Review
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Evaluation expression icaabcd biofilm formation genes in staphylococcus aureus by real-time PCR

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Abstract

Staphylococcus aureus is one of the most significant pathogens that cause several nosocomial and community infections. Adhesion to surfaces and biofilm formation is considered the main step in staphylococcal infection. This study aimed to investigate the expression of ica(intercellular adhesion) genes in clinical isolates of S.aureus. Material and Method: A total of 93 clinical S. aureus isolates were collected from hospitals in Tehran. Quantitative biofilm formation was determined by using Congo red agar (CRA). Out of 93 isolates, only 18 (19.35%) isolates had positive biofilm formation, and they were examined for expression the icaABCD genes by Real-time PCR method. Results: The Congo red agar assay results showed that attachment abilities in 4 (4.3%) strains were strong and in 14 (15.05%) strains were moderate. All isolates were positive for icaABCD genes expression. The average gene expression for 18 strains was as follows for icaA (9.332), icaB (1.485), icaC (17.612) and icaD (3.390), respectively. Discussion: The data obtained suggest that clinical S. aureus isolated from the evaluated patients has a potential for biofilm formation. S. aureus clinical strains have different capacity to produce biofilm. This may be caused by a difference in the expression of biofilm genes and heterogeneity in genetic origins.

Keywords

Staphylococcus aureus; Biofilm; icaABCD Genes Expression

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Aim

The collection of microbial cells that are connected tightly to the surface with a polysaccharide matrix being microbial originally is called Biofilm. Biofilms can consist of one species or a mixture of several species, being formed in different places possessing losses and benefits [1]. Biofilms were explained regarding bacteria in 1978 by Custer ton. The stressed situation causes the bacteria to connect to different surfaces. Generally, the microorganisms live by the side of solid surfaces, in nature. The connection with solid surfaces brings about the biofilm formation [2]. The bacteria's through biofilm forming causes resistance against antimicrobial agents, resistance against the host immune system and maintenance of physical and chemical conditions suitable for growth; so it induces resistance of biofilms in unfavorable conditions. Also, the synergistic and helping relations among bacteria or biofilm influences their resistance against unfavorable conditions [3]. Staphylococcus aureus is an optional anaerobic gram-positive coccus, considered to be the most important species in Staphylococcus genus medically. The so mentioned bacteria causes a wide range of infections, including simple skin infections (like acne, pimples, anthracoid, sties and abscess) and life-threatening diseases (like pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, and septicemia) [4, 5]. The bacteria contains genes, coding pathogenic factors, some of them are located on chromosome and some are moving as genetic elements. The pathogenic factors being coded, induce bacterial colonization in the host, influx to damaged mucus and skin, spreading through the body and invade from host defense mechanisms. Some of the factors include:

Peptidoglycan, lipoteichoic acid, protein, hemolysin, enterotoxin, clamp factor, and biofilm formation. The above – mentioned bacteria can produce polysaccharide and protein factors connected to the surface, through this production process, the bacteria is effective in biofilm production [6, 7]. In fact, a Staphylococcus biofilm is a group of microorganisms related to a network of internal canals in glycoprotein and extracellular polysaccharide matrix named extracellular polymeric substance. The Extracellular polymeric substance is composed of polysaccharide, protein, phospholipid, teichoic acid, and other hydrate and polymeric materials containing water of 85 to 95 %, making it able to adhere to pathogenic agents on the internal surface of carriers and food industry equipment surface, the process causes different pathogenic microbes to be transferred. Locus ica (intercellular adhesion) is composed of genes ica A, ica B, ica C, ica D, in which PIA is synthesized by synthesis mediated protein. The genes ica A and ica D are the most effective form among locus ica in the formation of biofilm in Staphylococcus Aureus and Epidermis. The gene ica A encodes N-acetyl glucosaminyl transferees, that is the involved enzyme in the synthesis of N- acetylglucosamine PCR oligo [5, 8]. It is reported that ica D has a vital role in expressing the maximum N- acetyl glucosaminyl transferees and it makes the phenotypic expression of capsular polysaccharide possible [5,9]. The study aimed to examine the expression of biofilm formation genes in clinical isolates of Staphylococcus Aureus isolated from clinical samples of Shariati Hospital in Tehran using the method Real-time PCR.

