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Variations in the levels of acute-phase proteins and lactoferrin in serum and milk during bovine subclinical mastitis

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Research Article

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Abstract

Variations in the levels of acute phase proteins and lactoferrin in serum and milk for diagnosis of subclinical mastitis in dairy cows are described in this research paper. Milking animals from two organized dairy farms in Kerala, India, were screened by California Mastitis Test (CMT), Electrical Conductivity test (EC) and Somatic Cell Count (SCC) test to identify animals affected with sub clinical mastitis (SCM). The concentrations of acute phase proteins (APP) Haptoglobin (Hp), C- reactive protein (CRP), Albumin, Lactoferrin (Lf) and α- 1 acid glycoprotein (AGP) in milk and Hp, Albumin, Serum Amyloid A (SAA) and CRP in the serum of 40 normal cows and 40 cows affected with sub clinical mastitis were assessed. Solid phase ELISA was employed for assessment of all parameters except the albumin levels, for which spectrophotometry was used. The values of Hp in milk; and SAA, AGP and Lf in serum, were significantly elevated in the group with sub clinical mastitis. Such variations were found to be independent of the specific bacterial organism causing the disease. These results show that significant variations exist in the levels of acute phase proteins Hp, AGP and Lf in milk, and SAA in serum of animals affected with subclinical bovine mastitis that are not affected by specific bacterial etiology.

Mastitis is the most economically important disease in the dairy industry, and subclinical mastitis represents the major portfolio of mastitis cases in the world (Hegde *et al*., 2013). While the diagnosis of clinical mastitis associated with demonstrable changes in the udder and secreted milk is relatively simple (Safi *et al*., 2009), many challenges are faced in the accurate diagnosis of subclinical mastitis due to its insidious nature. The cow-side diagnostic tests currently in practice targeting changes in the cellular content and pH of the milk are less proficient and incompatible with most of the modern milking systems (Eckersall *et al*., 2006). Failure in prompt identification, isolation and treatment of animals affected with sub clinical mastitis during the early stages of the disease can lead to delay in management and control of the disease, which in turn will facilitate further spread of the pathogens within the herd. Thus, there is scope for identifying and evaluating specific biomarkers capable of detecting subclinical mastitis at a very early stage of the disease (Akerstedt *et al*., 2007).

Assessment of changes in the levels of protein constituents of milk and serum as biomarkers for detection of subclinical mastitis has attracted considerable attention in the last decade (Eckersall *et al*., 2001). The potential of acute phase proteins (APP) in this context has been recognized (Biggadike *et al*., 2002). Mastitis essentially is an inflammatory response of the body against irritants, principally instigated by bacterial, parasitic, algal, and potentially viral and/or environmental elements (Wellenberg *et al*., 2002). Bacterial invasion of the mammary gland leads to a cascade of inflammatory events including an increase in the concentrations of acute-phase proteins in the blood and milk (Simões *et al*., 2017). This increase may precede the onset of clinical signs, making them suitable to be used as specific biomarkers for early detection of subclinical mastitis.

The most important APPs concerned with bovine mastitis are haptoglobin (Hp) and serum amyloid A (SAA) (Thomas *et al*., 2015), the concentrations of which were reported to increase several fold in the serum and milk during both clinical and subclinical cases. Levels of other APPs like α-1 acid glycoprotein (AGP) (Guha *et al*., 2013) and C- reactive protein (CRP) (Thomas *et al*., 2015) also increase considerably during mammary gland inflammation. There are certain negative APPs also like albumin and transferrin whose concentrations in serum decrease as a result of inflammation. Milk components other than APP have also been investigated as potential biomarkers. Lactoferrin (Lf) in milk was found to be one such reliable biomarker for diagnosis of subclinical mastitis by Sadek *et al*. (2016).

Subclinical mastitis caused by bacteria has a multifactorial etiology. Even though *Staphylococci* predominates, *Streptococci, Corynebacterium* and *Mycoplasma* are also

implicated in many instances (Gerardo *et al*., 2009). Differences in pathogenesis among these bacteria could be reflected in the inflammatory response of the body towards their presence, as the essential driver of irritation can decide the kind of cell reaction that happens against it in the body (Sadek *et al*., 2016).

