Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 1589-1592

Received: 15-03-2018
Accepted: 20-04-2018

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Association of somatic cell count and hs-CRP acute phase protein with bovine subclinical mastitis in Holstein Frisian cross bred cattle of Kashmir

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Abstract
The current study, carried between June 2017 to March 2018, was directed in Holstein Frisian dairy cattle under second lactation with an aim to evaluate bovine hs-CRP secretions in the milk and serum of normal and subclinical mastitis (SCM) cows after screening by CMT and milk electric conductivity (EC) test. The study includes a total of 72 cows comprising of 10 in normal and 62 in subclinical group. The mean values of SCC in normal and subclinical groups were significantly different ($p \leq 0.001$). The mean values (with standard error) of somatic cell count (SCC) in normal and subclinical groups for SCC were1.820 ± 0.148 and 4.850 ± 0.512 respectively. Quantitative analysis of bovine hs-CRP by ELISA showed significant difference between normal (26.0363±11.14) and subclinical (267.603±30.17) serum samples while as no evident results were seen in milk samples of same animals.

Keywords: Subclinical mastitis, hs-CRP, biomarker and Somatic cell count

1. Introduction
Bovine mastitis, defined as inflammation of the mammary gland, is characterized by physical and biochemical alterations of body fluids including milk [1, 2]. Based on severity, mastitis can be clinical or subclinical. In contrast to clinical mastitis, subclinical mastitis (SCM) lacks clear clinical signs and therefore not easy to diagnose [3]. The major losses caused by SCM include reduced milk production, discarded milk, premature culling, reduced conception rates, and cost with therapy [4]. Normal milk contains macrophages and lymphocytes as dominating somatic cells while as during mastitis normal milk contains polymorphonuclear (PMN) leukocytes dominate, constituting approximately 90–95% of the cells which reflects in the hike of somatic cell count (SCC) in milk [5]. SCM is accompanied by a large influx of blood leukocytes into the inflamed mammary gland, thereby, resulting in an increase in the milk SCC [6]. During mammary immune response or acute phase response (APR) of host against any pathogenic insult leads to change in physiological as well as biochemical status of udder characterized primarily by change in concentrations of acute phase proteins (APPs) [7]. APPs are recognized as important potential diagnostic biomarkers for inflammatory conditions in both human as well as veterinary medicine [7]. Interest in APPs as potential biomarkers in veterinary medicine involves the evaluation of their concentration and modifications, as well as, their interaction as a part of the host response [8]. APPs, C reactive protein (CRP) and high sensitivity CRP (hs-CRP) are currently used as diagnostic markers in human medicine to evaluate individuals for risk of cardio vascular disease (CVD), but due to high sensitivity hs-CRP is regarded as better than CRP. The hs-CRP test accurately detects lower levels of the protein (0.5 to 10 mg/L in human) than the standard CRP test. Bovine CRP is regarded as minor APP and rapid reacting first line APP against bacterial infection in serum [9] while as no literature is available till May 2018, related to role of bovine hs-CRP in inflammatory conditions of bovines. In current study the quantitative estimation of hs CRP has been taken into consideration to find any possible association of hs-CRP with bovine subclinical mastitis.

2. Materials and methods

The present study was carried out in two districts of the Kashmir Valley namely Srinagar and Ganderbal from March 2017 to February 2018. Lactating crossbred Holstein Frisian dairy cattle with and without subclinical mastitis were selected for current study. The animals were in second lactation. Study animals (n=72) were grouped in two groups viz., normal comprising of 10 and SCM comprising of 62 dairy animals after screening their composite milk (milk from all functional quarters of cow`s udder collected in common container) samples (CMS) by California Mastitis Test (CMT) and milk electric conductivity (EC) tests. Diagnosis for SCM was performed by somatic cell count (SCC) using portable digital somatic cell counter (DeLaval®, Sweden).

