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DETECTION OF ENTEROTOXIGENIC METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN DAIRY DESSERTS BY MULTIPLEX - PCR

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ABSTRACT

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The aim of this study is to evaluate the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) in dairy desserts samples and to investigate the antimicrobial resistance and molecular characteristics of these strains using PCR. A total of 120 samples comprising sweetened whipped cream, mehallabeia, ice cream and rice with milk were randomly collected from confectioneries, dairy shops, primitive restaurants and supermarkets in Sohag city, Egypt (30 samples each) and examined for presence of *S. aureus*. The results revealed that *S. aureus* could be detected in 23.3% of sweetened whipped cream, 6.7% of mehallabeia, 16.7% of ice cream and 3.3% of rice with milk samples. The results of antibiogram testing revealed that the highest percentage 9 (7.5%) of *S. aureus* isolates showed a complete resistance and 4(3.3%) showed intermediate resistance. However, the lowest percentage 2(1.7%) of the isolates were sensitive to methicillin. Eight out of nine strains that showed complete resistance using antibiotic sensitivity test identified as MRSA by detection of *mecA* gene by PCR (five from sweetened whipped cream, one from mehallbeia and two from ice cream samples). Furthermore, some classical enterotoxins gene profile of complete resistant strain were investigated by using M-PCR. The enterotoxins were detected in four strains only, and three different toxinotypes were recorded. The most frequent ones were "*sea*" gene, and followed by "*sed & seb*" from sweetened whipped cream and ice cream samples, while no *"sec"* gene could be detected from all samples. It is emphasized that the presence of *S. aureus* and their SEs genes in dairy desserts may be regarded as a potential risk for human health.

Key words: *Staph aureus, Dairy desserts, MRSA, SEs, M-PCR.*

INTRODUCTION

Staphylococcus aureus is involved in a wide variety of humans and animals diseases and its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance (Argudin *et al.,* 2010). *S. aureus* is considered a major foodborne pathogen (Hennekinne *et al.,* 2010). Some strains are able to produce enterotoxins within a foodstuff, causing staphylococcal food-poisoning (SFP), (Argudin *et al.,* 2010). In 2006, *S. aureus* toxins were responsible for 49% of 482 human food-borne outbreaks caused by bacterial toxins reported by EU Member States (EFSA, 2007), where 10^6 cells/g of enteroxiginic *S. aureus* strains or more in food is sufficient to produce amount of enterotoxins to cause intoxication (Zinke *et al.,* 2012). *S. aureus* enterotoxins (SEs) have been divided into 5 serological "classical types" (*sea, seb, sec, sed, and see*), and among them sea is considered as the main cause of SFP outbreaks in the United States, Japan, France, and UK (Argudin *et al.,* 2010). In the last few years, six new types of SEs (*seg, seh, sei, ser, ses, set*) and ten staphylococcal-like (SEl) designated as (*selj* to *selv*) proteins have been described (Hennekinne *et al.,* 2010).

Milk is a good substrate for *S. aureus* growth and dairy products are common sources of staphylococcal food-poisoning (Scherrer *et al.,* 2004; Morandi *et al.,* 2007). Presence of *S. aureus* on the skin and mucosae of food-producing animals and the frequent association of the pathogen with mastitis, often leads to contamination of milk (Jablonski and Bohach, 1997). Moreover, one third of people are considered as asymptomatic carriers. The organism finds its way into food through hands (infected wounds, skin

lesions) or by coughing and sneezing (Asperger & Zangerl 2003). The lack of proper hygienic measures during food processing would also increase the counts of *S. aureus*, especially in manually prepared foods as ready to eat dairy desserts.

