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# Determination of Acute Phase Proteins Using a Rapid Immunological Method for Detection of Subclinical Mastitis in Dairy Animals

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**Abstract:** A total of 145 dairy animals were examined in Kafr EL-Sheikh governorate, using California mastitis test (CMT) for subclinical mastitis (SCM). Somatic cell count (SCC), bacteriological examination and measurements of acute phase proteins (APPs) as biomarkers for SCM included milk serum amyloid A (mSAA), haptoglobin (Hp) and lactoferrin (Lf) using ELISA were investigated. These biomarkers were quantified alongside SCC to confirm the immunological status of each suspected animal. The obtained results revealed that 250/567 quarter milk samples were positive for CMT (40% and 47.3% for cows and buffalos, respectively). SCC ranged from $1.71X10^5$ to $10X10^5$ cells /ml and from $1.40\ X10^5$ to $12.00\ X10^5$ cells /ml for cows and buffaloes, respectively. The most frequently bacteria isolated as single or mixed infections were *E.coli*, environmental streptococci, *Staphylococcus aureus* (*S. aureus*) and coagulase negative staphylococci (CNS) with a total prevalence of 43.2%, 32.8%, 24% and 22 %, respectively. In cows, strong significant positive correlations were found between SCC and APPs; mSAA (r = 0.683), Hp (r = 0.813) and Lf (r = 0.693). Additionally, in buffaloes, highly significant positive correlations were found between SCC and each of mSAA (r = 0.792), Hp (r = 0.478) and Lf (r = 0.356). Therefore, APPs may be planned as a routine examination of lactating animals for detection of SCM using ELISA technique as it is sensitive, rapid, saving time and labor.

**Key words:** Bovine SCM · SCC · IMI Pathogens · mSAA · Hp · Lf

## INTRODUCTION

SCM is a major problem affecting dairy animals all over the world. In addition, the bacterial contamination of milk from affected cows render it unfit for human consumption and provide a mechanism of spread of many zoonotic diseases [1].

Buffalo is recognized as the world's second most important milk producing species after cows [2]. In buffalo milk production, Egypt ranked the 4th in the world, after India, Pakistan and China, respectively [3].

The presence of clinical mastitis is quite easy to asses; whereas the diagnosis of the SCM can be more difficult and requires diagnostic methods including CMT and SCC. The CMT is more perfect, efficient and reliable than other tests [4].

SCC is still used as an indicator of milk quality [1]. The legal SCC threshold for bovine milk acceptance in Germany, Canada and the USA are $1\times10^5$; $5\times10^5$ and $7.5\times10^5$ cells/ml, respectively [5,6]. SCC was mostly in agreement with bacterial culture examination to detect SCM in riverine buffalo [7]

In the last decade there has been an increasing interest in the possibility of using APPs in veterinary diagnosis. They were classified into positive APPs (including; SAA, Hp and Lf) and negative APPs (including; Albumin, Transferrin and Transthyretin) [8].

APPs are serum molecules synthesized by mammary tissue [9]. Therefore, their use in milk provided a more specific and sensitive diagnostic method for mastitis than bacteriological methods and they were less influenced by the physiological stage of the cow than SCC [10].

Recent research has suggested the usefulness of Lf indicator associated to SCC to detect the presence of mastitis and added that, the knowledge of milk Lf could also improve the milk nutritional quality [11].

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This study aimed to (a) elucidate the prevalence of SCM in dairy cows and buffaloes in Kafr EL-Sheikh governorate (b) find out the frequently causative agents of SCM (c) measurement APPs (mSAA, Hp and Lf) by using ELISA test for detection of SCM in dairy animals (d) investigate the correlation between SCC and APPs.

## MATERIALS AND METHODS

**Animals:** A total of 145 apparently healthy dairy animals (64 cows and 81 of buffaloes) were examined from small holder farms in Kafr EL-Sheikh governorate, Egypt.

