Animal Health Research Institute,
Assiut Branch.

STUDYING THE OCCURRENCE OF CLUMPING FACTOR GENE IN *STAPH AUREUS* ISOLATED FROM CASES OF SUBCLINICAL MASTITIS AND THE EFFECT OF SUCH PATHOGEN ON MILK COMPOSITION

(With 5 Tables and 2 Figures)

By

KH.A.S. EL-KHABAZ; M.F. HUSSIEN;
EMAN M. ABD-EL NASER and HANAA A. AHMED*

* Dept. of Biotechnology, Animal Health Research Institute

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دراسة تواجد جين عامل التجمع فى ميكروب المكور العنقودى الذهبى المعزول من حالات التهاب الضرع الخفى وتأثير هذا الميكروب على مكونات اللبن

خالد أحمد سيد الخباز ، محمود فرغلى حسين ، إيمان محمد عبد الناصر
هناء عبد القادر أحمد

اجريت هذه الدراسة على عدد ٣٥ بقرة حلابة حيث تم تجميع عدد ١٢٠ عينة لبن منها (كل عينة ممثلة لاحد ارباع الضرع) تم فحص هذه العينات فى البداية باستخدام اختبار الكاليفورنيا لاكتشاف المصاب منها بالتهاب الضرع الخفى وقد كانت ٣٨ عينة (تمثل عدد ٢١ حيوان) ايجابية للاختبار ومن ثم تم زراعتها على المستنبت البكتيرى الخاص فى محاولة لعزل ميكروب المكور العنقودى الذهبى وقد تم عزل عدد ٦ عترات لهذا الميكروب حيث تم تأكيدها بواسطة الاختبارات البيوكيميائية الخاصة وعند اخضاع العترات المعزولة لاختبار سلسلة تفاعل انزيم البلمرة المتعدد للتعرف على مدى وجود بعض جينات العوامل الممرضة وجد ان ٥ من العترات المعزولة تحتوى على جين التجلط (الكواجيولاز) بنسبة ٨٣.٣% وان ٢ فقط من هذه العترات تحتوى على جين عامل التجمع بنسبة ٣٣.٣%. هذا وبدراسة التغيرات الكيميائية فى مكونات اللبن نتيجة لاصابة الضرع بهذا الميكروب وجد انه فى حالات إلتهاب الضرع الخفى في الأبقار هناك إرتفاع معنوى إحصائى فى مستوى البروتين الكلى فى مصل اللبن والألبيومين وكذلك الصوديوم والكلوريد بينما يوجد نقص معنوى في كلا من الكالسيوم والفسفور والبوتاسيوم. وقد ناقش البحث الاهمية الوبائية والصحية والاقتصادية لحالات التهاب الضرع الناتج عن الاصابة بهذا الميكروب.

SUMMARY

120 milk samples (represent 35 cows) were randomly collected from different small holder farms, firstly tested for subclinical mastitis by CMT. 38 CMT positive samples were subjected for conventional methods of isolation and identificacion of *Staph aureus*. 6 isolates of *Staph aureus* were isolated which were subjected for genotypical characterization for the presence of clumping factor and coagulase genes by PCR assay using oligonucleotide primers that amplified genes encoding cluming factor (*clfa*)gene, and (*coa*)gene. The results of PCR assay revealed that 5 isolates of *Staph aureus* were positive for coagulase gene, while 2 isolates were positive for clumping factor gene. The chemical analysis of milk showed that there were significant statistical increase in total whey protein, albumin, sodium and chloride in cows' milk samples with subclinical mastitis. While, there was a significant decrease in both calcium, phosphorous and potassium levels in comparing with the normal milk. The present study was carried out to study the presence of some virulence factors genes in *Staph aureus* isolated from bovine subclinical mastitis which is very important prerequisites for implementation of effective control programs to face the economic losses due to subclinical mastitis caused by this microorganism.

