j.Egypt.vet.med.Assoc 77, no 2. 175 – 187 (2017)

STAPHYLOCOCCUS AUREUS IN RAW MILK: PHENOTYPIC AND MOLECULAR STUDIES

By

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ABSTRACT

A total of 494 staphylococci isolates, obtained from fifty raw milk samples. Identification of these isolates was carried out using combined phenotypic and molecular methods. Only 188 isolates were coagulase positive from 44 positive samples that confirmed using Latex Slide Agglutination Test, Staphytect plus. One hundred and fifty eight isolates were *Staphylococcus aureus* identified using Mannitol Salt Agar Test. Only 117/158 isolates were positive encoding genes for *S. aureus* using multiplex PCR. Selection of random isolates from different raw milk samples were examined for Staphylococcal Enterotoxin production and the percentage of positive isolates for toxin production was (8.1) of SEE toxin and (2.7) of SEC toxin. The sensitivity and specificity of the tube coagulase test was markedly improved when TNase was introduced as a combination test for routine identification of *Staphylococcus aureus* (100% specificity). The investigation highlighted that *S. aureus* is very common and constitute a potential risk for consumers' health.

Keywords:

Raw milk, *S.aureus*, Staphylococcal Enterotoxin, Staphytect plus, Multiplex PCR.

INTRODUCTION

Milk is a probable vehicle for the transmission of bacteria, including staphylococci to humans. In staphylococcal food poisoning, the most usual source of contamination is handlers of food**.** The most common causative agent of food poisoning is *Staphylococcus aureus*, which associated with the consumption of raw milk and milk products**.** Foodstuff contamination may occur directly from infected food-producing animals or may result from bad hygiene during production, or storage of food **(Asao *et al.,* 2003; Normanno *et al.,* 2007; Huong *et al.,* 2010; Spanu *et al.,* 2012 and Vazquez-Sanchez *et al.,* 2012).** One hundreds to two

hundreds ng of Staphylococcal Enterotoxin (SE) can produce intoxication symptoms. Staphylococcal enterotoxins are highly thermostable, resistant to many proteolytic enzymes and different environmental conditions**.** Latex agglutination is a very popular, sensitive, and rapid method currently have applied for accurate identification of *S. aureus* by using simultaneous detection of clumping factor and protein A**.** Enterotoxigenic *S. aureus* in milk possess a potential health hazard to consumers, it is considered as the world's third most important cause of food borne intoxication**.** The toxins of *S. aureus* are thermostable molecules**.** Five classic serological types were as A, B, C, D and E **(Bryan, 1980; Essers and Radebold, 1980; Betley *et al.,* 1992; Park *et al.,* 1994; Tirado and Schmidt, 2001; Zouharova and Rysanek, 2008; Ortega *et al.,* 2010 and Barker *et al.,* 2013).** The PCR-based approach is considered to be accurate, defined and rapid **(Bendahou *et al.,* 2009)**. This study aimed to investigate the prevalence level of *S. aureus* using phenotypic and molecular techniques in raw milk.

MATERIAL AND METHODS

Collection of samples:

Fifty raw milk samples were collected from different shops in Cairo and Giza Governorates and were transferred to the laboratory in an insulated ice box with a minimum of delay to be immediately examined and then Guaiac test was done according to **Schonberg (1956)**.

Preparation of samples:

Decimal serial dilutions were prepared according to **ISO (2001)**.

Enumeration and isolation of Total Coagulase Positive Staphylococci according to ISO (2003).

Phonotypical identification of the isolated Staphylococci according to Voss *et al.* (2009).

Essay for hemolytic activity according to Quinn *et al.* (1994).

Mannitol Salt Agar (MSA) Test according to Addis *et al.* (2011). The colonies that were identified by coagulase test as Coagulase Positive Staphylococci were streaked on MSA plates, incubated at 37°C and examined after 48 hour.

Latex Slide Agglutination Test; Staphytect plus (Oxoid, 2010). One drop of test latex was dispensed onto one of the circles on the reaction card and one drop of control latex onto another circle. Using a loop, smear the equivalent of 5 sized suspect staphylococcal colonies onto a circle from a culture media plate was picked up and mixed this in the control latex reagent and was spread to cover the circle and proceeded in the same way with the test latex.

