Journal of Advanced Veterinary Research

https://advetresearch.com

Detection of Biofilm and some Enterotoxins of *Staphylococcus aureus* Isolates in Ice Cream

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ARTICLE INFO

Original Research

Received:
05 August 2021

Accepted:
26 September 2021

Keywords:

Biofilm, Enterotoxins, Food, *S. aureus*, PCR

ABSTRACT

Staphylococcus aureus is the most bacteria that have ability to form a biofilm and secret different types of enterotoxins that cause food poisoning in humans. Biofilms is a community of microorganisms encased in a matrix of extracellular polysaccharide (slime), called polysaccharide intercellular adhesion (PIA). They have related to a diversity of chronic and persistent infections. This study aims to detect the ability of *S. aureus* isolated from ice cream to form biofilm by Congo red agar (CRA), microliter plate, and PCR and the ability of *S. aureus* to produce enterotoxins by PCR. 15 *S. aureus* isolates were grown on CRA and microtiter plate method then subjected for detection of *icaA* and *icaD* genes by PCR and for the presence of enterotoxins genes (*sea*, *seb*, *sec*, *sed*, and *see*) which are responsible for *S. aureus* biofilm formation and *Staphylococcus* food poisoning. 73.3% of the isolates were biofilm producers on Congo red agar, 60% of the isolates were positive for biofilm production using microtiter plate method and by PCR technique, all the isolates 100% had *icaD* gene and 86.6% had *icaA* gene. The enterotoxin *seb* gene was detected in 5 (33.3%) *S. aureus* isolates, the enterotoxin *see* gene was detected in 4 (26.6%) *S. aureus* isolates while *sea*, *seb* and *sed* gens were not detected in any *S. aureus* isolates. In conclusion all aureus isolates were positive for *icaD* gene and some of *S. aureus* isolates were positive for *icaA* gene which are responsible for biofilm formation and some *S. aureus* isolates were positive for enterotoxin B and enterotoxin E, which responsible for food intoxication so the ice-cream considered a potential source for food intoxication and persistent infection caused by *S. aureus*.

J. Adv. Vet. Res. (2021), 11 (4), 230-236

Introduction

Milk and dairy products are the source of nutrients required for the growth of many microorganisms, including Staphylococci (Farzana *et al*., 2004). Bacterial Milk contamination usually occurs throughout the milking process, and this depends on the sanitary condition of the environment and utensils used for milking and the milker's hands (Smith *et al*., 2007). Also, it can gain access to milk by direct excretion from udders with clinical or subclinical Staphylococcal mastitis (Peles *et al*. 2007). *Staphylococci* have been described as bacteria that may attach to the contact surfaces in both milk and meat processing industries, form biofilms and survive on them. Their attachment to food contact surfaces in food processing plants, and subsequent biofilm formation pose a risk of contamination in milk and meat products. Bacterial contamination of foodstuffs can lead to their decay or transmission of diseases (Schlegelová *et al*., 2008). The formation of biofilms is increasingly recognized as an important factor in the virulence of *Staphylococcus* (Oliveira *et al*., 2006). Bacteria in the biofilm are highly resistant to both innate and specific host defense mechanisms due to their extracellular polysaccharide matrix and low metabolic rate, among other things, they are not susceptible to phagocytes and antibiotic-resistant macrophages, which only attack dividing cells. These local conditions are conducive to the continued existence of bacteria for months to years, and periodic outbreaks (Potera, 1999).

The implications of biofilm formation for infections and drug resistance have triggered increased interest in the characterization of the genes involved in biofilm formation. The intercellular adhesion (*ica*) locus consists of the genes *icaADBC*, and among the *ica* genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) (Cramton *et al*., 1999).

S. aureus is one of the major bacterial pathogens which cause food poisoning (Yu-Cheng *et al*., 2008). Staphylococcal food poisoning is a mild intoxication occurring after the ingestion of food containing Staphylococcal enterotoxins (SEs) (Chiang *et al*., 2008)

There were five major classical SEs types, named; *SEA, SEB, SEC, SED,* and *SEE*. However, new genes encoding enterotoxins are currently being identified, for example, from *SEG* to *SEU*.

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One or more of these genes are thought to be involved in Staphylococcal food poisoning (Afifi *et al*., 2011).

Staphylococcal (SE) enterotoxin is part of the pyrogenic protein associated with many human diseases. These proteins are resistant to thermal inactivation and to gastrointestinal proteases, causing emesis and diarrhea, and can act as superantigens (Pinchuk *et al*., 2010). A wide variety of enterotoxin and enterotoxin-like coding genes (more than 20 SEs) have been categorized to have a significant role in stages of host colonizing, gastroenteritis infections, and invasion of skin, mucus, and host defense mechanisms (Argudín *et al*., 2010). Most genes coding for SEs is located on mobile elements such as plasmids, bacteriophages or pathogenicity islands (Lindsay *et al*., 1998; Zhang *et al*., 1998). Therefore, horizontal transmission between strains is not uncommon.