Key words: *Milk, subclinical mastitis, clumping factor gene, Staph aureus.*

INTRODUCTION

Subclinical mastitis, without any signs of inflammation compared with clinical mastitis is accounts for the majority of bovine mastitis cases in dairy herds (Oliver *et al*., 2004). *Staphylococcus aureus* is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking. *S. aureus* produces a spectrum of extra cellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism (Momtaz *et al.,* 2010)

S. aureus seems to be the predominant organism causing subclinical mastitis (Kader *et al.,* 2002) and it may predispose the herd for infection by coliforms or other pathogens (Ibtisam *et al.,* 1993).

S. aureus is usually considered the most common contagious pathogen and has been reported to infect 7 to 40% of all cows (Fox and Gay, 1993).

Staph aureus has a capacity to produce a large number of putative virulence factors (Fitzgerald *et. al.,* 2000). Some of these factors may be of more importance than others in different diseases or at different stages of the pathogenesis of particular infections, as not all factors are produced by each strain (Kalorey *et al.,* 2007). Natural populations of *S. aureus* have shown considerable variability in genome content (Phonimdaeng *et al.,* 1990 and Fitzgerald *et al.,* 2003), this variability has contributed to the emergence of distinct epidemiologic profiles that are dependent on the strains prevalent in a herd, which suggests the need to identify such strains or subtypes before applying specific measures to control mastitis (Zecconi and Piccinini 1999).

Molecular epidemiological analysis of the bovine *S. aureus* population suggested that small number of clonal types were responsible for most infections and those strains had abroad geographic distribution (Fitzgerald *et al.,* 1997 and Salasia *et al.,* 2004)

The ability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections (El-Sayed *et al.,* 2005). *S. aureus* possesses various adhesion genes, including *clfA*, *fnbA*, and *cna* (Smeltzer *et al.,* 1997). Genetic characterization of mastitis-causing *S. aureus* isolates is vital for an effective mastitis control program, especially for developing a vaccine against *S. aureus* (LI *et al*., 2009).

Kalorey *et al.* (2007) stated that *clfA* gene play an important role in the pathogenesis of bovine mastitis. The role of *ClfA* as a virulence factor was shown in an endocarditis model, where the *clfA*-defective mutant produced about 50% less endocarditis than the parent strain (Moreillon *et al.,* 1995).

The *coa* gene is one of the most important virulence factors for *S. aureus* (Goh *et al.,* 1992). Expression of this gene is thought to enhance bacterial growth and promote infection in the face of host defence mechanisms, such as phagocytosis (Aarestrup *et al.,* 1995).

Mastitis, particularly the subclinical type, influences the total milk output and modifies milk composition and technological usability. Subclinical mastitis is associated with altered protein quality, change in fatty acid composition, lactose, ion and mineral concentration, increased enzymatic activity, and a higher pH of raw milk (Auldist *et al.,* 1996 and Coulon *et al.,* 2002). Mastitis is accompanied by significant modifications of milk chemical composition (Anwer *et al.,* 2003) with both a reduced synthesis and altered cell permeability. Such

modifications affect protein and mineral fractions, in particular, carry major consequences for milk appear Linked with the technological properties (Batavani *et al.,* 2007) and appear linked with the mastitis germ.

The present study was conducted to phenotypically and genptypically characterize *S. aureus* isolates in milk samples from cows with subclinical mastis in addition to studying the changes in milk composition associated with such pathogen.

MATERIALS and METHOSD

Milk samples

120 quarter milk samples were collected from 35 dairy cows selected randomly from small different farms. Animals were physically and clinically investigated to exclude clinical mastitis. The milk samples were tested by California mastitis test (CMT) for subclinical mastitis according to Schalm *et al.* (1971). CMT scored from one to five corresponding to no reaction, trace, mild reaction, moderate reaction, strong reaction, respectively. The positive samples were subjected to bacteriological examination.