The card for up to 20 seconds was picked up and rocked and agglutination under normal lighting conditions was observed.

Identification of the isolated Coagulase Positive Staphylococci with multiplex PCR:

DNA extraction and purification from bacterial cultures according to **Reischl *et al.* (1994)** A rapid boiling procedure was used to prepare template DNA from bacterial strains, Two to 5 loops of Staphylococci isolates taken from the Brain Heart Infusion (BHI) agar plate were collected and suspended in two hundreds ul of lysis buffer comprised of 1% Triton X-I 00, 0,5% Tween 20,10 mM Tris-HCl (pH 8,0) and 1 mM EDTA After boiling for 10 min, the suspension was centrifuged for 2 minutes, to sediment debris of bacteria, the supernatant was aspirated and from which 5 ul was used directly for PCR amplification.

Sequences of primers (Bio Basic Inc., Canada) for the Staphylococcus genus (16SrRNA) and thermostable nuclease gene for *S. aureus* (*nuc*) with product size.

Primer name	Primer sequence 5'-3' (reference)	Product size	Specificity
16SrRNAf	5' GTA GGT GGCAAGCGTTAT CC 3' 5' CGC ACA TCA GCG TCA G 3' (Monday and Bohach, 1999)	228bp	Staphylococcus genus specific primers
16SrRNAr			
nuc 1	5'-GCGATTGATGGT GATACGGTT-3' 5'-AGCCAAGCCTTGACGAACTAAAGC-3 (Brakstad *et al.* 1992)	279 bp	*S.aureus* specific primers
nuc 2			

Primers and temperature used for the detection of Staphylococcal Enterotoxins genes.

Gene	Primer name	Primer sequence 5'- 3' (reference)	Product size	Annealing temperature (˚c)
Sea	SEA-1	5' TTGGAAACGGTTAAAACGAA 3'	120bp	50
	SEA-2	5' GAACCTTCCCATCAAAAACA 3' (Johnson *et al.*, 1991)		
Seb	SEB-1	5' TCGCATCAAACTGACAAACG 3'	478bp	50
	SEB-2	5' GCAGGTACTCCTATAAGTGCC 3 (Johnson *et al.*, 1991)		
Sec	SEC-1	5' GACATAAAAGCTAGGAATTT 3'	257bp	50
	SEC-2	5' AAATCGGATTAACATTATCC 3' (Johnson *et al.*, 1991)		
Sed	SED-1	5' CTAGTTTGGTTAATATCTCCT 3'	317bp	50
	SED-2	5' TAATGCTATATCTTATAGGG 3' (Johnson *et al.*, 1991)		
See	SEE-1	5' AGGTTTTTTCACAGGTCATCC 3'	209bp	50

	SEE-2	**5' CTTTTTTTTCTTCGGTCAATC 3' (Mehrotra *et al.*, 2000)**		

Multiplex Polymerase Chain Reaction (PCR) for detection of staphylococci and *Staphylococcus aureus* according to Hanaa and Samah (2011).

It was established using a total volume of 25 ul reaction mixtures contained 5 ul of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science), The amplification cycles were carried out in a PTC -100 Thermocycler (MJ Research, USA), reaction conditions were optimized to be 94°C for 4 minutes, as first denaturation, followed by 35 cycles of 94°C for sixty seconds, 55°C for 60 seconds and 72 °C for 60 seconds, A final extension step at 72°C for 10 minutes, was followed, DNA isolated from *S.aureus* ATCC 25923 & 29213 was used as positive controls, while water was used as negative control. Amplification products were electrophoresed in 1.5% agarose gel containing 0.5X Tris-Borate EDTA at 70 volts for 60 minutes and visualized under ultraviolet light. Amplification of both 228 and 279 bp bands indicated the isolate to be *S. aureus*.

Multiplex Polymerase Chain Reaction (PCR) for detection of genes encoding Staphylococcal Enterotoxins according to Rall *et al*. (2008).