**Milk Samples:** The examined udders were thoroughly washed, dried with a single paper towel and the teats were sanitized with 70% ethanol. The first few jets were discarded and a milk sample from each quarter was tested by CMT. The results were interpreted as negative, 1+, 2+ and 3+ as described by Schalm *et al.* [12]. Forty ml of CMT positive quarter milk samples were collected in sterile bottles, kept at 4°C and transported immediately to the laboratory. Each sample was divided into 3 parts; one part was directed to SCC using Bently Soma Count 150 (U.S.A), the 2nd part was incubated at 37°C for 18 h for bacteriological examination and the 3rd part was stored in a freezer (-20 °C) till ELISA testing.

**Bacteriological Examination:** Loop-full from pre-incubated milk samples were streaked on MacConkey agar, Mannitol salt agar, Baired Parker and Edward's agar and incubated at 37°C for 24-48 h. Suspected colonies were identified according to Quinn *et al*. [13].

**Immunological Measurement by ELISA:** ELISA test was carried out [after separation of milk serum (whey) from CMT positive quarter milk samples using cooling centrifuge ($10^4$ rpm for15min)] using anti-bovine 96-well micro-titer plate ELISA kits (Sunredbio Co., Shanghai, China) for quantitative determination of mSAA, Hp and Lf.

**Statistical Analysis:** All statistical analyses were computed. The correlations between SCC and concentrations of mSAA, Hp and Lf in tested milk samples were performed using ANOVA test. Significance was assessed for $P < 0.05$ (*), $P < 0.001$ (**) and $P < 0.0001$ (***).

## RESULTS AND DISCUSSION

The health status of the mammary glands of 64 dairy cows and 81 buffaloes was carried out using CMT. The results showed that, 13 quarters were blind (6 and 7 quarters for cows and buffaloes, respectively) and 250/567 (44.1%) quarters were positive for SCM. It was found that, 100/250 (40%) cow's quarters were positive; 41, 46 and 13 quarters (41%, 46% and 13%) showed degrees of CMT (3+), (2+) and (1+), respectively. However, 150/317 (47.3%) buffalo's quarters were positive; 19, 66 and 65 quarters (12.7%, 44% and 43.3%) showed degrees of CMT (3+), (2+) and (1+), respectively. The highest intensity of CMT reaction (46% and 44%) lied within the score (2+) for cows and buffaloes, respectively (Table 1). Nearly similar records were obtained by Ayano *et al*. [14] (41.02%). Higher incidence (73.0 %) was obtained by Kamal *et al*. [15], while lower incidence rate was obtained by Hussain *et al*. [16] in buffalo quarters (15.2 %). It was concluded that CMT is still the superior rapid, cheap and easy to be used as screening diagnostic aid for SCM.

SCC ranged from $1.71x10^5$ to $10x10^5$ cells/ml and from $1.4x10^5$ to $1.2\ x10^6$ cells/ml, with mean values of $4.02x10^5 \pm 16.35$ and $3.16x10^5 \pm 17.28$ for cows and buffaloes, respectively (Table 2). CMT positive quarter milk samples that have SCC cells/ml $>400 \times 10^3$ were 40 (40%) and 25 (16.7%) in cows and buffaloes, respectively. Nearly, similar records were conducted by Ibrahim [17] who reported SSC higher than $400 \times 10^3$cells/ml in 39.72 % and 25.62% of cows and buffalos, respectively. It has been shown that IMIs in cows were associated with high SCC. This may be due to differences in phagocytic activity of the neutrophils in various species and also diversity in concentrations of hydrolases enzymes between cattle and buffaloes [18].

Major bacterial pathogens were recovered from 71% (177/250) of positive CMT quarter milk samples {80/100 in cows (80%) and 97/150 in buffaloes (65%)}. The most frequently bacteria isolated from the examined milk samples were *E. coli,* environmental streptococci, *S. aureus* and CNS (as single and/or mixed infection) with a total prevalence of 43.2%, 32.8%, 24% and 22% {64%, 46%, 17% and 15% for cows and 29.3%, 24%, 28.7% and 26.67% for buffaloes} (Table 3). Concerning buffaloes, our results agreed clearly with Ahmed *et al*. [19] who recorded high incidence rate of bacteria from SCM buffalo's milk (76.9%) and the most prevalent bacteria were *E. coli*, *S. uberis* and *S. aureus*. About half of the total isolated