Staphylococcal enterotoxin B (*seb*) is the toxin mostly associated with typical food poisoning. It has also been shown to cause a non-menstrual toxic shock syndrome (TSS) (CDC, 2014). *Seb* has been studied as a potential biological warfare agent because it is easily disper*sed*; it is very stable and when inhaled at very high doses, it can cause extensive systemic damage, multiple organ failure, and even shock and death. However, *seb* is classified as disabling because in most cases, aerosol exposure does not lead to death, but a temporary but at the same time severe disabling disease that lasts up to 2 weeks. (Ulrich *et al*., 1997). So, the purpose of this study was to detect the ability of *S. aureus* to form biofilm phenotypically and genotypically and the ability of these strains to secrete enterotoxins genetically.

Materials and methods

Bacterial isolates

Fifteen *S. aureus* isolates were isolated from ice-cream samples and identified through culture on mannitol salt agar and biochemical identification and confirmed by PCR technique according to Samir *et al*. (2019). These isolates were subjected for biofilm formation phenotypically and genotypically and enterotoxins secretion by PCR.

Screening of Staphylococcus aureus for biofilm production by congo red agar (CRA) plate.

Production of biofilm from all isolates was studied by the cultivation of the *S. aureus* isolates on CRA plates, CRA prepared by adding 0.8 g of congo red dye and 36 g of sucrose to 1L of BHI (Oxoid), then plates were incubated at 37 °C for 24 h. After incubation, black colonies with a dry crystalline consistency were established as biofilm positive and non-biofilm producing strains produce red smooth colonies (Gundogan *et al*., 2006; Krukowski *et al*., 2008).

Using the microtiter plate method to determine quantitative biofilm formation

The overnight culture was diluted 1:200 with tryptic soya broth containing 0.25% glucose, and 200 μℓ of each dilution were seeded per well in a sterile 96-well polystyrene microtiter plate and incubated at 37 °C for 18 h. After washing 3 times in phosphate buffered saline (pH 7), wells are dried for 1 h at 60 °C and attached biofilm was stained with 1% crystal violet for 15 minutes. After rinsing 3 times with distilled water, drying was done at room temperature and the absorbance of the adherent biofilm was measured at 490 nm in a microplate reader (Cucarella *et al*., 2001). The Interpretation of biofilm production was calculated according to Stepanovic *et al*. (2007): The average OD values were calculated for all tested strains and negative controls since all tests were performed in triplicate and repeated three times. Second, the cut-off value (ODc) was established. It is defined as three standard deviations (SD) above the mean OD of the negative control: = average OD of negative control + 3x standard deviation (SD) of negative control. Then the average OD values of all tested strains compared with the ODc as follow; if OD ≤ ODc = no biofilm producer; ODc< OD ≤ 2×ODc= weak biofilm producer; 2× ODc <OD≤4×ODc= moderate biofilm producer; 4×ODc <OD= strong biofilm producer.

PCR method for the identification of icaA and icaD genes and enterotoxins genes

Extraction of DNA

The extractions of the genomic DNA were conducted according to instruction of QIAamp DNA mini kit (Qiagen, Germany, GmbH).

Polymerase chain reaction (PCR)

The primers for the amplification of *icaA* and *icaD* genes and enterotoxins genes were designed as mentioned in Table 1.

Table 1. Primers used in this study

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
icaA	F-CCT AAC TAA CGA AAG GTA G R-AAG ATA TAG CGA TAA GTG C	1315 bp	Ciftci *et al*. (2009)
icaD	F-AAA CGT AAG AGA GGT GG R-GGC AAT ATG ATC AAG ATA	381 bp	
sea	F-GGTTATCAATGTGCGGGTGG R- CGGCACTTTTTTCTCTTCGG	102 bp	Mehrotra *et al. (*2000)
seb	F-GTATGGTGGTGTAACTGAGC R-CCAAATAGTGACGAGTTAGG	164 bp	
sec	F-AGATGAAGTAGTTGATGTGTATGG R- CACACTTTTAGAATCAACCG	451 bp	
sed	F- CCAATAATAGGAGAAAATAAAAG R- ATTGGTATTTTTTTTCGTTC	278 bp	
see	F- AGGTTTTTTCACAGGTCATCC R- CTTTTTTTTCTTCGGTCAATC	209 bp	

Uniplex PCR for detection biofilm genes

Primers for *icaA* and *icaD* were used in 25 µl reaction volume consisted of, 1 µl of each primer, 12.5 µl Emerald Amp GT PCR master mix, 4.5 µl PCR grade water, and 6µl of template DNA. The reaction was done in a thermocycler (Applied Biosystem 2720) bas*ed* on the conditions mentioned in Table 2.

Preparation of Sea, Sed and *See* multiplex PCR reaction

Primers for *Sea, Sed* and *See* were used in 50 µl reaction volume consisted of, 1µl of each primer (20 pml), 25 µl Emerald Amp GT PCR master mix, 11 µl PCR grade water, and 8 µl of template DNA. The reaction was done in thermal cycler (Applied Biosystem 2720) bas*ed* on the conditions mentioned in Table 2.

