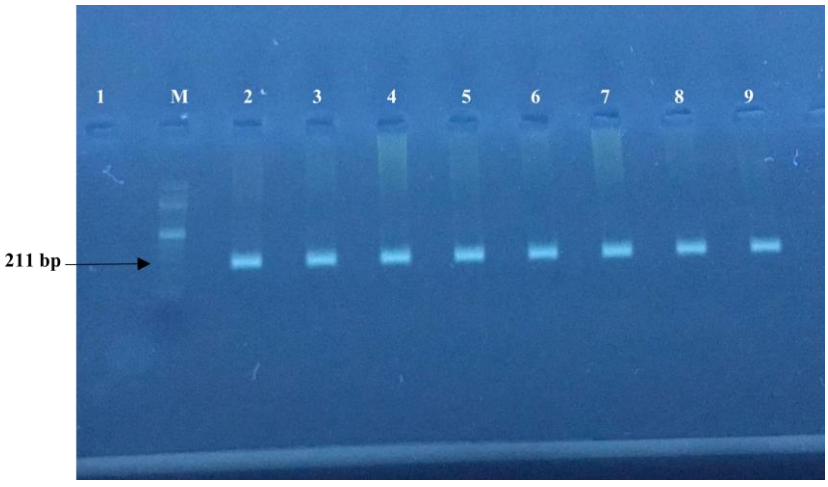

Figure 4: Agarose gel (1.5%) with amplicon of S. aureus isolates' icaD gene.

Other genes involved in biofilm formation in S. aureus were also studied i.e. clfB and fnbB. The results showed that all S. aureus isolates were negative for both these genes as shown in figure 5 and 6 respectively.

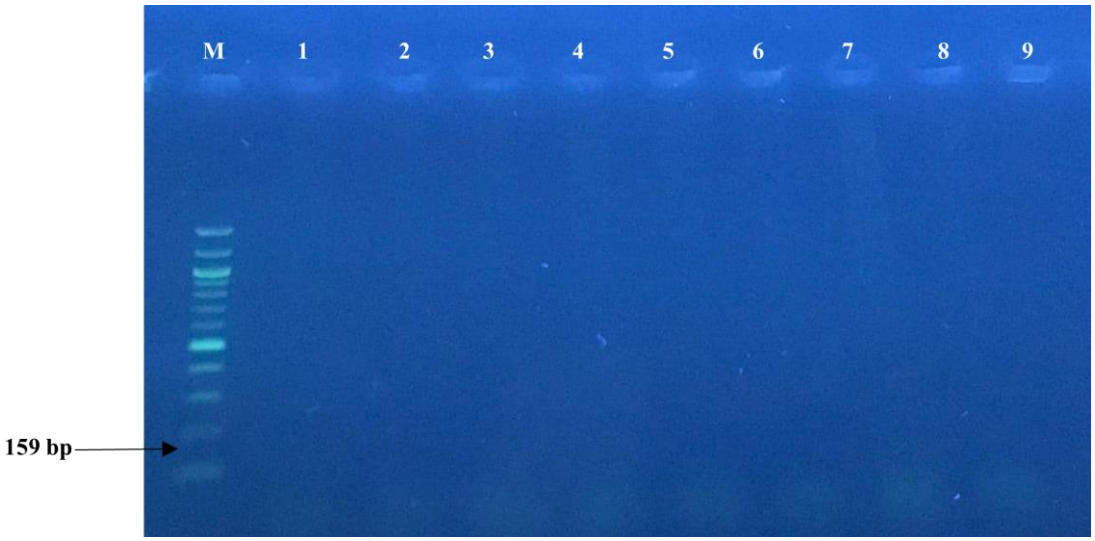

Figure 5: Agarose gel (1.5%) with no amplicon of S. aureus isolates' clfB gene.