Table 1: Relation between positive CMT and degree of quarter attack

| | | | | | Intensity of the reaction | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 3+ | | 2+ | | 1+ | |
| Species | No. of examined animals | No. of quarter milk samples | CMT +ve samples % | | No. | %• | No. | %• | No. | %• |
| Cows | 64 | 250 | 100 | 40.0 | 41 | 41.0 | 46 | 46.0 | 13 | 13.0 |
| Buffaloes | 81 | 317 | 150 | 47.3 | 19 | 12.7 | 66 | 44.0 | 65 | 43.3 |
| Total | 145 | 567 | 250 | 44.1 | 60 | 24.0 | 112 | 44.8 | 78 | 31.2 |

•: The percentages were calculated according to the number of CMT positive samples.

Table 2: Frequency distribution of mastitic quarter milk samples based on SCC

| | | SCC (cells/ ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ≤ 200.000 | | > 200.000 -400.000 | | > 400.000-800.000 | | >800.000 | |
| Species | CMT +ve samples | No. | %• | No. | %• | No. | %• | No. | % |
| Cows | 100 | 10 | 10.0 | 50 | 50.0 | 31 | 31.0 | 9 | 9.0 |
| Buffaloes | 150 | 61 | 40.7 | 64 | 42.7 | 15 | 10.0 | 10 | 6.7 |
| Total | 250 | 71 | 28.4 | 114 | 45.6 | 46 | 18.4 | 19 | 7.60 |

Table 3: The most common bacteria isolated from CMT positive samples.

| | | | | Mixed infection | | | | | | | | Single infection | | | | | | Others | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal species | CMT +ve samples | Total isolated organisms | | *E. coli*+ Environmental Streptococci | | *E. coli*+ *S. aureus* | | Environmental Streptococci + *S. aureus* | | *E. coli* + Environmental Streptococci + *S. aureus* | | *E. coli* | | Environmental Streptococci | | *S. aureus* | | CNS (with or without others) | |
| | | No. | %• | No. | %• | No. | %• | No. | %• | No. | %• | No. | %• | No. | %• | No. | %• | No. | %• |
| Cows | 100 | 80 | 80 | 31 | 31 | 5 | 5 | 5 | 5 | 3 | 3 | 25 | 25 | 7 | 7 | 4 | 4 | 15 | 15 |
| Buffaloes | 150 | 97 | 65 | 6 | 4 | 9 | 6 | 9 | 6 | 1 | 0.67 | 28 | 18.67 | 20 | 13.3 | 24 | 16 | 40 | 26.67 |
| Total | 250 | 177 | 71 | 37 | 14.8 | 14 | 5.6 | 14 | 5.6 | 4 | 1.6 | 53 | 21.2 | 27 | 10.8 | 28 | 11.2 | 55 | 22 |

pathogens (43.2%) was related to *E. coli,* that because *E. coli* is one of the most prevalent bacteria in SCM in dairy animals, this may be returned to fecal contamination and lack of hygienic measures in these farms. Also, about quarter of the total isolated pathogens (24%) was related to *S. aureus*; is one of the most prevalent bacteria in SCM in dairy cows and this may be returned to milkers hands which consider the main tool of cross infection, in addition to lack of hygiene in milking pallor. Moreover, about third of the total isolated pathogens (32.8%) was related to environmental streptococci. In the last decade, the CNS have become frequently identified in cases of subclinical mastitis, De Vliegher *et al*. [20].

The differences in the susceptibility between cows and buffaloes to IMIs were discussed by different researchers. Uppal *et al*. [21] attributed that buffaloes are more resistant to mastitis than cows that due to buffalo has a tighter teat opening than that of cow which may account for its lower susceptibility to mastitis.