Each polymerase chain reaction contained 2.5 ml PCR Buffer 10x, 1.0 mM MgCl, 200 mM dNTP, 1 U Taq DNA Polymerase, 10 pmol of each primer, and 3 ml DNA. The final volume was adjusted to 25 ml by adding sterile nuclease free water. DNA amplification was performed in a PTC-100 thermal cycler (MJ Research, USA) using the following conditions: initial denaturation for 5 min at 94˚C allowed by 30 cycles of denaturation (94˚C for 2 min), annealing (50˚C for 1 min) and extension (72˚C for 1 min). A final extension step (72˚C for 5 min) was performed after the completion of the cycles.As positive controls,PCRs containing template DNA obtained from the standard strains *Staphylococcus aureus* ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), FRI 361 (SED) and ATCC 27664 (SEE) were carried out in parallel. Amplification products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. Amplification of 120 bp fragment confirmed the strain to be SEA producer, amplification of the 487 bp fragment confirmed it to be SEB producer, the 257 bp fragment confirmed it to be SEC producer, the 317 bp fragment confirmed it to be SED producer and the 209 bp fragment confirmed it to be SEE producer.

The procedures of agarose gel electrophoresis for identification of the PCR products were carried out according to **Sambrook *et al.* (1989).**

RESULTS

Table (1): Statistical analytical results of Total Staphylococci Count and Total Coagulase Positive Staphylococci Count in the examined raw milk samples.

Parameters (CFU/ml)	Total no. of examined samples	Positive samples		Min.	Max.	Mean	± S.E.M.
		No.	%				
Total Staphylococci Count (TSC)	50	50	100.00	$1.5x10^5$	$2.5x10^8$	$7x10^6$	$1.4x10^5$
Total Coagulase Positive Staphylococci Count (TCPSC)	50	44	88.00	$3.1x10^4$	$1x10^8$	$3.0 x10^6$	$6.8x10^4$

Table(2): Identification of Coagulase Positive Staphylococci isolates of the examined samples.

Tested Coagulase Positive Staphylococci isolates		Hemolytic isolates						Non-hemolytic isolates (γ)		Thermostable nuclease (TNase)		Staphytect test	
		Alpha hemolytic (α)		Beta hemolytic (β)		Alpha & Beta hemolytic (α & β)							
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
188	100.00	13	6.9	140	74.5	7	3.7	28	14.9	152	80.9	188	100.00

Table (3): Mannitol Salt Agar test results for differentiation between Coagulase Positive Staphylococci isolated from samples.

Tested Coagulase Positive Staphylococci isolates		*Staphylococcus aureus*		*Staphylococcus intermedius*		*Staphylococcus hyicus*	
No.	%	No.	%	No.	%	No.	%
188	100.00	108	57.4	50	26.6	30	16.00

Table (4): Result of multiplex PCR method detecting the *16s rRNA* (228bp) and *nuc* (279bp) of isolated *S. aureus.*

Tested Coagulase Positive *Staphylococcus aureus*	***16s rRNA* PCR**		***nuc* PCR**	
No.	**No.**	**%**	**No.**	**%**
158	**158**	**100.00**	**117**	**74.05**

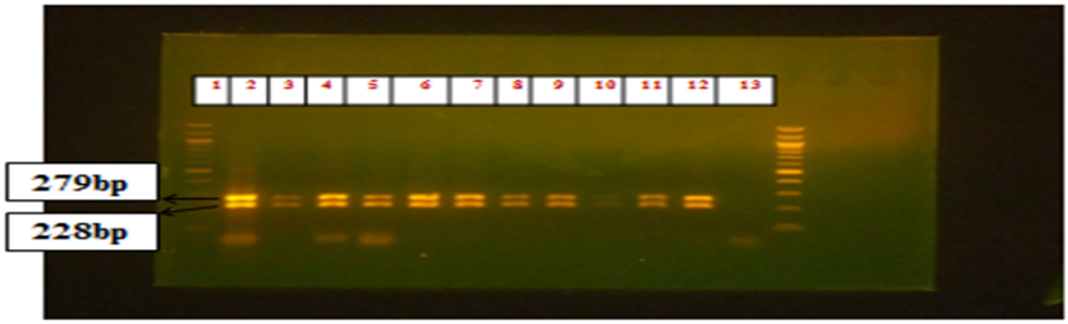

Fig. (1): Multiplex PCR assay detecting the *16SrRNA* (228bp) and *nuc* (279bp) genes.