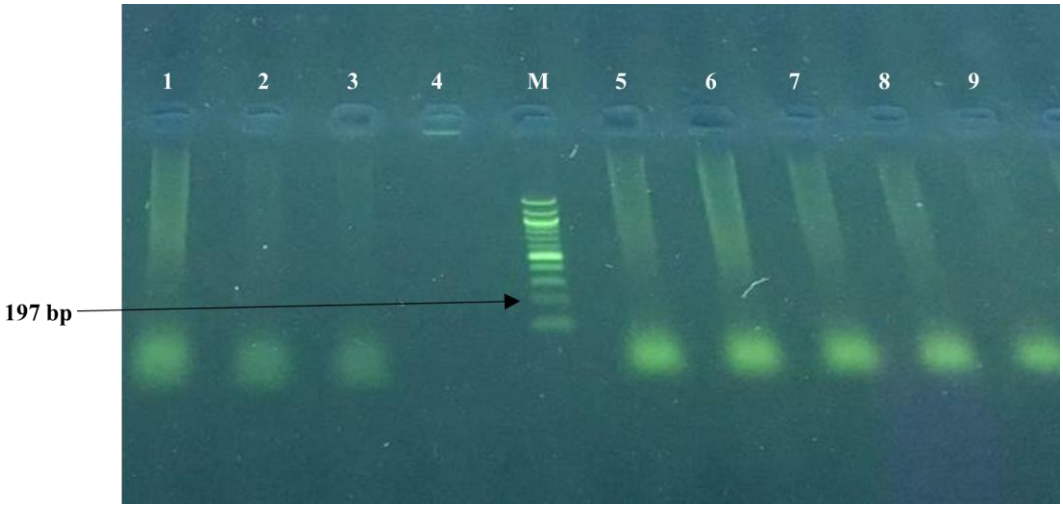

Figure 6: Agarose gel (1.5%) with no amplicon of S. aureus isolates' fnbB gene.

The results of this study indicated the presence of icaA, icaB, icaC and icaD genes in 4 (44.4%) of S. aureus biofilm forming isolates, whereas 6 (66.7%) isolates were found to have icaA, icaC and icaD genes.

Discussion

Surface-associated bacteria communities are called biofilms which usually affect various environments such as water pipes and indwelling devices in hospitals. Investigating the molecular processes which affect the development and maintenance of these biofilms in hospital devices has been increased.[24] In clinical environments, these biofilm producing bacteria cause extraordinarily high levels of resistance to a wide spectrum of antimicrobial agents in comparison to their planktonic counterparts.[25] Nosocomial pathogenic agents can infect patients by forming biofilms not only on materials that have been inserted into the body, like implants but also on biotic surfaces such as burn wounds.[26]

Current research was performed to investigate the capability of production of biofilms and the presence of operon icaABCD and adherence genes in S. aureus isolates from clinical samples of hospitalized patients. Results of this study showed that isolates of S. aureus were identified in 29 (19.3%) of 150 samples of which 9 (24.1%) isolates were biofilm producing i.e. they produced black colonies on CRA. These findings are in accordance with

other studies which revealed a high prevalence of biofilm forming S.aureus isolates in clinical samples.[17, 27, 28] However some studies also reported contrasting results. [29] It has been observed that icaABCD genes encoding polysaccharide inter cellular adhesion (PIA), are more able to produce biofilms among *S. aureus.* Many chronic infections related to *S. aureus* are due to ica dependent biofilm formation i.e. they developed by production of a "PIA- PNSG/ poly- beta-1, 6-N-acetylglucosamine polymer by the "N-acetyl glucose aminyl transferase enzyme. This enzyme is however encoded by icaA and D genes".[30] The development of biofilms is affected by different environmental aspects for example; glucose, lactose or proteases present in culture media, its surface area and kind of surface i.e. rough or smooth, the porosity and charge on the surface. Moreover, different types of samples, genome of S. aureus clinical isolate and the presence of foreign bodies can also cause variations.[31] *Staphylococcus Spp.* can produce a biofilm on almost any synthetic polymer used for prosthetic appliances. Moreover, they can also bind to the human cell receptors and matrix proteins. The most common infectious agent of skin and soft tissue is an opportunistic pathogen S. aureus. It is possible for this bacteria to penetrate the skin through the surgical puncture or wound barriers and induce infection. In addition, it has the tendency to attach to tissues and indwelling surgical equipment and create a biofilm on them.[32] Biofilms are able to withstand antibiotics concentration of about10-10,000 fold higher than those appropriate to inhibit the growth of free floating bacteria.[33] Thus periodic production of S. aureus biofilm and its pattern of resistance to antibiotics might occur, which will contribute to the treatment of wound infection as early as possible.[34]

