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DETECTION OF INTRACELLULAR ADHESION (*ica*) GENES AND BIOFILM FORMATION IN *STAPHYLOCOCCUS SPP*. ISOLATED FROM DIFFERENT CLINICAL SAMPLES

*Heba Khlaf Yassin Al-Mtory, Maysaa S. Al-Shukri & Huda H. Al-Hassnawi
Microbiology Department, College of Medicine, Babylon University, Iraq
*Corresponding author email: hebaalmtory1990@gmail.com

ABSTRACT
This study was designed to detect the intracellular adhesion (*ica*) genes and biofilm formation in *Staphylococcus spp*. 32 isolates of *Staphylococcus spp*. were isolated from 100 clinical samples (urine, burn, wound and ear swab) collected from Al-Hilla Teaching Hospital, isolates were identified by traditional biochemical tests. Some important virulence factor to *Staphylococcus spp*. was detected like adherence activity to epithelial cell, biofilm formation and the effect of green and black tea on biofilm formation and also detection of *ica* operon (*icaABCD*) by using molecular techniques include PCR.

KEY WORDS: *Staphylococcus spp, icaABCD,* Biofilm, adherence activity, green and black tea.

INTRODUCTION

The genus *Staphylococcus* is composed of Gram-positive bacteria with diameters of 0.5-1.5 µm, characterized by individual cocci that divide in more than one plane to form grape-like clusters. These bacteria are non-motile, nonspore forming facultative anaerobes, featuring a complex nutritional requirement for growth (Costa *et al*., 2013). *Staphylococcus aureus* is one of the main causes of hospital and community-acquired infections which can result in serious consequences (Diekema *et al*., 2001). *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal foodborne diseases (SFD). *Staphylococcus epidermidis* is responsible for a variety of infections such as bacteremia, eye infection, urinary tract infection and prosthetic and natural valvular endocarditis (Von *et al.*, 2002). Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan *et al*., 2002). Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing. Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation (Thomas, 2007). Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold (Stewart and Costerton, 2001). Biofilm formation is influenced by a number of factors among which, the most important is synthesis of the polysaccharide intercellular adhesion (PIA) by the organism (Gotz, 2002). The enzymes required for PIA synthesis are encoded within the *icaADBC* operon, mutation of which results in a reduced capacity to form biofilm in both *S. aureus* and *S. epidermidis* (Eftekhar and Dadaei, 2011). PIA biosynthesis is accomplished by the products of the *ica* gene locus, which comprises an N-acetylglucosamine transferase (*icaA* and *icaD*), a PIA deacetylase (*icaB*), a putative PIA exporter (*icaC*), and a regulatory gene (*icaR*) (Vuong *et al.*, 2004). Expression of the *ica* gene locus is regulated by a variety of environmental factors and regulatory proteins. *ica* A, C and D are located in the membrane fraction; *icaB* is mainly present in the culture supernatant, also *icaA* contains four transmembrane helices and has *N*acetylglucosaminyl- transferase activity with UDP-*N*acetylglucosamine as substrate. Certain domains of the amino acid sequence show similarity to the chitinase (NodC) of rhizobia and the hyaluronan synthase (HasA) of *Streptococcus pyogenes*. *icaA* alone has only low transferase activity; when *icaA* is co-expressed with *icaD*, the transferase activity increase ≈20-fold. *icaD* might be a chaperone that directs the correct folding and membrane insertion of *icaA* and, in addition, might act as a link between *icaA* and *icaC*. The *ica* gene expression is regulated by the *icaR* component and also it's noticeable that *icaD* is necessary for *icaA* activity however the partial role of *icaD* is still unknown (Lou *et al*., 2011). Plants produce many biologically active substances, providing defense against environmental microbes, and used as perspective sources of compounds influencing on biofilm formation and dispersion. Plant polyphenols (flavonoids and tannins) attract a special interest because they are common constitutes of food and perform beneficial effects on human health (Crozier *et al*., 2009). These compounds are known to have antioxidant properties due to their ability to scavenge radicals and chelate iron (Perron and Brumaghim, 2009). At the same time in certain conditions polyphenols expose pro-oxidant action owing to production of reactive oxygen species during

autooxidation (Smith *et al*., 2003; Tang and Halliwell, 2010). The mechanism of antioxidant action of polyphenols includes upregulation of antioxidant and detoxification enzymes and modulation of cell signaling and gene expression (Eberhardt and Jeffery, 2006; Smirnova *et al*., 2009).

