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DETECTION OF STAPHYLOCOCCI IN MILK SAMPLES IN RETAILS IN KAFR ELSHEIKH GOVERNORATE, EGYPT

Rana Tawfik Abd El-Rahman[1], Dalia Talat[2], Treza Mourice Azer[1] and Madiha Salah Ibrahim[2]*

[1]Laboratory of Directorate of Veterinary Medicine, Kafr El-Sheikh, Egypt.
[2]Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt.

***Corresponding Author: Dr. Madiha Salah Ibrahim**

Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt.

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ABSTRACT

The present work was undertaken to study the prevalence, antimicrobial susceptibility and molecular characterization of *Staphylococcus aureus* in milk samples in retails in Kafr Elsheikh governorate, Egypt. A total of 150 raw milk samples were examined, 66 *Staphylococcus* isolates were detected (44%). The incidence of *S. aureus* among a total of 66 samples was 34 isolates with a percentage of 51.51%. The antimicrobial susceptibility pattern of recovered *S. aureus* isolates revealed that the highest resistance was exhibited against neomycin; 100% and kanamycin; 97.1%. On the other hand, the most effective drugs were oxacillin, ciprofloxacin and cephalotin with bacterial resistance percentages of 91.2%, 82.3% and 79.4%, respectively. PCR was applied to detect *mec A* gene. Methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in 40% of chosen *S. aureus* isolates. Multiplex PCR for Staphylococcal enterotoxin genes (*Sea*, *Seb*, *Sec*, *Sed*) revealed that genes coding core *Sea* and *Sec* were more frequent in a percent of 40% of tested isolates, whereas *Seb* was 20% and *Sed* was completely absent. This study indicated that the quality of raw milk is low especially if introduced in non-heat-treated milk products so, more efforts are required to maintain consumer health.

KEYWORDS: *Staphylococcus aureus*, Antimicrobial resistance, MRSA, *mec*A, Staphylococcal enterotoxin.

INTRODUCTION

Milk is considered the most perfect food for human from his birth to senility. It is a very nutritional food that is rich in protein, lipids, milk sugar (lactose), vitamins (A, B) and minerals such as calcium **(Sheehan et al., 2009)**. Milk is an ideal rich media that help and support the growth of the microorganisms. Milk is highly susceptible to a variety of microorganism such as *Staphylococcus* species especially *S. aureus* **(Kalla *et al.*, 2015)**. The presence of *S. aureus* in milk is an indicator of poor sanitary conditions like unsanitary milk utensils, milking cow workers, environment surrounding milking process and transportation process **(Hill *et al.*, 2012)**. Contamination could be minimized by proper heat treatment. *Staphylococci* are normally present on the skin and mucous membranes of animals and humans, which has more several subspecies of which the most important one is *S. aureus* **(Chu *et al.*, 2012)**. *S. aureus* may be pathogenic or non-pathogenic and the pathogenic strains are usually coagulase-positive **(Bradley, 2002)**. *S. aureus* causes a wide range of inflammatory infections in dairy ruminant responsible for 30% to 40% of mastitis cases (**Asperger and Zangeri, 2003)**.

One important character of *S. aureus* is it is ability to secret enterotoxin **(Rahimi and Alian 2013)**, which lead to mild intoxication after ingestion of contaminated food and illness symptoms as vomiting, nausea, diarrhea and abdominal pains. There are many factors affecting toxin severity such as pH, cell count, temperature and presence of microflora **(Pelisser *et al.*, 2009)**.

S. aureus has the ability to produce 23 types of enterotoxin (SEs). The most common of them are *Sea*, *Seb*, *Sec*, *Sed*, *and See*. Staphylococcal enterotoxin lead to many diseases as food poisoning, toxic-shock syndrome and septicemia. Multiplex PCR assays were used for detection of enterotoxin-coding genes and the toxic shock syndrome toxin gene **(Seyoum *et al.*, 2016)**. In 1960, the first record *S. aureus* showed resistance to methicillin, by the time MRSA developed multiple resistance and became the main nosocomial pathogen worldwide **(David and Daum, 2010)**. MRSA infection in dairy product can be transferred to the human that lead to harmful toxic effects **(Loo *et al.*, 2007)**.