There has been increased concern about antibiotic resistant strains of *S. aureus*. Development of resistance has been attributed to the extensive therapeutic use of antimicrobials or to their administration as growth promoters in food animal production (Normanno *et al.,* 2007). Isolates of *S. aureus* are frequently resistant to methicillin and essentially all other β-lactam antibiotics. An organism with this type of resistance is referred to as methicillin-resistant *S. aureus* (MRSA) (Lee, 2003). MRSA infections are more difficult to treat with standard antibiotics and thus is more dangerous.

In 2009, the European Food Safety Authority underlined the increasing concern for Public Health represented by the presence of methicillin-resistant *S. aureus* (MRSA) in food producing animals, and recommended that further work should be performed on sampling, detection and quantification of MRSA carriage in both humans and animals, as well as on the contamination of food and the environment (EFSA, 2009). MRSA strains have been isolated in many countries from cows' or small ruminants' milk and various dairy products (Juhasz-Kaszanyitzky *et al.,* 2007; Normanno *et al.,* 2007; Turutoglu *et al.;* 2006, Ateba *et al.,* 2010; Hata *et al.,* 2010; Spanu *et al.,* 2010; Nam *et al.,* 2011; Vyletělova *et al.,* 2011; Ünal *et al.,* 2012; Medveďová *et al.,* 2014; Thabet *et al.,* 2014 and Carfora *et al.,* 2015).

The aim of this work was to study the occurrence of *S. aureus* in ready to eat dairy desserts produced locally in Sohag city. The isolates were studied in terms of: (i) methecillin susceptibility for MRSA screening; (ii) Detected MRSA isolates were further genomically characterized; (iii) SEs gene profiles detection by multiplex PCR (M-PCR).

MATERIALS and METHODS

1. Sample collection:

A total of 120 samples of ready to eat dairy desserts including sweetened whipped cream, mehallabeia, ice cream and rice with milk (30 samples each) were collected from confectioneries, dairy shops, primitive restaurants and supermarkets in Sohag city, Egypt.

2. Isolation and identification of *S. aureus*: according to Bennett and Lancette (2001)

All the samples were prepared and enriched on Staphylococci broth for 20 h at 35 ºC and then inoculated onto Baird Parker Medium (Oxide, Basingstoke, England), and incubated aerobically at 37 ºC for 24 h. The isolates were identified using established microbiological methods which included colony morphology, Gram staining and biochemical testing [catalase, coagulase, D-Nase and sugar fermentation (glucose, sucrose, lactose, mannitol)].

3. Antimicrobial susceptibility testing :

Antimicrobial susceptibility was tested by the single diffusion method according to Amita *et al.* (2008). Sensitivity disc of methicillin (oxacillin) at concentration of 1ug was used to determine the susceptibility of the isolated *Staphylococcus aureus* organism (Difco Laboratories and BioMerieux, France).

The antimicrobial susceptibility test was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards "NCCLS" (2001). The zones of inhibition (ZI) were measured and recorded after 24 h of incubation at 35º C, and the observations were interpreted. The isolates resistant to methicillin (ZI: 10 mm or less) were regarded as methicillin-resistant *Staphylococcus aureus* (MRSA), while those with zone of inhibition (11-12mm) were regarded as intermediate resistance and 13 mm or more were termed susceptible.

4. Molecular characterization:

a) PCR detection of MRSA:

Suspected MRSA (positive methicillin resistant of sensitivity test) isolates were further confirmed by molecular method. The detection of *mecA* gene by PCR assay was performed using primers and protocol described by Jonas *et al.* (2002).

b) SEs (*S. aureus* enterotoxins) gene detection:

Suspected MRSA isolates were investigated for the presence of genes coding for selected SEs (*sea, seb, sec, sed*), according to what described by Mehrotra *et al.* (2000) by using multiplex PCR protocols (M-PCR).