MATERIALS & METHODS

This study included 100 patients (aged 7 days - 50 years) collected from different clinical sites (urine, burn, wound and ear swabs) who admitted to Al-Hilla Teaching Hospital, during a period extending from 1 November 2015 to 29 February 2016. Out of 100 samples, a total of 24 (24%) *Staphylococcus aureus* and 8 (8%) of *S. epidermidis* isolates were recovered. The specimens were collected from patients for bacteriological analysis in a proper way to avoid any possible contamination agar then incubated at 37ºC for 24hrs.

Adherence activity: The ability of *Staphylococcus spp.*to adhere to epithelial cells is one of important virulence properties of these bacteria and detected according to (Avila-Compose *et al*., 2000; Mataveki *et al*., 2004).

Biofilm Formation

A number of tests are available in clinical laboratories to detect biofilm production by *Staphylococcus.* Methods include Tissue culture plate method, and Tube method (Christensen *et al*., 1985). Tube method: is a qualitative method for biofilm detection while Tissue Culture Plate Method (TCP): is quantitative test considered the gold-standard method for biofilm detection.

Effect of plant extract on biofilm formation: Present study has shown that green and black tea extracts can effect on *Staphylococcus spp.* biofilm formation according to (Samoilova *et al*., 2014).

Molecular detection

1. DNA extraction and purification: This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (UK). The suspension containing DNA was stored at-20 C until used as template for PCR.

2. Primer Sequences: The primer sequences and PCR conditions that are used in the study are listed in Table (1).

TABLE 1: The primer sequences and PCR condition

Gene's name	Primer sequence (5´- 3´)	Condition	Size Bp	Reference
ica A	F: ACACTTGCTGGCGCAGTCAA	94˚c 5min 1x 94˚c 1min	188	Alfatemi *et al*., 2014
	R:TCTGGAACCAACATCCAACA	52˚c30sec 30x 72˚c 1.30min 72˚c 10min1x		
ica B	F:AGAATCGTGAAGTATAGAAAATT	94˚c 5min 1x 94˚c 1min	900	Alfatemi *et al*., 2014
	R:TCTAATCTTTTTCATGGAATCCGT	55˚c 1min30x 72˚c 1.30min 72˚c 5min1x		
ica C	F:ATGGGACGGATTCCATGAAAAAGA	94˚c 5min 1x 94˚c 1min	1100	Alfatemi *et al*., 2014
	R:TAATAAGCATTAATGTTCAATT	55˚c 1min30x 72˚c 1.30 min 72˚c 5min1x		
Ica D	F:ATGGTCAAGCCCAGACAGAG	94˚c 5min 1x 94˚c 1min	189	Alfatemi *et al*., 2014
	R:AGTATTTTCAATGTTTAAAGC	55˚c 1min30x 72˚c 1.30min 72˚c 5min1x		

RESULTS

Isolation of *Staphylococcus spp.*

The isolates of *S. aureus* 24(24%) were distributed as following: 10 (41.66%) isolates from wounds and urine samples for each, 3 (12.5%) from burn, and 1(4.16%) from ear swabs. While *S. epidermidis* 8(8%) isolates were found as following: 3 isolates (37.5%) from wounds, 2 (25%) isolates from burns and urine samples for each, and 1(12.5%) from ear sample.

Detection adherence ability of *Staphylococci spp.* to Epithelial Cells

The result showed that all *Staphylococcal* isolates (*S. aureus* and *S. epidermidis*) have ability to adhere to oral epithelial cells in percentage (100%), and all of the adherent bacteria were slime producers.

Biofilm Formation by *Staphylococcus spp*:

1. Tube method (TM): The results detected that all *S. aureus* isolates were biofilm producers, while 4 isolates of *S. epidermidis* (50%) were biofilm producers and 4 isolates (50%) were not biofilm producers.

2. Tissue culture plate method (TCP): According to mean of OD value at 570nm the results were interpreted as none, moderate and high biofilm producer when the mean of OD value was (<0.120, 0.120-0.240, and >0.240) respectively. The results revealed that all Staphylococcal isolates were biofilm producer, high biofilm formation were account for (100%) while there are no isolates that express moderate and non-biofilm formation for both *S. aureus* and *S. epidermidis*. As shown in Table (2).

TABLE 2: Biofilm production by *Staphylococcal* isolates

Bacterial isolate No.	Biofilm (OD at 570nm)			
	Strong	Moderate	% of biofilm Formation	Weak
S. aureus (24)	24	0	100 %	0
S. epidermidis (8)	8	0	100%	0

The effect of some plant extract on biofilm formation (green and black tea):

The result revealed that the majority of isolates (87.12%) have a variable effect in biofilm formation after green and black tea was added except (7) isolates increased in biofilm formation (21.8%), this may be due to source of isolation and environmental condition. As shown in Table (3).

