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Original article

Antibiogram and phylogenetic diversity of enterotoxigenic *Staphylococcus aureus* strains from milk products and public health implications

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ABSTRACT

Food poisoning caused by *Staphylococcus aureus* (*S. aureus*) toxins is considered one of the foremost public health threat that usually occurs through the ingestion of raw milk contaminated with staphylococcal enterotoxins. The current study spotlights on the prevalence, antibiogram and genetic diversity of *S. aureus* enterotoxin genes. One hundred and fifty of raw milk (90) and ice cream (60) samples were randomly collected from local markets from Sadat city, Egypt. *S. aureus* was recovered from 44% of raw milk and 20% of ice cream samples. The identification for the obtained *S. aureus* isolates was confirmed through targeting the *nuc* gene. Antibiogram pattern of 32 *S. aureus* isolates showed high resistance to Cefoxitin, Sulpha/Trimethoprim, Tetracycline, Norfloxacin, Penicillin and Cephradine. However, high susceptibility to Gentamycin and Vancomycin were observed. Multiplex PCR was a competent practise for the recognition of Staphylococcus enterotoxin (SE) genes (*SEA*, *SEB* and *SED*). The phylogenetic analysis of the *SED* gene of enterotoxigenic *S. aureus* strains showed identical similarity with 100% to each other and high similarity with other international isolates in GenBank from different localities and sources. The frequency of enterotoxigenic *S. aureus* strains in milk products could have serious hazardous effects on humans. These results suggested possible strains transmission between different geographical areas through the food and milk product trades.

1. Introduction

Staphylococcus aureus (*S. aureus*) is classified as the third most predominant pathogen responsible for food poisoning outbreaks in humans and is considered a major contaminant for many dairy products (Rabelloet al., 2007; Kadariya et al., 2014; Dittmann et al., 2017). It remains a major problematic agent in food poisoning outbreaks due to its powerful heat-stable enterotoxins (ICMSF, 1996). Moreover, Stewart (2005) and Elbehiry et al. (2017) reported that *S. aureus* is a common pathogenic agent in food-producing animals, causing diseases such as subclinical mastitis. Several

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environmental vehicles, including air, dust, and surface food products, are considered a serious source of contamination by *S. aureus* enterotoxins. Many surveillance studies have reported the existence and contamination of food products by *S. aureus* enterotoxins (De Buyser et al., 2001; Denayer et al., 2017). Milk and its products often become contaminated at the time of cheese or dairy product manufacturing, causing a serious and potential public health hazard to human consumers (Vimercati et al., 2006; Velázquez-Ordoñez et al., 2019).

The pathogenicity of *S. aureus* is promoted via several virulence factors, including Staphylococcus enterotoxins (SEs), haemolysins, fibronectin-binding proteins, and toxic shock syndrome toxin-1; these factors have a critical role in *S. aureus* pathogenicity (Puah et al., 2016). To date, approximately twenty-two SE types have been described and identified from food poisoning cases designated A-V (Argudín et al., 2010), causing vomiting, headache, abdominal colic and diarrhoea shortly after the consumption of contaminated foods (Le Loir et al., 2003). The role of SE genes as virulence determinants in *S. aureus* is thought to be regulated by the accessory gene regulator (*agr*) gene (Kornblum et al., 1990; Jenul and Horswill, 2018) in conjunction with the staphylococcal accessory regulator (sar) gene (Novick, 2000).

Due to the popular and intensive use of raw milk in Egypt by human consumers and the serious and public health importance of raw milk and its products, this investigation designed to detect the frequency, antibiogram pattern and genetic relationship among enterotoxigenic *S. aureus* strains recovered from milk and ice cream specimens from local Egyptian markets and their effect on public health.

2. Materials and methods

2.1. Collection of samples

One hundred and fifty milk product (90 raw milk and 60 ice cream) specimens were collected from local market/street vendors in different areas in Sadat city. Each raw milk sample weighed approximately 10 ml and each ice cream sample weighed 10 g; the samples were preserved in a sterile container and transported to the laboratory.