Primer sequences of *S.aureus* used for PCR systems are shown in the following Table:

Target genes	Oligonucleotide sequence (5′ → 3′)
***mecA* (F)**	**5′** TAGAAATGACTGAAC GTCCG **′3**
***mecA* (R)**	**5′** TTGCGATCA ATGTTACCGTAG **′3**
***sea* (F)**	**5′** TTGGAAACGGTTAAAACGAA**′3**
***sea* (R)**	**5′** GAACCTTCCCATCAAAAACA **′3**
***seb* (F)**	**5′** TCGCATCAAACTGACAAACG **′3**
***seb* (R)**	**5′** GCGGTACTCTATAAGTGCC **′3**
***sec* (F)**	5′ GACATAAAAGCTAGGAATTT ′3
***sec* (R)**	**5′** AAATCGGATTAACATTATCC **′3**
***sed* (F)**	**5′** CTAGTTTGGTAATATCTCCT **′3**
***sed* (R)**	**5′** TAATGCTATATCTTATAGGG **′3**

RESULTS

Table 1: Incidence and antimicrobial profile of *S. aureus* isolated from dairy desserts samples

Samples	Positive biochemical *S. aureus*		Resistant strains		Intermediate strains		Sensitive strains	
	No./30	%	No./30	%	No./30	%	No./30	%
Sweetened whipped cream	7	23.3%	5	16.7	1	3.3	1	3.3
Mehallabeia	2	6.7%	1	3.3	1	3.3	__	__
Ice cream	5	16.7%	3	10	1	3.3	1	3.3
Rice with milk	1	3.3%	__	__	1	3.3	__	__
Total	15	12.5%	9	7.5	4	3.3	2	1.7

Table 2: Frequency distribution and enterotoxins gene profile of MRSA in the examined samples

Samples	Positive MRSA		SEs gene profile of positive MRSA					
	Sensitivity test	PCR	sea	seb	sec	sed	sea &b	Total
Sweetened whipped cream	5 (16.7%)	5 (16.7%)	1	__	__	__	1	2
Mehallabeia	1 (3.3%)	1 (3.3%)	__	__	__	__	__	__
Ice cream	3 (10%)	2 (6.7%)	1	__	__	1	__	2
Rice with milk	__	__	__	__	__	__	__	__
Total	9 (7.5%)	8 (6.7%)	2	__	__	1	1	4

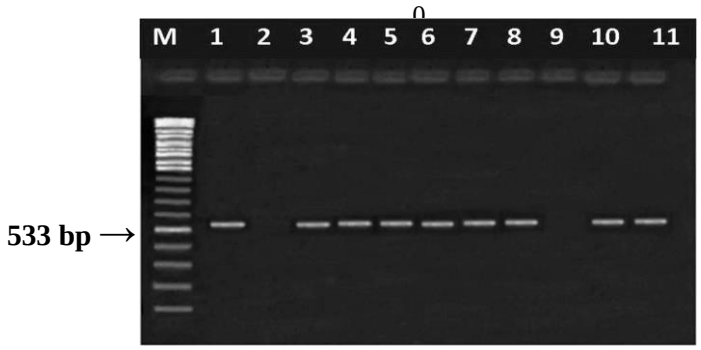

Photo 1: Agarose gel electrophoresis of PCR amplification products of *mecA* gene for characterization of Methicillin Resistant *Staphylococcus aureus* (MRSA).

Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control positive for *mecA gene* of *S. aureus*. Lane 2: Control negative for *mecA* gene of *S. aureus*. Lanes 3, 4, 5, 6 and 7: Positive *S. aureus* strains for *mecA* gene from sweetened whipped cream samples. Lane 8: Positive *S. aureus* strains for *mecA gene* from mehallbeia samples. Lanes 10 and 11: Positive *S. aureus* strains for *mecA* gene from ice cream samples. Lane 9: Negative *S. aureus* strain for *mecA* gene ice cream samples.

