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Biofilm-producing ability of *Staphylococcus* spp isolated from different foodstuff products

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Key words: Biofilm formation, Staphylococcus spp, icaA, icaD
Parole chiave: Produzione di biofilm, Staphylococcus spp, icaA, icaD

Abstract

Background: *In recent times, microbial-biofilm contamination has attracted considerable attention to the food industry. Pathogenic microorganisms can attach to food surfaces, grow on them, and form biofilm that cause an increase in the food safety risk. The mechanisms of biofilm formation have become an important issue in the food-processing industry, therefore, the aim of this study is to determine the biofilm formation and profiles of genes involved in biofilm production of staphylococci isolated from various foodstuff products.*
Materials and methods. *This cross-sectional study was conducted at some grocery stores and confectionaries from September 2015 to October 2016 in different areas of Isfahan, Iran. Staphylococcus spp were isolated from different foodstuff samples including sweet pastries, cakes and similar baked goods, dairy products such as cheese and yogurt, meat products such as sausages, and hamburgers. Standard microbiological methods were used for identification of Staphylococcus spp isolates. Antibiotic susceptibility pattern was determined by the disc diffusion method and icaA/icaD genes have been investigated as PCR target because of their role in the expression of intercellular adhesions involved in biofilm formation by S. aureus.*
Results. *From a total of 194 different foodstuffs samples, 84 Staphylococcus spp were isolated. Out of the 84 Staphylococcus isolates, 95.2% (80/84) were positive to the ability of biofilm formation. Overall, 35.7% (30/84) and 26.2% (22/84) of Staphylococcus spp isolates were positive for icaA and icaD genes, respectively.*
Conclusion. *The results of the present study indicate that the remarkable rate of biofilm formation with the emergence of antibiotic resistance still remains a significant risk for the food safety, especially in foodstuff samples.*

Introduction

Staphylococcus spp are non-flagellated, Gram-positive and among these, some are pathogenic to humans causing many serious community- and hospital-associated diseases, so it has been considered as a major problem for public health for a long time (1-3). It is obvious that *Staphylococcus aureus* can contaminate food if not protected properly. Some strains can produce enterotoxins while growing in foods which resulted in food poisoning (4). So far, the isolates of *S. aureus* have been gathered from meat and its products, milk and dairy products, and chicken (5).

The structured communities of microorganisms that can attach and develop

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on surfaces are defined as biofilms. Now, biofilms are recognized as microbial cells which are associated with surface assemblage and surrounded by hydrated extracellular polymeric substances (EPS) (6). The main components of EPS are included polysaccharides, proteins, phospholipids, nucleic acids, and teichoic acids. The ratio of these components is extremely variable and depending on the response to environmental factors (7). A biofilm community may contain single and/or multiple species of bacteria and build a single layer or three-dimensional structures. Biofilm formation of *S. aureus* is affected by a number of factors such as slime production, colony spreading, and cell surface hydrophobicity. *S. aureus* is able to produce a multilayered biofilm located in a matrix in throughout where the heterogeneous protein is expressed and formed at least two types of biofilms: *ica*-dependent, mediated by polysaccharide intercellular adhesion (PIA)/poly-N-acetyl-1,6-b-glucosamine (PNAG), and mediated by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as elastin-binding protein (Ebps) and collagen-binding protein (Cna) (7).

In the last few years, biofilm formation has attracted considerable attention from the food industry. Pathogenic microorganisms can attach to food surfaces, grow on them, and form biofilms that cause an increase in the food safety risk. Therefore, the mechanisms of biofilm formation have become an important issue in the food-processing industry from several years. Some studies reported biofilm formation by *S. aureus* from foods (8).

Bridier et al. show that *S. aureus* strains by various origin (five clinical, two isolated from water, two unknown, and one milk isolate from ewes with mastitis) can produce high biovolumes and high substratum coverage biofilms (9).

The risk of *S. aureus* contamination of foods previously treated to break down the resident microbial load is a matter of great concern for food industry and public health. It is thought that biofilm formation by this microorganism can increase the contamination of processed food products. Literature reports that biofilm is considered as a part of *S. aureus* normal life cycle in the environment, so that the planktonic cells attach to solid surfaces, proliferating and accumulating in multilayer cell clusters embedded in an organic polymer matrix (10, 11). The aim of this study is to determine the biofilm formation and profiles of genes involved in biofilm production of Staphylococci isolated from various foodstuffs products.

