Phenotypic and genotypic characterization of S. *aureus* isolated from clinical and subclinical bovine mastitis

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ABSTRACT

In order to determine the prevalence of ***S. aureus*** in clinical and subclinical bovine mastitis as well as the relationship between the presence of protein A gene and the udder inflammatory response . A total of 400 quarter milk samples were collected from 100 lactating Friesian cows at Ismailia Governorate . The percentage of clinical mastitis was (19.5%.), while the percentage of subclinical mastitis according to C.M.T was (42.85%) . The percentage of ***S.aureus*** in clinical mastitis was (28.2%) , while in in subclinical mastitis was (22.46%).The identification of ***S,aureus*** was confirmed by PCR amplification of species-specific parts of the gene encoding the 16S rRNA. Twenty nine ***S.aureus*** isolates were subjected to PCR for detection of X region of protein A (spA) gene,10 isolates from clinical mastitis and 19 isolates subclinical mastitiC milk specimens (+++ve CMT and ++ve CMT) were (100%) positive . Results indicate that there was a positive relationship between the presence of protein A gene of ***S.aureus*** and the inflammation of udder in clinical mastitis as well as the high grades of subclinical mastitis.

INTRODUCTION

Bovine mastitis is an important and a persistent infection in the bovine population producing economic losses; drop in milk production, increased cost of treatment and culling process ***(Singh and Bansal, 2004).***

The major reservoirs of ***S. aureus*** are found in infected udders, teat canals, and teat lesions, but these bacteria also have been located on teat skin, muzzles, and nostrils. The bacteria are spread to uninfected quarters by teat cup liners, milkers' hands, washcloths, and flies. ***Staphylococci*** do not persist on healthy teat skin but readily colonize damaged skin and teat lesions. The organisms multiply in infected lesions and result in increased chance of teat canal colonization and subsequent udder infection. Heifers infected during

gestation that carry infections through calving represent an important reservoir from which ***S. aureus*** can spread to uninfected herd mates. There is considerable debate surrounding the route of ***S. aureus*** infection in heifers prior to first calving, but calves fed colostrum from an ***S.aureus***-infected dam is a likely source. ***(Petersson and Mullarky 2010)***

S. aureus has a capacity to produce a large number of potential virulence factors, including a variety of exotoxins and cell surface-associated proteins ***(Fitzgerald et al, 2000; Foster, 2005; Kalorey et al, 2007).*** One of the major surface proteins is staphylococcal protein A (Spa), which bacterial cell wall product that binds immunoglobulin G and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes **.** The decrease of protein A on the cell surface of ***S. aureus*** resulted in a greater number of free receptor sites for complement C3b and in an increase in phagocytosis**.** The gene encoding protein A (*spa*) is composed of some functionally distinct regions: IgG Fc binding region (*spa*- IgG), X region (*spa*-X) and at C terminus, a sequence required for cell wall attachment. The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains. ***(Gao and Stewart, 2004)***

The objective of this study was to determine the prevalence of *S.aureus* in bovine clinical and subclinical mastitis (using CMT and bacteriological examination), detection of protein A (spA) gene in clinical and subclinical mastitis milk samples as well as the relationship between the presence of protein A gene and the udder inflammatory response with different degrees.

MATERIAL AND METHODS

Milk samples

A total of 400 milk samples were collected from apparently normal quarters (322 milk samples) and clinical cases of mastitis (78 quarter milk samples) of 100 lactating Friesian cows from Ismailia Governorate.

California mastitis test (CMT)

Apparently normal milk samples were subjected to CMT (screening test) for the detection of subclinical mastitis. Milk samples with CMT scores (suspect ±), +, ++ and +++ were prepared for bacteriological examination.

Isolation and identification of *S.aureus*:

Milk samples were collected from clinical mastitis quarter and positive C.M.T quarter milk samples were incubated aerobically for 24 hours at 37 °C to achieve potential bacterial growth and a loopfull was taken from each sample and streaked onto Nutrient agar, blood agar and Mannitol salt agar plates . All plates were incubated at 37°C

for 24-48 hours and examined for bacterial growth. Bacterial colonies were identified morphologically using Gram's stain as well as biochemically using methods described by ***(Quinn et al, 1994).***

PCR detection of 16SrRNA gene and the protein A (spA) gene of *S.aureus*

Twenty nine isolates were subjected to PCR for detection of 16S rRNA and spA genes.