This study aimed to investigate the prevalence, antimicrobial susceptibility and molecular characterization of *Staphylococcus aureus* in milk samples. Further, to detect the prevalence of the MRSA in raw milk samples.

MATERIALS AND METHODS

Samples

A total of 150 raw milk samples (80 samples from street peddlers and 70 sample from farmers) were aseptically collected from various locations in Kafr Elsheikh governorate, Egypt. The milk samples were immediately transported to the laboratory on ice for direct bacteriological examination.

Isolation and identification of *Staphylococcus aureus*

Each milk sample was enriched in peptone water followed by direct plating on Baird Parker agar as a selective medium for *Staphylococcus* at 37°C for 48 hr. After identification based on colony morphology and microscopic morphology, all positive samples were subjected to coagulase test and biochemical characterization according to **MacFaddin, (2000)**.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disc diffusion method for the isolated *S. aureus* against 14 different types of antibiotic discs according to Clinical and Laboratory Standards Institute (CLSI) guidelines **(CLSI, 2010)**.

Molecular characterization

DNA was extracted from *S. aureus* according to **Mehrotra *et al.*, (2000)**. Polymerase chain reaction **(**PCR) was applied on 5 identified *S. aureus* isolates for detection of *mec A*. The *mec A* gene was amplified as described by **Buhlmann *et al.* (2008)**. The primers' sequence used were F: 5′TAGAAATGACTGAAC GTCCG ′3 and R: 5′ TTGCGATCA ATGTTACCGTAG ′3 with a product size of 533 bp.

Detection of Staphylococcal enterotoxins (*Sea, Seb, Sec* and *Sed*)

Multiplex PCR was applied on 5 *S. aureus* isolates for the detection of Staphylococcal enterotoxin genes (*Sea, Seb, Sec* and *Sed*) using the primers shown in Table 1. PCR Master Mix and PCR conditions were performed according to **Rall *et al.* (2008)**.

Table (1): Primers used for the detection of Staphylococcal enterotoxin genes.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
sea (F)	TTGGAAACGGTTAAAACGAA	120	Rall *et al.* (2008)
sea (R)	GAACCTTCCCATCAAAAACA		
seb (F)	TCGCATCAAACTGACAAACG	478	
seb (R)	GCAGGTACTCTATAAGTGCC		
sec (F)	GACATAAAAGCTAGGAATTT	257	
sec (R)	AAATCGGATTAACATTATCC		
sed (F)	CTAGTTTGGTAATATCTCCT	317	
sed (R)	TAATGCTATATCTTATAGGG		

RESULTS

Prevalence of Staphylococci in milk samples

Sixty-six *Staphylococci* isolates were obtained out of 150 samples according to morphological and cultural characteristics. Biochemical identification revealed that isolation rate of *S. aureus* among a total of 66 positive samples was 34 isolates with a percentage of 22.66%, *S. intermedius* 2.6%, *S. epidermidis* 8%, *S. capitus* 2%, *S. xylosus* 1.3%, *S. saprophytiscus* 4.6%, and mixed culture 2.6 % as shown in table 2.

Table (2): Isolation and identification of Staphylococci from milk samples.

Total No of milk samples	Total No of Staphylococci	Types of Staphylococci	No of isolates (%)*
150	66	*S. aureus*	34 **(**22.66**)**
		S. epidermidis	12 **(**8**)**
		S. intermedis	4 **(**2.6**)**
		S. capitus	3 **(**2**)**
		S. saprophyticus	7 **(**4.6**)**
		S. xylosus	2 **(**1.3**)**
		Mixed culture	4 **(**2.6%**)**

*percentage was calculated in relation to the total number of collected samples.

