Immunological and Bacteriological Findings Associated with Subclinical Mastitis in Dairy Farm

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Abstract: In the present study we examined quarter milk samples obtained from 120 cows (480 quarter) using the California mastitis test (CMT) and Somatic cell count (SCC). A quarter was considered to have subclinical mastitis if it had a positive California Mastitis Test and was subsequently confirmed to have a somatic cell count 150 x10^3 - < 200 x10^3 cells/ml. Any cow with one or more quarters with subclinical mastitis was considered to have subclinical mastitis at the cow level. The predominant microorganisms isolated from quarters meeting the subclinical mastitis definition were contagious pathogens, including *Staphylococcus* (23.75%), *Streptococcus* (21.5%) and *E. coli* (2.5%). Also we study the correlation level of lysozyme in udder milk in comparison of California Mastitis Test (CMT) and Somatic cell count (SCC) in diagnosis of subclinical mastitis (SM). The lysozyme level in milk is a biomarker for subclinical mastitis.
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1. Introduction

Mastitis remains one of the most costly and serious problems of the dairy industry worldwide **(Fetrow *et al.*, 1991, Heald *et al.*, 2000; Seegers *et al.*, 2003)** since approximately 20% of the subsequent milk yield is lost following moderate to severe cases of mastitis occurring during lactation **(Berry and Hillerton, 2001).** Mastitis-related losses are associated with reduction in yield, increased treatment costs, discarded milk, increase in culling and associated dairy cow replacement rates, and financial penalties for exceeding legal milk quality limits **(Dohoo and Martin, 1984; Fetrow *et al.*, 1991; Hortet and Seegers, 1998; Bennedfgaard *et al.*, 2003).** Mastitis is an inflammation of the mammary gland and udder tissue which can occur clinical (CM) and Subclinical (SM) forms **(Gonzales *et al.*, 2008).**

Subclinical mastitis (SM) is a condition in which there are no detectable inflammatory change in the udder and no observable abnormality in the milk, however it reduces reproductive performance and milk production,and adversely affects milk quality of lactating cows, similar to clinical mastitis (CM) **(Plastridge 1985; Batavani *et al.*, 2007).** Furthermore, SM cows are reservoirs for bacteria that can later cause CM. So a mastitis control program should pay more emphasis on monitoring and prevention of SM in dairy herds.

Subclinical mastitis is usually characterized by a rise in somatic cell counts (SCC) and a 15–45% drop in daily milk production, in the absence of visible changes to milk itself or the udder **(Dohoo and Meek, 1982; Dohoo *et al.*, 1984; Schukken *et al.*, 1995),** As the proportion of dairy cows affected by subclinical mastitis can be high, losses associated with reduced daily milk production and the off-farm purchaser with holding milk quality premiums can be significant **(Thurmond, 1993).** However, as IMI are usually followed by an influx of leucocytes into the milk, an increase in its SCC has been used widely as indicating mastitis. Therefore quarter, individual cow and bulk milk SCC are all used commonly to monitor mastitis and milk quality **(Schukken *et al.*, 1989; Bradley and Green, 1999; Green *et al.*, 2002b; Berry and Meaney, 2006).**

The California mastitis test (CMT), first described and used **(Schalm and Noorlander, 1957),** has been accepted as a quick ,simple test to predict SCC from individual quarters or composite milk **(Sanford *et al.*, 2006).** Whilst an increase in CMT score corresponds with an increase in SCC, it is uncertain whether or not CMT or SCC scores can reflect accurately IMI due to specific pathogens. If either SCC or CMT could be used reliably to identify subclinical mastitis in lactating cows, they might be useful in identifying such affected quarters that require antibiotic treatment and early drying off **(Barkema *et al.*, 1998**). High SCC during lactation or around the dry period are therefore considered to be predictive for the development of CM **(Beaudeau *et al.*, 1998; Rupp *et al.*, 2000 Rupp and Boichard, 2000; Peeler *et al.*, 2003; Green *et al.*, 2004a,b; Whist and Steras, 2007).**