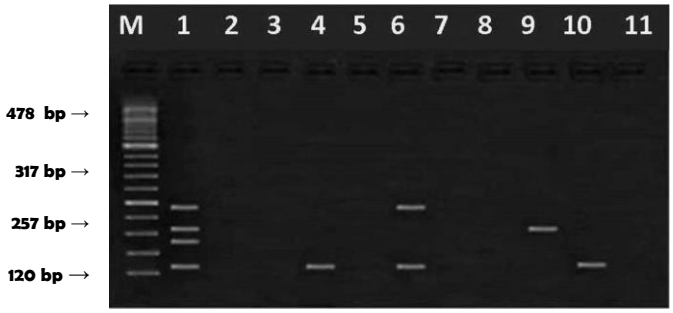

Photo 2: Agarose gel electrophoresis of multiplex PCR of *sea* (120 bp), *seb* (478 bp), *sec* (257bp) and *sed* (317 bp) enterotoxins genes for MRSA isolates.

Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control positive for *sea, seb, sec and sed* genes. Lane 2: Control negative for *sea, seb, sec and sed* genes. Lanes 4 & 10: Positive *S.aureus* strains for *sea* gene from sweetened whipped cream & ice cream samples. Lane 9: Positive *S.aureus* strain for *sed* genes from ice cream samples. Lane 6: Positive *S.aureus* strain for *sea* and *seb* genes from sweetened whipped cream samples. Lanes 3, 5, 7, 8 & 11: Negative *S.aureus* strains for enterotoxins.

DISCUSSION

Incidence of *S. aureus* in dairy desserts samples:
From the current study, it is clear that 15 (12.5%) out of 26 *Staphylococcal spp*. isolated from total 120 samples were identified as *S. aureus* which were distributed in, 7(23.3%), 2(6.7%), 5(16.7%) and 1(3.3%) of sweetened whipped cream, mehallbeia, ice cream and rice with milk examined samples, respectively (Tables 1&2).While a higher percentage (52%) of *S. aureus* isolated from dairy desserts was recorded by Ertas *et al.* (2010). The highest percentage of *S. aureus* were isolated from sweetened whipped cream and ice cream examined samples may be attributed to that these products do not subjected to heat during manufacture in the opposite to mehallbeia and rice with milk examined samples which subjected to heat during manufacture. The high percentage of *S. aureus* in ice cream samples is in agreement with the results obtained by Thabet *et al.* (2014).

Antimicrobial susceptibility:
Methicillin-resistant *Staphylococcus aureus* is an important hospital and community associated pathogen worldwide (Ho *et al.*, 2008). Foods may serve as an important reservoir and source of community-acquired MRSA (Jones *et al.*, 2002). In recent years, MRSA strains have been identified in various foods including bovine milk, ice cream and ready-to-eat foods **(**Kwon *et al.*, 2006; Fessler *et al.*, 2011; Gucukoglu *et al.*, 2013; Thabet *et al.*, 2014 and Carfora *et al.*, 2015).

The antibiotic resistance profile of MRSA recorded in Tables 1&2 showed that a highest percentage, 9 (7.5%) out of 15 isolates of *S. aureus*, were resistant to methicillin which were recovered from sweetened whipped cream, mehallbeia and ice cream samples in percentages of 16.7, 3.3 and 10%, respectively. While, the intermediate resistance profile was shown in one (3.3%) isolate from each of the examined

dairy desserts products with 3.3% of total percentage. On the other hand, the sensitive one was the lowest percentage which was represented by 2(1.7%) and recovered from sweetened whipped cream and ice cream examined samples (one sample of each).

From the present study, it was noted that high percentages of MRSA could be isolated from sweetened whipped cream and ice cream and this poses a potential health risk to consumers. The possible explanation for the significant occurrence of MRSA in these samples may be due to unrestricted and uncontrolled use of antibiotics in animals and farming, besides unsatisfactory health status of cattle herds. Secondly, a greater percentage of contaminations during industry are extensively managed, but they still exist in the contaminated environment (Le Loir *et al.,* 2003; Lowy, 2003; Ono *et al.,* 2008 and Strastkova *et al.,* 2009). In another study, Wang *et al.* (2014) stated that although MRSA prevalence in retail foods is relatively low, the risk of its transmission through the food chain, especially by uncooked food, cannot be disregarded.