Materials and methods

Bacterial isolates and identification

This cross-sectional study was conducted between September 2015 to October 2016. In total, 194 staphylococcal isolates were obtained from different foodstuff samples including sweet pastries, cakes and similar baked goods, dairy products such as cheese and yogurt, meat products such as sausages, and hamburgers at some grocery stores and confectionary in different areas of Isfahan, Iran.

All isolates were analyzed for the presence of staphylococci on Baird Parker agar containing egg yolk tellurite emulsion (HI Media, India) and suspected Staphylococcus colonies were recognized using the conventional microbiological methods such as colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, DNase, tube coagulase tests, and susceptibility to novobiocin and polymyxin B. The confirmed isolates were stored at -70°C for further analysis (12).

Antimicrobial susceptibility test

The antibiotic susceptibility pattern was performed based on disk diffusion method on Mueller–Hinton agar (Himedia, India)

according to the Clinical and Laboratory Standards Institute (CLSI) recommendation for penicillin (10 U), cefoxitin (30 U), gentamicin (10 U), tetracycline (30 U), ciprofloxacin (5 U), clindamycin (2 U), trimethoprim-sulfamethoxazole (1.25/23.75 U), chloramphenicol (30 U), rifampin (5 U), levofloxacin (5 U), and erythromycin disks (MAST, Merseyside, UK) (13).

Biofilm Formation Assay

A modified microtiter plate test was carried out to determine the biofilm formation according to the protocol of O'Toole (14). Briefly, an overnight culture of each strain was grown in tryptic soy broth (TSB, Oxoid) plus 0.25% glucose (Merck, Germany) at 37°C. The cultures were diluted 1:100 in TSB medium, 200 mL of each dilution was distributed in flat-bottom 96-well polystyrene plates and incubated at 37°C for 24 hours without agitation. After 24 hours of incubation at 37°C, the content of each well was aspirated, and each well was washed with sterile physiological saline to remove all non-adherent cells. The attached bacteria were fixed with absolute ethanol for 10 minutes. The plates were stained using crystal violet (1% W/V) solution in water for about 10-15 minutes. After staining, the wells were washed three times with distilled water. The wells were destained with 200 μL of 30% glacial acetic acid in water and the absorbance of each well was measured at 570 nm using an ELISA reader (Stat Fax-2100). Each test was performed in triplicate. The adherence ability of the tested strains was classified into four categories according to OD values Negative: ODs < 0.500; Positive: ODs 0.500-1.500; strongly positive: ODs > 1.500. All tests were carried out in triplicates (14).

Detection of biofilm formation genes

Genomic DNA was extracted using a simple boiling method (15). PCR was performed to detect *icaA*/*icaD* genes using the elsewhere described primers (16). The conditions for PCR amplification were: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 54 °C for *icaA*, and *icaD* and extension at 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes. Amplification products were analyzed using 1.5% agarose gel with KBC power load dye (CinnaGen Co. Iran).

Results

A total of 84 confirmed Staphylococcus isolates were collected from different foodstuffs samples. Out of the 84 Staphylococcus isolates, 95.2% (80/84) were positive to the ability of biofilm formation and were divided into four groups based on their ability to form biofilms. Anyway, 57.5% (46/80) of *Staphylococcus* spp strains were classified as strong or moderate in their ability to form biofilms.

This study demonstrated that the highest frequency of biofilm formation was found in pastries products 47.6% (40/84), followed by meat products 36.9% (31/84) and dairy products 10.7% (9/84). The details of biofilm-forming ability by *Staphylococcus* spp isolates in foodstuff samples are shown in Table 1.

According to PCR amplification, the most frequently detected biofilm formation gene among the Staphylococcus spp strains was *icaA,* 35.7% (30/84), meanwhile, 26.2% (22/84) of isolates carried the *icaA/D* genes, simultaneously. The results also showed that 36.4% of *icaA*/*icaD*-positive isolates have a strong ability of biofilm formation. The distribution of biofilm formation genes in the Staphylococcus spp. isolates based on biofilm formation ability and type of foodstuff samples are presented in Table 2.