Lane 1: molecular size marker (100bp DNA ladder)

Lane 2: positive control

Lanes 3-12: representative Staphylococcal isolates

Lane 13: negative control.

Table (5): Result of multiplex PCR method detecting the genes encoding Staphylococcal Enterotoxins of isolated *S. aureus.*

Examined isolates	**Tested *nuc* PCR positive**		***SEA* PCR**		***SEB* PCR**		***SEC PCR***		***SED PCR***		***SEE PCR***	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
	37	**100**	**0**	**0.00**	**0**	**0.00**	**1**	**2.70**	**0**	**0.00**	**3**	**8.11**

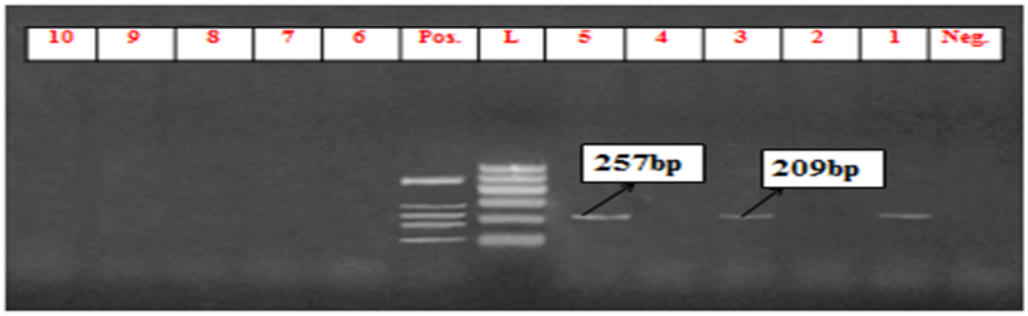

Fig. (2): Multiplex PCR assay detecting the genes encoding Staphylococcal Enterotoxins SEA (120bp), SEB (478bp), SEC (257bp), SED (317bp) and SEE (209bp).

L: molecular size marker (100bp DNA ladder)

Pos.: positive control

1-10: representative Staphylococcal isolates

Neg.: negative control

Table (6): Sensitivity, Specificity, Positive Predictive value (PPV) and Negative Predictive value (NPV) for the used diagnostic tests of isolated *S. aureus*.

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Coagulase	**100.00**	**50.00**	**77.27**	**100.00**
TNase	**62.50**	**100.00**	**85.00**	**100.00**
β-heamolytic	**100.00**	**50.00**	**77.27**	**100.00**
Mannitol	**100.00**	**50.00**	**77.27**	**100.00**
Staphytect plus	**100.00**	**50.00**	**77.27**	**100.00**

DISCUSSION

Staphylococcus species may be found on skin and mucous membranes of healthy warm-blooded animals, as well as in soil, air and water. They can grow in any food article if the conditions are suitable for their growth and multiplication **(Asperger and Zangerl, 2001and Cunha and Calsolari, 2008)**. The results given in (Table 1) explore that 100 % of the examined raw milk samples were positive for Staphylococci with a minimum of $1.5x10^5$ CFU/ ml, a maximum of $2.5x10^8$ CFU/ ml and a mean value of $7x10^6$ CFU/ ml. The public health significance of Staphylococci isolated from milk is important because it could be a source of toxins for humans **(Ebrahimi *et al.*, 2010)**. In (Table 1) also reveal that 88% of the