The most common mastitis pathogens are found either in the udder (contagious pathogens) or the cow's

surroundings (environmental pathogens), such as bedding, manure, soil, etc. Contagious mastitis pathogens (*Staphylococcus aureus, Streptococcus agalactiae)* are spread from infected udders to "clean" udders during the milking process through contaminated teat cup liners, milkers' hands, paper or cloth towels used to wash or dry more than one cow, and possibly by flies. Although new infections by environmental pathogens (other *streptococci such* as *Str. uberis* and *Str. dysgalactiae* and coliforms such as *Escherichia coli* and *Klebsiella*) can occur during milking, primary exposure appears to be between milking. Coliform infections are usually associated with an unsanitary environment (manure and/or dirty, wet conditions), while *Klebsiella* are found in sawdust that contains bark or soil.

Defense of the mammary gland against mastitis-causing pathogens is mediated by several anatomical, cellular and soluble protective factors **(Sordillo *et al.*, 1997).** Many soluble factors present in milk act in collaboration with innate and immune defense mechanisms. Of these are lysozyme **(Sordillo *et al.*, 1990, Losnedahl *et al.*, 1996).** Lysozyme serves as nonspecific innate opsonin by binding to the bacterial surface to reduce the negative charge and facilitate phagocytosis of this bacterium before opsonins from the acquired immune systems enter the scene.

The purpose of the study to correlate the level of lysozyme in udder milk in comparison of California Mastitis Test (CMT) and Somatic cell count (SCC) in diagnosis of subclinical mastitis (SM).

2. Material and Methods

2.1. Sampling procedure

A total of 480 apparently normal quarter milk samples were collected from 120 machine milking Friesian lactating cows of different age and number & stages of lactation from Nobaria farm at Alexandria governorate. Examination of udder and teats was done according to (**Radostitis *et al.*, 1994**). Quarter milk samples were collected, after cleaning the teats with cotton wool and disinfected with 70% alcohol. The initial foremilk streams were discard, approximately 30 ml milk from each quarter were collected and added into sterile universals. First, it was inspected for the presence of clots and any discoloration then, it was divided into three portions. The first comprising 15 ml was used for quarter SCC estimation, lysozyme and nitric oxide , the second of 10 ml was were transported at 4-8□C to the laboratory for bacteriological examination. and the remainder was used to carry out the California mastitis test (CMT) on the farm. No preservatives were added.

2.2. Blood samples

A total of 120 blood samples were taken by the jugular vein puncture from the same Friesian lactating cows into vacotainer tubes and send to the lab. as soon as where serum was separated and kept frozen until use.

2.3. Mastitis markers

The California Mastitis Test (CMT) was developed to sample individual quarters to determine the presence of subclinical mastitis. The California Mastitis Test (CMT) is a rapid, accurate, cow-side test to help determine somatic cell counts (SCC) in a specific cow. A small sample of milk (approximately half of a teaspoon) from each quarter was collected into a plastic paddle having 4 shallow cups marked A, B, C, and D. 2 mL of California mastitis reagent **(Schalm and Noorlander, 1957)** was added to the milk. The paddle was rotated to mix the contents. In approximately 10 s, the score was recorded while continuing to rotate the paddle.The test result was interpreted based on the thickness of the gel formed by CMT reagent and milk mixture, and scored as negative (0), trace (T), weak positive (+), distinctive positive (++), and strong positive (+++). Quarters with CMT score of (+) or above were judged as positive. Cows were considered positive for SM when at least one quarter turned out to be positive for CMT.

2.4. Somatic cell count:

The relevant portion of each milk sample was examined for somatic cell count (SCC) according to (**Zecconi *et al.*, 2002),** using automatic reader (Bently Soma count U.S.A).