Bacteriological examination:-

A- ***Isolation of S.aureus***

The milk samples were incubated at 37 ºC for 18-24h and 10 ml of the milk samples were transferred into sterile small centrifuge tubes. The tubes were centrifuged at 3000rpm for 20 min and then the cream and supernatant were discarded to obtain the sediment, loopful from the milk sediment was inoculated into 10% Nacl broth (A.P.H.A., 1985), then incubated at 37 ºC for 24h. From the incubated tubes loopfuls were streaked onto the surface of mannitol salt agar plates (Bailey and Scott, 1994),the inoculated plates were incubated aerobically at 37 for 24h.

The mannitol fermenting pure cultures (surrounded by a yellow hallo) were streaked on blood agar plates and incubated for detection of haemolysis

B- ***Identification of S. aureus:***

Colonies of *S. aureus* on blood agar which is golden, brown, yellow or pink, domed 1-3 mm in diameter (Collins *et al.,* 1991), and identified by Gram's stain as cocci arranged in clusters or bunches, colonies confirmed biochemically according to (Quinn *et al.,* 1994) using catalase and coagulase tests (slide method).

C- Identification of *S.aureus* genotypically by PCR assay for the presence of coagulase and clumping factor genes:

DNA extraction: 2ml of previously enriched *S. aureus* isolates were centrifuged at 14 000 RPM, then the sediment is suspended in 50 µl of distilled water. The cellular suspension was brought to boil during 10 min, and immediately was centrifuged at 14,000 RPM for 5 min. The supernatant was directly used for the PCR assay (Franco *et al.,* 2008).

Oligonucleotide primers used encoding coagulase positive (*coa*) gene were *Coa* -1 CGA GAC CAA GAT TCA ACA AG and *Coa* -2 AAA GAA AAC CAC TCA CAT CA with initial denaturation at 94^{o}C for 10 min followed by 35 cycle of 94^{o}C for 1min, 58^{o}C for 1min and 72^{o}C for 1min, with final extension of 10 min at 72^{o}C (Aslantas *et al.,* 2007) and clumping factor A *(clfA*) gene forward: GGC TTC AGT GCT TGT AGG, reverse: TTT TCA GGG TCA ATA TAA GC with initial denaturation at 94^{o}C for 10 min followed by 35 cycle of 94^{o}C for 1min, 57^{o}C for 1min and 72^{o}C for 1min, with final extension of 10 min at 72^{o}C (Kalorey *et al.,* 2007). The PCR products were electrophoresed on 1.5% agarose gel using GeneRuler 100 bp plus DNA Ladder (Fermentas)

Table 1: Primers used for amplification of some *S. aureus* genes

Gene	Sequences
Coa	CGA GAC CAA GAT TCA ACA AG AAA GAA AAC CAC TCA CAT CA
clfA	GGC TTC AGT GCT TGT AGG TTT TCA GGG TCA ATA TAA GC

Chemical analysis:

Sample preparation:

Fresh raw milk was obtained from cows. Within an hour after milking, milk was skimmed by centrifugation at 3000 r.p.m for 15 min to remove their creams and cells. Samples were then treated with 0.1 M., hydrochloric acid at the controlled pH of 4.8 for casein precipitation. Treated samples were recentrifuged and the supernatants (Whey) were collected. Total protein, albumin, calcium, phosphorous, and chloride, levels were measured by using spectrophotometer through reagent kits supplied commercially by (STANBIO laboratories). Sodium and potassium measured by flame photometer.

Statistical Analysis:

Student's t-test was carried out to fined the differences between the results of mastitic and non mastitic milk samples. The results were given as mean ± SEM.