Research on the utilization of the potential of APP as biomarkers for diagnosis of subclinical mastitis have so far involved only small study populations with one or two APPs being studied at a time. Another ambiguity has been on whether the variations in the levels of acute phase proteins during mastitis were confined to the milk or serum alone, or for both (Fathi and Farahzadi, 2011, Tothova *et al*., 2014). This study sought to provide comprehensive data on potential use of Hp, SAA, AGP, CRP and Lf both in serum and milk as biomarkers for diagnosis of subclinical mastitis in a larger sample size of dairy cattle. The specificity of these markers with regard to the etiological agent was also investigated to further refine their diagnostic value.

Materials and methods

Animals

The study was carried out in two medium-sized organized dairy farms of Thrissur district, Kerala State, S. India, in 110 lactating cows. The animals were cross-breds of Holstein-Friesian and Jersey breeds with the local non-descript cattle. They were of different parity and in various stages of lactation. Routine clinical examination was done on all animals before collecting samples and all udder quarters were checked to assess their apparent health status.

Diagnosis of subclinical mastitis

California mastitis test (CMT) and measurement of electrical conductivity (EC) of milk samples from individual quarters were done by direct stripping of milk into the designated wells of the CMT paddle, and the cups of a Draminsky^{R} multi quarter EC meter. Somatic cell count (SCC) was done by direct, manual microscopic cell counting using modified Newman's staining to reduce the reported errors due to clots and flakes encountered in automated counting (Eckersall *et al*., 2001).

Grouping of animals

Milk was collected individually from all lactating quarters of the 110 animals and subjected to CMT, SCC and EC tests. Forty cows with CMT score below two, EC score above 300 and SCC below 200 000 cells/ml in milk from each of all the milking quarters were grouped as healthy cows (Group 1) and 40 cows with CMT score above two, EC score below 300 and SCC above 200 000 cells/ml in milk from each of all the secreting quarters were grouped as sub-clinically infected (Group 2) (Sharma *et al*., 2010). Determination of APP levels in milk was done using milk from the quarter with the lowest SCC score for Group 1 animals and the highest SCC score for Group 2 animals. Milk and blood samples were collected from all the 80 selected animals. Serum was separated and stored at −20°C along with milk samples until further use.

Culture, isolation and identification of pathogens

Direct isolation of bacteria was done from milk by streaking on brain heart infusion (BHI) agar plates followed by incubation at 37°C for 24 h. Plates were examined after 24 h for growth. Isolated colonies were selected and representative samples were inoculated into BHI broth and incubated at 37° C for 24 h. The isolates were identified based on polymerase chain reaction (PCR) for the etiological agents for bovine subclinical mastitis as below. Details of primers used are given in Online Supplementary Table S5.

Pathogen specific variation of APP

Group 2 was subdivided into three subgroups, namely A with only Staphylococcal infection, B with only Streptococcal infection and C with mixed infection of both *Staphylococcus* and *Streptococcus* spp.

Measurement of acute-phase protein levels and albumin

The levels of APP, Hp and CRP were measured in both milk and serum samples. Serum amyloid A was measured in serum samples alone and AGP and Lf levels were determined from milk samples only. Solid-phase ELISA was used for the quantitative analysis of the APP levels using commercially available ELISA kits specific for each APP, developed by Life Diagnostics, USA, according to the manufacturer's instructions. A standard curve was constructed for each ELISA and concentrations of that particular APP in the samples tested were determined from the curve using quadratic equations. The values obtained were multiplied with the dilution factor to get the concentration in ng/ ml of sample. The levels of albumin in serum samples were estimated using Liquick Cor-ALBUMIN kits (Cormay Group, India). The test was based on spectrophotometry for measuring the color intensity of the complex formed between serum albumin and bromocresol green in the presence of succinate buffer (Doumas *et al*., 1971). Milk samples were centrifuged at 3000 g for 15 min at 4° C to separate skim milk. Skim milk was centrifuged with 0.1 N HCl to separate milk serum (whey). Then the albumin level was estimated like that of the blood serum.

Statistical analysis

The concentrations of the APP obtained were not in normal distribution, so they were transformed into natural logarithm prior to statistical analysis. Independent sample t test was used for comparing the means of the healthy and infected groups. The pathogen specific variation of the APP was analyzed by one way ANOVA.