2.5. Bacterial culture and identification

From each milk sample that was positive for subclinical mastitis (SM) a sub sample of 0.01 ml was mixed, using a platinum handle, onto freshly prepared 5% blood agar plates for culture and isolation according to **(Koneman *et al.*, 1997).** The inoculated plates were incubated aerobically at 37□C for 24 h. *Staph. aureus* was identified by colony morphology/characteristics on the selective media Mannitol-Salt-Agar and a coagulase reaction (coagulase- positive) **(Carter, 1994).** Milk sample were also inoculated onto Edward's agar and onto MacConkey agar, respectively, for the selective isolation of streptococci and coliforms as described by **(Smith *et al.*, 1985).** Plates were incubated at 37□C and examined for bacterial growth 24 and 48 h later. Bacteria were identified tentatively by gross colony morphology and Gram stain, and further confirmatory tests used as necessary **(Cowan, 1974; Quin *et al.*, 1994).** Briefly, after Gram staining and microscopic examination, Gram-positive cocci were tested for the presence of catalase to differentiate *streptococci from staphylococci*. The tube coagulase test **(Boerlin *et a.*, 2003)** was used to identify *Staph. aureus* and other coagulase-positive *staphylococci* from coagulase-negative *staphylococci . Strept. Agalactiae, Strept. uberis and Strept. agalactiae* were identified according

to their ability to split aesculin and on the basis of Lancefield Group polysaccharide antigens, present in the streptococcal cell walls. *All Gram –negative bacteria were tested for the production of oxidase. E. coli* bacteria was then tested for the production of indole , urease.

2.6. Whey samples preparation:

Whey samples were prepared by addition of 5.0µI of a 10|% rennet solution/ml milk and incubation in a water bath at 32□C for 15 to 30 min. for casein precipitation. Casein was removed together with the fat by centrifugation in an Eppendorf 3200 r. p. m., table centrifuge (Eppendorf Geratebau, Hamburg, West Germany).

2.7. Detection of lysozyme concentration in milk samples:

Lysozyme concentration in whey and serum was quantified by the modified lysoplate assay as described by (**Lie and Solbu, 1986**). The test was carried in 1% agarose gel containing *Micrococcus lysodeikticus*. A500 mg uniform suspension of Micrococcus lysodeikticus cells was prepared in 0.067 M phosphate buffer, pH 6.24, at room temperature. Working lysozyme standards were prepared freshly by diluting 3.0 ml of stock lysozyme solution to 10.0 ml with 8.5 g/l sodium chloride to prepare 120 mg/ml. The wells were filled with a volume of 25 ml of skim milk samples. Each filled plate contained the 5 working lysozyme standards as well as the sample to be assayed. At the end of the incubation period, the clear zone ring diameters were measured to the nearest 0.1 mm with an enlarger viewer (Kalesttad Laboratories, Inc, Austin, TX). For each lysoplate, the lysozyme concentrations in the samples were determined from a plotted standard curve against the corresponding clear zone ring diameter on the linear axis.

2.8. Statistical analysis:

In our study, the obtained results were statistically analyzed using Costate computer program version 3.03 **copy right (1986)** Cottorot software.

3. Results:

At each sample point California mastitis test (CMT), Somatic cell count and bacteriological data were available for analysis from a total of 480 quarters (120 Cow). The number of milk sample with California mastitis test score (0), trace, weak positive, distinctive positive and strong positive were 292(60.8%),41(8.5%), 49(10.2%), 54(11.3%) and 44(9.2%) quarters respectively, table (1).

Table (1): Results of California mastitis test.

Total number of examined samples	CMT Score	Reaction	Numbers	%
480	0	Negative	292	60.8
	1	Trace	41	8.5
	2	Weak positive	49	10.2
	3	Distinct positive	54	11.3
	4	Strong positive	44	9.2

Also our result revealed that, The SCC was 83 (17.3%) samples ranged between 400x10³- <500x10³ cells/ml, 91(19%) samples ranged between 350x10³ - <400 x10³ cells/ml and 50 (10.4%) samples ranged between 200x10³ - < 350x10³ cells/ml and 256(53.3%) samples contain SCC below 150X10³ cells/ml, table(2).