RESULTS

Table 2: Quarter –wise prevalence of subclinical mastitis in cows milk samples based on the results of CMT and Bacteriological examination

No of quarters	CMT positive		Positive samples for *S. aureus*		CMT negative		Negative smples for *S. aureus*	
120	No	%	No	%	No	%	No	%
	38	31.7	6	5	82	68.3	114	95

Table 3: Prevalence of subclinical mastitis in cows based on CMT

No. of cows	CMT positive		CMT negative	
35	No.	%	No.	%
	21	60	14	40

Table 4: Incidence of coagulase (*coa*) and clumping factor (*clfA*) genes in staph aureus isolates

S.aureus isolates	+ve isolates for *coa* gene		+ve isolates for *clfA* gene	
No	No	%	No	%
6	5	83.3	2	33.3

Table 5: Changes in some chemical constituents of milk as a result of subclinical mastitis

Parameters	units	Normal milk	SCM milk
Total protein	g/L	31.4±1.3	34.3±1.5*
Albumin	g/dI	2.87±0.09	5.81±0.14*
Calcium	mg/dl	107.4±1.26	90.8±1.91*
Phosphorous	mg/dl	25.51±0.32	19.30±0.22*
Chloride	mmol/L	28 ± 21	35 ± 27*
Sodium	mg/dl	49.72±1.20	87.97±4.32*
Potassium	mg/dl	155.74±1.9	139.56±2.1*

*Significant at $p< 0.01$

DISCUSSION

S. aureus has been recognized as a pathogen in human and animal. Sublinical mastitis causes considerable loss to the dairy industry of which *S. aureus* is probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured.

Out of 120 quarter cows milk samples examined 38(31.7%) were positive for subclinical mastitis based on the results of CMT (Table 2). These results were lower than that recorded by Sharma and Rai (1977), Ismail and Hatem (1998) and Nazem and Azab (1998) as they recorded 40.43, 67.7 and 75.25%, respectively, while the results of this study are in accordance for some extent to that mentioned by Sadek (2008) 28.5%. The subclinical mastitis incidence varied widely due to changing management condition (Radostitis *et al.,* 2000).

Depending on the results of CMT, animal prevalence of subclinical mastitis has been illustrated in (Table 3), out of 35 dairy cows examined, 21animals (60%) gave positive results, nearly similar results were recorded by Sexena *et al.* (1993) 64%, while lower results were recorded by Li *et al.* (2009) 54.3%, Tijare *et al.* (1999) 16.6% and Sadek (2008) 59.05%.

S. aureus is one of the contagious organisms that well adopted to survive in the udder and usually establish mild subclinical infection for long duration (El- khodery and Hoedemakes, 2005) and can spread from infected quarter to another quarter (El–Balkemy *et al.,* 1997). Staphyococci typically colonize the broken skin and can enter the udder through abrasions of the teat (Dhakal, 1997).

Generally *S. aureus* is commonly isolated from subclinical mastitis cases (Abdel–Khalek and El–Sherbini, 2005) due to its ability to develop sophisticated system to avoid phagocytosis or macrophages (Vanfuth and Zwet, 1986).

The prevalence of subclinical mastitis caused by *S. aureus* were studied by many investigators, the obtained results (5%) come in coincides with the results of Fox and Gay (1993) as they stated that *S. aureus* mastitis in cattle ranged from 7- 40 % , while it was lower for high extent than that recorded by Janosi and Balty(2004). Attia *et al.* (2003) and Shitandi and Kihumbu, (2004) which were, 80%, 60% and 45.6%, respectively.

Prevalence and etiology of subclinical mastitis in dairy animal show that coagulase-negative staphylococci are the most prevalent, ranging from 25% to 93% (mean value approximately 78%) of bacterial

isolates. *S. aureus* prevalence ranges from 3% to 37% (mean value approximately 4%) of the bacterial isolates (Janosi and Balty (2004).

Several genotypic techniques have been developed in the last decades. The coagulase protein is an important virulence factor for *S. aureus*. The coagulase gene amplification has been considered a simple and accurate method for typing. This method is found to be technically simple with a good reproducibility and discriminatory power (Karahan and Cetinkaya, 2007). The *coa* gene has polymorphic repeat regions that can be used for differentiating *S. aureus* isolates (van Belkum *et al.,* 1998).

