Prevalence and Genetic Characterization of *S. Aureus* Strains Isolated from Raw Milk and Its Products

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Abstract

Raw milk and milk products could be contaminated with bacterial pathogens such as *S.aureus*, which could be transmitted to human causing severe illness. In order to throw the light on the prevalence as well as molecular typing of *S.aureus* isolated from raw milk and milk, a total of 202 samples of raw milk (n=122) and milk products (n=80) were collected from Ismailia, Port-said and Suez Governorates, Egypt. The collected samples were subjected to bacteriological examination. The prevalence of *S. aureus* was (14%) in raw milk samples and (5%) in milk products samples. Antimicrobial susceptibility for isolated strains was carried out where (85.7%) of the isolated strains were sensitive to Azithromycin. PCR protocol was used for detection of *Spa, Coa, Sea, Seb, Sec, Sed* and *See* genes. *Spa* gene and *Coa* gene were detected in (90%) and (80%) of the tested *S.aureus* strains, respectively. Concerning *S.aureus* enterotoxins genes, 40% of the tested strains were positive for *Sed* gene, while 30% were positive to *See* gene. The combination of both phenotypic and genotypic analysis is a valuable diagnostic tool for identification of *S.aureus* in raw milk and its products.

Key words: *S.aureus*, raw milk, *Spa* gene, *Coa* gene, Enterotoxins genes

Introduction

A variety of agents predominantly bacteria are considered major causes of milk borne illnesses worldwide. Raw milk is an ideal growth medium for several microorganisms. Milk and its derivate are considered vehicles for *S.aureus* infection in humans ***(Zecconi and Hahn, 2000).*** *S.aureus* grows at a wide temperature range between 6 to 48°C with optimum of 35 to 41°C. It tolerates a pH between 4 to 10 with optimum of 6 to 7, a salt concentration of 0 to 20%, and a water activity (Wa) level of 0.83 to 0.99 with optimum at 0.99 ***(Cretenet et al., 2011).*** These growth characteristics enable the bacterium to grow in a wide range of foodstuffs including milk and dairy products

(Meyrand et al., 1998; Le Loir et al., 2003).

Staphylococcal food poisoning is caused by the ingestion of food containing pre-formed toxins secreted by the bacteria. These enterotoxins are heat-stable and resistant to the action of digestive enzymes ***(Brooks et al., 2001; Enany et al., 2018).***

Although pasteurization is likely to destroy all pathogens, there is concern when raw milk is consumed or when pasteurization is incomplete or faulty. *S.aureus* produces several staphylococcal virulence factors, including enterotoxins (SEA to SEE and SEG to SEQ), and other toxins, such as exfoliative toxin A and B, and toxic shock syndrome toxin (TSST-1) ***(Fagundes and Oliveira, 2004)***, these toxins are known to cause nausea, vomiting and abdominal cramps when ingested by human and are responsible for staphylococcal food poisoning outbreak ***(Loncarevic et al., 2004 and Kerouanton et al., 2007).*** Symptoms generally have a rapid onset, appearing around 3 hours after ingestion (range 1–6 hours) and recovery is usually between 1–3 days ***(Stewart, 2003).***

Recently, many authors proposed the usage of PCR in the detection of foodborne pathogens to achieve the time-consuming classical techniques based on classical culturing ***(Gravet et al., 1999).*** They are easily handled and rapid, sensitive and specific and also constitute a very valuable tools in routine applications. Several pathogens can be diagnosed simultaneously in one step by multiplex PCR. These multiplex methods are of relevance to food microbiology have been used in detection of food borne pathogens with special interest to enterotoxigenic strains of *S.aureus* ***(Becker et al., 1998; Olsen, 2000).*** This study was planned to highlight on the prevalence and genetic characterization of *S.aureus* in raw milk and milk products.

Materials and Methods

Sampling

A total of 202 milk and milk products samples (Yoghurt, Kariesh cheese, low salted cheese, high salted cheese (Domiati), Feta cheese and Istanboly cheese) were collected from apparently healthy lactating cows and buffaloes and different marketing areas in Ismailia, Port Said, and Suez Governorates, Egypt as illustrated in Tables (1 and 2). Samples were collected aseptically and transported in an icebox to the lab for further processing and microbiological analysis.