Table (2): Distribution of SCC in 480 quarter milk samples.

SCC (Cellsx10³)	No. of examined samples	%
400-<500	83	17.3
350-<400	91	19.0
200-<350	50	10.4
<200	256	53.3
Total	480	100

On screening milk samples described in table (3) by using California mastitis test (CMT), and Somatic cell count (SCC), revealed that subclinical mastitis detected in a prevalence rate of 30.6% and 46.0% respectively, while on assessing the state of bacteriological examination of tested milk sample a prevalence rate of 23.75%, 21.5% and 2.5 % indicating presence of *Staphylococcus, Streptococcus* and *Escherichia coli* respectively.

Table (3): Screening of collected milk samples by using CMT, SCC and Bacteriological examination.

Test	Milk sample examination											
	CMT		SCC(x10³ cells/ml)		Bacteriological examination							
No. 480	No.	%	No.	%	*Staph.*	%	*Strept.*	%	*E. coli*	%	Total	%
Positive	147	30.6	224	46.7	114	23.75	103	21.5	12	2.5	229	47.7
Negative	333	69.4	256	53.3	366	76.25	377	78.5	468	97.5	251	52.3

*The % was calculated according to the 480 quarters examined.

Table (4): Correlation between lysozyme and subclinical mastitis (No. of positive SCC) in quarter milk samples:

Degree	No. of quarter samples	Milk lysozyme µ/ml
Subclinical	224	117.49 +/-6.4
Negative	256	11.14+/-0.86

Table (5): Correlation between lysozyme and subclinical mastitis (No. of positive SCC cases) in blood samples:

Degree	No. of blood samples	Blood lysozymeµ/ml
Subclinical	56	70.65 +/- 6.8
Negative	64	27.21+/-5.7

Table (6):Relationship between lysozyme, isolation of bacterial pathogens, CMT scores and SCC.

Lysozyme µ/ml		Bacteriological examination of 480 milk samples			CMT score	SCC(x10³ cells/ml
Milk	Blood	Isolates	No.	%		
11.14±0.86	27.21±5.7	*Staph.aureus* *S. intermedius* *S. hyicus* *S. agalactiae* *S. dysgalactiae* *S. uberis* *E.coli*	4 0 0 1 6 0 0	0.8 0 0 0.2 1.3 0 0	Negative and Trace	<150
67.2±5.9	59.19±2.3	*Staph.aureus* *S. intermedius* *S. hyicus* *S. agalactiae* *S. dysgalactiae* *S. uberis* *E.coli*	8 4 0 1 3 6 0	1.7 0.8 0 0.2 0.6 1.3 0	+	150<200
112±4.7	77.09±3.8	*Staph.aureus* *S. intermedius* *S. hyicus* *S. agalactiae* *S. dysgalactiae* *S. uberis* *E.coli*	17 2 8 13 10 5 3	3.5 0.4 1.7 2.7 2.1 1.0 0.6	++	200<400
181.49±2.2	91.18± 2.7	*Staph.aureus.* *S. intermedius.* *S. hyicus.* *S.agalactiae* *S. dysgalactiae* *S. uberis* *E.coli*	52 7 11 21 28 9 9	10.8 1.5 2.3 4.4 5.8 1.9 1.9	+++	400<500

4. Discussion

Mastitis occurs when white blood cells (leukocytes), are released into the mammary gland, usually in response to an invasion of bacteria of the teat canal.