2.1 Collection and processing of milk and blood samples

Before collection of CMS, udders of animals were cleaned with cotton swabs soaked with 70% ethanol. Teats were dried with second paper towel. After discarding the initial streaks of fore milk, CMS of assigned animals were collected by full hand stripping method. CMS of about 17 mL was collected from each animal out of which 14 mL volume was used for milk screening tests, 1 mL for SCC using SCC cassette (DeLaval ®), whereas 1.5 mL milk was collected in 2 mL Eppendorf tube and stored at -80 ºC for hs-CRP analysis. Blood sample of about 5 mL was also aseptically drawn from each animal by acupuncturing jugular vein. The blood samples were collected in clot activator vacutainer (RAPID™) for serum extraction. The requisite serum was harvested and transferred into 1.5 mL microfuge tube and stored at -80 ºC for hs-CRP analysis.

2.2 Screening tests

Immediately after collection, CMS were subjected to measurement of EC as reported by Razak *et al.*, 2015 [10], using portable EC meter (Eutech®, Singapore) at an average temperature of 26 ºC. The second cow side test performed for screening of subclinical mastitis was CMT as reported by Kandeel *et al*., 2018 [11]. The CMT results were scored as 0, 1, and 2 depending on the degree thickness of gel formation. About 14 mL of CMS was poured into the paddle and an equal volume of CMT reagent was added into it. The paddle was swirled to thoroughly mix the contents for about 15-25 seconds. According to the degree of thickness of gel formed, the reaction was scored as "0" for normal, "1" for mild or 2 for severe SCM.

2.3 Diagnosis of subclinical mastitis

Diagnosis of SCM was exercised by somatic cell count (SCC) using automatic portable somatic cell counter (DeLaval® Sweden) as reported by Kawai *et al*., 2017 [12]. Immediately after performing the screening tests, SCC was performed by loading commercially available DeLaval SCC cassette with milk sample. Every new DeLaval SCC cassette was loaded with every new milk sample (About 60 uL) within minimum possible time without exposing the cassette to direct sunlight. Reading of SCC ($\times 10^5$ cells/mL) was displayed within 45 seconds after installation of sample loaded cassette in DeLaval somatic cell counter.

2.4 Estimation of Bovine hs CRP from serum

Bovine hs CRP protein was estimated in milk and serum of stored samples by using bovine specific hsCRP ELISA kit (QayeeBio, South Korea) as per the manufacturer`s instructions. The data from screening and biochemical tests was statistically assessed by independent "t" test using SPSS (2016).

3. Results

Electrical conductivity (EC) of milk from uninfected quarters (normal milk), at a temperature of 25 ºC, is typically between 4.0 and 5.0 mS/cm as found by Wong *et al*., 1988 [13]. In the present study EC of milk samples was calculated by digital EC meter in mS (mili-Siemens) at an average temperature of 26 ºC. In present study, the mean (with standard error) of the EC in normal and subclinical mastitis groups were 4.611±0.264 and 5.551±0.158 respectively. These results of electric conductivity are significantly different ($p \leq 0.001$) in two groups. The mean values (with standard error) of HF of normal and subclinical group for SCC were 1.820 ± 0.148 and 3.176 ± 0.236 respectively. In HF animals, the mean values of SCC in both normal and subclinical groups were significantly different ($p \leq 0.001$).

In present study hs-CRP was determined in milk and serum by using commercial reagent kit based on double sandwich ELISA method. In the current study the mean values (with SE) of hs-CRP acute phase protein was significantly different in serum samples between normal (26.03 ± 11.14) and subclinical mastitis (267.60 ± 30.17) while as no observable reading was found in milk samples. The assay range of kit was between 6.25 ng/ml to 400 ng/ml.

Table 1: Mean values (± standard error-SE) of composite milk sample parameters and hs-CRP in bovine mastitis using independent "t" test.