A- Preparation of samples:

Milk samples

Milk samples were incubated aerobically at 37ºC for 24 hours, and then centrifuged at 3000 r.p.m. for 20 minutes. The cream and supernatant were discarded. A loopfull from the sediment was cultured in peptone water for 24 h at 37ºC ***(Quinn et al., 2002).***

Cheeses:

Twenty-five grams of cheese

sample were added to 225 ml. of sterile 2% sodium citrate in 18x30 cm polyethylene bags. The bags were locked in to jaws of stomacher and blended for 2 min. ***(APHA, 1992).*** A loopful from each collected sample was streaked onto the surface of different culture media.

Yoghurt:

Each sample was thoroughly homogenized in a sterile stomacher's bag ***(APHA, 1992).***

B- Isolation of *S.aureus*:

The processed samples were inoculated in peptone water for 24 h at 37ºC and then a loopful was taken and inoculated on nutrient agar, 5% sheep Blood agar, Mannitol salt agar and Baird parker agar media. All inoculated plates were incubated at 37ºC for 24-48hrs and examined daily for bacterial growth ***(Quinn et al., 2002).***

C- Identification of *S.aureus:*

The suspected colonies were examined for their morphological characters using Gram's stain, hemolytic activity on blood agar, and biochemical characters according to ***(Quinn et al., 2002).***

D- Antimicrobial susceptibility

The antibiotic sensitivity of the isolated strains was tested against 9 antibiotics (Amikacin, Gentamycin , Cefoxitin, Unasyn "penillin + sulbactam", cefotaxime , Augmentin" amoxicillin + clavulanic acid", Tetracyclin Azithromycin and Penicillin) according to the procedures ***of (Finegold and Martin, 1982)*** using disc diffusion technique. The susceptibility of the tested strains was determined according to the diameter of the inhibition zone.

II- Molecular typing of *S.aureus* isolates

A- Extraction of DNA from *S. aureus* isolates according to ***(Sambrook et al., 1989)*** using QIAamp DNA mini kit.

B- Preparation of PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit.

C- Oligonucleotide primers sequences of enterotoxigenic genes according to ***(Sambrook et al., 1989)*** using **Midland Certified Reagent Company_ oilgos (USA).**

D*- Agarose gel electrophoresis *(Sambrook et al., 1989)

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethedium bromide was added and mixed thoroughly.

Antibiotic sensitivity of the isolated *S. aureus* strains

Antibiotic sensitivity of 21 *S.aureus* strains revealed that 18 (85.7%) were sensitive to Azithromycin, followed by 16 (76%) for Gentamycin, 15 (71.4%) for Amikacin, 14 (66.6%) for Cefoxitin. On the other hand 18(86%) were resistant to Unasyn, followed by 17 (81%) for cefotaxime, 13 (62%) for each Augmentin and Penicillin as described in Table (6).

Molecular typing of virulence genes of isolated *S.aureus* strains

Protein A (*Spa*) gene:

PCR protocol was used for amplification and detection of Protein A (*Spa*) gene, where (90%) of the tested strains were positive for *Spa* gene with specific amplicon size 226 bp as illustrated in Figure (1).

Coagulase (*Coa*) gene:

PCR protocol used for amplification and detection of Coagulase (*Coa*) gene, where (80%) of the tested strains were positive *Coa* gene with specific amplicon size 570 bp as shown in Figure (2).

Enterotoxins genes:

Multiples PCR was used for detections of Enterotoxins genes (70%) tested isolates were enterotoxigenic by using multiplex PCR, where 40% of the tested strains were positive for *Sed* gene, while 30% were positive to *See* gene as illustrated in Figure (3).

Table (1): *Number and distribution of raw milk samples collected from different Governorates:*

Raw milk samples		
Governorate	**Area of collection**	**Number of milk samples**
Ismailia	Al-Salhia (farms)	44
	Al-Kssassin (farms)	8
	Kilo 11 (farms)	9
	Markets	9
	Dairy shops	2
Port said	Private Farm	20
Suez	Markets	30
Total		122

Table (2): Number of milk products samples collected from markets in Ismailia governorate.