In order to implement early diagnosis is a must for reduction of production losses and for enhancing the prospects of recovery. Due to the long time of the routinely used culture methods, it is very important to apply rapid, recent up-to-date and specific other methods such as ELISA. Applications of APPs in bovine medicine have largely focused on diseases with economic importance; bovine mastitis is the most important one of these Tothova *et al*. [8]. Our immunoassay cleared a linear relationship for mSAA, Hp and Lf. The mentioned results in Table (4) showed that, mSAA concentrations for cow's and buffalo's milk samples ranged from 8.24 to 24.86 µg/ml with mean of 13.63±0.31 and from 0.50 to 21.15 µg/ml with mean of 13.29±0.24, respectively. Hp concentrations for cows and buffaloes ranged from 0.09 to 0.56 mg/ml with mean of 0.334±0.01 and from 0.03 to 0.76 mg/ml with mean of 0.24±0.01, respectively. Lf concentrations for cows and buffaloes ranged from 0.16 to 2.45 mg/ml with mean of 1.19±0.07 and from 0.05 to 1.91 mg/ml with mean of 1.02±0.03, respectively. The frequency distribution of mSAA, Hp and Lf concentrations for cows and buffaloes were investigated in Table (5) that means of the overall three APPs increased by increasing of SCC. Several studies strongly agreed with our previously sorted results, Kováĉ *et al*. [10] illustrated that in cows with

Table 4: Statistical analytical results of mSAA, Hp and Lf values in the examined quarter milk samples

| | | | ELISA results | | |
|---|---|---|---|---|---|
| Species | CMT +ve samples | Minimum Maximum Mean | mSAA (µg/ml) | Hp (mg/ml) | Lf (mg/ml) |
| Cows | 100 | Minimum | 8.24 | 0.09 | 0.16 |
| | | Maximum | 24.86 | 0.56 | 2.45 |
| | | Mean ± SE | 13.63+0.31 | 0.334±0.01 | 1.19±0.07 |
| Buffaloes | 150 | Minimum | 0.50 | 0.03 | 0.05 |
| | | Maximum | 21.15 | 0.76 | 1.91 |
| | | Mean + SE | 13.29+0.24 | 0.24±0.01 | 1.02±0.03 |
| Total | 250 | Minimum | 0.50 | 0.03 | 0.05 |
| | | Maximum | 24.86 | 0.76 | 2.45 |
| | | Mean ± SE | 13.43+0.19 | 0.28±0.01 | 1.09 ± 0.03 |

Table 5: Frequency distribution of mastitic quarter milk samples based on mSAA, Hp and Lf

| | | mSAA (µg/ml) | | | | Hp (mg/ml) | | | | Lf (mg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ≤ 13.64 | | > 13.64 | | ≤ 0.176 | | > 0.176 | | ≤ 0.3 | | > 0.3 | |
| Species | CMT +ve samples | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Cows | 100 | 56 | 56.0 | 44 | 44.0 | 6 | 6.0 | 94 | 94.0 | 25 | 25.0 | 75 | 75.0 |
| Buffaloes | 150 | 91 | 60.7 | 59 | 39.3 | 42 | 28.0 | 108 | 72.0 | 15 | 10.0 | 135 | 90.0 |
| Total | 250 | 147 | 58.8 | 103 | 41.2 | 48 | 19.2 | 202 | 80.8 | 40 | 16.0 | 210 | 84.0 |

Table 6: Coefficient of correlation between SCC and mSAA, Lf and Hp concentrations in the examined quarter milk samples

| Quarter milk samples | R value/Values | mSAA Conc. | Hp Conc. | Lf Conc. |
|---|---|---|---|---|
| Cows | R value | 0.683*** | 0.813*** | 0.693*** |
| Buffalo | R value | 0.792*** | 0.479*** | 0.356*** |
| All samples | R value | 0.738** | 0.640*** | 0.529*** |