1-Extraction of DNA from ***S.aureus*** isolates by boiling method according to ***(Van Eys et al, 1989)***

2-Polymerase chain reaction:

DNA samples were tested in 50 µl reaction volume in a 0.2 ml PCR tube , containing PCR buffer (50 mM Kcl , 10 mM tris - Hcl , 1mM $Mgcl_2$) each dNTPS (Deoxy nucleotide Triphosphate) 200 uM each (dATP , dGTP , dCTP and dTTP) , [Two primer pairs each at 50 picomol / reaction] and 0.5 of taq DNA polymerase . Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done. A negative control PCR reaction with no template also was included in this assay.16SrRNA primers were prepared as described by ***(Monday and Bohach, 1999),*** while spA gene primers were synthesized as described by ***(Akineden et al, 2001).***

Table (1*): list of primers used for PCR assay*

Primer	Primer Sequence.	Molecular weight (bp)	Annealing temp.
16S rRNA. F	**GTA GGT GGC AAG CGT TAT CC**	**228**	**64°c**
16S rRNA. R	**CGC ACA TCA GCG TCA G**		
spa-F	**CAA GCA CCA AAA GAG GAA**	**140, 270 and 290**	**60°c**
spa-R	**CAC CAG GTT TAA CGA CAT**		

PCR Protocol: Initial Denaturation at 94 ^{o}C for 4 min, **Denaturation** at 94 ^{o}C for 1 min, **Annealing** at 64 ^{o}C for 16SrRNA primer and 60 ^{o}C for (spA) gene primer for 1 min, **Extension** at 72 ^{o}C for 1 min . Cycles repeated for 39 times and proceeded by initial denaturation at 95 ^{o}C for 5 min. and followed by final extension at 72 for 10 min.

3-Screening of PCR products: ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. The gel was photographed in order to obtain a permanent record using UVP BioSpectrum Imaging Systems, UVP® LLC.

RESULTS

Incidence of clinical and subclinical mastitis in examined quarter

Table (2): *Prevalence of subclinical mastitis in examined quarter milk samples of cows according to California mastitis test (CMT)*

No. of examined milk samples	No. of +ve CMT samples	% of +ve CMT samples	No. of -ve CMT samples	% of -ve CMT samples
322	138	42.85 %	184	57.14 %

Table (3): *The percentage of S.aureus isolated from clinical mastitis milk samples*

Total clinical mastitis milk samples	No. of +ve samples for *S.aureus*	% of +ve samples	No. of –ve samples	% of –ve samples
78	22	28.2%	56	71.79%

Table (4) *The percentage of S.aureus isolated from subclinical mastitis milk samples*

Total subclinical mastitis milk samples	No. of *S.aureus* isolates	% of *S.aureus* isolates	No. of other bacterial isolates	% of other bacterial isolates
138	31	22.46 %	107	77.536 %

PCR results:

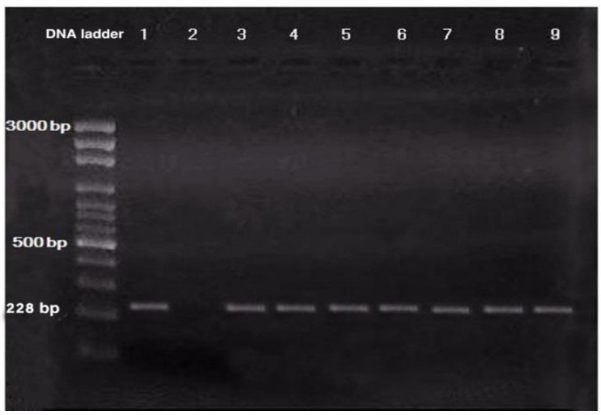

Fig. **(1) illustrated the positive amplification of 228 bp fragment of 16S rRNA gene from the extracted DNA of *S.aureus* isolates**.