Subclinical mastitis means that, although there are no visible or palpable external changes, the infection is present and the inflammation is occurred in the udder **(Blowey and Edmondson, 1995**). Subclinical mastitis leads to undesirable effect on milk constituents and its nutritional value. Economic losses are due to discarded abnormal milk and milk withheld from cows treated with antibiotic, costs of drugs, veterinary services and increased labor costs. In addition the problems related to antibiotic residues in human foods, milk, and subsequently affect the dairy manufactures, nutritional quality of milk and degrading of milk supplies due to high bacterial or somatic cell count **(Bramely *et al.,* 1996).** Regarding public health, mastitis is considered of vital importance due to its association with many zoonotic diseases in which milk acts as a source of infection **(APHA, 1992).**

The CMT is a rapid and inexpensive test to indirectly determine the somatic cell concentration in milk **(Midleton *et al.,* 2004)** and is a practical, easy method for demonstrating subclinical mastitis by testing milk samples on-farm **(Rindsig *et al.,* 1979; & Dingwell *et al.,* 2003).** In the present work, The number of milk sample with California mastitis test score (0), trace, weak positive, distinctive positive and strong positive were 292(60.8%), 41(8.5%), 49(10.2%), 54(11.3%) and 44(9.2%) quarters respectively, table (1). Also our result revealed that, the SCC was 83 (17.3%) samples ranged between $400x10^3$- $<500x10^3$ cells/ml, 91(19%) samples ranged between $350x10^3$ - $<400 x10^3$ cells/ml and 50 (10.4%) samples ranged between $200x10^3$ - $< 350x10^3$ cells/ml and 256(53.3%) samples contain SCC below $150X10^3$ cells/ml, table(2).

On testing the collected milk samples were collected as a quarter milk samples revealing presence of subclinical mastitis cases revealed that SCC (46.7%) found to be more sensitive indicator for detecting subclinical mastitis than CMT (30.6%) table (3), but CMT remain quit sufficient to be reliable test to be applied during milking process. So combining both CMT and SCC found to be essential to have a reliable screening data for milking animals. Previous studies by other workers had reported variable degrees of sensitivity and specificity for the CMT and SCC scores. Using CMT score 1 (trace), **(Middleton *et al.,* 2004)** reported low sensitivity and high specificity, while others reported high sensitivity and low specificity **(Sanford *et al.,* 2006)** or low sensitivity and low specificity **(Sargeant *et al.,* 2001).**However, differences in CMT or SCC scores could also be associated with other factors such as age of cows and environmental factors. some studies have shown that SCC scores may be affected by the position of the quarter, with hind quarters having higher SCC compared to front quarters **(Harmon, 1994)** or right quarters having higher SCC than left quarters **(Dhakal, 2006).** When SCC are elevated, they consist primarily of leukocytes or white blood cells which include macrophages, lymphocytes, and PMN. During inflammation, the major increase in SCC is because of the influx of PMN into milk. At this time, over 90% of the cells may be PMN.

In our present study, the number of bacterial isolates *Staphylococcus, streptococcus and E. coli* were 114 (23.75%), 103 (21.5%) and 15 (2.5%) respectively.

Our results suggest that overall there was a significant association between the frequency of isolation of pathogens (46.7%) and the SCC (47.7%) and CMT(30.6%) score in milk . These results agree with those reported by **(Omore *et al.,* 1996) and (Kivaria *et al.,* 2004),** which suggested that CMT scores of 2 + or more were associated with an increased risk of infection with *S. aureus*. Our studied showed that, *S. aureus* was isolated 52(10.8) when CMT score (+++),17 (3.5) when CMT score(++) 8 (1.7) when CMT score(+) and 4 (0.8) in case of negative and trace CMT, also *S. intermedius* was 7 (1.5%), 2 (0.4%) ,4 (0.8%) and 0 when CMT score were (+++) ,(++), (+), and negative or trace respectively while S.hyicus was isolated 11 (2.3%),8 (1.7%), 0 and 0 when CMT score were (+++) ,(++), (+), and negative or trace respectively table (6), The present results also indicated that quarters infected with a major pathogen were more likely to have higher CMT scores than those infected with either minor pathogens or uninfected. **(Sargeant *et al.*, 2001)** reported that the CMT had a useful surveillance role in dairy herd monitoring programs to detect cows with IMI caused by major pathogens.