Antimicrobial susceptibility of *S. aureus* isolates

Antimicrobial susceptibility of 34 isolated *S. aureus* was assessed against 14 different antibiotics as shown in Table.3. Multiple antimicrobial resistance (MAR) was also detected as shown in Table.5.

Table (3): The antimicrobial resistance patterns of the *S. aureus* isolates.

Antimicrobial agent	Sensitivity disc con. (µg)	S		I		R	
		NO	%	NO	%	NO	%
Neomycin (N)	30	0	0	0	0	34	100
Kanamycin (K)	30	0	0	1	2.9	33	97.1
Sulphamethoxazol (SXT)	25	0	0	3	8.8	31	91.2
Oxytetracycline (T)	30	1	2.9	3	8.8	30	88.2
Chloramphenicol (C)	30	2	5.9	5	14.7	27	79.4
Ampicillin (AM)	10	5	14.7	4	11.8	25	73.5
Erythromycin (E)	15	5	14.7	7	20.6	22	64.7
Norfloxacin (NOR)	10	7	20.6	9	26.5	18	52.9
Cloxacillin (CL)	5	11	32.4	8	23.5	15	44.1
Gentamicin (G)	10	14	41.2	6	17.6	14	41.2
Enrofloxacin (En)	5	19	55.9	6	17.6	9	26.5
Cephalotin (CN)	30	27	79.4	3	8.8	4	11.8
Ciprofloxacin (CP)	5	28	82.3	3	8.8	3	8.8
Oxacillin (OX)	1	31	91.2	2	5.9	1	2.9

Table (4): Multiple antimicrobial resistance (MAR) index of *S. aureus* isolates

No. of *S. aureus* isolates	MAR index
1	1
2	0.9
1	0.8
10	0.7
1	0.6
7	0.5
3	0.4
2	0.3
4	0.2
2	0.1
1	0.07

Prevalence of MRSA (*mec A*) gene in *S. aureus*:
As shown in Fig. 1, two out of five isolates (40%) were positive to the *mec* A gene.

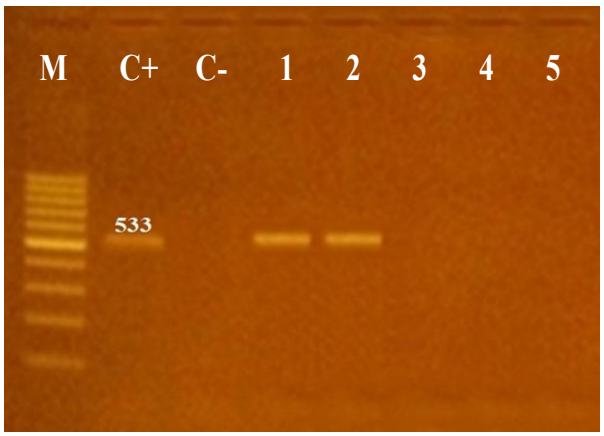

Fig. 1: Agarose gel electrophoresis showing PCR amplification of *mec*A gene (533bp) of Methicillin Resistant *S. aureus* (MRSA). M; 100 bp ladder as molecular size DNA marker, C+; Control positive S. *aureus* for *mec*A gene, C-; Control negative, 1 and 2; Positive *S. aureus* isolates, 3, 4, 5; Negative *S. aureus* isolates.

Detection of Staphylococcal enterotoxin genes
Multiplex PCR showed the presence of *Sea* with a percentage of 40%, *Seb* 20%, *Sec* 40% and absence of *Sed* as shown in Fig.2.

