Bacteriological and molecular studies on *staphylococcus aureus* isolated from raw milk

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A B S T R A C T

The present study was performed on a total of 100 raw buffalo Milk samples from different sources at El-Menofiya Governorate that the inspected samples were taken in ice box within an a hour for bacteriological examination. The results revealed that 80 out of 100 samples (80%) were positive for staphylococcus spp. Out of those 80 samples, 42(53%) were coagulase positive Staphylococci spp while 38(47%) were coagulase Negative Staphylococci spp. Out of those 42 coagulase positive Staphylococci spp**,**31 were (73.8%) *Staph. aureus.* In vitro Gentamycin, Trimethoprim / sulphamethazole, Ampicillin, Cephadrine were the most proper antibiotic against isolated *Staph. aureus.* By using PCR Spa virulence gene was detected in two studied strains while, enterotoxin E was detected only in one strain out of 6 studied strains. Meanwhile, hIyA and enterotoxin (Sea, Seb, Sec, Sed) virulence genes were not detected in all studied strains.

Keywords: *Staphylococcus aureus*, raw milk, PCR

(http://www.bvmj.bu.edu.eg) **(BVMJ-28(1): 88-97, 2015)**

1. INTRODUCTION

Staphylococci spp. often represent as a part of normal bacterial flora of skin and mucosal surfaces of the respiratory, upper alimentary and urogenital tract of mammals and birds. Thus staphylococci are easily spread between animals and under certain conditions to humans by skin contact with excretions which contain *Staphylococci spp.,* such as saliva, or aerosols released during sneezing and coughing. Moreover, *Staphylococci spp.* may be spread by animal products such as non-pasteurized milk (Werckenthin et al., 2001).

Staphylococcus species are aerobically growing Gram-positive cocci. Isolation of *Staphylococcus* species is usually not difficult since *Staphylococc i*not fastidious organism and will grow on commonly media and under variety of conditions (Rowlinson et al., 2006). *Staphylococcus aureus* is recognized to cause health care associated and community-acquired infections in every region of the world (Yilmaz et al., 2007). It is recognized worldwide as an important food-borne pathogen because of its ability to produce a wide range of extracellular toxin proteins and virulence factors typically resulting in sudden onset of nausea, violent vomiting, abdominal cramps and sometimes diarrhea. (Rosengren et al., 2013). The extracellular protein toxins and virulence factors of Staph.aureus, which are thought to contribute to the pathogenicity of the organism. *Staphylococcal* enterotoxins (SEs) are serologically grouped into five major classical types, which are SEA, SEB, SEC, SED, and SEE in addition to toxic shock syndrome toxin (TSST -1) which causes toxic shock syndrome in human. SEA and SEB are usually more common in milk and milk products (Chiang et al., 2006). For epidemiological surveillance, the methods most frequently used for the detection of *Staphylococcal* toxins are

immune diffusion, agglutination, radioimmunoassay, and enzyme-linked immune sorbent assay. The techniques used to identify toxin genotypes, DNA hybridization and PCR which are very successful and reliable (Johnson et al., 1991). Polymerase chain reaction (PCR) technology is the most promising method due to its rapidity, economical convenience and sensitivity, since it can detect a few microorganisms in clinical samples. Recently, specific oligonucleotide primers for PCR have been described for analysis of *Staph. aureus* strains for the presence of toxin genes (Johnson et al., 1991 and Becker et al., 1998). Thus, the present study was planned for bacteriological characterization of *Staph. aureus* isolates from raw milk and detection of virulence genes of the isolated strains using Polymerase chain Reaction.

2. MATERIAL AND METHODS

2.1. Samples

A total of 100 buffalo's milk samples were collected from small scale shops and individual house hold at Meniofiya Governorate. The samples were transferred in ice box directly with an hour to the laboratory with a minimum delay to be bacteriologically examined.

2.2. Bacteriological examination

Milk samples were incubated aerobically at 37°C for 24 hr then centrifuged at 3000 rpm for 20 min., the cream and supernatant fluids were discarded. A loopfull from the sediment was streaked into the surface of 7% salted nutrient agar; baird-parker agar; mannitol salt agar, and milk salted agar. All plates were incubated for 24-48 hours at 37ºC.The developed colonies were picked up and sub cultured for purification. The purified colonies were morphologically identified by Gram stain and biochemical tests(oxidase test, catalase test,coagulase test and V-P test)(Quinn et al., 2002 and Arora, 2003).