TABLE 3: Effect of green and black tea on biofilm formation of *Staphylococcal* isolates at by measuring the absorbance at OD (570nm**)**

Isolates no. of *S. aureus*	Before adding the tea	After adding the green tea	After adding the black tea
1	3.500	2.913	3.219
2	1.302	0.355	1.145
3	1.237	0.403	0.850
4	1.214	0.189	0.299
5	1.455	0.522	0.799
6	2.564	2.922	2.426
7	1.272	0.369	0.292
8	1.076	0.395	0.390
9	2.245	2.871	2.670
10	1.099	3.098	3.184
11	1.321	0.315	0.535
12	0.981	2.960	2.578
13	1.109	0.291	0.342
14	3.500	3.113	2.687
15	1.117	0.214	0.247
16	1.996	2.001	2.372
17	3.500	1.973	2.538
18	1.217	0.347	0.790
19	1.332	3.363	2.854
20	1.103	0.238	0.377
21	1.126	0.511	0.389
22	1.142	0.294	0.313
23	1.785	2.664	0.606
24	1.314	0.437	0.542
Isolates no. of *S.epidermidis*	Before adding the tea	After adding the green tea	After adding the black tea
1	1.011	0.420	0.506
2	1.009	0.475	0.662
3	0.990	0.381	0.650
4	1.125	0.594	0.546
5	1.313	0.617	0.416
6	1.814	0.292	0.495
7	1.398	0.732	0.655
8	1.018	0.624	0.381

Molecular detection of *ica* operon:

1. *ica A*: The result show that out of 24 *S. aureus* 23 (95.8%) isolates gave positive result for *icaA* gene at 188 bp in PCR amplification when compared with allelic ladder, as shown in figure (1). And *S. epidermidis* show result in 7(87.5%) isolates gave positive result.

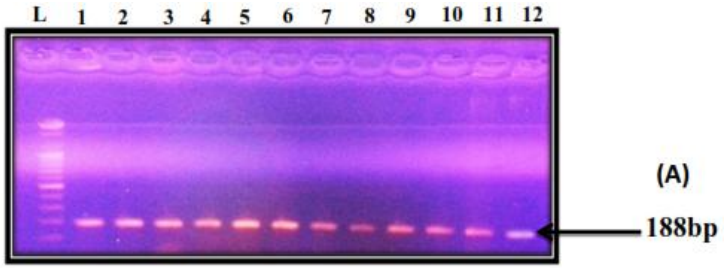
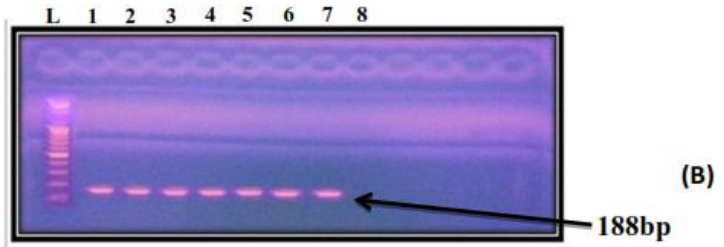


FIGURE 1: A- gel electrophoresis of *icaA* gene that the positive result of *S. aureus* represents 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 isolates from left to right. L: Ladder with 2000 bp.



B- gel electrophoresis of *icaA* gene with positive result of *S. epidermidis* represents 1, 2, 3, 4, 5, 6, 7 isolates from left to right**.**

2. *ica B*: The result of PCR amplification to specific *ica B* primers indicated that (91.6%) of *S. aureus* isolates gave positive result at 900bp when compared with allelic ladder, as shown in Figure (2).

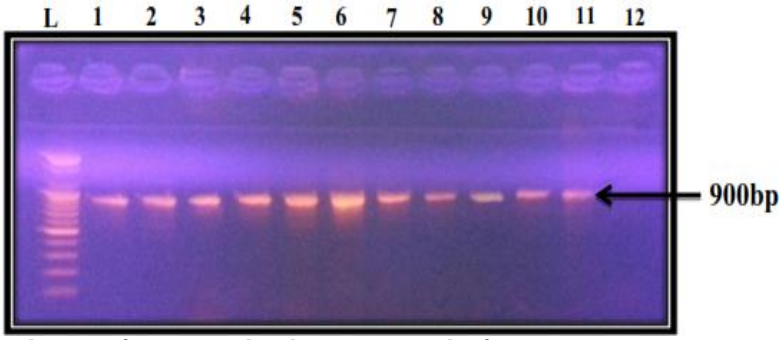


FIGURE 2: gel electrophoresis of *icaB* gene that the positive result of *S. aureus* represents 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 isolates from left to right at 900pb. L: Ladder with 2000 bp.