P < 0.0001. Significant*

SCM, strong elevations of both mSAA and Hp in milk were observed, indicating an activation of the acute phase response in these cows. The concentrations of mSAA in the SCM were about 100 times higher than in control milk samples [22]. In a study performed by Akerstedt *et al.* [23] for the detection of mSAA and Hp in different milk samples, they found a large proportion (53%) of the animals had detectable concentrations of APPs and mSAA was detected more frequently and at higher concentrations than Hp. Many data from investigations of the expression of mRNA for M-SAA3 and Hp suggested that an extra-hepatic synthesis of the APPs; mammary tissue can be a source of the APPs in bovine milk [9]. Therefore, mSAA is believed to be a more sensitive indicator of mastitis, which accumulates in milk only during mammary inflammation. In addition, the concentrations of mSAA found in samples from mammary quarters without clinical changes were also relatively high, as the uninfected quarters had to have very low or undetectable concentrations of mSAA. These results suggested that some quarters might be affected by inflammatory processes, while returning negative results in the CMT. Elevated concentrations of mSAA and Hp in quarters with mastitis compared to healthy quarters were also reported by several researchers [23, 24]. Therefore different authors suggested mSAA and/or Hp as useful early and very accurate markers of mastitis [25, 26].

The concentrations of Lf in milk varied from 1.15 to 485.63 µg /ml in healthy cows; however, its levels can rapidly increase in cows with SCM. This Lf raise in milk can be understood as an immunological response to bacterial infection, Moreover, in clinical mastitis, Lf concentrations in milk can be 13 times as high as those of healthy cows and in chronic mastitis, 7 times as high [27]. Presently, an increased in Lf concentrations with increased SCC can be understood as an immunological response to bacterial infection [28].

SCC is the gold standard in diagnosis of SCM, also is an important parameter in quality programs of dairy cooperatives. As routine SCC analysis is usually restricted to central laboratories, much effort has been invested in the search for alternative biomarkers of mastitis and milk quality, including the presence of positive APPs. In the present study, we studied the

correlations between SCC and APPs concentrations as shown in Table (6). Strong significant positive correlations were found between the severity of mammary infection according to SCC and APPs in the examined cow's quarter milk samples; mSAA (r = 0.683); Hp (r = 0.813) and Lf (r = 0.693), Also, in the examined buffalo's quarter milk samples; mSAA (r = 0.792); Hp (r = 0.479) and Lf (r = 0.356). Our results came in accordance to great extend with varieties of previous studies. O'Mahony *et al*. [29] reported a significant correlation between concentrations of mSAA and both SCC and CMT. Kováĉ *et al*. [10] observed high significant correlations either between milk Hp values and SCC or mSAA concentrations and SCC (r = 0.83, r = 0.81, respectively), those were consistent with our results. Moreover, there were significant differences in concentrations of Hp, milk amyloid A (MAA) and SCC between healthy cattle with clinical and SCM [30]. Lastly, the presence of Hp in milk was suggested as an indicator for unfavorable changes in milk [26]. Further, MAA may be a more sensitive marker than SCC as it was less influenced by other physiological factors [30].

Milk Lf concentrations tended to be correlated with the SCC score (r = 0.375) that nearly equal to our result, This finding suggested that Lf may be helpful as an indicator for IMIs in dairy cows [31]. Considering the results of ours and other studies, the increase of Lf concentrations, proportional to SCC qualify Lf as a positive APP in milk and may indicate that Lf might be used as a good indicator of SCM in dairy buffalos as in dairy cows.

Generally, the main function of APPs is to defend against pathological damage, some APPs (Hp, SAA, C-reactive protein) have scavenging activities and bind metabolites released from cellular degradation, so they can re-enter host metabolic processes rather than be utilized by pathogen [32]. Lf plays a key role in the defense mechanisms of the mammary gland, contributing to the prevention of infectious microbiological diseases and characterized by anti-bacterial activity on different types of bacteria that cause bovine mastitis (*E. coli, S. aureus*, *S.agalactiae*, *M. bovis, M. bovigentalium* and *P. aeruginosa*) [33, 34].

## CONCLUSION

It was concluded that SCM in dairy animals constituted a serious problem, therefore, programs for control it may be planned around the routine ELISA examination of lactating animals for detection of APPs. Consequently early detection of SCM can be obtained rapidly for, preventing their conversion towards clinical form, protecting the herd health from infection transmission between dairy animals, maintain milk hygiene and sustain the consumer health.

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