The antimicrobial resistance patterns of *icaA*/*D*-positive isolates are shown in Table 3. Overall, 84 *Staphylococcus* spp

Table 1 - *Staphylococcus* spp strains grouped according to their biofilm forming ability

Biofilm-forming ability	Origin		
	Meat products	Pastries	Dairy products
Strong	14 (43.7)	10 (25)	4 (33.3)
Moderate	4 (12.5)	13 (32.5)	1 (8.4)
Weak	13 (40.6)	17 (42.5)	4 (33.3)
Negative	1 (3.2)	-	3 (25)
Total	32 (100)	40 (100)	12 (100)

Table 2 - The distribution of biofilm formation genes in the *Staphylococcus* spp isolates based on biofilm formation ability and types of products

Biofilm formation	*icaA*	*icaD*	*icaA+icaD*
	30 (35.7%)	22 (26.2%)	22 (26.2%)
Strong	10 (33.3)	8 (36.4)	8 (36.4)
Moderate	7 (23.3)	6 (27.3)	6 (27.3)
Weak	12 (40)	7 (31.8)	7 (31.8)
Negative	1 (3.4)	1 (4.5)	1 (4.5)
Products	No. (%)	No. (%)	No. (%)
Meat	9 (30)	9 (41)	9 (41)
Pastry	15 (50)	8 (36.4)	8 (36.4)
Dairy	6 (20)	5 (23)	5 (23)
Total	30 (100)	22 (100)	22 (100)

Table 3 - The antimicrobial resistance patterns of *icaA*/*D*-positive isolates

Antibiotics	*icaA*- positive n = 30		*icaD*-positive n = 22	
	Susceptible No. (%)	Resistant No. (%)	Susceptible No. (%)	Resistant No. (%)
Rifampicin	30 (100)	0	22 (100)	0
Levofloxacin	30 (100)	0	22 (100)	0
Erythromycin	27 (90)	3 (10)	19 (86.4)	3 (13.6)
Penicillin	10 (33.3)	20 (66.7)	6 (27.3)	16 (53.3)
Chloramphenicol	29 (96.7)	1 (3.3)	21 (95.5)	1 (4.6)
Ciprofloxacin	30 (100)	0	22 (100)	0
Gentamicin	30 (100)	0	22 (100)	0
Tetracycline	26 (86.6)	4 (13.3)	19 (86.4)	3 (3.6)
Co-trimoxazole	30 (100)	0	22 (100)	0
Clindamycin	28 (93.3)	2 (6.7)	22 (100)	0

isolates were sensitive to rifampicin, levofloxacin, ciprofloxacin, gentamicin, and co-trimoxazole, whereas, the higher rates of antibiotic resistance were against penicillin and tetracycline.

Discussion

Pathogenic microorganisms can adhere, grow on and form biofilms on food products, equipment, and processing environments; therefore, the presence of microorganisms on food and equipment for foodstuff industry, increases the food safety risk and has become an important challenge in the public health care in the past several years (17, 18). Furthermore, humans are at a risk of being infected with foodborne bacteria via the food products from animal reservoirs (10, 19). Although there is little information on biofilm production by staphylococci in different foodstuff samples, the main aim of the present study was to determine the biofilm production and profiles of genes involved in biofilm production by *Staphylococci* isolated from various foodstuffs. We found that 95.2% of *Staphylococcus* spp obtained from samples were biofilm producer using the Microtiter-Plate Test (MTP) method. Several studies previously applied MTP method to assay biofilm formation among staphylococci isolates in Iran and other parts of the world that revealed various results based on the source of isolation, type of species, and geographic regions (19-22). The results of our study were in accordance with the findings reported by Kim et al which found that all *S. aureus* strains, isolated from various food origins, were biofilm producer (11). In contrast, Gundogan et al showed that 70% of Staphylococcus isolates had biofilm formation capacity revealed by the microplate method (23).