Preparation of *Seb* and *Sec* duplex PCR reaction

Primers for *Seb* and *Sec* were used in 50 µl reaction volume consisted of, 1 µl of each primer (20 pml), 25 µl Emerald Amp GT PCR master mix, 13 µl PCR grade water, and 8 µl of template DNA. The reaction was done in thermal cycler (Applied Biosystem 2720) bas*ed* on the conditions mentioned in Table 2.

Investigation of the PCR products according to Sambrook *et al.* (1989) with modification

The products of the uniplex, duplex, and multiplex PCR were separated by electrophoresis on a 1.5% agarose gel (AppliChem, Germany, GmbH) in 1× TBE buffer at room temperature using a gradient of 5V/cm. For gel analysis, 20 µl of each product was loaded in each gel well. A 100-bp DNA ladder (Qiagen, Germany) was used to find out the amplicon sizes. The gel was visualized by a gel documentation system (Alpha Innotech, Biometra).

Results

Detection of biofilm-producing phenotype by Congo red agar method and microtiter plate method

Bas*ed* on Congo red agar plate, 11 out of 15 *S. aureus* isolates were produced biofilm and showed black colonies, while 4 out of 15 isolates were no biofilm-producing showing red colonies (Table 3 and Fig. 1). However, by microtiter plate method, 2 isolates out of 15 *S. aureus* isolates, were strong biofilm producers, 7 isolates were moderate biofilm producers, and 6 isolates were weak biofilm producers (Table 3).

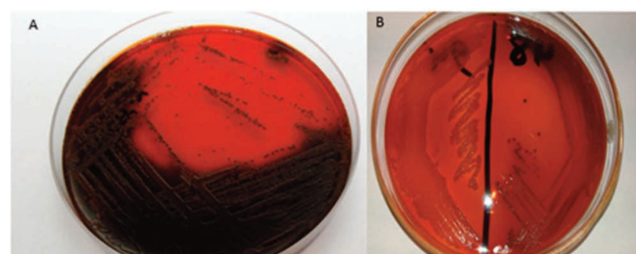

Fig. 1. A) *S. aureus* isolate showing black colonies on Congo red agar, B) Two *S. aureus* isolates showing red colonies on Congo red agar.

PCR detection of icaA and icaD

All 15 isolates were examined for the presence of *icaA* and

Table 2. Temperature and program conditions during PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
IcaA	94˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.	35	72˚C 12 min.
IcaD	94˚C 5 min.	94˚C 30 sec.	49˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
Sea, *Sed* and *See*	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
Seb and *Sec*	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.

Table 3. Results of phenotypic detection of biofilm production

	Phenotypic detection									
Method	Congo red agar				Microtitre plate					
Results	Black colonies		Red colonies		Strong producer		Moderate producer		Weak producer	
	No	%	No.	%	No.	%	No.	%	No.	%
	11	73.30%	4	26.60%	2	13.3	7	46.6	6	40
Total	15									

Table 4. Results of genotypic detection of biofilm production

Genes	Genotypic detection							
	Ica A				*Ica D*			
Results	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
	13	86.6	2	13.3	15	100	0	0
Total	15							

icaD genes. Among the 15 isolates, 13 isolates revealed the presence of *icaA* gene as shown in Table 4, Fig. 2, and all the 15 isolates have *icaD* gene as shown in Fig. 3.

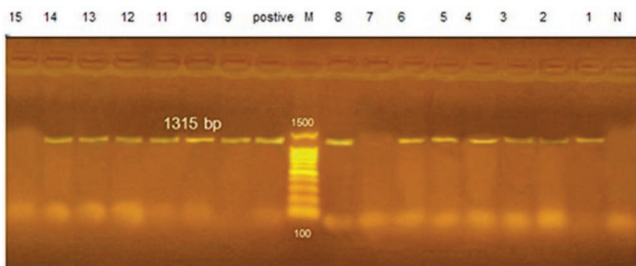

Fig. 2. PCR results of *icaA* gene among *S. aureus* isolates. Lane L: DNA ladder, Lane pos: control positive, Lane neg: control negative, lane 1,2,3,5,6,8,9,10,11,12,13 and 14 positives for *icaA*. Lane 7, 15 negatives for *icaA*.

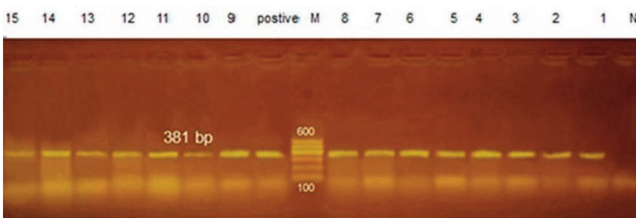

Fig. 3. PCR results of *icaD* gene among *S. aureus* isolates. Lane L: DNA ladder, Lane pos: control positive, Lane neg: control negative, lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14,15 positive for *icaD*.

Occurrence of enterotoxin genes among S. aureus isolates

All the 15 *S. aureus* isolates were examined for the presence of enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*). Among the 15 *S. aureus* isolates, two enterotoxin genes (*seb* and *see*) were detected. The enterotoxin *seb* gene was detected in 5 (33.3%) *S. aureus* isolates as shown in Fig. 4, However, the enterotoxin *see* gene was detected in 4 (26.6%) *S. aureus* isolates (Fig. 5).