Material and Method

Sample collection and evaluation of biofilm formation by phenotypic methods:

In a cross-sectional study, 93 isolates of Staphylococcus Aureus were collected separated from patients referring to Hospital in (year). All the isolated had been proved using biochemical tests including catalase, coagulase, oxidase, fermenting the sugar mannitol, DNase, and sensitivity to novobiocin. The number of 18 isolates from whole were able to produce biofilm. The phenotypic formation of biofilm was studied in Staphylococcus Aureus using the medium of CRA (Merck, Germany) containing 36 gr sucrose (sigma, USA) to evaluate its formation. The isolates have been incubated at 37°C for 24 hours and room temperature for a night after culturing. The black colonies in isolates were identified as biofilm forming strains, Red almost black colonies as weak strains of biofilm formation and the bright red colonies as strains unable to produce biofilm [10].

Draining expression of ica ABCD by Real-time PCR:

Real-time PCR was used to quantitatively analysis of ica ABCD and 16 sr RNA (as an internal controlling gene) individually, by the use of ferment as a kit and the presented primers in table 1. For this purpose, RNA was extracted using micro kit RNeasy (Ki agene). Reverse transcription reaction was done for cDNA synthesis using Hexamer primers produced by Ki agene company. According to protocol, Master mix including synthesized cDNA and primers (PCR mixture: 0.3 ⊠M each primer, 0.2 ⊠M each d NTP, 1.5 ⊠M Mgcl2, 1x PCR buffer, 1.5 Utaq polymerase) were mixed in an appropriate volume, and the reaction was done by Bio-Rad Real-time PCR device. The reaction volume was 25 ⊠L and included CDNA, sweep primer, master mix, and double distilled sterile water. Temperature cycling included initial denaturing in 95°C for 5 minutes, followed by 40 cycles in 95°C for 20 Seconds, 60°C for 20 seconds and 72°C for 20 seconds, and a final cycle of 72°C for 5 minutes. The expression of ica ABCD was compared with expression of 16sr RNA as housekeeping gene. The reaction was done 3 times for each sample, and the average resulted was considered to be the expression quantity of the sample. The relative reference formula of expression (was used to determine the target gene expression. is obtained through subtraction of of the clinical sample from of the standard sample of Staphylococcus Aureus ATCC 25923.

Table 1. Primer sequences used for icaABCD genes for Real-Time-PCR (8)

primer sequence	Gene	Gene size
5-GAGGTAAAGCCAACGCACTC-3 5-CCTGTAACCGCACCAAGTTT-3	icaA	188
5-ATACCGGCGACTGGGTTTAT-3 5-TTGCAAATCGTGGGTATGTGT-3	icaB	190
5-CTTGGGTATTTGCACGCATT-3 5-GCAATATCATGCCGACACCT-3	icaC	192
5-ACCCAACGCTAAAATCATCG-3 5-GCGAAAATGCCCATAGTTTC-3	icaD	198

Results

Biofilm formation using the medium CRA showed that 18 isolates (19.3570) were able to form biofilm in which the black colonies (strong biofilm) were numbered as 4 strains (4.3 %) (Figure 1) and the black and Red ones (moderate biofilm) as 14 strains (15.05) (Figure 2). 16sr RNA gene showed positive results in all strains as the internal controlling gene and housekeeping one, suggesting accuracy in PCR reaction individually. In examining the presence or absence of ica ABCD in 18 strains under the study, in this essay, it was shown that all the strains possessed it. Also, the results from Real-time PCR using cDNA suggested that all the clinical strains expressed ica ABCD (fig-

ure 3). The results from analysis of ica ABCD expression using qRT- PCR for standard strains and 18 clinical isolates is shown in table 2. The curve of melting point for ica ABCD is demonstrated in figure 3. The average of gene expression for 18 isolates of ica A was obtained as (9.332), and for ica B as (1.485), for ica C as (17.612) and for ica D as (3.390). The graph 1 shows expression amount of the 4 genes in 18 isolates of staphylococcus aureus.