Results

Culture and isolation-culture of 146 quarter milk samples (QMS) from 40 cows in Group 2 produced bacterial growth in 132 from which 261 pure isolates were obtained (Table 1). Eighty nine isolates (34%) showed positive result for catalase indicating Staphylococcus organisms and remaining 172 (66%) were Streptococci. Genus and species specific PCR of 89 catalase positive samples recognized Staphylococci spp. of which 37 (14%) were *S. aureus* and 52 (20%) were other Staphylococci spp. Species specific PCR for 172 catalase-negative isolates revealed 98 (38%) *S. agalactiae*, 48 (18%) *S. dysagalactiae* and 26 (10%) *S. uberis*. Fifty-six samples yielded only a single genus of bacteria of which 12 were *Staphylococcus* and 44 *Streptococcus*. Species level characterization revealed single infections with *S. aureus, S. agalactiae, S. dysagalactiae* and *S. uberis* in seven, 17, eight and two isolates respectively. In all other samples multiple infections were identified.

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Table 1. Number of different species of bacteria isolated and identified in the milk samples from 40 cows in Group 2

Sl. No	Organisms	No. of isolates
1	*Staphylococcus aureus*	37
2	Other *Staphylococci*	52
3	*Streptococcus agalactiae*	98
4	*Streptococcus dysagalactiae*	48
5	*Streptococcus uberis*	26
	Total	261

APP concentrations and pathogen specificity

Comparison of Groups 1 (healthy) and 2 (sublinical mastitis) are given in Table 2, and the pathogen-specific APP data for Group 2 are in Table 3. In this latter case no significant differences were noticed between the three sub-groups.

The mean of Hp levels in the milk of infected cows (Group 2) was significantly higher ($P < 0.05$) than that of the healthy cows (Group 1) whereas significant differences were not observed between the mean values of Hp in serum. Mean values of AGP and SAA were significantly higher ($P < 0.01$ and $P < 0.001$ respectively) in the subclinical mastitis group, as was Lf ($P < 0.001$) Significant differences were not detected in the mean values of either CRP or albumin in milk or serum.

Table 2. Comparison between the levels of acute-phase proteins: Hp, CRP, SAA, AGP, albumin, and Lf in the serum and milk of 40 healthy cows (Group 1) and 40 cows with subclinical mastitis (Group 2)

Sl. No.	Parameters	Group 1, healthy	Group 2, infected	*P* value
		Mean ± SE		
1	Hp in milk (Ln)	6.05 ± 0.16[a]	7.96 ± 0.26[b]	<0.001
2	Hp in serum (Ln)	10.04 ± 0.12[a]	10.22 ± 0.15[a]	n.s.
3	CRP in milk (Ln)	6.55 ± 0.04[a]	6.59 ± 0.026[a]	n.s.
4	CRP in serum (Ln)	12.41 ± 0.20[a]	12.79 ± 0.10[a]	n.s.
5	SAA in serum (Ln)	7.29 ± 0.25[a]	8.09 ± 0.24[b]	<0.05
6	AGP in milk (Ln)	9.15 ± 0.17[a]	9.99 ± 0.17[b]	<0.01
7	Lactoferrin in milk (Ln)	12.42 ± 0.14[a]	13.64 ± 0.17[b]	<0.001
8	Albumin in milk (g/dl)	0.38 ± 0.03[a]	0.43 ± 0.04[a]	n.s.
9	Albumin in serum (g/dl)	2.58 ± 0.09[a]	2.48 ± 0.09[a]	n.s.

Means with different superscripts differ significantly, n.s. is non significant ($P > 0.05$)
Hp- Haptoglobin, CRP- C-reactive protein, SAA- serum amyloid A, AGP-α-1 Acid glycoprotein, Ln- natural logarithm

Discussion

The large sample size of the research, and inclusion of all the major acute phase proteins have provided comprehensive information on the potential use of lactoferrin and acute phase proteins as potential bio-markers for diagnosis of sub-clinical mastitis in dairy cattle. Levels of lactoferrin and three acute phase proteins, namely Hp, AGP and SAA were significantly elevated in dairy cows affected with the disease, while the CRP and albumin levels were not. Interestingly, these changes were found to be independent of the specific etiology for the disease.

