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Interrelationship of milk acute-phase proteins and casein percentage in cows and buffaloes subclinical mastitis

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Abstract

A total number of 62 clinically healthy dairy animals of three farms located in Kafr El Sheikh governorate, Egypt, were subjected to field screening surveys of subclinical mastitis (SCM) using California mastitis test (CMT). The obtained results revealed that 38.80% of quarter milk samples were positive to CMT. The most frequently major causative agents isolated from the positive CMT samples were *Escherichia coli*, *Staphylococcus aureus,* and environmental *streptococcus* spp. Acute-phase proteins (APPs), as immunological biomarkers for SCM, including milk serum amyloid A (mSAA) and haptoglobin (Hp) were measured using ELISA. A significant positive correlation was found between the severity of the mammary infection of cow's quarter milk samples represented in somatic cell count (SCC) and each of APPs and pH values. The correlation coefficient (R) between SCC and mSAA, Hp and pH were 0.54, 0.38 and 0.73, respectively. On the other hand, there was a significant negative correlation between casein percentage in milk of SCM cases, and each of APPs, pH and the presence of bacterial pathogens in the milk samples. The obtained results threw light on the inter-relationship between SCC, mSAA, pH value and casein percentage in milk of cows and buffalo suffered from SCM. The percentage of casein in milk is considered a significant accurate tool for diagnosis of SCM and this finding offers the farmers a cheap and fast selection for diagnosis of such disease. These results presented a specific structured view on the efficacy of different diagnostic tools of SCM in dairy herds.

Introduction

Subclinical mastitis (SCM) is asymptomatic inflammation of the mammary gland characterized by non -visible changes in milk and non-inflammatory signs on the udder or the animal. The disease is associated with reduction in milk production, altering milk composition and the presence of inflammatory components and bacteria in milk rendering it of low quality and unfit for processing.[1,2] In addition, the bacterial contamination of milk from affected cows render it unfit for human consumption and provide a mechanism of spread of zoonotic diseases like tuberculosis, sore-throat, Q-fever, brucellosis and leptospirosis.[3] The main causative bacteria of SCM in the Egyptian dairy farms are *S. aureus, E. coli,* and *environmental streptoccoi*.[4] Invisible changes in the milk of SCM cases can be recognized indirectly by several diagnostic methods including California mastitis test (CMT), somatic cell count (SCC) and pH level. These tests are preferred as screening tests for SCM because they are easily applicable and represent satisfying results in a short time.[5,6] All of these tests are subjected to variation due to lack of specificity resulted from the concomitance of physic-chemical modifications of milk and bacterial count.[3,7]

Various techniques have been suggested and evaluated as alternatives to SCC, including changes in milk proteins such as acute-phase proteins (APPs) to allow early identification of SCM. APPs are serum molecules synthesized by many cell categories, especially hepato-cytes.[8] Therefore, the use of APPs such as milk amyloid A (mSAA) and Haptoglobin (Hp) in milk provide a more specific and sensitive diagnostic method for mastitis

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than bacteriological methods and it is less influenced by the physiological stage of the cow than SCC and may be a useful marker of milk quality.[9,10] Hp and mSAA are the two most important APPs of cattle which are produced in high amount following bacterial or viral disease, however, have relatively low amount or absent in the healthy cows.[11] The presence of APPs in serum and milk sample as potential indicators of mastitis in both natural and experimental conditions have also been reported.[12]

Caseins are milk proteins secreted by cells of the mammary gland. They constitute approximately 78.00-82.00% of bovine milk proteins and are divided into four main groups: αS1-casein, αS2-casein, β-casein, γ-casein and κ-casein, forming supramolecular structures known as micelles.[13] The protein composition of cow's milk is an important factor for the profitability of the dairy industry. An increase in the proportion of casein, in particular α- and β- casein (α- and β-CN) results in better product yield, especially in cheese, while in subclinical mastitis, casein is subjected to degradation and mastitic milk contains low amounts of such protein**.** An increase in the levels of γ-casein in mastitic milk is originated from proteolytic degradation of gel forming casein; β-CN.[13]

Up to our knowledge, there are no previous studies to compare the inter-relationship between SCC, casein percentage, mSAA, Hp, pH and bacterial pathogens in SCM cases especially in buffaloes. This inter-relationship upon detect could offer the farmers an idea on the quickest, cheapest and more accurate tool for SCM diagnosis. The objectives of this study are to carry out a comparative study for the interrelationship of different protein fractions of milk including casein and APPs in SCM cases of cows and buffaloes in Kafr El Sheikh governorate, Egypt, and to study the diagnostic values of these protein fractions in SCM.

Materials and Methods

Animals. In winter 2016, a total number of 62 dairy animals; 40 cows and 22 buffaloes, were examined in Kafr El Sheikh governorate, Egypt. All dairy animals were apparently healthy without obvious signs of mastitis and no visible changes to the milk composition. All animals were subjected to clinical and physical examinations with special interest in the udder and teats.