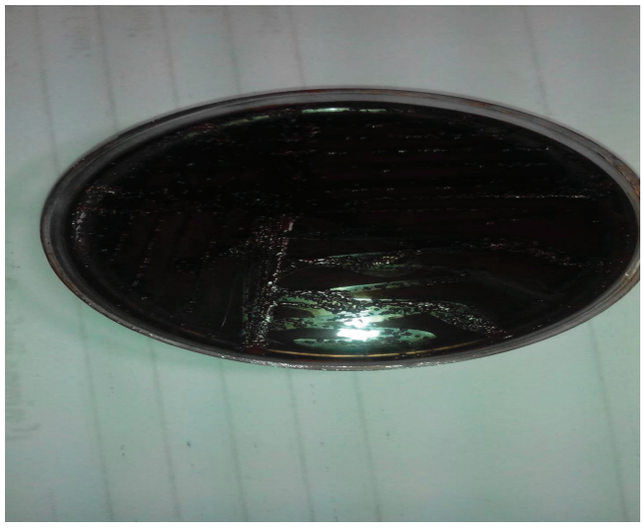

Figure 1. Biofilm formation on Congo red agar medium (strongly)

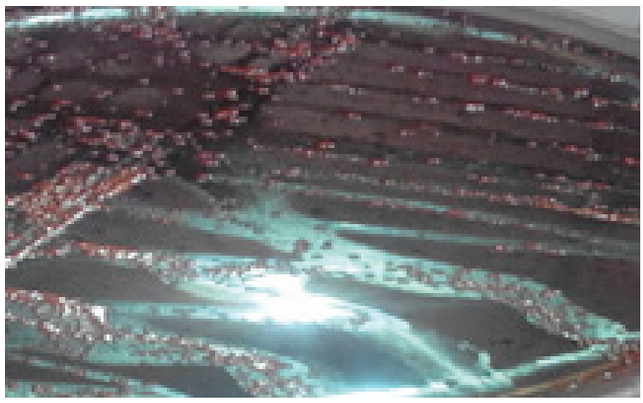

Figure 2. Biofilm formation on Congo red agar medium (on average)

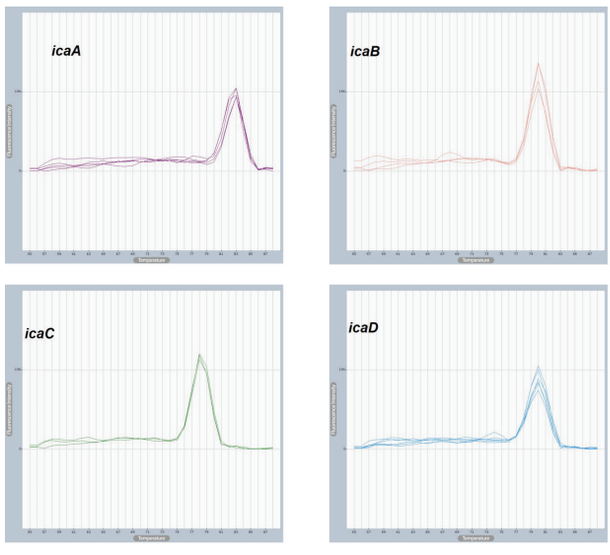

Figure 3. The melting curve for genes icaABCD

Chart 1. IcaABCD gene expression in 18 clinical S. aureus isolates

Table 2. IcaABCD analysis of gene expression in biofilm-producing S. aureus 18 strains