examined samples were positive for Coagulase Positive Staphylococci with a minimum of $3.1x10^4$ CFU/ ml, a maximum of $1x10^8$CFU/ ml and a mean value of $3.0 x10^6$ CFU/ ml. Nearly similar findings were reported by **(Tsegmed *et al.,* 2007; Alnakip, 2009; Tahoun, 2009 and Kamal *et al.,* 2013)**. Lower findings reported by **(Normanno *et al.*, 2005; Hanaa and Abd-El Rahman, 2008 and Ebrahimi and Taheri, 2009)**. The high TCPSC indicates that consumption of this milk poses a health risk and frequent occurrence of staphylococcal food poisoning, especially in children. Counts more than 10^5 CFU/ ml in milk increase probability of staphylococcal toxins production that are resistant to boiling, when buying raw milk, and to the pasteurization processes **(Hempen *et al.,* 2004; Tebaldi *et al.,* 2008 and De-Oliveira*et al.,*2011)**.The Coagulase Positive Staphylococci (CPS) isolated from examined raw milk samples were 188 isolates in a percentage of 38.1, while TNase and coagulase positive isolates were in a percentage of 80.9 (Table 2). Result showed that, the most coagulase positive considered TNase positive. CPS alpha, beta, alpha and beta and gamma hemolytic isolates were found in a percentage of 6.9, 74.5, 3.7 and 14.9 respectively. The β hemolytic isolates of Staphylococci are usually associated with CPS in the examined samples as shown in above result. One hundreds % of total Coagulase Positive Staphylococci isolates were positive with latex agglutination test (staphytect plus). Nearly similar findings were reported by **(Takeshige *et al.,* 1983; Mastunga *et al.,* 1993; Ebrahimi and Taheri, 2009 and Malinowski *et al.,* 2011).** Staphylococci diseases caused by virulence factors including the different hemolysins, which are important for virulence of the *S.aureus* **(Ebrahimi *et al.,* 2010)**. In addition, Mannitol Salt Agar is a selective and indicator medium for *S. aureus* that ferment mannitol and turn the indicator to yellow colour **(Abd EL-Tawab *et al.,* 2015)**. The data represented in (Table 3) show that Coagulase Positive *S. aureus* was found in a percentage of 57.4. In (Table 4) and Fig. (1); the performed multiplex PCR method confirmed that 158 isolates of Coagulase Positive Staphylococci gave successful amplification of the 228 bp PCR product of the Staphylococcal specific 16S rRNA gene. Using the same multiplex PCR, 117 isolates were confirmed to be *S.aureus* by amplification of 279 bp PCR product of the *S.aureus* specific *nuc* gene. These findings were nearly similar to **(Hanaa and Samah, 2011 and Sohad *et al.* 2013)**. In (Table 5) and Fig. (2); showed that, the percentage of positive isolates for toxin production was (8.1) of SEE toxin and (2.70) of SEC toxin based on selection of random isolates from different raw milk samples. This was observed that coagulase, β-heamolytic, mannitol and staphytect plus tests were the highest sensitivity

(100%) and moderate specificity (50%), while TNase was higher sensitivity (62.5%) and the highest 100% specificity (Table 6). These findings similar to that obtained by **(Weist *et al.*, 2006 and Kateete *et al.*, 2010),** while lower results obtained by **(Han *et al.*, 2007)**. The sensitivity and specificity of test combinations for detecting *Staphylococcus aureus*, aiming at improving the performance of the molecular tests was evaluated. There is no single phenotypic test alone that can guarantee reliable results in the identification of *Staphylococcus aureus*. The accuracy of PCR in the detection of *S. aureus* in milk samples was more than the cultural methods. With comparison between phenotypic and molecular identification of CPS isolates; multiplex PCR was accurate, rapid and specific more than phenotypic methods which found to be time consuming and labor intensive.

CONCLUSION

From the observed result of this study, it is noticed that, the high prevalence of *S.aureus* in raw milk were influenced by the poor hygienic conditions during milking and handling, as well as post-milking environmental contaminants. The presence of *S. aureus* in raw milk showed that consumption of raw milk might be a potential risk of foodborne intoxication. Therefore, consumers should avoid the consumption of raw milk. PCR assay could be used as a supplement, or as an alternative, to conventional bacteriological procedures currently used for rapid detection of *S. aureus* in milk samples. Further surveillance on prevalence of *S. aureus* is required to recognize foods that may represent health risk and to ensure the effective treatment of staphylococcal food poisoning.

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