Milk samples and chemical examinations. The examined udders were thoroughly washed, dried with a single paper towel and the teats were sanitized with 70.00% ethanol. After that, the first few jets of milk were discarded and a milk sample from each quarter was tested by CMT (Kerbl GmbH, Buchbach, Germany). According to the grade of gel formation, results were interpreted as negative, +1, +2, and +3, as described by Schalm *et al.*[14] Fifty mL of CMT positive milk samples from each quarter were collected in sterile bottles then kept at 4.00 ˚C and transported immediately to the laboratory. Each sample was divided into 4 parts: One part was directed to SCC and casein percentage using a somatic cell counter (Soma Count 150; Bentley Instruments Inc., Chaska, USA) and MilkoScan (Foss Co., Hilleroed, Denmark), respectively, pH of the second part was measured using digital pH meter (6173 pH; Jenco Instruments, San Diego, USA). The third part was incubated at 37.00 ˚C for 24 hr for bacteriological examination, and the fourth part was stored in a freezer (~ – 20.00 ˚C) till ELISA testing. Some CMT negative milk samples were collected to be used as the control for ELISA assay.

Bacteriological examination. Loopfulls from pre-incubated milk samples were streaked on MacConkey agar for *E. coli*, Baird Parker for *S. aureus* and Edward agar (Oxoid Ltd., Hampshire, UK) for Streptococci and incubated at 37.00 ˚C for 24 - 48 hr. Suspected colonies were identified biochemically according to Quinn *et al.* for *E. coli* IMViC (Indole, Methyl red, Voges-Proskauer and citrate).[15] For *S. aureus* (Catalase, Oxidase, Urease, Citrate, Coagulase) and for S*treptococci* spp. (VAN, vancomycin susceptibility (S) screening test, GAS (gas production in MRS broth(Oxoid, Ltd. Hampshire, UK)) Bile Esculin (BE), hydrolysis of esculin in the presence of bile, growth in broth containing 6.50% NaCL were performed, respectively.

ELISA. Milk samples were subjected to immunological measurement of APPs including mSAA and Hp using ELISA test after separation of milk serum (whey) using cooling centrifuge (10,000 rpm for 15 min). The bovine ELISA plate kits for mSAA and kits for Hp were purchased from Sunredbio Co. (Shanghai, China). The kits, a quantitative laboratory tests, came with polyclonal antibodies immobilized on a 96-well microtiter plate. The procedure was followed according to the instructions provided with the kits.

Statistical Analysis. All statistical analyses were computed using SPSS software (version 18.0; SPSS Inc., Chicago, USA). Statistical analyses were performed to evaluate the correlation between SCC, casein percentage, pH and mSAA, Hp and types of bacterial pathogens in milk samples. Descriptive statistics were expressed as the mean ± standard error. Normality of data distribution was assessed by a Shapiro-Wilk test. Since data were not normally distributed, a non-parametric Spearman correlation was tested. The threshold for statistical significance was considered to be $p < 0.05$.

Results

The prevalence of SCM among quarters of examined animals was estimated at 38.80% where 90 quarters were positive to CMT out of 232 (40.00% and 36.60% among quarters of examined cows and buffaloes, respectively), (Table 1). *E. coli* and environmental *Streptococcus* spp.

were the most prevalent pathogens isolated from SCM cases in cows (65.00% and 55.00%, respectively). While, *E. coli* and *S. aureus* were mostly common in SCM cases of buffaloes (43.30% and 20.00%, respectively), (Table 1). The overall means of SCC, pH and casein percentage among SCM cases in the present study were 364 × 10^3 cells mL^{-1}, 6.73% and 2.61%, respectively (Table 2). While the mean ± SE concentrations of mSAA and Hp among these cases were 13.60 ±_3.05 µg mL^{-1} and 0.30 ± 0.12 mg mL^{-1}, respectively (13.60 ± 0.38 and 0.34 ± 0.01 in cows and 13.61 ± 0.58 and 0.22 ± 0.01 in buffaloes). The frequency distribution of mSAA and Hp among quarters of SCM cases in buffaloes and cows are shown in Table 3. There was a significant positive correlation between concentrations of mSAA and Hp among SCM cases of both cows and buffaloes (r = 0.165; $p < 0.01$ and r = 0.763; $p < 0.0001$, respectively). The results showed that there was a positive correlation between the SCC and each of pH values, mSAA and Hp concentrations in milk of SCM cases in both cows and buffaloes (Table 4). Instead, there was a negative correlation between casein percentage in milk of SCM cases of cows and buffalo and each of pH value, presence of microbial pathogens in such milk, concentrations of either Hp or mSSA in such milk (Table 5).