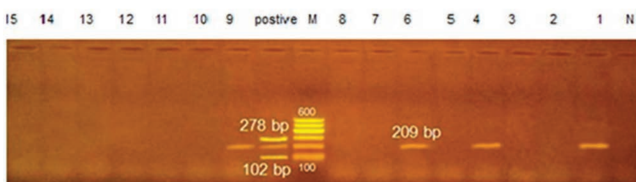

Fig. 4. PCR results of enterotoxin genes (*sea*, *sed* and *see*) among Staph aureus isolates. Lane M: DNA marker, Lane pos: control positive, Lane neg: control negative, lane 1,4,6 and 9 positives for *see*. Lane 2,3,5,7,8,10,11,12,13,14,15 negative for presence of *sea*, *sed* or *see*.

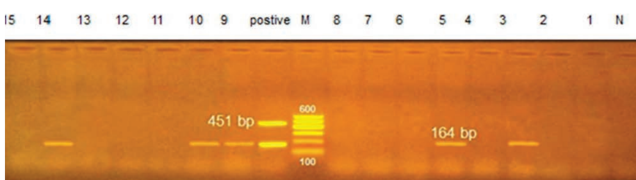

Fig. 5. PCR results of enterotoxin genes (*seb*, *sec*) among *S. aureus* isolates. Lane M: DNA marker, Lane pos: control positive, Lane neg: control negative, lane 3,5,9,10,14 positive for *seb*. Lane 1,2,4,6,7,8,11,12,13,15 negative for *seb* or *sec*.

Discussion

S. aureus with biofilm formation helps bacteria to survive in the host and is responsible for persistent or chronic infection (Christensen *et al*., 1985; Bernardi *et al*., 2007). They exhibit resistance to antibiotics by various methods including restriction of penetration of antibiotics into biofilms, decreasing the growth rate, and expression of resistance genes (Kim, 2001).

Several studies have shown that mucus and biofilm formed in *S. aureus* strains that cause catheter-associated and nosocomial infections is associated with the presence of the *icaA* and *icaD* genes (Arciola *et al*., 2001; 2002). In this study, the results of a PCR test for the *icaA* and *icaD* genes and phenotypic tests were important to develop diagnostic tests for biofilm-producing microorganisms.

There are various methods for biofilm detection. (Christensen *et al*.,1985). In this study, we evaluated the 15 *S. aureus* isolates for their ability to form biofilms by three screening methods (CRA, MTP and PCR).

The obtained results showed that among the 15 isolates of *S. aureus*, 11 (73.3 %) are biofilm producers against 4 (26.6%) non-biofilm producers by CRA method similar results were reported by Namvar *et al*. (2013), who reported that 65% positive results with Congo red agar and by Gowrishankar *et al*. (2016), who reported that 77.8% of *S. aureus* isolates tested positive for slime using the CRA method. Also, this result agrees with the previous reports by Kouidhi *et al*. (2010); Arciola *et al*. (2002) and Ammendolia *et al*. (1999), where 88.9%, 60.8, and 50 of *S. aureus* were found to be positive for slime production, respectively. However, (Mathur *et al*.,2006; Taj *et al*., 2012; Ba *et al*., 2014) reported a smaller number of biofilm production by Staphylococci species by this method they found positive results with CRA in 1.97%, 3.47%, and 4.47% respectively. As researchers have only recently found that PIA/PNAG (polysaccharide intracellular adhesions/poly N-acetyl glucosamine) have little input in the biofilm matrix of *S. aureus*, so cannot be detected by the CRA method (Taj *et al*., 2012; Knobloch *et al*., 2002).

The microtiter plate test is a convenient and economical quantitative technique for the identification of critical factors and optimal culture conditions for biofilm formation. This technique is used for the direct detection of polysaccharide production because spectrophotometric measurements provide quantitative information on the ability of bacterial strains to rapidly grow while adhering to the substratum (Stepanovic *et al*., 2000).

This study showed that the results of biofilm production by microtiter plate method were 13.3% strong biofilm producer, 46.6% moderate biofilm producer, and 40% weak biofilm producer. These results nearly agree with Mathur *et al*. (2006), who reported that 14.47 % strong and 39.4 % moderate biofilm producer, while 46.0 % of isolates were weak or no biofilm was detected. Also, the results in this study agreed with Kot *et al*. (2013), who found that weak biofilm formers were 48.6%, while 40% and 11.4% of strains were moderate and strong biofilm producers, respectively.

The data for the MTP test were not in agreement with Darwish and Asfour (2013), who observed that the results of biofilm production by MTP method were 52.5%, 27.5%, and 20% of *S. aureus* isolates that had strong, moderate, and weak biofilm producers, respectively.