2.2. Phenotypic characterization of S. aureus isolates

The processed samples were inserted onto Baird-Parker agar (Oxoid Ltd.) and 7–10% defibrinated blood agar and then incubated at 37 °C for two successive days. The doubtful colonies of *S. aureus* were subjected to catalase, coagulase (APHA, 2004), gram staining, haemolytic activity, DNase agar (Murray et al., 2003) and Congo red medium to evaluate biofilm activity (Freeman et al., 1989).

2.3. Antibiogram profile of S. aureus isolates

The obtained *S. aureus* isolates were subjected to antibiotic susceptibility testing against 11 antibiotics on MHA medium according to (Finegold and Martin, 1982). Penicillin (P), Cefoxitin (FOX), Cephradine (CE), Tetracycline (TE), Erythromycin (E), Ciprofloxacin (CIP), Amoxicillin/Clavulanate acid (AX), Gentamycin (CN), Sulpha-Trimethoprim (SXT), Vancomycin (VA), CE, Norofloxacin (NOR).

2.4. Multiplex PCR for the recognition of enterotoxin genes

2.4.1. DNA extraction

From each sample, DNA was extracted by QIAamp DNA mini kits purchased from Qiagen Company, Germany. The extraction process was carried out based on the constructor's recommendations.

2.4.2. Oligonucleotide primers and PCR cycling conditions

As shown in Table 1, the primers used for detection of *nuc* and enterotoxins genes were obtained from Metabion (Germany). The PCR conditions were as follows: for *nuc* gene: primary denaturation was carried out for 5 min at 94 °C; 35 cycles of secondary denaturation were done for 30 secs at 94 °C; annealing was performed for 30 secs at 55 °C; then extension was done for 1 min at 72 °C and a final extension was accomplished for 10 min at 72 °C. Meanwhile for enterotoxins genes: primary denaturation for 5 min at 94 °C; 35 cycles of secondary denaturation for 30 secs at 94 °C; annealing for 45 secs at 50 °C; extension for 45 secs at 72 °C; and a final extension for 10 min at 72 °C. For multiplex PCR, the primers were added to a reaction of 50-μl comprising 25 μl of Master Mix (Takara, Japan), 7 μl of DNA template, 1 μl of each primer at 20 pmol concentrations, and 16 μl of water.

2.5. Phylogenetic analysis of the S. aureus SED gene from milk

A QIAquick extraction kit was used for purification of the PCR products. A Big Dye Terminator V3.1 cycle sequencing kit purchased from the Company of Perkin-Elmer was used for the sequence reaction, and a Centrisep spin column was applied for purification of the products. The sequences of DNA were created by genetic analyser (Applied Biosystems, USA). To establish sequence identity with existing GenBank accessions, a basic local alignment search tool (BLAST®) analysis was applied (Altschul et al., 1990). The MegAlign module of Lasergene DNAStar was applied to create the phylogenetic tree (Thompson et al., 1994), and by using maximum likelihood, neighbour-joining and maximum parsimony methods in MEGA6, the phylogenetic analyses were carried out (Tamura et al., 2013). The accession numbers of enterotoxigenic *S. aureus* strains form raw milk and ice cream samples MF359584 and MF359585 respectively.

3. Results

3.1. Prevalence of S. aureus isolates

Out of 90 and 60 raw milk and ice cream samples, the incidence rates of *S. aureus* were 44% and 20%, respectively. Other staphylococci were 26% and 10%, respectively, as revealed in Table 2. The obtained isolates of *S. aureus* were subjected to biochemical characterization tests, including catalase, coagulase, haemolysis, and DNase testing as well as biofilm activity testing.

Table 1
Primer sequences applied for *nuc* and enterotoxins genes in *S. aureus.*

Target gene	Primer sequences (5′-3′)	bp	Reference
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTA AAGC	270	Louie et al. (2002)
Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTCTCTTCGG	102	Mehrotra et al. (2000)
Seb	CGGCACTTTTCTCTTCGG CCAAATAGTGACGTTAGG	164	Mehrotra et al. (2000)
Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAACCG	451	Mehrotra et al. (2000)
Sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278	Mehrotra et al. (2000)
See	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209	Mehrotra et al. (2000)

Table 2
Prevalence of *S. aureus* isolates from raw milk and ice cream samples.