Table 1. Results of CMT and pathogens associated with SCM cases among examined animals at Kafr El Sheikh governorate, Egypt, in 2016. Data are presented as number (percentage of CMT positive quarter milk samples).

Species	Examined animals	Examined quarters	CMT positive samples	Intensity of the reaction to CMT			Identified pathogens		
				1+	2+	3+	*E. coli*	*S. aureus*	Environmental *streptococcus*
Cow	40	150	60 (40.00)	11(18.30)	27(45.00)	22(36.70)	39(65.00)	8(13.30)	33(55.00)
Buffalo	22	82	30 (36.60)	7(23.30)	15(50.00)	8(26.70)	13(43.30)	6(20.00)	6(20.00)
Total	62	232	90(38.80)	18(20.00)	42(46.70)	30(33.30)	52(57.80)	14(15.60)	39(43.30)

Table 2. Somatic cell count (SCC), pH and casein (%) of CMT positive milk samples of animals at Kafr El Sheikh governorate, Egypt, in 2016. Data are presented as minimum - maximum (mean ± standard error).

Species	CMT positive samples	SCC (×10^3 cells mL^{-1})	pH	Casein (%)
Cow	60	105 - 696 (353.70 ± 168.20)	6.26 - 7.13 (6.71 ± 0.21)	1.62 - 2.87 (2.44 ± 0.37)
Buffalo	30	195 - 873 (387.50 ± 206.30)	6.25 - 7.29 (6.76 ± 0.04)	2.30 - 3.31 (2.94 ± 0.04)
Total	90	105 - 873 (364.00 ± 181.30)	6.25 - 7.29 (6.73 ± 0.22)	1.62 - 3.31 (2.61 ± 0.41)

Table 3. Distribution of milk serum amyloid A (mSAA) and haptoglobin (Hp) in CMT positive samples of examined animals at Kafr El Sheikh governorate, Egypt, in 2016. Data are presented as number (percentage of CMT positive quarter milk samples).

Species	CMT positive samples	mSAA (µg mL^{-1})		Hp (mg mL^{-1})	
		≤ 13.64	> 13.64	≤ 0.176	> 0.176
Cow	**60**	32 (53.30)	28 (46.70)	45 (75.00)	15 (25.00)
Buffalo	**30**	19 (63.30)	11 (36.70)	30 (100.00)	0 (0.00)
Total	**90**	51 (56.70)	39 (43.30)	75 (83.30)	15 (16.70)

The percentages were calculated according to the number of CMT positive quarter milk samples.

Table 4. Correlation between SCC and milk serum amyloid A (mSAA), haptoglobin (Hp) and pH in CMT positive milk at Kafr El Sheikh governorate, Egypt, in 2016.

Species	Statistical values	mSAA concentrations	Hp concentrations	pH value
Cow	r	0.360*	0.404**	0.695**
	p	0.005	0.001	0.0001
Buffalo	r	0.851**	0.832**	0.800**
	p	0.0001	0.0001	0.0001
Total	r	0.549**	0.389**	0.735**
	p	0.0001	0.0001	0.0001

* means significance level at $p < 0.005$ and ** means significance level at $p < 0.0005$.

Table 5. Correlation between casein (%) and pathogens, pH, mSAA and Hp of CMT positive milk at Kafr El Sheikh governorate, Egypt, in 2016.

Species	Statistical values	*E. coli*	*S. aureus*	Environmental *streptococcus*	pH value	mSAA	Hp
Cow	r	–0.057	–0.108	–0.051	–0.525-	–0.348**	–0.298*
	p	0.660	0.410	0.697	0.001	0.006	0.021
Buffalo	r	–0.255	–0.441*	0.017	–0.651***	–0.700***	–0.641**
	p	0.175	0.015	0.192	0.001	0.001	0.001
Total	r	–0.264	–0.114	–0.217	–0.391**	–0.358***	–0.582***
	p	0.005	0.28	0.03	0.001	0.0001	0.0001

* means significance level at $p < 0.05$, ** means significance level at $p < 0.005$ and *** means significance level at $p < 0.0005$.

Discussion

Subclinical mastitis is an economically persistent worldwide problem affecting the dairy sector. Early and feasible diagnostic tools for such a problem is much-required demand for monitoring herd performance and identifying management interventions. There are many diagnostic tools invented for early diagnosis of SCM and in the current study, we aimed to assess the efficacy of these tools for diagnosis of SCM, measuring the protein fractions concentrations of milk including casein and APPs, pH and SCC.