Etiological agents of SCM like *Staphylococcus* spp. and *Streptococcus* spp. cause formation of micro abscesses, pin point necrotic changes and fibrosis which can lead to tissue injury leading to increase in Hp levels in milk. Elevation of Hp in milk subsequent to mammary tissue injury is caused by local expression of Hp mRNA rather than influx from the serum, making it a specific marker for inflammatory changes in mammary tissue. This is borne out by the findings of this study where only the levels of milk Hp, and not the serum Hp, were found to be significantly enhanced. Our results also points to the non-specificity of serum Hp levels as a true indicator for SCM as Hp values among the healthy cows were actually relatively high, possibly due to co-morbidities. Kovac *et al.* (2007) report Hp values in the serum of healthy cows to be below detectable levels, but such high levels of animal health are yet to be achieved by the Indian dairy industry. Safi *et al.* (2009) find an insignificant increase in the serum Hp concentration in case of SCM, whereas Eckersall *et al.* (2006) describe the increase in serum Hp of cows with SCM to be significant. Only the β subunit of haptoglobulin is present in milk during subclinical matitis while the serum contains all three subunits (Upadhyaya *et al.*, 2016). Hence, the specificty of the commercial ELISA kits for Hp could be increased if they were to target only the β subunits, instead of targeting both beta and alpha chains as done now.

SAA is a group of three proteins produced by the liver as a response to acute inflammatory changes after induction by pro-inflammatory cytokines like IL-1, IL-6 and TNF alpha. Significant increase in SAA levels among affected cows in our study supplement the findings of Eckersall *et al.* (2001) and Razak *et al.* (2015), but Gerardo *et al.* (2009) report the SAA levels to be of little use for discriminating between subclinical and clinical mastitis. It could be that the larger size and homogeneity of our samples nullified the influence of external factors like hidden inflammatory diseases suspected in their study. We obtained a low correlation between SCC and SAA values (data not shown), which contradicts the theory of ingress of SAA into milk during mastitis (O'Mahony *et al.*, 2006).

AGP is produced mainly in liver and secreted. Extra hepatic production of AGP does occur (Ceciliani *et al.*, 2007) and there is evidence of influx of serum AGP into organs for maintenance of homeostasis by reducing the tissue damage associated with the inflammatory process (Murata *et al.*, 2004). The significant elevation of AGP detected among Group 2 animals in our study points to the potential of AGP being a sensitive indicator of chronic inflammatory changes associated with SCM in cattle.

C-reactive protein (CRP) is a pentamer, which has the capacity to bind with non-self antigens, thus playing an important role in the recognition and protection against infections and clearance of damaged tissue (Mold *et al.*, 2002). Only a non-significant numerical increase in CRP levels in Group 2 animals was detected in the present study. According to Panicker *et al.* (2014) the CRP levels increase only by very moderate levels during infections in cows, although Thomas (2015) consider CRP in milk to be a potential marker for mastitis detection while their second paper (Thomas, 2015) reports that M-SAA3 and CRP do not correlate with Hp or SCC or between themselves. Significant increase of CRP in blood in human mastitis (Fetherston *et al.*, 2006) and canine clinical mastitis (Vasiu *et al.*, 2017) have also been

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Table 3. Comparison of variation of acute-phase proteins against the bacterial isolates in the milk of 40 cows from Group 2

Sl. No.	Parameters (Mean ± SE)	Pathogen-specific variation			*P* value
		Group 2A	Group 2B	Group 2C	
1	Hp in milk (Ln)	7.43 ± 1.08	7.30 ± 0.715	8.110 ± 0.267	n.s.
2	Hp in serum (Ln)	11.04 ± 0.87	10.31 ± 0.527	10.11 ± 0.14	n.s.
3	CRP in milk (Ln)	6.70 ± 0.05	6.63 ± 0.09	6.57 ± 0.03	n.s.
4	CRP in serum (Ln)	13.06 ± 0.05	12.95 ± 0.4	12.74 ± 0.12	n.s.
5	SAA in serum (Ln)	8.15 ± 0.64	8.69 ± 0.71	8.00 ± 0.28	n.s.
6	AGP in milk (Ln)	9.53 ± 0.32	9.64 ± 0.55	10.09 ± 0.20	n.s.
7	Albumin in milk (g/dl)	0.55 ± 0.18	0.38 ± 0.12	0.42 ± 0.04	n.s.
8	Albumin in serum (g/dl)	2.42 ± 0.33	2.30 ± 0.29	2.50 ± 0.10	n.s.