Milk products samples	
Type	**Number of samples**
Yoghurt	20
Kariesh cheese	20
Low salted cheese	16
High salted cheese	10
Feta cheese	8
Istanboly cheese	6
Total	80

I- Isolation and identification of *S.aureus*

Table (3): *list of primers used for PCR assay*

Genes		Sequence	Amplified product	Reference
Entero-toxigenic genes	*Sea*	F: GGTTATCAATGTGCGGGTGG	102 bp	**Mehrotra et al., (2000)**
		R: CGGCACTTTTTTCTCTTCGG		
	Seb	F: GTATGGTGGTGTAACTGAGC	164 bp	
		R: CCAAATAGTGACGAGTTAGG		
	Sec	F: AGATGAAGTAGTTGATGTGTATGG	451 bp	
		R: CACACTTTTAGAATCAACCG		
	Sed	F: CCAATAATAGGAGAAAATAAAAG	278 bp	
		R: ATTGGTATTTTTTTTCGTTC		
	See	F: AGGTTTTTTCACAGGTCATCC	209 bp	
		R: CTTTTTTTTCTTCGGTCAATC		
Coa		F: ATA GAG ATG CTG GTA CAG G	Four different types of bands may be detected 350 bp, 430 bp 570 bp, 630 bp	**Iyer and Kumosani (2011)**
		R: GCT TCC GAT TGT TCG ATG C		
Spa		F: TCA ACA AAG AAC AAC AAA ATGC	226 bp	**Wada et al., (2010)**
		R: GCT TTC GGT GCT TGA GAT TC		

Table (4): *Cycling conditions of Enterotoxins, Coa and Spa genes*

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Enterotoxins genes (mPCR)	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
Coa	94˚C 10 min.	94˚C 1 min.	55˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.
Spa	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Results

Table (5) *Prevalence of S.aureus in raw milk and milk products*

Type of examined samples	No of examined samples	Positive samples for *S. aureus*		Negative samples	
		No.	%	No.	%
Raw milk	122	17	14	105	86
Milk products	80	4	5	76	95
Total	202	21	10.4	181	89.6

Table (6): *Interpretation of antibiotic sensitivity of the isolated S.aureus strains isolated from raw milk and milk Products:*

Antibiotics	Disc Conc.	*S.aureus* isolates (n=21)					
		Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Amikacin (AK)	30 µg	15	71.4	0	0	6	28.6
Gentamycin (CN)	10 µg	16	76	0	0	5	24
Cefoxitin (FOX)	30 µg	14	66.6	0	0	7	33.4
Unasyn (penillin+sulbactam) (SAM)	20 µg	3	14	0	0	18	86
cefotaxime (CTX)	30 µg	4	19	0	0	17	81
Augmentin amoxicillin+clavulanic acid (AMC)	30 µg	8	38	0	0	13	62
Tetracyclin (TE)	30 µg	10	47.6	4	19	7	33.4
Azithromycin (AZM)	15 µg	18	85.7	3	14.3	0	0
Penicillin (P)	10 units	7	33.3	1	4.7	13	62

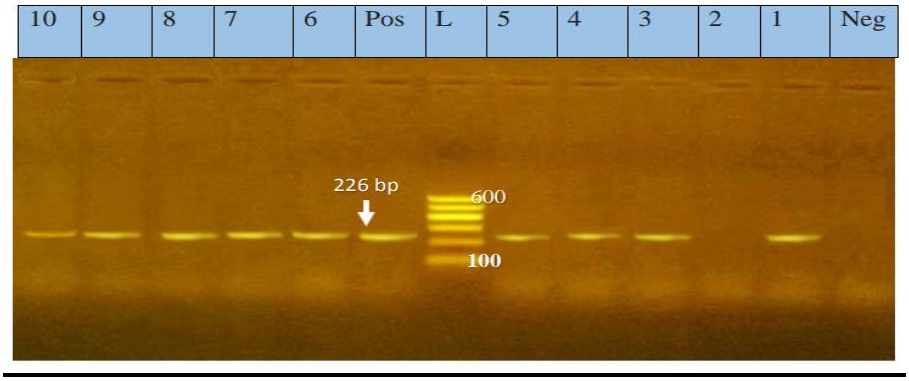

Figure (1): Electrophoretic pattern of Protein A (*Spa*) gene of *S. aureus*: Protein A (SPA) gene. Lane L: 100-600 bp DNA Ladder.
Neg: Negative control. Pos: Positive control (at 226 bp).
Lane 1,3,4,5,6,7,8,9,10 : (SPA) gene (Positive at 226 bp).
Lane 2: (Negative).