- (100 bp DNA ladder)
- Lane 1: control positive ***S.aureus*** strain according to

(EL-Gammal, 2011)

- Lan**e** 2: control negative
- Lane 3-9: showed positive ***S.aureus*** isolates

Table(5) :*Prevalence of SPA gene in isolated S.aureus*

10 from 22 clinical mastitis isolates				
No. of samples subjected to PCR for SPA gene detection	**No. of +ve SPA gene**	**% of +ve SPA gene**	**No.-ve SPA gene**	**% of .-ve SPA gene**
10 isolates from 22	10 isolates	100%	0	0%
19 from 31 subclinical mastitis isolates				
No. of samples subjected to PCR for SPA gene detection	**No. of +ve SPA gene**	**% of +ve SPA gene**	**No.-ve SPAgene**	**% of .-ve SPA gene**
5 isolates from 9 +++ve CMT	5 isolates	100%	0	0%
10 isolates from 18 ++ve CMT	10 isolates	100%	0	0%
All 4 isolates +ve CMT	1 isolate	25%	3 isolates	75%

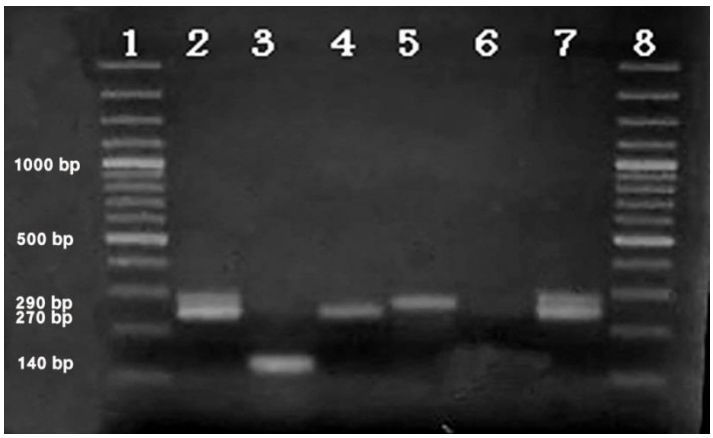

Fig.(2) *illustrated the positive amplification of 140 bp, 270 bp and 290 bp fragment spA gene from the extracted DNA of S.aureus isolated from quarter milk samples. (clinical and subclinical isolates)*

- Lane 1 : (100 bp DNA ladder)
- Lanes 2 and 7: +ve clinical mastitis isolates with double specific bands (at 270bp and 290 bp.)
- Lane 3 : +ve isolates (+veCMT subclinical mastitis) with specific band (140 bp),
- Lane 4 : +ve isolate (+++ veCMT subclinical mastitis) with specific band (270 bp)
- Lane 5: +ve isolate (++ veCMT subclinical mastitis) with specific band (290 bp).
- Lane 6: -ve isolate for protein A (**spA**) gene (+ve CMT subclinical mastitis)
- Lanes 8 : (100 bp DNA ladder)

DISCUSSION

Bovine mastitis is an important and a persistent infection in the bovine population producing economic losses; drop in milk production, increased cost of treatment and culling process.

In the present work, the percentage of clinical mastitis was (19.5%.) which agree with the finding of ***Petrovski et al (2009)*** . It seems that in Egypt there is high incidence of clinical mastitis compared to their finding , the high sample size and may be the herd area explains that low level of clinical mastitis in their situation .

As shown in Table (2), the percentage of subclinical mastitis according to C.M.T was (42.85%) . High incidence of subclinical mastitis (47.7%) was reported by **El Gammal (2011).** In other countries high figures of subclinical mastitis ranging from (26.7%) to (63.1%) have been reported by several authors ***(Chang et al, 2005 and Dego and Tareke, 2003).*** The high rate of subclinical mastitis can be explained by the fact that most cases of mastitis occur in subclinical forms, and the diseased animals continues for a time , to be a dangerous source of infection until increasing the clinical symptoms alert it to the dairy workers.

As shown in Tables (3) and (4) the percentage of ***S.aureus*** clinical mastitis was (28.2%) while the percentage of ***S.aureus*** from subclinical mastitis was (22.46 %). These results agreed with those of ***(Bedane et al ,2012)*** . ***S.aureus*** is responsible for approximately 30% to 40% of all mastitis cases. ***S. aureus*** can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk ***(Scherrer et al, 2004).***

In this study PCR protocol used for amplification and detection of 16S rRNA genes of ***S.aureus*** isolates as a confirm diagnosis of these isolates; 10 isolates from 22 ***S.aureus*** isolated from clinical mastitis milk samples and 15 isolates from 31 ***S.aureus*** isolated from subclinical mastitis milk samples were subjected to PCR for detection of 16S rRNA gene and all (100%) were +ve for that gene (Fig. 1) .These results agreed with those obtained by ***Løvseth et al (2004).*** 16S rRNA gene is species-specific gene used in identification of ***S.aureus (Monday and Bohach, 1999).***