Name samples	icaA	icaB	icaC	icaD
N	1	1	1	1
U1	6597/0	0196/0	521/0	806/0
U2	556/10	5368/1	1/77	735/5
U3	8898/0	1445/0	099/0	339/0
u4	832/13	2116/0	979/0	515/1
W1	556/10	5800/1	0055/0	747/0
W2	765/18	0178/0	223/16	550/0
W3	81712/12	5315/0	285/0	0656/0
W4	426417/5	856/4	78/40	273/30
W5	784/22	1698/4	62/85	563/4
W6	010/4	004/1	051/2	005/1
T1	502/5	705/1	111/0	8969/9
T2	221/14	829/0	182/3	705/1
T3	282/8	7526/0	784/22	5987/0
W7	784/13	169/2	627/58	563/2
W8	010/4	0428/1	0518/2	005/1
T4	502/3	705/2	012 /1	989/1
Abse	382/10	515/0	404/2	569/3

Discussion

Biofilm matrix includes exopolysaccharides, protein, lipid, and nucleic acids. The matrix has known structural role in defense and protection, and causes new genetic features and provides food and combined metabolic activity. The most important microorganisms that are effective in polymicrobial biofilm formation contain Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, some members of Enterobacteriaceae family, and the yeast Candida albicans. The first step in infectivity of S. aureus is its adhesion to surfaces such as medical devices, host tissues and so on; that is attributed to a combination of extracellular factors like the ability to connect or to form biofilm [11]. This step is mediated by cell–adhesive polysaccharides or PIA in which protein in intracellular adhesion agents ica A, ica B, ica C and ica D are involved in its production. The collection is set on an operon [12, 8]. This polysaccharide is synthesized following the expression of related enzymes by ica A. Ica participation with locus increases synthesis in polysaccharide and induces capsule phenotype. Role of ica B is to De–acetyle polysaccharide before adhesion to the cell membrane and ica C genes encode a membrane protein helping in elongation and transudation of polysaccharide from the coll. The expression and operon ica ABCD increase by regulatory systems like sar A and sigma B. On the other hand, ica R as a strong negative controller can decrease expression of genes in the operon through adhesion to promoter area [13]. Therefore, the amount of ica ABCD genes is obvious according to their direct effect on biofilm formation and pathogenicity of Staphylococcus aureus mac than before, to achieve a solution to combat this phenomenon. The paper is aimed to study the expression of operon ica ABCD in Staphylococcus aureus strains that form the biofilm. The previous studies suggested an important relation between biofilm formation and presence of ica ADBC among S. aureus clinical isolates [14 -16]. Although less attention was paid to the expression of these genes and fewer studies reported the ability of S. aureus clinical isolates in the expression of ica [3, 8]. Park et al. observed no difference in studying the presence

of operon ica and the relation with biofilm formation on those S. aureus ones, isolated from clinical samples and saprophytic strains; all possessing this gene [17]. However, none of the strains produced biofilm, and it seems similar to the present study in this regard. Atshan et al. suggested in a study on S. aureus in 2013 that expression of operon induces production of PIA and leads intracellular adhesion, some S. aureus strains were also observed not producing biofilm, but possessing ica [8]. It is found out in this paper that biofilm formation occurs just when ica D and ica A are expressed simultaneously. It was observed that ica C average along with ica A expression were more influential in forming the biofilm in this respect, it was inconsistent with the results of the study of Atshan [8]. This difference and inconsistency of expression under study may lie on other factors including bacteria's physiological conditions and on the difference in bacteria's genetic origin. Identifying possible sources and ways of infection may be effective in prevention from the initial biofilm formation, due to the significance of S. aureus in medical departments and its ability in creating multiple infections.

Conclusion

Clinical isolates of Staphylococcus aureus were able to form biofilms, and the expression of all four ica genes in these isolates were high and therefore, and a significant relation was observed between biofilm formation and presence of ica ADBC.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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