Group 2A – Only *Staphylococcus*, group 2B – Only *Streptococcus*, Group 2C – Both *Staphylococcus* and *Streptococcus*
N.S. non significant ($P > 0.05$)
Hp- Haptoglobin, CRP- C-reactive protein, SAA- serum amyloid A, AGP-α-1 Acid glycoprotein, Ln- natural logarithm

reported. All these studies were conducted with sample sizes that were confined to a small location and were much smaller than ours, and could have been influenced by inflammatory responses against co-morbidities in one or a few animals in the study population. Another interpretation could be that the synthesis of different acute phase proteins is not uniform, and cows in different stages of inflammatory response may be having different levels of specific proteins. Zalewska *et al.* (2020) did not find any significant differences between the SAA and Hp levels in the cistern lining epithelium of cows affected with persistent infection of coagulase negative staphylococci and healthy cows, leading them to conclude that during chronic infections, the APP production is downgraded to some extent to ensure only minimal damage to the host cells.

Even though numerous APP have been identified, the rise in their serum levels is neither common nor uniform during inflammatory processes due to different diseases. The seat of infection during subclinical mastitis is limited to the mammary gland alone, favouring increase in production of APP that also have extra hepatic sources, like the mammary gland for AGP, Hp and Lf. The levels of only Hp and AGP in milk and SAA in serum were found to increase significantly during SCM while milk and serum concentrations of CRP and albumin remained insignificant. This is in agreement with the results of a study by Thomas *et al.* (2015) when the samples were collected without prejudice to the clinical condition of the cows. Thomas *et al.* (2018), using an experimental infection had found the profile of the three APP (CRP, Hp and SAA-3), to closely mirror each other during clinical mastitis. Dissimilarities in the onset and magnitude of fold increase of each APP in infected milk were observed in that study, indicating a possible influence of the stage of infection on the APP profile. Interestingly, the variations in levels were found to be insufficient to differentiate between clinical and subclinical mastitis. This points to the necessity of identifying the specific APP as biomarker for each disease conditions.

Different bacteria elicit their pathogenicity using different virulence factors that induce variable cytokines, thereby eliciting diverse inflammatory signs and damage to udder tissue in mastitis cases (Pyörälä and Syväjärvi, 1987; Schukken *et al.*, 2003; Thomas *et al.*, 2018; Dalanezi *et al.*, 2020). C-reactive protein concentration was found to vary significantly with the causative pathogen of mastitis indicating variable levels of stimulation of its secretion by the virulence factors of different pathogens (Thomas *et al.*, 2018). The micro-abscess formation due to coagulase activity of staphylococci assisting their invasion and persistence in the alveoli, and high anti-oxidant activity by streptococci are the major pathogenic mechanisms of SCM. In our study, the majority (32/40) of animals had mixed infection with both staphylococci and streptococci whereas only eight had individual infections. There was increased concentration of APP in milk and serum in all these three subgroups but there was no significant difference between the subgroups. Pyorala *et al.* (2011) found the concentrations of APP to be low in mastitis caused by CNS and streptococci while a rise was noticed for *S.aureus* group. This is not borne out by our findings and the probable reason could be that the secretion of acute phase proteins in all persistent infections may be downgraded in the body so as to protect permanent damage to the host cells (Zalewska *et al.*, 2020). Another reason could be the similarity in the confinement of pathogenesis to udder tissue by both of these pathogens.

Lactoferrin is an iron binding protein which functions as an innate defense factor in mastitis by preventing infection of mammary glands. The high levels of lactoferrin present in milk during the immediate post-calving period decreases to base levels as lactation progresses (Shimazaki and Kawai, 2017). Excess secretion of lactoferrin from the mammary epithelial cells during acute clinical mastitis has been reported (Huang *et al.*, 2012). The concentrations of Lf in milk quickly increase in cows with subclinical and clinical mastitis (Galfi *et al.*, 2016). The levels of lactoferrin in milk from Group 2 animals in our study were significantly higher, and we were able to establish a positive correlation between lactoferrin levels and Hp, SAA, as well as AGP levels (data not shown), indicating potential for lactoferrin to be used as a biomarker for detection of subclinical mastitis at very early stages of subclinical mastitis.

Batavani *et al.* (2007) have reported increased milk albumin and Singh *et al.* (2014) reported decreased serum albumin in case of SCM in dairy cows, but in the present study neither milk nor serum albumin was affected by health status, which supports Razak *et al.* (2015).

We conclude that serum levels of lactoferrin and three acute phase proteins, namely Hp, AGP and SAA are significantly increased during subclinical mastitis in dairy cattle, which can potentially provide a useful tool for early diagnosis of the disease.

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