Raw milk (90)				Ice cream (60)			
S. aureus		Other staphylococci		*S. aureus*		Other staphylococci	
N	%	N	%	N	%	N	%
22	44	13	26	10	20	5	10

The % was estimated according to the total number of isolates (50).

3.2. Antibiogram profile of S. aureus isolates from milk products

The antibiogram pattern of 32 *S. aureus* isolates was determined. The results indicated that most of the *S. aureus* isolates exhibited high resistance to Cefoxitin, Sulpha/Trimethoprim, Tetracycline, Penicillin, Norfloxacin and Cephradine, with percentages of 78.1%, 71.9%, 65.6%, 62.5% and 59.4%, respectively. However, the same isolates, with 53.1% of each isolate, showed high sensitivity to Gentamycin and Vancomycin, as shown in Table 3.

3.3. Molecular identification of isolated S. aureus by nuc gene

Successful amplification and targeting the nuc gene was carried out using specific primer sets at 270 bp. Among tested ten isolates of *S. aureus*, the nuc gene was recorded in seven strains (7/10) as illustrated in Fig. 1.

3.4. Multiplex PCR for the recognition of enterotoxin genes in S. aureus strains

Ten strains of *S. aureus* were carefully chosen for the recognition of five classical enterotoxin genes (*SEA*, *SEB*, *SEC*, *SED*, and *SEE*). The successful amplification of the *SEA* and *SED* genes, at 102 bp and 278 bp, correspondingly, was performed; there was a 60% prevalence rate among the tested *S. aureus* isolates. The successful amplification of the *SEB* gene, at 160 bp, was performed, with a prevalence rate of 30%; there was no recognition of the *SEC* and *SEE* genes in any of the tested samples, as shown in Fig. 2.

3.5. Phylogenetic analysis of the SED gene in field S. aureus

The sequencing of the *SED* gene was carried out to analyse the similarity amongst the *S. aureus* isolates from the raw milk and ice cream samples. Figs. 3 and 4 showed an identical similarity between the two analysed isolates accession numbers (MF359584) and (MF359585) from the raw milk and ice cream samples, respectively. The phylogenetic tree is classified into two main clusters. The first cluster has several isolates. The second cluster included our two isolates (MF359584 and MF359585) which showed 100% similarity with each other and with the (KX168622.1) isolate of human origin from Switzerland. Additionally, high homology and similarity (99.3%) to numerous isolates of rabbit origin from Switzerland (KX168621.1), from a patient's wound in Iran (KF007920.1), and from the United Kingdom (GQ900426.1) were observed. Furthermore, there was high genetic similarity to three clinical isolates from the USA (GQ900416.1; GQ900406.1; GQ900377.1) and one isolate from Sweden (FR714929.1). However, a distinct segregation from (DQ630750.1), isolated from food poisoning cases in India, was noted.

4. Discussion

The normal compositions of milk products enhance the growth of several microorganisms, resulting in severe serious food poisoning hazards to humans (Soomro et al., 2003; Velázquez-Ordoñez et al., 2019). *S. aureus* is a public health pathogen implicated in food-borne illness in humans. For example, approximately 13,420 food-borne cases were reported in Japan (Hennekinne et al., 2012). In the current study, *S. aureus* was recovered from 44% and 20% of 90 raw milk and 60 ice cream specimens, correspondingly. Similar results in Egypt were reported by (Saly et al., 2019), who detected *S. aureus* in 46% of the examined milk and milk products.