The difference between the results of CRA and MTP methods can be attributed to the fact that phenotypic expression of biofilm formation is very sensitive to in vitro conditions, so various methods can be used for detection. In addition, both of these tests measure the same phenomenon, but in different ways. CRA has been used as an indirect indicator of polysaccharide production (Baselga *et al*., 1993; Stevens *et al*., 2009). A combination of phenotypic and genotypic methods would be recommended for identifying biofilm-producing strains,

the detection of two biofilm-related genes and their incidence in *Staphylococcus* isolates were investigated using PCR reactions.

The intercellular adhesion (*ica*) locus, consisting of the genes *icaADBC*, has been reported to have a potential role of biofilm formation (Vasudevan *et al*., 2003). Among the ica genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* (Gotz, 2002). The gene encoded by ica has been shown to cause the intercellular biosynthesis of PIA molecules, and It can participate in the biofilm accumulation stage. Various studies have demonstrated the key role of the ica gene as a virulence factor in staphylococcal infections (Namvar *et al*., 2013).

In this study, the prevalence of *icaA* and *icaD* genes in *S. aureus* was 86.6% and 100%, respectively, which nearly agree with Marques *et al*., (2017), who detected that *icaA* and *icaD* in 85% and 95% isolates, respectively. Castelani *et al*. (2015) reported that PCR detected the *icaA* and *icaD* genes in 98% and 100% of *S. aureus* isolates, respectively. Furthermore, Namvar *et al*. (2013) found that all isolates were positive for the *icaD* gene determined by PCR. In addition, results from this study are consistent with Notcovich *et al*. (2018), who stated that the PCR results showed that all but one of the isolates were positive for both *icaA* and *icaD* genes, with the one exception being negative for *icaA*.

Through comparison of genotypic and phenotypic biofilm characteristics of the isolates revealed that two isolates showed a good correlation between phenotypic detection by Congo red agar producing black colonies and by microtiter plate method that showed strong adherence and also, genotypically, they have both genes responsible for biofilm formation.

It has been reported that failure of staphylococcal strains that possess the ica locus to form biofilm in vitro could be due to point mutations in the locus and/or other unidentified factors negatively regulate the synthesis of polysaccharide cell adhesion or affect the formation of biofilms (Cramton *et al*., 1999).

There is some experimental evidence that the development of new clones, called biofilm negative, has *icaA* and *icaD* genes (Arciola *et al*., 2001). It has been shown that the expression of the *icaADBC* operon is a highly variable factor that is regulated by phasic changes and rearrangements of the genome. It has been suggested that variable biofilm expression helps bacteria adapt to the environmental change conditions of incubation (Costerton *et al*., 1999; Rachid *et al*., 2000).

It has previously been demonstrated that the phenotypic expression of biofilm formation is highly sensitive to in vitro conditions (Baselga *et al*., 1993) and hence can be detected variably by different methods. Therefore, a combination of various methods (phenotypic and genotypic) would be useful for identifying biofilm producing *S. aureus*.

The present study showed that one of the isolates was biofilm producer phenotypically but genotypically have only *icaD*, this suggesting that biofilm can be formed even when only one ica loci gene is present (De Almeida *et al*., 2017) These results are like those obtained by Darwish and Asfour (2013) with bovine milk samples. These authors reported that the prevalence rates of *icaA* and *icaD* genes were 15.0 and 62.5%, respectively. Furthermore, Ciftci *et al*. (2009) found that 16 (27.1%) and 38 (64.4%) out of 59 strains were positive for *icaA* and *icaD* genes, respectively. The lower detection of *icaA*, and therefore the differences in the prevalence rates can be attributed to the variation in DNA sequences. This may lead to failed amplification of the genes in some isolates and consequently false negative results (Tormo *et al*., 2005; Ferrer *et al*., 2012; Darwish and Asfour, 2013).

In this study, the 4 isolates which did not produce slime factor on CRA plate in vitro, were positive for both *icaA* and *icaD* genes. This suggests that certain environmental conditions or the presence of other genes may affect the possibility of phenotypic behavior on the Congo red agar plate, that may show colonies that did not fully express the ica gene (Ciftci *et al*., 2009).

One of the aims of this study is to evaluate the presence of staphylococcal enterotoxins genes in the staphylococcal isolates. The 15 *S. aureus* isolates were tested for the presence of *sea*, *seb*, *sec*, *sed*, *see* genes by PCR. The data illustrated that 60% of the isolates have enterotoxin genes. This agrees with Normanno *et al*. (2007), who reported a 59.8% prevalence of se genes in food samples, and with Nazari *et al*. (2014), who reported that the gene encoding enterotoxin was found in 53.8% of *S. aureus* isolates that were positive for at least one enterotoxin gene. and also, with Pereira *et al*. (2009), who found the prevalence of enterotoxigenic strains was 68.2%. Also, the results nearly agree with Omoe *et al*. (2002), who found that 77.4% *S. aureus* isolates were positive for one or more se genes, and with Mashouf *et al*. (2015), who reported that the prevalence of SEs was 77.6% among the tested isolates. According to research, genes can be located in plasmids (*sed* and *sej*), bacteriophages (*sea* and *see*), and pathogenic islands. (*seb* and *sec*), and chromosomes (*seg, seh,* and *sei*); therefore enterotoxin-producing *S. aureus* carry several se genes (Alibayov *et al*., 2014).