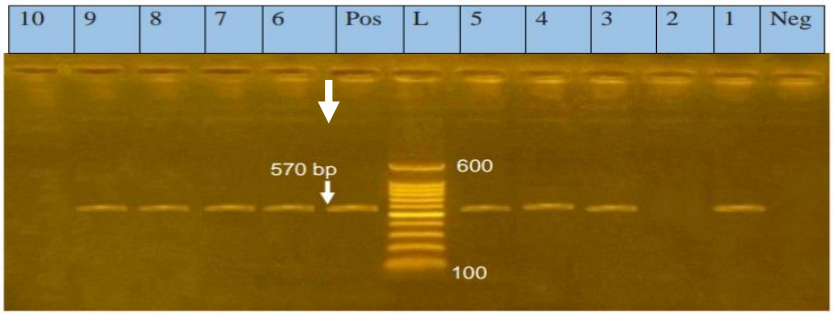

Figure (2): Electrophoretic pattern of Coagulase (*Coa*) gene of *S. aureus*: Coagulase (coa) gene. Lane L: 100-600 bp DNA Ladder.
Neg: Negative control. Pos: Positive control (at 570 bp).
Lane 1, 3, 4, 5, 6, 7, 8, 9: Coagulase gene (Positive at 570 bp).
Lane 2, 10: Coagulase gene (Negative).

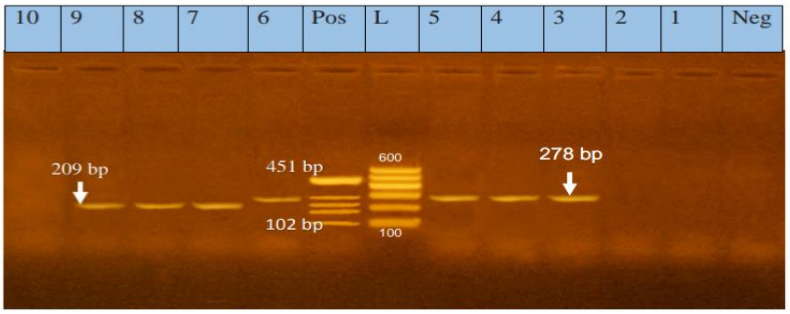

Figure (3): Electrophoretic pattern of Enterotoxins (A, B, C, D and E) genes of *S. aureus*:
Enterotoxin genes by multiplex PCR. Lane L: 100-600 bp DNA Ladder.
Neg: Negative control. Pos: Positive control (at 102-451 bp).
Lane 1, 2, 10: Enterotoxin gene (Negative).
Lane 3, 4, 5,6: (*Sed*) positive (at 278 bp).
Lane 7, 8, 9: (*See*) positive (at 209 bp).

Discussion

In dairy cattle, *S.aureus* is frequently associated with subclinical mastitis and may contaminate milk and other dairy products ***(John, 2006).***

In the presented study, the prevalence of total *S.aureus* in raw milk and its products was in percentage of 10.4% (21 isolates / 202). While the prevalence of *S.aureus* in raw milk were in percentage of 14% (17 isolates / 122) and in milk products 5% (4 isolates / 80). These results agreed with that obtained by ***(Youssef 2005 and Nashwa et al., 2014)*** and disagreed with ***(Lílian et al. 2011)*** who reported higher incidence of *S.aureus* in raw milk (68%).

In regards to the distribution of positive results of *S.aureus* from raw milk samples, (11.1%) were recovered from Ismailia, (30%) from Suez and in Port-said no samples were isolated. This variation was largely attributed to the changing management conditions and using of different diagnostic tests ***(koreen et al., 2004).***

In regards to the results of antibiotic susceptibility testing, most of *S.aureus* strains isolated were sensitive to Azithromycin (85.7%) followed by Gentamycin (76%), Amikacin (71.4%) and Cefoxitin (66.6%). On the other hand (86%) were resistant to Unasyn, followed by (81%) for cefotaxime and (62%) for each Augmentin and Penicillin. These results are supported by **(*Islam et al., 2016).*** While higher resistance to penicillin obtained by ***(Abera et al., 2010) and (Shi et al., 2010)*** in a percentage of (94.4%) and (87.6%) respectively. In this work**,** PCR protocol was used for amplification and detection of *Coa*, *Spa* and enterotoxin genes, (80%) of isolates were *Coa* gene positive and (90%) were *Spa* gene positive. These findings were agree and supported by ***(Yadav et al., 2015). While (Ashraf et al., 2015)*** recorded very low results (2%) for SPA gene and Lower result for *Coa* gene reported by ***(Ahlam et al., 2013)*** by percentage of (8.8%). *S.aureus* is characterized as coagulase-positive staphylococci and known as the main pathogen of mastitis infections in dairy animals**.** Although the coagulase tube test is the standard phenotypic routine test used to identify *S.aureus* in biological samples, several groups have implemented the molecular analysis of the coagulase gene as an accurate defined test ***(Windria et al., 2016).***

In the present study, (70%) of *S.aureus* strains were entero-toxigenic. These results are agreed with the results obtained by ***(Rall et al., 2008)*** who recorded that (68.4%) of *S.aureus* strains were toxigenic, and disagreed with those obtained by ***(Ghaleb et al., 2005)*** who recorded (37%) enterotoxigenic *S.aureus* strains. Concerning the type of enterotoxins, this study revealed two types of SEs, type D (40%) and type E (30%). ***(Gihan et al., 2012)*** supported our findings while **(*Rall et al., 2008)*** recorded lower results.