S. epidermidis show positive result to *icaB* gene in 7(87.5%) with PCR amplification, as shown in Figure (3).

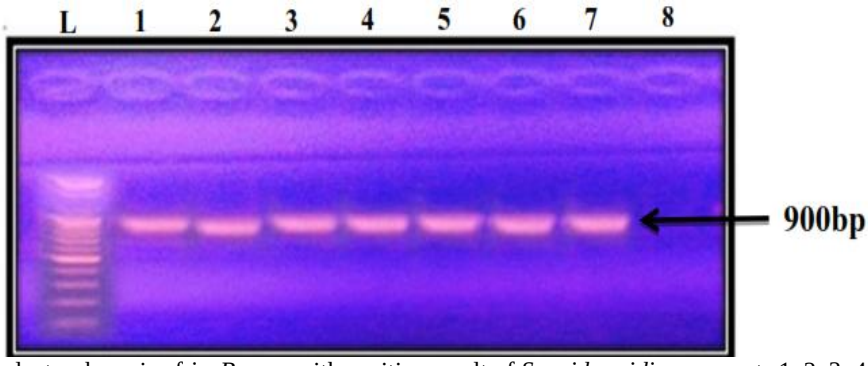


FIGURE 3: gel electrophoresis of *icaB* gene with positive result of *S. epidermidis* represents 1, 2, 3, 4, 5, 6, 7 isolates from left to right at 900pb. L: Ladder with 2000 bp.

3. *icaC*: A total of (24) isolates of *S. aureus*, it was found that (11) (45.8%) isolates contain this gene with base pair 1100 when compare with allelic leader Figure (1-4). While *S. epidermidis* show that (4 of 8) contain the *icaC* gene in (50%), as shown in Figure (5).

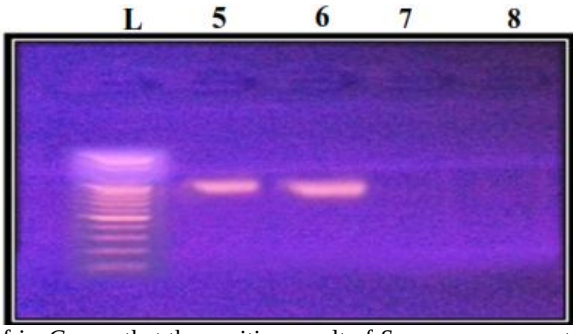


FIGURE 4: gel electrophoresis of *icaC* gene that the positive result of *S. aureus* represents 1, 2, 5, 6, 7 isolates from left to right at 1100pb. L: Ladder with 2000 bp.

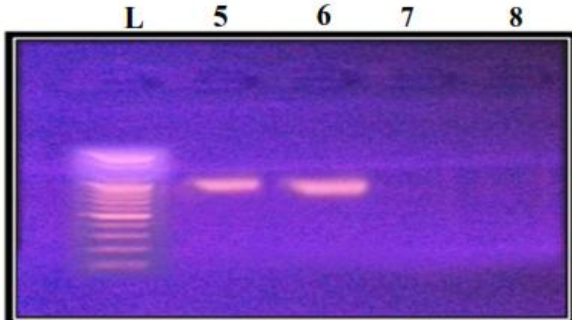


FIGURE 5: gel electrophoresis of *icaC* gene with positive result of *S. epidermidis* represents 1, 3, 5, 6 isolates from left to right at 1100pb.

icaD

The result showed that out of 24 isolates of *S. aureus* only 23 isolates contain this gene with 198bp in (95.8%) Figure (6), while all isolates of *S. epidermidis* contain this gene (100%), as shown in Figure (7).

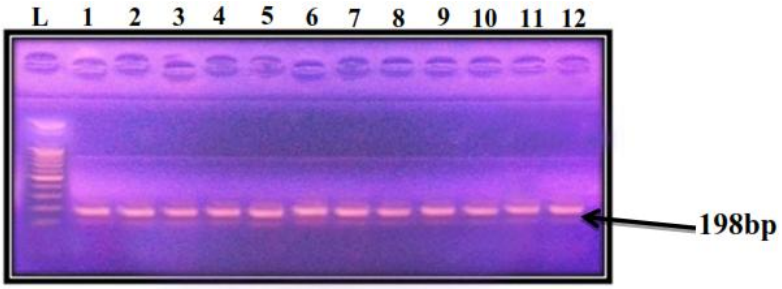


FIGURE 6: gel electrophoresis of *icaD* gene that the positive result of *S. aureus* represents 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 isolates from left to right at 198 pb. L: Ladder with 2000 bp.