The CMT is still rapid, cheap and feasible diagnostic method used for screening SCM.[16] It was developed by Schalm and Noorlander[17] and is routinely used to evaluate the content of leucocytes and epithelial cells, defined as the somatic cells (SC) in the milk. It is based on the interaction between an anionic surfactant and the DNA of the somatic cells present in the milk sample submitted to evaluation.[18] Thus, CMT indicates increasing SCC as well as damaging of secretory cells of the mammary gland in the response to SCM. The magnitude of the inflammatory response may be influenced by the causative pathogen, stage of lactation, age, immune status of the animal, genetics and nutritional status,[19] therefore, CMT sometimes suffers lack of specificity to diagnosis of SCM. However, the high sensitivity of CMT makes it a gold standard test for detection of SCM and intra-mammary infection as declared by Kandeel *et al.*[6] Therefore, CMT has been used for detection of SCM cases in the current study. On the other hand, some recent studies showed that CMT is not suitable for large number of samples and it should be replaced by more sensitive techniques like SCC.[20]

The pH value ranged from 6.4 to 6.6 for cow milk and 6.70 to 6.80 for buffalo milk, respectively.[6,21,22] Ahmad *et al.* reported that milk pH increased 0.13 per unit increase in CMT score, therefore, it could serve as an indicator to assess the udder health condition in dairy animals.[23] In the current study, the pH of milk was increased in SCM cases, however, in some animals this increment was insignificant. These findings may be attributed to this fact that many of the common mastitis pathogens are capable of fermenting lactose, the lower concentration of lactose in mastitis and milk may be partly due to the activities of these organisms which may increase lactose fermentation and lower pH with high scores of CMT.

This could also decrease the sensitivity of such approach for diagnosis of SCM and agrees with Kandeel *et al.* who concluded that pH was less sensitive tool for diagnosis of SCM and did not provide a clinically useful method for diagnosis of SCM.[6]

Measurement of APPs in cows and buffalo's milk is an important tool for diagnosis of diseases with economic importance such as SCM.[10] It is believed that milk amyloid mSAA is a more sensitive indicator of mastitis because of accumulation in the milk only during mammary inflammation. The usefulness of mSAA in the diagnosis of clinical and subclinical mastitis was investigated by Kováč *et al.* who recorded significant higher mSAA concentrations in cow's milk samples from quarters with clinical changes as well as in milk positive to CMT obtained from quarters without clinical signs of mastitis.[24] Several studies strongly agreed with our results of the significant correlation between SCM and increase in APPs concentrations in milk. Kováč *et al.* illustrated that cows with SCM had an elevated amount of both measured APPs (mSAA and Hp) in milk indicating an activation of the acute phase response in animal.[9] The normal values of mSAA and Hp in cows and buffalo milk are 2.82 μg mL^{-1}, 0.52 mg mL^{-1} and 1.20 μg mL^{-1}, 0.11 mg mL^{-1}, respectively.[25,26] The significant increase in APPs in our study was in agreement with the findings of Kovačević-Filipović *et al.* who found that the concentrations of mSAA in the SCM milk samples was about 100 times higher than in control milk samples.[27] The use of APPs in serum and milk samples as potential indicators of mastitis in both natural and experimental conditions have also been reported by Eckersall and Bell,[12] thus, highlighting their significance as biomarkers of mastitis.

The SCC is a useful predictor for intra-mammary infection, stress and tissue injury, therefore, it is an important component in the assessment of aspects of quality, hygiene and mastitis control.[3] It is considered one of the most accurate and specific tools for diagnosis of SCM despite being affected by age of the animal, stage of lactation, storage and transportation of milk and parity.[3] The universal standard for SCC in SCM cases should be > 2.00×10^5 cells mL^{-1}.[20] In the current study, SCC had a significant positive correlation with APPs levels and pH in SCM cases milk. This correlation between the SCC and other diagnostic tools like APPs and pH has been proven in recent studies. On the other hand, lack of specificity of SCC and the lack of sensitivity of pH make the detection of APPs in the SCM a more useful diagnostic tool.[28]

An increase in the SCC is associated with increase in the protein content and decrease of casein percentage in SCM cases milk.[29,30] The reason for such findings may be attributed to that during mastitis. There is increase in the permeability of blood-milk barrier which results in an increased influx of proteins and enzymes from serum of blood and leads to an increase in proteolysis.[31] Furthermore, increased SCC in milk results in elevated activation of plasminogen into plasmin, that in turn leads to proteolysis of some proteins chains, primarily β-casein.[32,33] Our finding of the low casein% in SCM milk was in agreement with the finding of Ottalwar *et al.* [21] and Bobbo *et al.*[34] who found a significant negative correlation between the count of SCC and milk casein percentage in SCM cases associated with intramammary infection.

In conclusion, this study showed the importance of the use of APPs as potential biomarkers of cows and buffalo mastitis, however, it is time and financial consuming tool for diagnosis of SCM. Milk casein percentage measurement reflects the SCC in milk and offers fast, accurate and economic tools for the diagnosis of SCM.

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Conflict of interest

The authors declared that there is no conflict of interest.

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