3. Molecular analysis of suspected MRSA isolates:
mecA is responsible for resisting to methicillin and other beta lactam antibiotics and is localized in the *S. aureus* chromosome. *mecA* encodes penicillin-binding protein 2a (PBP2a), which differs from other penicillin-binding proteins as its active site does not bind methicillin or other beta lactam antibiotics (Lowy, 2003). As such, PBP2a can continue to catalyze the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of antibiotics. As a consequence of the inability of PBP2a to interact with beta lactammoieties, acquisition of *mecA* confers resistance to all beta lactam antibiotics in addition to methicillin (Lowy, 2003 and Sahebnasagh *et al.,* 2011).

Molecular analysis of the suspected MRSA isolates by polymerase chain reaction (PCR) was carried out to detect *mecA*, which is the gold standard for detecting methicillin-resistance. Eight out of nine suspected MRSA isolates (5 from sweetened whipped cream, 1 from mehallbeia and 2 from ice cream) were positive to the presence of *mecA* gene (Table 2 & Photo 1). These results go parallel to the results of sensitivity test indicating that most of strains (88.9%) showed complete resistance carried *mecA* gene. The same results were obtained by Thabet *et al.* (2014). On the other hand, Adesida *et al.* (2005) stated that PCR assays for detection of MRSA do not always give indisputable results, some isolates have been found to be *mecA* negative in the PCR, but resistant to methicillin/oxacillin. Also, some isolates have been found to be *mecA* positive, but susceptible to both methicillin and oxacillin (Olonitola *et al.,* 2007). Finally, measures should be taken to prevent the transmission of MRSA among animals, humans, and the farm environment.

4. SEs gene profiles detection by multiplex PCR:
Another troubling aspect of food-associated MRSA is that MRSA frequently contain staphylococcal enterotoxin genes, including genes encoding for enterotoxins most often associated with food poisoning (*sea, seb, sec, sed*) (EFSA, 2008). Different combinations of staphylococcal enterotoxin genes are associated with different MRSA clones, but the reasons of this association remain unclear. Increased prevalence of MRSA amongst *S. aureus* strains could lead to a higher prevalence of toxinogenic *S. aureus* (Ferry *et al.,* 2006; Tristan *et al.,* 2007 and EFSA, 2008). Clinically, food poisoning caused by MRSA should be no different than that caused by other *S. aureus* strains (Weese, 2010).

The analysis of the SEs gene profiles (*sea- sed*) was carried out on those suspected MRSA isolates obtained from this study. Results in Table 2 & photo 2 illustrated that only four of suspected MRSA harbor staphylococcal enterotoxins, by which three of them were also harbor *mecA* gene, these isolates were recovered from two sweetened whipped cream and two ice cream examined samples. These results confirm that enterotoxigenic *S. aureus* can be commonly found in milk and dairy products, as reported in other studies conducted in Northern Italy (Bianchi *et al.,* 2014) and Switzerland (Hummerjohann *et al.,* 2014), adopting a similar approach.

Toxinotypes composed by a single gene were observed in 3 isolates (75%), two represented by *sea* gene and one of *sed* gene, while combination of more than one toxin gene (*sea &seb*) occurred in one (25%) of isolates, which displaying a remarkable heterogeneity. From these findings, the most frequently SEs genes detected were *sea*, present in 3 of the isolates. It was found, that *sea* gene is the most frequent gene among isolates studied by Rall *et al.* (2008), Ertas *et al.* (2010) and Medveďová *et al.* (2014). However, Normanno *et al.* (2007); Ote *et al.* (2011) and Carfora *et al.* (2015) noticed the majority of strains with *sed* gene. It is assumed, that *sea* together with *sed* are the most frequent agents in SFP outbreaks (Rosengren *et al.,* 2010). Furthermore, *sea is* predominantly produced by the human strains, so the connection with food contamination during the manufacture is possible (Akineden *et al.,* 2008). Meanwhile, none of the isolates harbored *sec* gene in this study, the *sec* was the most predominant gene in other study (Trnčíková *et al.,* 2010; Zigo *et al.,* 2011). This disunity in SEs genes presence among different isolates of *S. aureus* may result from the different ecological niches and geographical origins

of strains, different cultivation and detection conditions and kinds of samples investigated.