In this study, *seb* enterotoxin was detected in 33.3% of *S. aureus* isolates. The result agreed with Nazari *et al*. (2014), who detected *seb* in 26.9% and disagree with Kitai *et al*. (2005) and Rasoul *et al*. (2015), who observed *seb* in 64.1% and 4.1% of *S. aureus* respectively. The enterotoxin *see* was detected in 26.6% of *S. aureus* isolated in this study. This nearly agrees with Asadollahi *et al*. (2014), who reported that *see* gene found in 31%. and disagree with El-Nagar *et al*. (2017), who found 4.8% of the isolates have *see*. Some studies reported that none of the isolates were positive for *see* (Rahimi *et al*., 2013; Nazari *et al*., 2014), which disagree with the obtained results.

Conclusion

According to the findings of this study, *S. aureus* isolated from ice cream were pathogenic, harbor *icaA* and *icaD* gene, which are responsible for biofilm formation and also some of *S. aureus* isolates carry *seb* and *see*, which cause food intoxication. Therefore, much more attention should be paid to hygienic measures during ice cream manufactures.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Afifi, A.N., Sadek, A., Aggour, G.M., El-Temawy, A.A., Nemr, M.M., 2011. Molecular Characterization of *Staphylococcus aureus* Enterotoxins in Milk and Some dairy Products. Egyptian Journal of Medical Microbiology 20, 107-116.

Alibayov, B., Zdeňková, K., Purkrtová, S., Demnerová, K., Karpíšková, R.,2014. Detection of some phenotypic and genotypic characteristics of *Staphylococcus aureus* isolated from food items in the Czech Republic. Ann. Microbiol. 64, 1587-1596.

Ammendolia, M, G., Di Rosa, R., Montanaro, L., Arciola, C, R., Baldassarri, L., 1999. Slime production and expression of the slime-associated antigen by staphylococcal clinical isolates. Journal of Clinical Microbiology 37, 3235–3238.

Arciola, C.R, Baldassarri, L., Montanaro, L., 2001. Presence of *icaA* and *icaD* and slime production in a collection of staphylococcal strains from catheter-associated infections. J. Clin. Microbiol.

39, 2151-2156.

Arciola, C.R., Campoccia, D., Gamberini, S., Cernellati, M., Donati, E., Montanaro, L., 2002. Detection of slime production by means of an optimized Congo red agar plate based on a colorimetric scale in *Staphylococcus epidermidis* clinical isolates genotyped for ica locus. Biomaterials 23, 4233-4239.

Argudín, M.A., Mendoza, M.C., Rodicio, M.R., 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins 2, 1751-1773.

Asadollahi, P., Delpisheh, A., Maleki, M.H., Jalilian, F.A., Alikhani, M.Y., Asadollahi, K., Soroush, S., Hematian, A., Emaneini, M., Taherikalani, M., 2014., Enterotoxin and Exfoliative Toxin Genes among Methicillin-Resistant *Staphylococcus aureus* isolates Recovered from Ilam, Iran. Avicenna Journal of Clinical Microbiology and Infection 1, 20208.

Ba, X., Harrison, E.M., Edwards, G.F., Holden, M.T.G., Larsen, A.R., Petersen, A., Skov, R.L., Peacock, S.J., Parkhill, J., Hohmes, M.A., 2014.Novel mutations in penicillin –binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the mec genes. Journal of Antimicrobial Chemotherapy No.69.

Baselga, R., Albizu, I., De la Cruz, M., Del Cacho, E., Barberan, M., Amorena, B., 1993. Phase variation of slime production in *Staphylococcus* aureus: implications in colonization and virulence. Infection and Immunity 61, 4857–4862.

Bernardi, A.C. A., Pizzolitto, E.L., Pizzolitto, A.C., 2007. Detection of slime production by coagulase-negative staphylococci isolated from central venous catheter. Rev Cien Farm 28, 57-66.

Castelani, L., Pilon, E.L., Martins, T., Pozzi, R.C., Arcaro, P.R.J., 2015. Investigation of biofilm production and *icaA* and *icaD* genes in *Staphylococcus aureus* isolated from heifers and cows with mastitis. Animal Science Journal 86, 340–344.

CDC., 2014. Toxic-shock syndrome. United States, 1970-1982. MMWR Morb Mortal Wkly Rep. 31, 201-204.

Chiang, Y.C., Liao, W.W., Fan, C.M., Pai, W.Y., Chiou, C.S., Tsen, H.Y., 2008. PCR detection of *Staphylococcus* enterotoxins N, O, P, Q, R, U and survey of SE types in *S. aureus* isolates from food poisoning cases in Taiwan. Int. J. Food Microbiol. 12,166-173.

Christensen, G.D., Simpsonv, W.A., Yonger, J.J., Baddor, L.M., Barrett, F.F., Melton, D.M., Beachey, E.H.,1985. Adherence of coagulase negative Staphylococci to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices. J. Clin. Microbiol. 22, 996-1006.

Ciftci, A., Findik, A., Onuk, A., Savasan, S., 2009. Detection of Methicillin resistance and slime factor production of *Staphylococcus aureus* in bovine mastitis. Brazilian Journal of Microbiology 40, 254-261.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322.