2.3. In-Vitro anti-microbial sensitivity test:

The isolated *Staph. aureus* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Martin,1982).

2.4. Detection of Virulence genes of isolated Staph. aureus by PCR:

Primers for detection of eight virulence gene that may play a role in virulence of *Staph.aureus*. These genes were protein A (spa),haemolysin(hlyA) and enterotoxins (sea, seb, sec, sed, see). It was applied on eight random isolated *Staph. aureus* following QIAamp® DNA Mini Kitinstructions (Catalogue no.M501DP100), Emerald Amp GT PCR mastermix (Takara) with Code No.RR310A and agarose gel electrophoreses(Sambrook et al., 1989).

3. RESULTS

Coagulase positive *Staph. aureus* was identified by morphological and culture characters as well as identical biochemical tests as following:

3.1. Colonial appearance

On salted nutrient agar *Staph. aureus* colonies appear smooth, low convex, glistening and densely opaque after incubation for 24 hours at the optimum growth temperature of 37°c, while on mannitol salt agar, they were yellow color surrounded by yellow halo with yellow colored medium. On baired Parker medium appear black Small 1mm colonies after 24 hours incubation surrounded by an opalescent ring and a clear zone. Pigmentation is characteristic of this species when grown aerobically appear as golden yellow colonies onto milk agar medium

3.2. Microscopic examination

Staphylococcus aureus appears as grapes like clusters under light microscope.

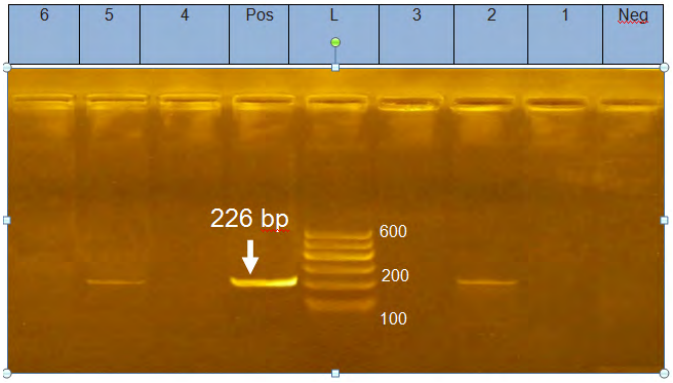


Photo (1): protein A (spa) gene. Lan M: 100-600bp DNA Ladder, Neg: Negative control. Pos: Positive control (at226bp), Lane 1, 3, 4, 6: S. aureus (Negative). Lan 2,5 : S. aureus(Positive).

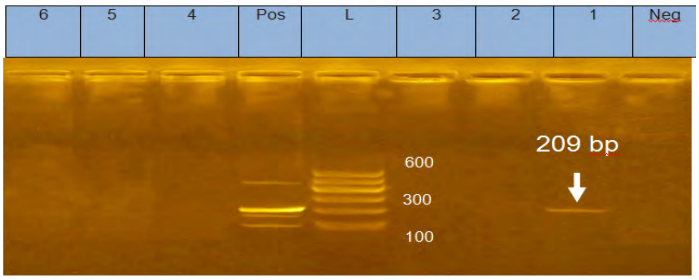
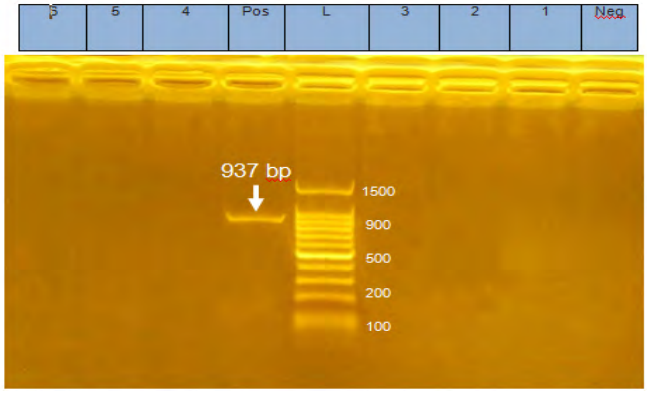


Photo (2): Enterotoxin e. See gene. **Post**: positive control (at 209bp). **Neg:** Negative control. **Lane 2, 3, 4, 5, 6: *S. aureus*** (Negative). **Lane 1 *S. aureus*** (positive)



Photo(3): haemolysin (hIy A). Lane M:100-1500bpDNA Ladder. Neg: Negative control. Pos: positive control (at 937 bp. Lane12, 3, 4, 5& 6 *S. aureus* (Neg).