Table (4) and photo (1) showing that six identified field isolates by biochemical tests were tested for the presence of *coa* gene. 5 samples were positive to this gene (83.3%) with different polymorphism. Two isolates gave one band at 200 bp, one gave two bands at 970 and 200 bp and one gave at one band at 970 bp and one isolate gave one band at 910 bp.

The variability in size and number of *Coa* bands seen in this study may be due to presence of structurally different gene forms of coagulase in *S. aureus*, allowing one strain to produce one or more of these variants (Goh *et al.,* 1992).

Studies carried out on PCR amplification of *coa* gene in different countries using the same primer pairs revealed extensive polymorphism with predominance of one or more of *coa* gene amplified products among *S. aureus* responsible for mastitis in cows and buffaloes. Annemuller *et al.* (1999) obtained four PCR products of 990, 900, 800, and 740 bp, with 990 bp being the predominant product. Lange *et al.* (1999) found seven PCR products ranging from 580 to 1060 bp Guler *et al.* (2005) obtained 1000-, 900-, 800-, and 700-bp PCR products while Katsuda *et al.* (2005) found five types of amplified products ranging from 420 + 20 bp to 820 + 20 bp. Vimercati *et al.* (2006) observed amplified products of *coa* gene ranging from 420 to 900 bp**.** Saei *et al.* (2009) observed five different PCR products with molecular weight ranging from 490-850 bp in a study in nine dairy herds. PCR amplification of the 30 end of the coa gene showed that 161 (80.5%) of *S. aureus* isolates were *coa* positive (Akineden *et al.,* 2001).

The proportion of *coa* positive isolates varied from 0% to100% by geographic location (Karahan and Cetinkaya, 2007). The predominance of one or more *coa* gene genotypes may be more beneficial in the control of *Staphylococcus aureus* mastitis since they were reported to be more resistant to neutrophil bactericidal activities

than rare genotypes (Su *et al.,* 1999). It also suggests a common source, host to host transmission i.e. contagious transmission, host adaptation of subsets of the population of *S. aureus* strains. Also, differences in distribution of coagulase gene variants *S. aureus* may reflect presence of virulence factors responsible for suppressing host defence mechanisms (Goh *et al.,* 1992).

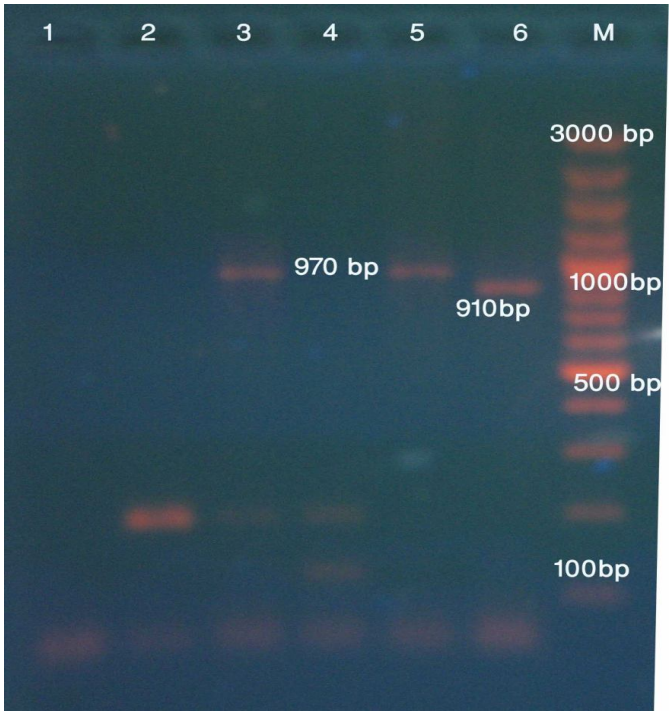

Photo (1): Electrophoretic pattern of coagulase (*coa*) gene in different isolates