Detection of SEs in sweetened whipped cream and ice cream examined samples may support the fact that said If food is cooked properly, MRSA cells will be killed however, as with enterotoxigenic MRSA strains, under condition of temperature abuse MRSA cells could grow in food produce heat-stable enterotoxins and cause foodborne intoxication.

In conclusion, the presence of enterotoxigenic *S. aureus* in dairy desserts showed that consumption of ready to eat dairy desserts might be a potential risk of food poisoning. Therefore, consumers should avoid the consumption of unpasteurized dairy products. Further surveillance on prevalence of *S. aureus* as well as emerging antimicrobial resistance is required to recognize foods that may represent health risk and to ensure the effective treatment of foodborne infections.

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الكشف عن المكور العنقودى الذهبى المفرز للسموم والمقاوم للمزيسيللين فى الحلاوى اللبنية باستخدام إختبار البلمرة المتعدد المتسلسل

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تم تجميع ٣٠ عينة لكل من القشدة المخفوقة والمحلاه والمهلبية والآيس كريم والأرز باللبن من محلات الحلويات والألبان والمطاعم والسوبر ماركت بمدينة سوهاج لمعرفة مدي تواجد المكور العنقودي الذهبي المقاوم للمزيسيللين مع الكشف عن جينات التسمم المعوى بها. وقد تبين من الفحص البكتريولوجي أن (٢٣,٣%) من عينات القشدة المخفوقة والمحلاه و (٦,٧%) من المهلبية و (١٦.٧%) من الآيس كريم و (٣,٣ %) من الآرز باللبن ملوثة بالمكور العنقودي الذهبي. وبإجراء اختبارالحساسية باستخدام المزيسيللين على السلالات التي تم عزلها وجد أن أعلى نسبة كانت للعترات المقاومة للمزيسيللين بنسبة (٧.٥%) من عينات القشدة المخفوقة والمحلاه والمهلبية والآيس كريم ، بينما (٣.٣%) كانت متوسطة المقاومة، فى حين أن أقل نسبة (١.٧%) كانت للعترات الحساسة للمزيسيللين. وقد تم فحص العينات المقاومة للمزيسيللين باستخدام اختبار البلمرة المتسلسل، وأوضحت النتائج وجود الجين المسئول عن هذه المقاومة فى ١٦.٧% ، ٣.٣% ، ٦.٧% ، من العترات المعزولة من عينات القشدة المخفوقة والمحلاه والمهلبية والآيس كريم. وبالكشف عن مدى تواجد المكور العنقودى الذهبى المفرز للسموم والمقاوم للمزيسيللين وجد انه تمثل فى ٤ عترات فقط والتى تم عزلها من كل من عينات القشدة المخفوقة والمحلاه والآيس كريم ، وكان الجين (*sea*) ألأكثر تواجدا ثم يليه الجين (*seb* و *sed*) بينما الجين (*sec*) لم يتمثل فى أى من العترات. من هذه الدراسة نوصي بضرورة غلي وبسترة اللبن قبل تناوله وكذلك استخدام البسترة قبل تصنيع منتجاته، وعدم شراء الآيس كريم إلا من مصادر موثوق فيها كالشركات الكبيرة لضمان سلامة منتجات الألبان لكي لايتعرض المستهلك لخطورة هذا النوع من الميكروبات.