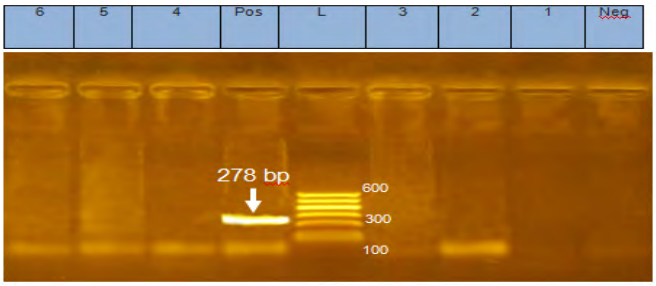


Photo (4) Enterotoxin d. Sed LaneM:100-600bp DNA Ladder. Neg.: Negative control. Pos.: positive control (at 278). Lane1, 2, 3, 4, 5, 6 S. aureus (negative)

Table (1): Incidence of staphylococci in the examined raw Buffalo Milk:-

No. of sample	Staphylococcus species	
	No. of positive	Percentage
	80	80%

Table (2): Catalase Test:-

No. of samples	Catalase Test
80	+ve
20	-ve

Table (3): Result of culture on mannitol salt agar:-

No. of positive	No. of positive Growth on MSA	No. of mannitol fermenter isolate
80	54	26
Percentage	67.8%	32.14%

Table (4): Result of pigmentation of positive sample on milk agar:-

Pigmentation	No. of positive	Percent
Golden yellow	9	11.25
Yellow creamy	38	47.5%
White	33	41.25%

Table (5): Result of Tellurium reduction and lipase activity on Baired Parker Media:-

No. of samples	Tellurite reduction	Lipase activity
80	80	42
Percent	100%	53%

Table (6): Result of the coagulase Test:

No. of samples	No. of positive	No. of Negative
80	42	38
Percent	53%	47%

Table (7): Result of V.P Test to differentiate between *S. aureus* and other coagulase positive staph as [*S. intermdieus*, *S. hyicus*]

No. of samples	No. of positive	No. of Negative
42	31	11
Percent	73.8%	26.19%

Table (8) Result of sensitivity Test:-

Antibiotic disc	Sensitive		Intermediate		Resistant	
SXT	18	58%	3	9.6%	8	25.8%
CN	28	90.3%	3	9.6%	-	-
AM	18	58%	7	22.5%	6	19.3%
DA	9	29.03%	11	35.4%	11	35.4%
RF	11	35.4%	7	22.5%	13	41.9%
CE	14	45.16%	4	12.9%	13	41.9%
FL	-	-	3	9.6%	28	90.3%
AX	9	29.03%	11	35.4%	11	35.4%

Table(9): The Results of PCR amplification of different used genes of *S. aureus*

sample	*hIg*	*Spa*	Enterotoxins				
			Sea	*Seb*	*Sec*	*Sed*	*See*
1	-	-	-	-	-	-	+
2	-	+	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	+	-	-	-	-	-
6	-	-	-	-	-	-	-
Total NO.	0	2	0	0	0	0	1
%	0.0	33.3	0.0	0.0	0.0	0.0	16.6