1- Negative control
2- *Staphylococcus aureus* field isolate (1)
3- *Staphylococcus aureus* field isolate (2)
4- *Staphylococcus aureus* field isolate (3)
5- *Staphylococcus aureus* field isolate (4)
6- *Staphylococcus aureus* field isolate (5)
7- Marker GeneRuler (Fermentas)

Clumping factor A (*ClfA)* is considered one of most important adhesion factors and has been identified as a virulence factor in an endocarditis model in human (McDevitt *et al.,* 1995). It is a cell wall-anchored *S. aureus* surface protein that has been shown to enhance

staphylococcal virulence in animal infection models. From Table (4) and photo (2) It is clear that clumping factor gene (*clf A*) was detected only in two isolates out of the tested six isolates with a characteristic band at 1042 bp in a percentage of 33.3% indicating no size polymorphism to this gene. These results agreed with Akineden et al. (2001) and Momtaz *et al.* (2010). These positive samples were also positive to the presence of *coa* gene. Presence of the *clfA* gene *Staphylococcus spp* virulence gene has its importance in development of severity of mastitis (Akineden *et al.,* 2001). The phenotypic and genotypic results of the present study might help to understand the distribution of prevalent *S. aureus* clones among bovine mastitis isolates of both countries and might help to control *S. aureus* infections in dairy herds.

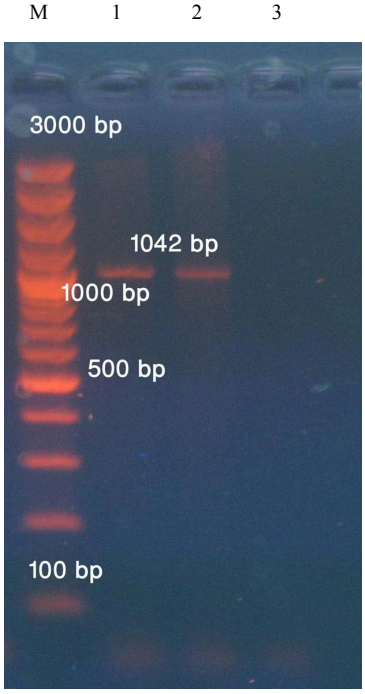

Photo 2: Electrophoretic pattern of clumping factor A (*clfA*) gene in different isolates

M-Marker GeneRuler (Fermentas)

1- *Staphylococcus aureus* positive field isolate (4)

2- *Staphylococcus aureus* positive field isolate (5)

3- Negative control

It is generally accepted that during subclinical mastitis, there is an increase in milk proteins (Leitner *et al.,* 2004) that has been attributed to the influx of blood borne proteins (such as serum albumin). According to Auldist and Hubble (1998) this increase in proteins of blood serum origin during mastitis is possibly due to disruption to the integrity of the mammary epithelia by microbial toxins and opening of the tight junctions.

The increase of albumin content during mastitis has been reported in cows Vijayalakshmi *et al.,* 2001; Coulon *et al.,* 2002 and Batavani *et al.,* 2007).

The significant increase of albumin in Subclinical mastitic milk suggest that a major source of the increase in the content of albumin in milk under inflammatory conditions is the mammary gland itself (Shamay *et al.,* 2005).

The levels of calcium and phosphorous is also affected by SCM. There were a significant ($p<0.01$) decrease in both calcium and phosphorous levels. The reduction in both calcium and phosphorus level in the case of intramammary infections have been reported by (Coulon *et al.,* 2002 and Batavani *et al.,* 2007).

Sub clinical mastitis changed the ionic environment. Sodium and chloride showed significant increase in contract, potassium; normally the predominant mineral in milk is declined. These increases in sodium and chloride and decrease in potassium levels have been confirmed by other authors as methods of monitoring udder health (Vijayalakshmi *et al.,* 2001 and Bruckmaier and Blum 2004).

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