In conclusion, *S.aureus* is continues to be one of the most common contagious bacterial pathogens which could contaminate milk and milk products causing food-borne illness in human. *S.aureus* could gained access to the raw milk due poor sanitary measures during milking, handling and distribution of raw milk. The routine application of antimicrobial susceptibility testing is necessary for selection of the antibiotic of choice. The enterotoxins *Sed* and *See* genes are frequent in *S.aureus* originated from raw milk. The combination of both phenotypic and genotypic analysis is a valuable diagnostic tool for identification of *S.aureus* in raw milk and its products.

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الملخص العربي

مدى إنتشار والتوصيف الجيني لعترات المكورات العنقودية الذهبية المعزولة من الألبان ومنتجاتها

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[٢]قسم البالثولوجيا الاكلينيكية-معهد بحوث صحة الحيوان-معمل الاسماعيليه

لقد خططت هذه الدراسه لإلقاء الضوء على مدى انتشار ميكروب المكورات العنقوديه الذهبيه فى ٢٠٢ عينة عشوائيه ١٢٢عينة من اللبن الخام و٨٠ عينه من منتجات الألبان(الزبادى , الجبن القريش , الجبن قليلة الملوحة , الجبن المملح , الجبن الفيتا , الجبن الاسطنبولى) . من مناطق بمحافظات القناه الاسماعيليه (منطقة الصالحيه , القصاصين , كيلو ١١ , وبعض الباعه الجائلين فى الأسواق ومحلات بيع الألبان) و بورسعيد (مزارع انتاج الألبان) و السويس (الأسواق).أظهرت نتائج الفحص البكتريولوجى والتصنيف البيوكيميائى أن ٢١ عينه (١٠,٤٪) من اجمالى العينات كانت ايجابيه للميكروب العنقودى الذهبى .

بالنسبة للبن الخام تم عزل ١٧ عتره من الميكروب بنسبة (١٣,٩٪) بينما تم عزل ٤ عترات فقط من منتجات الألبان (الجبن قليلة الملوحة) بنسبة (٥٪) .وقد تم دراسة حساسيه المضادات الحيويه لعترات الميكروب العنقودى الذهبى المعزول من الألبان و منتجاتها وذلك بإستخدام ٩ أنواع من المضادات الحيويه المختلفه فى المعمل وكانت النتائج كالتالى : أظهرت معظم العترات حساسيه لكل من ازسروميسين , جينتاميسين , أميكاسين و سيفوكسيتين .بينما أظهرت درجة مقاومه عاليه لكل من يوناسين , سيفوتاكسيم و بنسيلين .وقد تم استخدام جهاز تفاعل انزيم البلمره المتسلسل (بى سى ار) للتأكيد على وجود الميكروب العنقودى الذهبى عن طريق الكشف عن عوامل الضراوه (بروتين أ و انزيم الكوأجيلاز) فى ١٠ عترات من الميكروب الذهبى العنقودى (٨ عينات من اللبن الخام و ٢ عينه من الجبن قليلة الملوحة) ووجد أن عامل الضراوه (بروتين أ) موجود فى ٧عينات من اللبن الخام (٨٧,٥٪) و عينتان (٢)من الجبن قليلة الملوحة (١٠٠٪) .وعامل الضراوه (انزيم الكوأجيلاز) موجود فى ٧ عينات من اللبن الخام (٨٧,٥ %) و عينه واحده (١) من الجبن قليلة الملوحة (٥٠٪) .كما تم استخدام تفاعل انزيم البلمره المتسلسل المتعدد (بى سى ار مالتى بلكس) للكشف عن السم المعوى لميكروب المكورات العنقوديه الذهبيه ووجد أن ٧ عترات من١٠ (٧٠٪) لهم القدره على انتاج السم المعوى ,٦ عترات من ٨ من اللبن الخام (٧٥٪) و عتره واحده من ٢ من الجبن قليلة الملوحة (٥٠٪), ٤ منهم تحتوى على السم من النوع (D) و ٣ تحتوى على السم من النوع (E) .