3.3.Biochemical reactions

Staphylococcus aureus colonies were positive for coagulase, catalase, V-P test and negative oxidase testes. The results revealed that 80 out of 100 raw milk samples (80%) were positive for staphylococcus spp. as shown in Table(1). Out of those 80 sample, 42(53%) were coagulase positive *Staphylococci spp.* while 38(47%) were coagulase negative *Staphylococci spp.* as shown in Table (6). Out of those 42 coagulase positive *Staphylococci spp* were 31(73.8%) *Staph. aureus.* As shown in Table (7). In vitro sensitivity tests in Table (8) showed the isolated *Staph. aureus* were highly sensitive for Gentamycin, Trimethoprim / Sulphamethazole, Ampicillin, Cephadrine. However, they were resistant to Flumox, Rifamycin, Amoxicillin and Clindamycin. The PCR result detected that spa virulence gene was detected in two strains out of 6 studied strains (33.3%) The spa gene was giving product of 226bp (photo,1), while enterotoxin E was detected in one strain out of 6 studied strains (16.6%) giving product at 209 bp (photo, 2). Moreover, the hIyA gene and enterotoxin (sea, seb, sec, sed) virulence genes were not detected in all studied strain. The hlyA gene was not amplified in *Staph.aureus* strains and giving no product at 937 bp (photo, 3). The sea gene was not amplified in *Staph.aureus* strains and giving no product at102 bp (photo, 2).The seb gene was not amplified in all tested *Staph. aureus* strains and giving no product at 164 bp. The sec gene was not amplified in *Staph. Aureus* strains only and giving no product at 451 bp. The sed gene was not amplified in all *Staph. aureus* strains and giving no product at 278 bp(photo,4).

4. DISCUSSION

Staphylococcus aureus is recognized to cause health care associated and community- acquired infection in every region of the world. (Yilmaz et al., 2007). Enterotoxigenic *S.aureus* in milk possess a potential health hazard to consumers, the identification of such strain should be used

as a part of a risk analysis and milk product**.** (Zouharova &Rysanek., 2008). Phenotypic assays were by catalase, oxidase, coagulase, tellurite and acetoin production as well as by testing of coagulase positive *Staph.aureus* against antibiotics while molecular identification performed by detection of *S.aureus* enterotoxin {A- B-C-D- E} genes, haemolysin gene and protein A gene. As shown in (Table1) eighty *Staphylococci* isolates were isolated from 100 milk samples with a percentage of 80%. These nearly similar to those reported by Tsegmed et al., (2007) investigated the occurrence of *Staphylococci* in raw milk. *Staphylococci* were isolated from 72(74%) of the raw 97 raw milk samples. Also Ghaleb et al., (2005) recorded the incidence of staphylococci (68.3%). And Kamel (1993**)** isolate *staphylococcoi* at the rate of (62.8%) from 320 milk samples. They found that *Staph.aureus* in 57.5% on contrary, (Donkor et al., 2007) Cultured 96 raw milk samples and identified *Staphylococcus SPP* in 14.6% of samples. In this study, all *Staph .aureus* isolates were Gram- positive cocci arranged in clusters, they were coagulase producer as coagulase test is the main characteristic and most reliable phenotypic method used for classification of rabbit plasma. These results came paraller with those of Harmon et al., (1991**)** who reported that *Staph. aureus* strains were Gram positive cocci.*Staphylococcus aureus* strains were coagulase positive as recorded by Wladimir et al., (2000) the same result was confirmed by Howard &Kloss (1993). In this study *Staph. aureus* gives characteristic black-centered colonies surrounded by a halo zone onto Baired parker medium as shown in (Table5). In addition, mannitol salt agar is a selective and indicator medium Staph. *aureus* ferment mannitol form colonies that turn the indicator to yellow color as shown in (Table3). The obtained result was confirmed by Wilson & Miles (1975) who published that *Staph. aureus* gives characteristic black-centered colonies surrounded by an area of clearing, sometimes with an area of opacity within onto Baired Parker. Quinn et al (1994) who reported that mannitol salt agar and Baired Parker medium were used and specifically in food microbiology also Colle et al., (1996) and Mackei&Mccarthey (1996) reported that mannitol salt ager is a selective and indicator medium for *Staph. aureus*. In this study *Staph. aureus* represent (31%) from 80 isolates as shown in (Table7). These results disagree with Rall et al., (2008) who found *Staph .aureus* in 38(70.4%) out of 54 raw milk samples and Tenhagen et al.,(2009) who examined milk samples and found that coagulase negative *Staphylococci* (CNS) were the predominate group of bacteria isolated (46.8% of samples) while *Staph. aureus* could be isolated from (11.7%) of the examined milk samples, Ateba et al., (2010) examined a total of 28 milk samples were collected and screened for the presence of *Staph.aureus* and found that all samples were contaminated with *Staph.aureus* ,OldeRiekerinl et al., (2010) found that the prevalence of *Staph.aureus* in bulk tank milk was (74%) and Giannatale et al.,(2011) found that (14.0%) of 350 examined raw milk samples. Nearly similar result obtained by El-kholy et al.,(1994) who recorded that the incidence reached to (22.9%). Baudet&chieze (1994) recorded that the incidence reached to (30%) of the examined milk samples, Rampone et al.,(1993**)** found that *Staph. aureus* constituted (38.5%) of the isolates obtained from raw milk samples, Saini et al.,(1994) found that *Staph. aureus* constituted (34%) of the isolates, Fabre et al.,(1997)stated that *Staph. aureus* represent (29%). Saddek et al., (1999) recorded that the incidence reached to (29.1%) of the isolates, Andrade (2001) stated that *S.aureus* reached to (30.2%) and Janosi&Baltay (2004) recorded that the incidence reached (32.5%) of the examined raw milk samples. Higher incidence obtained by Gianneechini et al., (2002) recorded that the incidence reached (62.8%). On the other hand, Kivaria et al., (2006) recorded that the incidence of