Moreover, (Wang et al., 2017) isolated *S. aureus* from 46.2% of raw milk samples recovered from two dairy farms in China. Furthermore, (Liu et al., 2017) stated that the frequency rate of *S. aureus* was 22% in the raw milk of cows. In Brazil, a comparative study conducted by Rall et al., (2008) recovered *S. aureus* from 20.4% of 162 pasteurized and raw milk samples. In contrast, a lower incidence of 3.9% was reported in pasteurized milk samples in China (Dai et al., 2019). However, an increased prevalence was obtained by (Gündogan et al., 2006), who recorded the frequency rate of 56.6% from 180 pasteurized, raw milk, and ice cream samples. The variation between studies in the frequency of *S. aureus* recovered from milk and their products may be attributed to differences in sample sizes, origins, and geographic locations and may reflect the degree of applicable sanitary measures.

In this study, the amplification of *nuc* gene was carried out using specific primer sets for confirmatory identification of

Table 3
Antibiogram results of *S. aureus* isolates from milk products (raw milk and ice cream).

Antibiotics/Abbreviation	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Penicillin (P)	20	62.5	5	15.6	7	21.9
Cefoxitin (FOX)	25	78.1	7	21.9	–	–
Tetracycline (TE)	21	65.6	5	15.6	6	18.8
Erythromycin (E)	15	46.9	9	28.1	8	25
Ciprofloxacin (CIP)	18	56.3	11	34.3	3	9.4
Amoxicillin/clavulanate acid (AX)	11	34.3	6	18.8	15	46.9
Gentamycin (CN)	15	46.9	–	–	17	53.1
Sulpha/Trimethoprim (SXT)	23	71.9	–	–	9	28.1
Vancomycin (VA)	4	12.5	11	34.4	17	53.1
Cephradine CE	19	59.4	8	25	5	15.6
Norfloxacin (NOR)	20	62.5	8	25	4	12.5

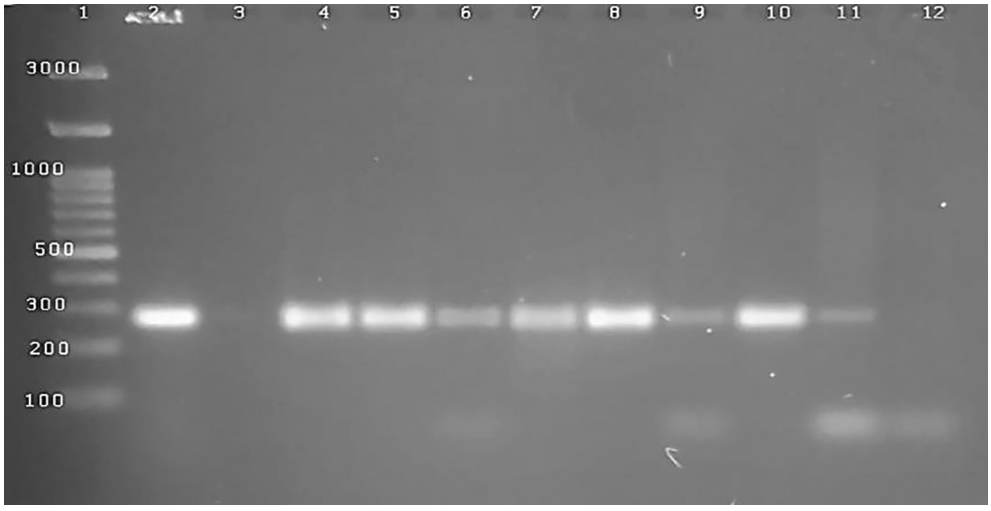

Fig. 1. 1.5% agrose gel electrophoresis of PCR product for 10 *S. aureus* isolates (3–7 isolated from raw milk**;** 8–12 from ice cream), the nuc genes were positive in 8 samples (4–11) at 27 bp., 2 control positive, 3,12 were negative samples.