Staphylococcus aureus is an important cause of mastitis in dairy cows. Infected cows udders are the main reservoir from which *S. aureus* is transmitted to other cows in the herd, and prevention of pathogen transmission from cow to cow reduces mastitis incidence **(Rivas *et al.,* 1997).** The most important species of *staphylococci* is *S. aureus* due to their pathogenicity and enterotoxin production causing food intoxication. The increase in incidence of *staphylococci* mastitis among animals is a serious source of *S. aureus*. Regarding the massive treatment of mastitic animals with antibiotics, resistant *S. aureus* strains have an opportunity to multiply inducing certain problems of public health significance among consumers of dairy products **(Tsung and Huang**

1993& Roberson et al., 1998). Prevention of bovine mastitis and production of high quality milk are strategic to favorable development of the dairy business and proper response to consumer demand **(Nagahata *et al.*, 2007).**

Streptococcus agalactiae is a contagious pathogen that can persist within the mammary gland and can be transmitted to healthy cows through poor milking hygiene **(McDonald, 1984).** Our result showed that, *Strept. dysagalactiae* were 28 (5.8%), 10 (2.1%), 3 (0.6%) and 6(1.3) when CMT were (+++), (++), (+) and negative or trace score respectively, while *Strept. agalactiae* were 21 (4.4%), 13 (2.7%), 1 (0.2%) and 1(0.2) when CMT were (+++), (++), (+) and negative or trace score respectively. Also *Strep. uberis* were 9 (1.9%), 5 (1%), 6 (1.3%) and 0 when CMT were (+++) ,(++), (+) and negative or trace score respectively. *E. Coli* were 9 (1.9%), 3 (0.6), 0 and 0 when CMT were (+++), (++), (+) and negative or trace score respectively.

A significant increase in the concentration of lysozyme was also observed. These findings suggest that other factors may be involved in the initial leukocyte recruitment into mammary glands after bacterial infection. Based on the study on healthy cows and cows with naturally occurring cases of subclinical mastitis, milk lysozyme was considered to be good indicator on the health of the udder quarters, and also to have a potential as indicators of subclinical mastitis. The large variation in lysozyme within quarter samples from cows with subclinical suggests that different mechanisms regulate migration of leukocytes into the udder compared to the influx and/or secretion of lysozyme into milk.

Lysozyme is strongly basic proteins and a bactericidal protein that cleaves peptidoglycans from the cell wall of Gram-positive bacteria. **(Reiter, 1978)** during subclinical mastitis, lysozyme is known to increase in bovine milk **(Schalm *et al.*, 1971 & Blood and Henderson 1986).** The limited lysozyme activity in normal cow milk increases due to high somatic cell counts. Lysozyme can limit the migration of neutrophils into damaged tissue and might function as an anti-inflammatory agent **(Peli *et al.*, 2002)**. In addition, this enzyme can synergize with antibodies, complement or lactoferrin.

our study revealed that, the lysozyme concentration in milk were 11.14+/- 0.86, equal to negative CM and equal to < 150 x10^3 SCC cells/ml. lysozyme concentration up to 67.2+/-5.9 in milk equal to (+ score) CM and equal to 150 x10^3< 200x10^3 SCC cells/ml. Also the lysozyme concentration was 112+/- 4.7 in milk equal to (++ score) CM and equal to 200x 10^3< 400 x10^3 SCC cells/ml. while lysozyme concentration was 181.49 +/- 2.2 in milk equal to (+++ score) CM and equal to 400x10^3< 500 x10^3 SCC cells/ml. our result concur with the results of **(Person *et al.*, 2007)** who reported that lysozyme was activated against many bacteria and increase in response to mastitis infection.

In conclusion, Our investigation suggest that, the lysozyme enzyme secreted in large quantities in the mammary gland in response to mastitis ,the lysozyme level in milk is a biomarker for subclinical mastitis , as well as measuring of lysozyme level in milk is more accurate than serum analysis for the diagnosis of subclinical mastitis in dairy farm.

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