Cramton, S.E, Gerke, C., Schnell, N.F., Nichols, W.W., Gotz, F., 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infec. Immun 67, 5427-5433.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penadés, J.R., 2001.Bap a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. 183, 2888-2896.

Darwish, S., Asfour, E.A.H., 2013. Investigation of biofilm forming ability in Staphylococci causing bovine mastitis using phenotypic and genotypic assays. The Scientific World Journal, Article ID 378492, 9 pages.

De Almeida, C.C., Pizauro, L.J.L., Junior, R.D.O., Pizauro, M.J., 2017. Identification and production of biofilm by *Staphylococcus aureus* isolated from buffalo milk and milking environment. Afr. J. Microbiol. Res. 11, 132-140.

El-Nagar, S., AbdEl-Azeem, M.W., Nasef, S.A., Sultan, S., 2017. Prevalence of Toxigenic and Methicillin Resistant Staphylococci in Poultry Chain Production. Journal of Advanced Veterinary Research 7, 33-38.

Farzana, K., Hussain Shah, S.N., Jabeen, F., 2004. Antibiotic resistance pattern agent various isolates of *Staphylococcus aureus* from raw milk samples. J. Sci. Res. 15, 145-151

Ferrer, C.P., Taponen, S. Pyo, S., 2012. Is the biofilm formation and slime producing ability of coagulase- negative staphylococci associated with the persistence and severity of intramammary infection? Vet. Microbiol. 158, 344-352.

Gotz, F., 2002 Microreview on *Staphylococcus* and biofilms, Molecular Microbiology 43, 1367–1378.

Gowrishankar, S., Kamaladevi, A., Balamurugan, K., Pandian, K. S., 2016. In Vitro and In Vivo Biofilm Characterization of Methicillin-Resistant *Staphylococcus aureus* from Patients Associated with Pharyngitis Infection. BioMed Research International, Article ID 1289157, 14 pages.

Gundogan, N., Citak, S., Turan, E., 2006. Slime production, DNase activity and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk, pasteurized milk and ice cream samples. Food Cont. 17, 389-392.

Kim, L., 2001. Riddle of biofilm resistance. Antimic. Ag Chemother. 45, 999-1007.

Kitai, S., Shimizu, A., Kawano, J., Sato, E., Nakano, C., Kitagawa, H., Fujio, K. Matsumura, K., Yasuda, R., Inamoto, T., 2005. Prevalence and characterization of *Staphylococcus aureus* and enterotoxigenic *Staphylococcus aureus* in retail raw chicken meat throughout Japan. J. Vet. Med. Sci. 64, 269-274.

Knobloch, J.K.M., Horstkotte, M.A., Rohde, H., Mack, D., 2002 Evaluation of different detection methods for biofilm formation in *Staphylococcus* aureus. Med. Microbiol. Immunol. 191, 101-106.

Kot, B., Binek, T., Piechota, M., Wolska, M.K., Zdunek, E., Płatkowska, K., 2013. Virulence factors and ability of staphylococci from bovine milk and the cowshed environment to biofilm formation. Polish Journal of Veterinary Sciences. 16, 639–645.

Kouidhi, B., Zmantar, T., Hentati, H., Bakhrouf, A., 2010.Cell surface hydrophobicity, biofilm formation, adhesives properties and molecular detection of adhesins genes in *Staphylococcus aureus* associated to dental caries, Microbial Pathogenesis 49, 14–22.

Krukowski, H., Szymankiewicz, M., Lisowski, A., 2008. Slime production by staphylococcus aureus strains isolated from cases of bovine mastitis. Polish Journal of Microbiology 57, 253-255.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N., Novick, R.P., 1998. The Gene for Toxic Shock Toxin Is Carried by a Family of Mobile Pathogenicity Islands in *Staphylococcus* aureus. Mol. Microbiol. 29, 527–543.

Marques, V.F., da Mottaa, C.C., Soares, B.D., de Melo, D.A., Coelho, S.D.D., Coelho, I.D., Barbosa, H.S., de Souza, M.M.S., 2017. Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. Brazilian Journal of Microbiology 4 8, 118–124.

Mashouf,Y.R., Hossein, M.S., Mousavi, M.S Arabestani, R.M., 2015. Prevalence of Enterotoxin Genes and Antibacterial Susceptibility Pattern of *Staphylococcus aureus* Strains Isolated from Animal Originated Foods in West of Iran. Oman Medical Journal. 30, 283–290.

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 Journal of Medical Microbiology 24, 25-34.

Mehrotra, M., Wang, G., Johnson, W.M., 2000. Multiplex PCR for Detection of Genes for *Staphylococcus aureus* Enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1, and Methicillin Resistance. Journal of Clinical microbiology. 38, 1032-1035.

Namvar, A.E., Asghari, B., Ezzatifar, F., Azizi, G., Lari, A.R., 2013. Detection of the intracellular adhesion gene cluster (*ica*) in clinical *Staphylococcus aureus* isolates. GMS. Hyg. infect. control. 8, Doc03.