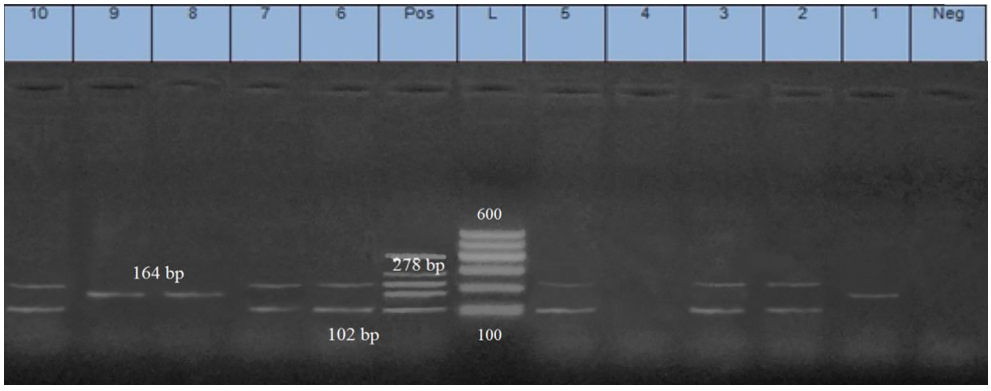

Fig. 2. Multiplex PCR for 10 strains of *S. aureus* (1–5 recovered from fresh milk**;** 5–10 from ice cream), 2, 3, 5, 6, 7 and 10 samples were positive to sea genes at 102 bp; 1, 8 & 9 samples, the *seb* genes was recognized at 164 bp and in samples number 2, 3, 5, 6, 7 & 10, the *sed* was identified at 278 bp.

S. aureus isolates. This result was previously supported by several surveillances (Hu et al., 2013; Sahebnasagh et al., 2014; Sudhaharan et al., 2015; Elbehiry et al., 2019) they discussed the actual role of *nuc* gene as potential and gold standard marker gene in detection of *S. aureus* isolates.

The misuse of antibiotics in dairy animals may have a serious hazard for the broadcast of resistant strains of bacteria to humans and the environment (Sharma et al., 2017). Our findings showed that *S. aureus* isolates exhibited high resistance to Cefoxitin, Sulphate/Trimethoprim, Tetracycline, Norfloxacin, Penicillin and Cephradine, at 78.1%, 71.9%, 65.6%, 62.5%, 62.5% and 59.4%, respectively. However, high susceptibility to Gentamycin and Vancomycin, at 53.1%, was recorded. Similar findings were reported in a recent study in Egypt (El Faramaway et al., 2019), who illustrated increased resistance in *S. aureus* clinical mastitis isolates to Penicillin (95.65%), Oxacillin (67.39%), Tetracycline (56.52%) and Erythromycin (52.17%); the highest sensitivity was to Gentamycin (71.74%) and Vancomycin (69.57%).

Moreover, (Abraha et al., 2017) recovered *S. aureus* isolates from raw milk that were highly susceptible to Gentamicin (100%) in Ethiopia. Furthermore, a study from Bangladesh (Islam et al., 2016) reported the tolerance of *S. aureus* strains from uncooked milk samples to Tetracycline (73.33%) and susceptibility to Tentamicin (100%). In contrast, (Akanbi et al., 2017) demonstrated high susceptibility to Tetracycline and Sulfamethoxazole-Trimethoprim (56.7%), Ciprofloxacin (66.7%) and Cefoxitin (76.7%). In addition, (Okpo et al., 2018) indicated that all tested *S. aureus* strains were sensitive to Ciprofloxacin, although 50% of these strains were unaffected by Tetracycline. Additionally, (Aliyu et al., 2018) tested 12 *S. aureus* isolates obtained from pasteurized milk in Nigeria that showed high susceptibility to Ciprofloxacin and high resistance to Amoxicillin, Erythromycin, and Norfloxacin.