In another study, Vasil et al et al (24) indicated that 75.4% of *S. epidermidis* and *S. aureus* isolated from sheep milk were able to form a biofilm; however, this rate is slightly lower than the result obtained in the present study. Our study also found that the biofilm formation by staphylococci isolated from meat products was higher than by strains isolated from other sources (pastries and dairy product). These findings were in accordance with the previous study, which has shown that 89% of clinical staphylococci isolates obtained from beef meat were biofilm positive, as determined by Christensen's tube method (25). Thiran et al (26) described a 45.8% prevalence of biofilm formation among *S. aureus* isolated in the dairy product in Switzerland and northern Italy; however, this finding was not consistent with our finding of the prevalence of biofilm formation among dairy product isolates. Bearing in mind these findings, the ability of Staphylococcus spp. isolates to produce biofilm is different depending on their origin. Previous studies have also described that the pathogen's origin, alternate temperatures during storage and transportation, unsuitable packaging of foodstuffs and poor food handling procedures are effective on biofilm formation. In our study, the frequency of *icaA* positive *Staphylococcus* spp isolates was found to be 35.7%, which is not in agreement with the results obtained by Tang et al (27), which showed a 87.5% *icaA/D* positivity in *S. aureus* strains isolated from different sou. In agreement with our results, previous studies have shown a high frequency of *icaA* genes among *S. aureus* strains isolated from clinical and environmental samples, which have demonstrated that formation of biofilm in Staphylococcus spp. is associated with the presence of *icaA* and *icaD* genes. In these studies, the foodstuffs such as pastries and meat products, which are most frequently handled during production and packaging process, showed the highest levels of biofilm formation and high rate of *icaA* and *icaD* positive isolates. According

to our results, a remarkable level of penicillin resistance has been detected in *icaA*/*icaD* positive *Staphylococcus* spp isolates, which were in agreement with the results of a study conducted by Dehkordi et al that reported 100% resistance against penicillin among methicillin-resistant *S. aureus* isolated from hospital foods (28). Furthermore, our results showed that all *icaA* and *icaD* positive isolates were sensitive to rifampicin, levofloxacin, ciprofloxacin, gentamicin, and clindamycin and trimethoprim/sulfamethoxazole. In addition, *icaA* was more frequent among isolated from pastries products, whereas, *icaD* was more frequent among isolated from meat products.

Conclusion

In the present study, the remarkable rate of biofilm formation and the emergence of antibiotics resistance among biofilm producing staphylococci isolates suggest their potential risk for the food safety. Also, these findings suggest that food processing and storage conditions are unsuitable and that a possible contamination by staphylococci producing biofilms are associated with manipulation of food products.

Conflicts of interest
The authors declare no conflicts of interest.

Riassunto

Produzione di biofilm da parte di ceppi di** Staphylococcus spp **isolati da diversi prodotti alimentari

Premessa. Recentemente la contaminazione degli alimenti con film microbici ha richiamato grande attenzione sull'industria alimentare. Microorganismi patogeni possono aderire alle superfici degli alimenti, moltiplicarsi e formare biofilm che aumentano i rischi per la salute dei consumatori. Tutto il processo della formazione dei biofilm è diventato un tema importante per l'industria della produzione alimentare, ed a questo proposito il presente studio ha inteso indagare questi eventi ed identificare i profili dei geni coinvolti nella produzione di biofilm da parte di *Staphylococcus* spp isolati da vari substrati alimentari.

Materiali e metodi. Questo studio trasversale è stato condotto in un certo numero di negozi di alimentari e di dolciumi di diversi quartieri di Isfahan, Iran, tra il settembre 2015 e l'ottobre 2016. Ceppi di *Staphylococcus* spp sono stati isolati da pasticcini, torte ed altri prodotti da forno, nonchè da latticini (formaggi e yogurt) e da carni lavorate (salsiocce ed hamburger). Il profilo di sensibilità agli antibiotici di questi ceppi è stato studiato con il metodo della diffusione da dischi, ed i geni *icaA*/*icaD* sono stati indagati mediante PCR per il loro ruolo nella espressione dell'adesione intercellulare coinvolta nella foermazione di biofilm da parte di *S. aureus*.

Risultati. Da un totale di 194 diversi campioni alimentari sono stati isolati 84 ceppi di *Staphylococcus* spp. Tra di essi il 95.2% (80/84) è risultato capace di produrre biofilm, mentre il 35.7% (30/84) ed il 26.2% (22/84) è risultato positivo, rispettivamente, per i geni *icaA* ed *icaD*.

Conclusioni. I risultati del presente studio indicano che la notevole frequenza con cui i batteri formano biofilm e l'emergere della resistenza antibiotica mantengono il significato di un notevole rischio per la sicurezza alimentare sostenuta.

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