Biofilms are highly diverse mixtures of bacteria which are kept closed through jra secreted extracellular matrix called extracellular polymeric substances (EPS). The major EPS in S. aureus and S. epidermidis are PIA and capsular polysaccharide/ adhesion (PS / A).[35, 36] The PIA is produced as a result of the enzymes expressed by the ica operon genes.[28] The present study focused on investigating the genes that code for adherence molecules and also the tendency of S. aureus isolates to produce biofilms. Results showed that amplicons of intercellular adhesion icaA and icaB genes were present in 7 (77.8%) and 5 (55.6%) of biofilm producing S. aureus isolates respectively. Whereas, amplicons of both icaC and D genes were found in 8 (88.9%) S. aureus isolates. Several studies demonstrated the presence of icaA and icaD genes in S. aureus biofilm producing isolates from patients with catheter associated infections.[36-40] Another study reported the presence of the ica operon in 75% of biofilm forming S. aureus isolates.[41] Some other studies also revealed that most of the S. aureus strains were harbored with icaD gene as compared to other ica genes.[42] On the other hand, another study showed the presence of both icaA and icaD genes in all S. aureus isolates.[43] Other investigations have found an association between production of biofilms and existence of these ica genes. [29, 44] From a clinical standpoint, understanding the primary adhesive mechanisms in infections may aid in the development of preventive and therapeutic measures such anti-adhesive coatings or anti-adhesion drugs.[37]

It is believed that the development of Staphylococcus biofilm is a two-stage process. The initial step involves the attachment of bacterial cells to a substrate's surface while in the second step, biofilm production involves the aggregation of bacteria using PIA[45] through adhesion of cells. The PIA is found to be regulated by gene locus consisting of four intercellular adhesion (icaADBC) genes arranged in the structure of an operon.[46] There are various genes which contribute to the formation and preservation of staphylococci-formed biofilms. The most investigated of these genes are the ica A and B genes which are required in production of PIA, which contains N-acetylglucosamine used for the primary constituent of the matrix of exopolysaccharides within biofilm.[13, 47, 48]

The icaA is needed for co-expression of icaD and N-acetylglucosamine which can enhance the capsular polysaccharide characteristics[30] and signaling the essential part the icaD locus which has been identified as a virulence factor in S. aureus pathogenesis.[49, 50] The ica expression has been been found associated with environmental factors.[51, 52] Numerous factors such as low doses of antibiotics, osmolality and anaerobic environments are recognized as expression enhancers for biofilm formation. Furthermore, the operon expression can be activated and deactivated by inserting and excising the insertion sequence (IS),[53, 54]

All S. aureus isolates in this study were negative for both clfB and fnbB genes. These genes are involved in the invasion and adhesion of bacteria to surfaces.[55-57] However, there are certain studies which showed contrasting results i.e. high prevalence of clfB and fnbB adhesion genes in S. aureus and their role in biofilm production.[15, 19, 39, 40, 58-60] Fibronectin is a glycoprotein which binds to the integrins, fibrin and collagen. These are cell membrane proteins which are also present in the plasma of the human body. During the coagulation of blood, the fibrinogen is converted to insoluble fibrin. Fibrinogen binding protein is regarded as a potent virulence gene in S. aureus illnesses because it is linked to fibrinogen and may interferes with platelet clumping and the formation of blood clots.[61]

The protein constituents of outer membrane of microorganisms recognize adhesive matrix molecules and are highly capable of interacting with laminin-

binding protein (lbp), collagen-binding protein (cbp), elastin-binding protein (ebp), fibrinogen-binding protein (fib) and fibronectin-binding proteins (A,B). Moreover, they also interact with host extracellular matrix proteins (fnbA and fnbB) and clumping factors A and B (clfA, clfB).[62] The primary constituent of the biofilm development in the S. aureus strains is a polysaccharide intercellular adhesion expressed by the ica operon (icaA, icaB, icaC, icaD).[63]

Conclusion

According to our findings, biofilm formation was present in S. aureus isolates with frequencies of the adhesion ica encoding genes. These results revealed the importance of ica operon in biofilm production. However, all biofilm producing isolates were negative for clfB and fnbB gene.

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