Nazari, R., Godarzi, H., Rahimi Baghi, F., Moeinrad, M., 2014 Enterotoxin gene profiles among *Staphylococcus aureus* isolated from raw milk. IJVR. 15, 409-412.

Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N.C., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisetti, E., Celano, G.V., 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. Int. J. Food Microbiol. 115, 290-296.

Notcovich, S., DeNicolo, G., Flint, H.S., Williamson, B.N., Gedye, K., Grinberg., A., Villalobos, L.N., 2018. Biofilm-Forming Potential of *Staphylococcus aureus* Isolated from Bovine Mastitis in New Zealand. Vet. Sci. 5, 8.

Oliveira, M., Bexiga, R., Nunes, S.F., Carneiro, C., Cavaco, L.M., Bernardo, F., Vilela, C.L., 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates, Vet Microbiol. 118, 133-140.

Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D.L., Ueda, S., Shinagawa K., 2002. Detection of *seg*, *seh* and *sei* genes in *S. aureus* isolates and determination of enterotoxin productivities of *S. aureus*

isolates harboring seg, seh or sei genes. J. Clin. Microbiol. 40, 857-862.

Peles, F., Wagner, M., Varga, L., Hein, I., Rieck, P., Gutser, K., Keresztúri, P., Kardos, G., Turcsányi, I., Béri, B., Szabo, A., 2007. Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. Int. J. Food Microbiol. 118,186-193.

Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., Teixeira, P., 2009. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. Food Microbiol. 3, 278-282.

Pinchuk, V.I., Beswick, J.E., Reyes, E.V., 2010. Staphylococcal enterotoxins. Toxins. 2, 2177-2197.

Potera, C., 1999. Forging a link between biofilms and disease. Science 283,1837–1839.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., Ziebuhr, W., 2000. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 44, 3357–3363.

Rahimi, E., Nonahal, F., Salehi, A.E., 2013. Detection of Classical Enterotoxins of *Staphylococcus aureus* Strains Isolated from Raw Meat in Esfahan, Iran. Health Scope. 2,95-98.

Rasoul, Y.M., Seyed, M.H., Seyed, M.M., Mohammad, R.A., 2015. Prevalence of enterotoxin genes and antibacterial susceptibility pattern of *Staphylococcus aureus* strains isolated from Animal Originated Foods in West of Iran. Oman Medical. 30, 283-290.

Sambrook, J., Fritscgh, E.F., Mentiates, T., 1989. Molecular coloning. A laboratory manual., edition 2, Cold spring Harbor Laboratotry press, New York.

Samir, H., Younis, W., Sultan, S., Abd Al-azeem, M.W., 2019.Isolation of *Staphylococcus* Aureus from Ice-Cream Samples. Journal of Veterinary and Animal Research 1, 1–7.

Schlegelová, J., Babák, V., Holasová, M., Dendis, M., 2008. The biofilm-positive *Staphylococcus epidermidis* isolates in raw materials, foodstuffs and on contact surfaces in processing plants. Folia Microbiol. 53,500-504.

Smith, K., Peter, K., Daniela, H., Melchior, S., 2007. Food borne pathogenic microorganisms and natural toxins. Food drug Administration Center Food Safety Applied Nutrition 10, 119-150.

Stepanovic, S., Vukovi, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 115,891-9.

Stepanovic, S., Vukovic, D., Daki, I., Savic, B., Vlahovic-Svabic, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40, 175-179.

Stevens, N.T., Greene, C.M., O'Gara, J.P., Humphreys, H., 2009. Biofilm characteristics of *Staphylococcus epidermidis* isolates associated with device-related meningitis, Journal of Medical Microbiology 58, 855–862.

Taj, Y., Essa, F., Aziz, F., Kazmi, S.U., 2012. Study on biofilm forming properties of clinical isolates of *Staphylococcus aureus*. J. infect. dev. Ctries 5, 403-409.

Tormo, M.Á., Knecht, E., Götz, F., Lasa, I., Penadés, J.R., 2005. Bap- dependent biofilm formation by pathogenic species of *Staphylococcus*: Evidence of horizontal gene transfer? Microbiology 151, 2465-2475.

Ulrich, R.G., Sidell, S., Taylor, T.J., 1997. Staphylococcal enterotoxin B and related pyogenic toxins. Textbook of Military Medicine. Part Ⅰ. Warfare, Weaponry and Casualty. Vol. 3, pp. 621-631

Vasudevan, P., Nair, M.K.M., Annamalai, T., Venkitanarayanan, K.S., 2003. Phenotypic and Genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92, 179-185.

Yu-Cheng, C.H., Wan-Wen, L., Chin-Ming, F., Wan-Yu, P., Chien-Shun, C.H., Hau-Yang, T., 2008. PCR detection of Staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. Int. J. Food Microbiol. 121, 66-73.

Zhang, S., Iandolo, J.J., Stewart, G.C., 1998. The Enterotoxin D Plasmid of *Staphylococcus aureus* Encodes a Second Enterotoxin Determinant (Sej). FEMS Microbiol. Lett. 168, 227–233.