The pathogenic and toxigenic effects of *S. aureus* mainly rely on the existence of some virulence determinants. Among these determinants, SE genes in *S. aureus* isolates in different food and milk products have an important and emerging role; these genes have been screening identified in many surveillance studies and represent a serious public health hazard (Wang et al., 2017). In our study, the *SEA* and *SED* genes were the predominant SE genes (60%) amongst the examined strains, followed by the *SEB* gene (30%). Similar to our results (Zhang et al., 2015), the predominance of the *SEA* and *SED* genes among *S. aureus* isolates from retail foods in China has been reported. Moreover, the predominance of the *SED* gene among *S. aureus* isolates of milk origin was previously demonstrated in several findings in Poland (McMillan et al., 2016; Liu et al., 2017). Furthermore, (Wang et al., 2017) demonstrated that more than 90% of *S. aureus* isolates enclosed the *SEA* and *SEB* genes, and 80% contained the *SED* gene. In a similar finding, the *SEA* gene was described as the major aetiological agent

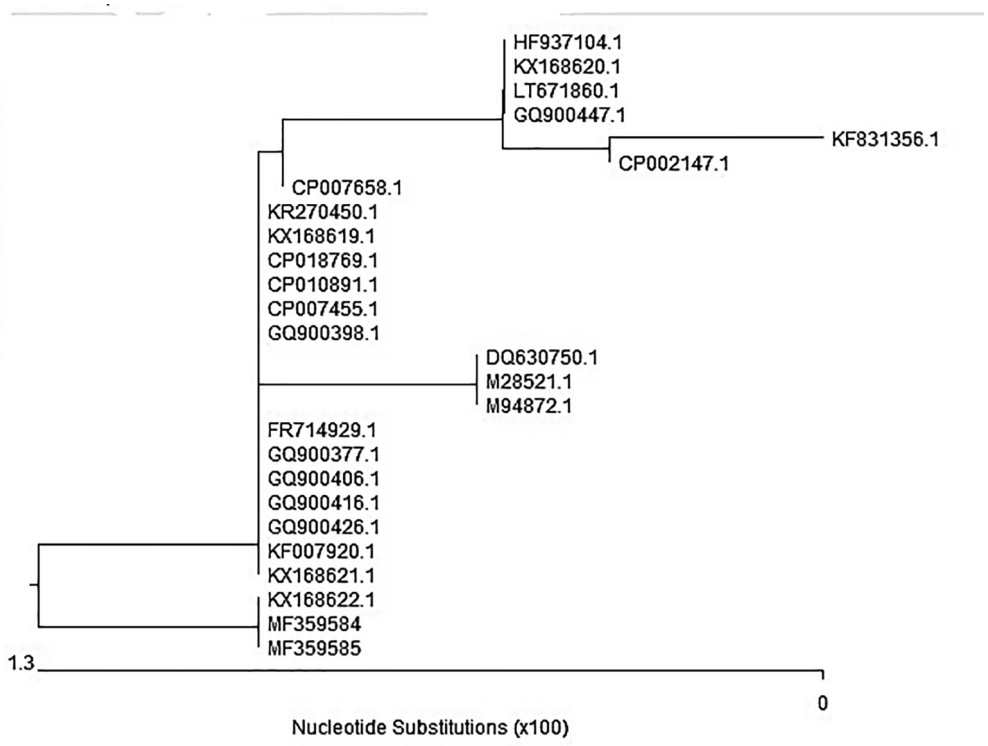

Fig. 3. Dendrogram showed the genetic homogeneity of two *S. aureus* isolates (MF359584) and (MF359585) from raw milk and ice cream respectively with other related international isolates.