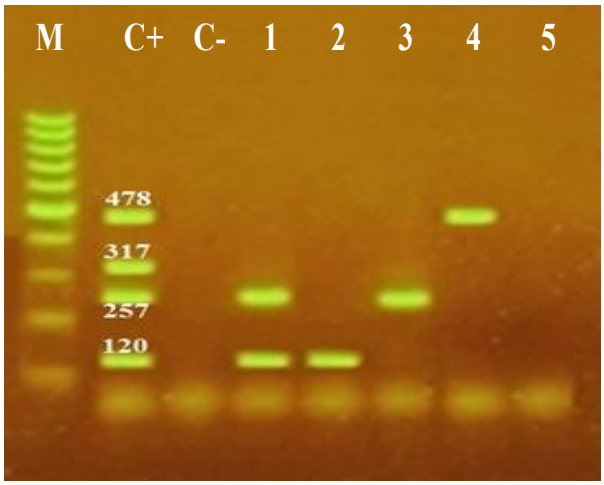

Fig. 2. Agarose gel electrophoresis of Multiplex PCR of S. aureus enterotoxin genes. Sea (120 bp), Seb (478 bp), Sec (257 bp) and Sed (317 bp). M; 100 bp ladder as molecular size DNA marker, C+; Control positive S. aureus for sea, seb, sec and sed genes, C-; Control negative, 1; Positive S. aureus isolates for sea and *sec* genes, 2; Positive *S. aureus* isolates for *sea* gene, 3; Positive *S. aureus* isolates for *sec* gene, 4; Positive *S. aureus* isolates for *seb* gene, 5; Negative *S. aureus* isolates.

DISCUSSION

In the present study, 150 raw milk samples were collected and examined. The results revealed the isolation of 66 *Staphylococcal* isolates in a percent of 44%. The results nearly agree with **Addis *et al.*, (2011)** who investigated the prevalence of *Staphylococcus* species in raw milk and recorded a 46%. While it is lower than that reported by **Mubarack *et al.*, (2010)** with a percent of 65% and 66.7% by **Hamza *et al.*, (2015)**.

The importance of the examination of milk for *S. aureus* is attributed to that, it is an important cause of mastitis in dairy animals **(Akineden et al., 2011**). The isolation rate of *S. aureus* (22.6%) was nearly similar to those obtained by **Singh *et al.*,** (**2011**) who isolated *S. aureus* from raw milk in a percent of 21.4%. Similar results were observed by **Keane *et al.*, (2013; Nassar, (2013**) at 23% and 22.7%, respectively. This is compared to 13% reported by **Mohamed, (2016)** in milk and milk products. On the contrary, lowest incidence rates of *S. aureus* of 9.13%, 15.5% and 13.13% were observed in raw milk by **Abebe et al., (2013); Shunda et al., (2013); Riva et al., (2015)**, respectively. The difference in the prevalence rates of *S. aureus* in raw milk samples may originate from the method of collection, storage and/or handling of the milk samples. Moreover, comparatively higher prevalence of *S. aureus* in raw milk samples was reported by **Gania et al., (2016**) who isolated *S. aureus* at a percent of 56%. Similar findings were previously reported by **El-Gendy, (2015); Khattab, (2016)** at 100% and 90.4%, respectively.

Various chemotherapeutic agents have been used for the prevention and control of bacterial diseases caused by *S. aureus.* Antimicrobial susceptibility of the 34 isolated *S. aureus* to 14 types of antimicrobial agents were detected. All isolates were found to be 100% resistant to neomycin. This result is nearly in agreement with those recorded by **Leskovec *et al.*, (2015**). Our results are higher than those reported by **Sarker, (2014**) who found *S. aureus* to be 66.66% resistant to neomycin. Also, high resistance rates were observed to kanamycin (97.1%) followed by Sulphamethoxazol (91.2%). On the other hand, **Tessema, (2016**) found that *S. aureus* were only 26.7% resistant to Sulfamethoxazole-trimethoprim. Furthermore, *S. aureus* showed 88.2%, 73.5% and 64.7% resistance to oxytetracycline, ampicillin and erythromycin, respectively. This disagrees with **Sarker, (2014**) who reported that *S. aureus* were found to be 66.66% resistant to erythromycin. Among, all antimicrobial agents used the most effective was oxacillin, ciprofloxacin and cephalotin, which exhibited bacterial sensitivity of 91.2%, 82.3% and 79.4%, respectively. In agreement with our study, *S. aureus* isolated from raw milk was found to be highly sensitive to ciprofloxacin (**Tanzin *et al.*,2016**).