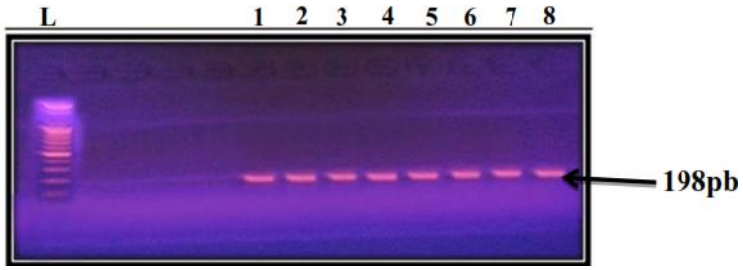


FIGURE 7: gel electrophoresis of *icaD* gene with positive result of *S. epidermidis* represents 1, 2, 3, 4, 5, 6, 7, 8 isolates from left to right at 198 pb.

DISCUSSION

Accordance with the preceding studies, TM cannot be suggested as general screening test to identify biofilm producing isolates (Mathur *et al*.,2006). According to results TCP is a quantitative and reliable method to detect biofilm forming microorganisms. When compared TM and TCP methods, the TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories. It has been shown that plant extracts (green and black tea) used in this study containing much polyphenols demonstrate antimicrobial activity and can reduce biofilm formation and adhesion of *Staphylococcus* and other bacterial species to the artificial surface and epithelial cells. Once a biofilm has been established, micromolar concentrations of all 3

polyphenolic compounds (EGCG, EGC, and ECG) were able to disrupt a preformed biofilm community within a period of 24 h (Wojnicz *et al*. 2012; Trentin *et al*. 2013). In this study we found that 11 (45.8%) of S. *aureus* isolates were positive for all *ica* genes, while 4 (50%) of *S. epidermidis* contain all *ica* genes. While other isolates contain two or three of *ica* genes, and all these isolates form strong biofilm when detected in phenotypic assay, this result suggesting that the difference between the phenotypic and the genotypic characterization of the strain may be explained by an alternative PIA-independent mechanism for biofilm formation in this isolate (Mirzaee *et al*., 2014). Among *ica* genes, the *icaA* and *icaD* have been reported to a play a significant role in biofilm formation. The *icaA* gene encodes *N*-acetyl glucosaminyl transferase, the enzyme involved in PIA synthesis. Further, *icaD* has been reported to a play a critical role in the maximal expression of *N*-acetylglucosaminyl transferase, leading to the full phenotypic expression of the capsular polysaccharide (Gotz, 2002) and (Arciola *et al*., 2001), for explain that these isolates that form biofilm despite the absence of *ica* gene these, some investigators reported the presence of certain genes in *ica*-negative biofilm-forming staphylococci, called the accumulation-associated protein (*aap*) (Rohde *et al*., 2005) and Bap homolog protein (*bhp*) genes (Tormo *et al*., 2005). These genes were found to induce an alternative PIA-independent mechanism of biofilm formation. However, Qin *et al*. (2007) found that some strain of *S. epidermidis* produce biofilm and they did not detect *ica* genes. They assumed that the biofilm-positive/*ica*-negative strain represents a newly emergent subpopulation of clinical strains, arising from selection by antibiotics in the nosocomial milieu, especially that epidemiological data show a tendency towards an increasing proportion of this subpopulation in staphylococci-associated infections. Møretrø *et al*. (2003) suggest that it is more appropriate to use the biofilm formation assay (tube methods, tissue culture plates, congo red agar) and that not related with the *ica* genes as one of the criteria for determining potentially virulent strains because biofilm formation on inert surfaces is highly sensitive to environmental and nutritional conditions, such as the presence of ethanol, iron, varying glucose and sodium chloride concentrations, among others. The absence of biofilm production in some staphylococcal isolates despite the presence of the *ica* operon, due to the insertion of a 1332-bp sequence element, known as IS256, in *icaA* causing its inactivation (Cho *et al*., 2002; Kiem *et al*., 2004).

CONCLUSION

TCP which is a phenotypic biofilm detection method remains a better tool for screening biofilm formation. The presence of *icaABCD* genes was not always associated with in vitro formation of biofilm. We have identified that plant extract (green and black tea) have inhibitory effect bacterial biofilm on different surface.

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