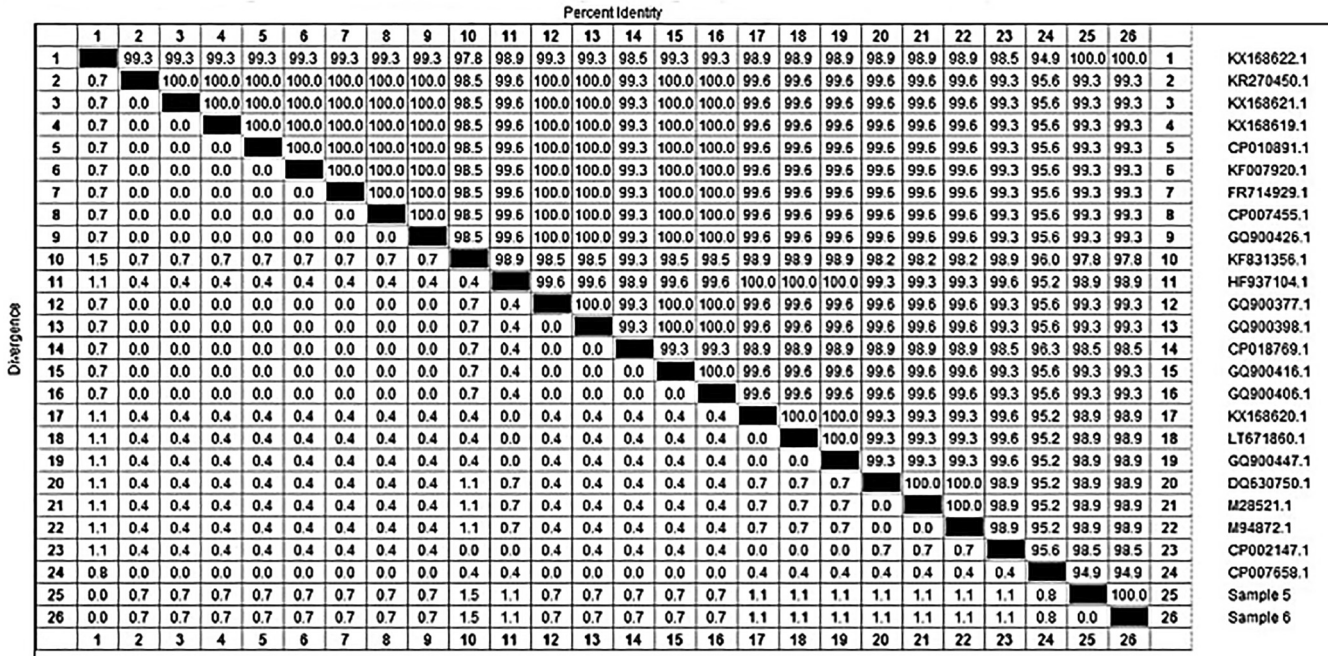

Fig. 4. Show the identical similarity between our isolates of accession number sample 5 (MF359584) and sample 6 (MF359585) within the other international isolates on Gene bank.

of *S. aureus* food poisoning eruptions (Argudín et al., 2010; Gholamzad et al., 2015). Nevertheless, (Veras et al., 2008) found that each of the SEs (*SEB*, *SEC* or *SED*) alone or in combination with other genes were concerned in food poisoning eruptions all over the world. On the other hand, the results of (Gholamzad et al., 2015; Chang et al., 2016) disagreed with our findings; these studies found that the *SEB* gene was the most common and prevalent *S. aureus* enterotoxin gene.

The phylogenetic analysis of the *SED* gene demonstrated 100% similarity between the two analysed isolates (accession numbers MF359584 and MF359585), and with other international isolates. Interestingly, (Patel, 2001) used the *rrs* gene for genotyping and

the genetic analysis of *S. aureus* isolates of milk origin that were not easily phenotypically identified. Also, (Herron-Olson et al., 2007) performed genetic characterization of the *tst* and sec genes of bovine strain RF122. Moreover, (Ikawaty et al., 2008) declared that the multiple-locus variable-number tandem-repeat analysis (MLVA) technique was effective for the detection of the close genetic relationship among human *S. aureus* isolates. The clear diversity between *S. aureus* enterotoxin genotypes from different farms and countries may be attributed to differences in geographical areas and isolate origins (Larsen et al., 2002; Monistero et al., 2018).

5. Conclusions

The current results spot highlights on the high prevalence of *S. aureus* enterotoxins among fresh milk and ice cream samples that contribute a serious public health hazardous to humans. In addition to, the close relationship between the circulating *S. aureus* strains recovered from these products in Egypt and other strains from the USA may reflect the possible transmission of these strains through the importation of dairy cows and milk products from these countries and highlight the important role of animal transport and milk product trading in spreading these circulating strains.

Author contributions

All authors contributed to the reagents/materials/analysis tools, collected the material, analysed the data and wrote and revised the manuscript.

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