Group	Milk EC (mS)	SCC (10^5cells/ml)	hs-CRP	
			Serum(ng/ml)	Milk(ng/ml)
Normal	4.611±0.264	1.820 ± 0.148	26.03 ± 11.14	0
SCM	5.551±0.158	3.176 ± 0.236	267.60 ± 30.17	0

EC: electric conductivity, mS: mili Siemens, SCC: somatic cell count, SCM: subclinical mastitis. *p value ≤0.001.*

4. Discussion

Mastitis is the most prevalent disease and the primary cause of economic losses in dairy cows. The major losses caused by mastitis include reduced milk production, discarded milk, premature culling, reduced conception rates, and cost with therapy [4]. Electric conductivity of milk was introduced as indicator of mastitis in 1943 [14]. Since then numerous studies have been carried out to evaluate the accuracy of EC for predicting infection status, and several authors have concluded that it has a potential for detecting mastitis [15]. Electrical conductivity of milk from uninfected quarters (normal milk), with a temperature of 25.8 ºC, is typically between 4.0 and 5.0 mS [13]. The mean values of milk EC in normal and subclinical mastitis groups in our study were significantly different. There were high mean values in subclinical group as compared to normal one. Our study is also in agreement with the study of [10, 15] suggesting that EC is good indicator of subclinical mastitis during early lactation in bovines. Our work is also in concomitance with [13] work which revealed that subclinical mastitis is associated with increase in mean values of EC in milk. The EC value depends on the concentration of anions and cations in milk. The

concentration of Na^+ and Cl^- increases in milk produced under mastitis disease; therefore, milk from mastitis cows typically shows higher EC when compared to milk from health cows [16]. The reason behind the increase in milk EC is due to damage of "leaky" tight junctions of epithelial cells of alveoli which leads to efflux of Na^+ and K^+ ions into milk thus increases the EC of milk. Furthermore, study carried out by [17] also supports our work which has shown that there is increase in EC during subclinical mastitis in Lithuania dairy cattle.

There was a significant difference between the mean values of SCC between apparently normal dairy cattle and SCM cattle suggesting that SCC is standard test for the diagnosis of SCM in crossbred HF dairy cattle. As per International Dairy Federation the normal level of SCC in milk of apparently healthy is equal or less than 200,000 cells/mL [18]. Our results are in concomitance with [19] which suggests that uninfected quarters of udder contains fewer somatic cells 100×10^3 cells/mL and that a quarter SCC with $>200 \times 10^3$ cells/mL is very likely to be infected. Additionally [20], indicated that quarter SCC $>100 \times 10^3$ cells/ mL are generally related to an inflammatory process in the mammary glands of Holstein cows.

Acute phase proteins high-sensitivity CRP is one of a growing number of cardiac risk markers in human. hs CRP is commonly used as low grade inflammation biomarker associated with cardio vascular diseases (CVD) in human medicine. Till date no literature is available related to association between bovine hs-CRP and inflammatory diseases of bovines. hs-CRP is same as CRP but is expressed in early stage of inflammation that too in minute quantity (which needs high sensitivity assay for estimation) hence cannot be estimated by standard CRP estimation assay [21]. Absence of hs-CRP in milk in our study is supported by [22] where they have concluded that hs-CRP is found in very minute quantity in serum that too at the early stage of inflammation, thus there may be least chance for this protein to gain access into the milk and maintain its concentration here. CRP protein in serum is already regarded as moderate APP in ruminants and also nonspecific biomarker of inflammation [7] hence its estimation during mastitis is not so worth compared to other specific biomarkers of bovine mastitis like serum amyloid A and haptoglobulin etc.

5. Conclusion

Taking the results of present study into consideration it may be concluded that SCC is more reliable marker for diagnosis of bovine subclinical mastitis as compared to hs-CRP. In present study our results of SCC in subclinical mastitis are in concomitance with. Acute phase proteins are non-specific biomarkers for disease diagnosis. Acute phase protein hs-CRP may not be associated with bovine subclinical mastitis as we find its presence in serum but not in milk which supports that hs-CRP in bovines is nonspecific marker of inflammation in bovine mastitis. Negligible or non-observable reading of hs-CRP in milk samples of subclinical mastitis group suggests that this inflammatory biomarker is not sensitive in milk or its extrahepatic synthesis in udder is also ruled out.

6. Acknowledgment

I am highly thankful to Department of Science and technology (Govt of India) for financial support of this work. The author is also thankful to Dr. Sajad Darzi (Veterinary Assistant surgeon) who provided us full support for collection of samples in local areas.

7. References

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