S. aureus isolates were analyzed by PCR for identification of MRSA. Our results showed that only 2 isolates were positive for *mec*A gene with a percent of 40%. This is almost similar to those reported by **Gania *et al.*, (2016**) who detected *mec A* in a percent of 44.1% of raw milk samples. While it is lower than that reported by **Al-Ashmawy *et al.*, (2016**) who detected 75% of MRSA in Mansoura City, Egypt. Also, **Al-Khafaji, (2013**) revealed that 93,81% of S. *aureus* isolates were MRSA. Our findings were also higher than those reported by **Matyi *et al.*,** (**2013), Khaji and Shahreza, (2016**) who reported that the prevalence of MRSA was 21.8% and 30%, respectively. Such differences could result from the differences in the sample size used in each study. However, it does indicate the need for more efficient control strategies to avoid spread of such bacteria.

Staphylococcal enterotoxin food poisoning (SEP) are groups of single chain polypeptides with low molecular weight, which is responsible for gastroenteritis disturbances as vomiting, diarrhea and abdominal cramps after consumption of contaminated milk **(Naffa *et al.*, 2006)**. Multiplex PCR for identification of enterotoxin genes (SEs) in *S. aureus* isolates showed that 80% of the isolates possess enterotoxin genes. These results are nearly similar to **Carfora *et al.*,** (**2015); Mashouf *et al.*, (2015**) who found 82.7% and 77.6% SEs gene in milk samples, respectively. On the other hand, low incidence of enterotoxin (21.7%) was observed by **Abd All *et al.*,** (**2010**). Moreover, **Tang *et al.*,** (**2012**) found that 95% of *S. aureus* isolates carried the enterotoxin genes. On the contrary, **Nasef and Dawod**, **(2016**) reported the absence of enterotoxin from all *S. aureus* isolated from mastitic cows. The most frequent SEs were *Sea* and *Sec* at 40% for each followed by *Seb* (20%) and absence of *Sed*. Similar finding was previously reported by **Tang *et al.*,** (**2012**) who detected *Sea* in 36.8% of milk samples. Also, **Abd El Tawab *et al.*, (2016**) reported that the most frequently detected enterotoxins in *S. aureus* isolates from milk were *Sea*, *Sec* and *Sed* at 45.45%, 36.36% and 36.36%, respectively. In Egypt**, Omara *et al.*,** (**2016**) reported that 6 (5.7%) of 105 isolates from milk and milk products were positive for *Seb* gene, 4 (3.8%) positive for S*ee* and 3 (2.9%) positive for *Sed*, while *Sea* and *Sec* genes were completely absent. Also, **EL-Seedy *et al.*,** (**2012**) reported that *Sec* gene is the most prevalent types of enterotoxins, followed by *Sea* (15.7%), *Seb* (11.8%), and *Sed* (zero) in *S. aureus* isolates from milk.

CONCLUSION

The current study reported that the raw milk samples were highly contaminated with *S. aureus* (22.66%), posing a high risk of food poising. This highlights the need for continuous surveillance of antimicrobial susceptibility of *S. aureus* to select the appropriate therapy and control the indiscriminate use of antimicrobials. Further, raw milk might be considered a potential vehicle for transmission of multidrug resistant MRSA that may cause severe infection in human due to wide variety of virulence factors. Routine hygienic practices in milk processing, cleaning of milkers' hands, cleaning of cows udders and teats, discarding the mastitic cows and post milking preservation could be very effective to increase the hygienic quality of raw milk and decrease the contamination of milk with microorganisms. Moreover, PCR technique is very effective, rapid and reliable for detection of *S. aureus* enterotoxin genes (*Sea*, *Seb*, *Sec* and *Sed*) and MRSA gene.

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