S.aureus (6.3%). Tenhagen et al., (2006) added that *S.aureus* was present in (5.7%) milk samples. The results of in-vitro sensitivity tests for the isolated *S.aureus* strains are presented in (Table8) revealed that Gentamycin, Trimethoprim, Sulphamethazole, Ampicillin, Cephradine, were sensitive with the highest *in-vitro* efficiency. Against isolated *S.aureus* but they were resistant to flummox, Amoxicillin,Rifamycin,
Clindamycin.These results were agreed with that obtained by (Jha et al., 1994 & Singh et al., 1994 & Kamel 1996 & Andrada et al., 2000 &Chowdhury et al., 2002). While it was dis-agreed with that recorded by Gentilini et al., (2000) who detected that resistance in 83 (40.3%), 24(11.6%), 16 (7.7%) and 7(3.4%)*Staph. aureus* isolates for Pencillin, Erythromycin, Pirlimycin and Gentamycin respectively. No resistance was detected for Oxacillin, Cephalothin and Ampicillin sublactam. Also Corti et al., (2003**)** recorded that (91%) of the *Staph. aureus* strains were sensitive to all Antibiotics tested only (9%) of the strains were resistant to Penicillin G and (7%) to Amplicillin. So, the present study was directed mainly to recognize some virulence genes that may play a role in virulence of *Staph. aureus.* By using one of the recent developments molecular biological techniques (PCR). These genes were protein A (Spa) that binds immunoglobulin G molecules by the **FC** region in serum, bacteria will bind IgG molecules with wrong way round by this non-immune mechanism resulting in prevention of opsonization and phagocytosis, haemolysin (hIy A) Which is extracellular, soluble, hydrophilic protein has haemolytic properties and enterotoxin (sea, seb, sec, sed and see) that cause diarrhea and vomiting when ingested and are responsible for *staphylococcal* food poisoning. The PCR results for *Staph. aureus (Table9)* showed that protein A(Spa) was detected in 2 out of 6 studied strains. While enterotoxin E (see) was detected in 1 out of 6 studied strains. Moreover haemolysin gene was not detected in all studied *S. aureus* strains also enterotoxin (A- B- C- D) were not detected in all studied *Staph. aureus* strains. These results disagreed with Shin et al., (2002) recorded that most *Staph. aureus* strains produced enterotoxin B and D.Also Yamashita et al., (2003) detected only *Staphylococcal* enterotoxin sea gene among se (a- e) genes by PCR in milk samples. Also Lim et al., (2004) detected SEA in 32 isolates, SEB in 3 isolates, SEC in 1 isolate and SEA in 1 isolates. In addition, Adwan et al., (2005) detected sea in 4 strains, seb in 20 strains, sec in 4 strains, sed in 3 strains and see in 3 strains and Peles et al., (2007) detected that toxin genes SEB, SEA and SEC were the most commonly detected whereas none of the isolates possessed the SEE, SHE, SEJ. These results go parallel with Sahekhtiariet al., (2011) detected see only in one isolate.

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