As shown in Table (5) PCR protocol used for amplification and detection of X region of protein A *(spA)* gene of ***S.aureus*** isolates to confirm the virulence of these isolates as the presence of protein A is an index of virulence; 10 isolates from 22 clinical mastitis milk specimens were subjected to PCR for detection of X region of protein A (spA) gene and all (100%) were +ve . Also 19 isolates from 31

subclinical mastitis milk specimens (5 isolates from 9 were +++ve CMT and 10 isolates from 18 were ++ve CMT) were subjected to PCR for detection of X region of protein A (spA) gene and all (100%) were +ve . While the 4 isolates which were + ve CMT gave a result as one isolate positive PCR (25%) and the 3 isolates were negative PCR of protein A (spA) gene . This results revealed that all clinical mastitis ***S.aureus*** samples which were subjected to PCR for detection of X region of protein A were 100% positive as well as higher degrees of subclinical mastitis samples . Figure (2) illustrated the positive amplification of 140 bp, 270 bp and 290 bp fragment of **spA** gene from the extracted DNA of ***S.aureus*** isolated from quarter milk samples(clinical and subclinical mastitis isolates). These results are agreed with those obtained by ***Akineden et al (2001).***

Protein A displayed gene polymorphisms and allowed a genotypic characterization of the bacteria. The repetitive region X of the *(SpA)* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains. The number of repeats has been related to the dissemination potential of ***S. aureus***. Strains with more than seven repeats in the X region tended to be epidemic, while the presence of seven or less repeats was indicative of a non-epidemic methicillin-resistant ***S. aureus*** strain as reported by ***Erskine and Burton (2003)***, ***Salasia et al (2004)*** *and* ***Shanmugam et at (2007).***

In this work results revealed that the relationship between ***S. aureus*** isolates and the grades of subclinical mastitis is statistically significant as much as the grade of subclinical mastitis increases the prevalence of ***S. aureus*** increases subsequently as showed in Table (6) and the results revealed also that the relationship between the virulence genes of ***S.aureus*** and the inflammation of udder in clinical mastitis as well as the high grades of subclinical mastitis is a positive relationship.

Finaly we concluded that ***S.aureus*** is one of the major bacterial causes of clinical and subclinical bovine mastitis. The relationship between protein A gene of ***S.aureus*** and the inflammation of udder in clinical mastitis as well as the high grades of subclinical mastitis is a positive relationship

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توصيف المظهري والجينى للمكور العنقودي الذهبي من التهاب الضرع البقري الاكلينيكي وتحت السريري

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تم تجميع عدد ٤٠٠ عينة لبن من ١٠٠ بقرة فريزيان وتم تحديد نسبة وجود بكتيريا المكور العنقودى الذهبى فى عينات اللبن وتحديد العلاقة بين التهاب الضرع ووجود جين الضراوة Protein A . و أظهرت النتائج انه من اجمالى عدد ال ٤٠٠ عينة لبن كانت نسبة الالتهاب الضرع الإكلينيكي 19.5% ونسبة التهاب الضرع السريري بعد تشخيصه باستخدام اختبار الكاليفورنيا 42.85% . نسبة وجود المكور العنقودي الذهبي من عينات التهاب الضرع الاكلينيكى كانت 28.2% ونسبة وجود المكور العنقودي الذهبي من عينات التهاب الضرع تحت السريري كانت 22.46% . تم تاكيد تصنيف الميكروب المكور العنقودى الذهبى باستخدام اختبار انزيم البلمرة المتسلسل بتحديد جين (Sr RNA١٦).وكل النتائج كانت (١٠٠٪) ايجابى. وتم استخدام اختبار انزيم البلمرة المتسلسل بتحديد جين (protein A) لعدد ٢٩ عينه وهى المسئولة عن ضراوة البكتيريا المعزولة , وكانت النتائج (١٠٠٪) ايجابية فى كل الحالات المعزولة من التهاب الضرع الاكلينيكى وحالات التهاب الضرع تحت السريري التى كانت ++ و +++ ايجابى فى اختبار الكاليفورنيا . كما اكدت النتائج على وجودعلاقة ايجابية بين التهاب الضرع ووجود جين الضراوة Protein A.