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Research Article

BIOMARKER OF CLINICAL DIAGNOSIS AND THERAPEUTIC MANAGEMENT OF SUBCLINICAL MASTITIS IN COW

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ABSTRACT: The timing of mastitis and sub-clinical mastitis outbreaks often gives important clues to the origin of herd problems. Infection rates are highest before calving, during early lactation, and near dry-off. The goal of every dairy farmer should be to minimize the number of organisms permitted to come into contact with the teats. To simplify the understanding of mastitis complexity, it is useful to detect the severity of the disease as well as diagnosis and therapeutic strategies. Serum amyloid A and haptoglobin are the two major acute-phase proteins and fibrinogen is a minor acute-phase protein in cattle. The therapeutic experiment was conducted by using four groups including Group A animals treated with only Bovimint, Group B animals with Bovimint + Mastotreet, and Group C with Bovimint + Mastotreet + Inj. Enrofloxacin, and Group D with Bovimint + Inj. Enrofloxacin. They are potentially useful as disease markers owing to their low concentration in normal animals, the rapid increase in their concentration during the acute phase of inflammation, and their rapid decrease with the resolution of the disease. To minimize economic losses due to high prevalence, its early detection with suitable tests and appropriate treatment regimen becomes most important. From the study, it was observed that treatment with Bovimint and Inj Enrofloxacin depicted the best recovery of the other treatment groups. Effective antimicrobial coverage along with local application on the udder would be a more suitable regimen of treatment against sub-clinical mastitis.

Key words: Sub clinical mastitis, Biomarker, Clinical diagnosis, Treatment.

INTRODUCTION

Animal husbandry and dairying play a prominent role in the rural economy in supplementing the income of rural households, particularly, the landless, small, and marginal farmers. However, it is threatened by mastitis and sub-clinical mastitis which continues to be a cause of significant economic loss to the dairy industry not only in India but also internationally. In addition, reported 36.69% and 16.78% prevalence of mastitis at a cow and quarter level, respectively in sub-clinical mastitis-affected crossbred cows (Sahiwal and Jersey) by cultural examination from Haryana. A study from Rajasthan showed 60.25% prevalence in cows and 39.00% in quarters by cultural examination, and the highest prevalence was found in 6th lactation every quarter and 3rd lactation on an animal basis (Chanawong *et al*. 2002). Sub-clinical mastitis is considered of vital importance because, in its sub-clinical form, a major part of the cow's udder is already affected and the quality and quantity of milk are reduced (Harmon 1994) and its association with many zoonotic diseases in which milk acts as a vehicle of pathogens causing tuberculosis and brucellosis (El-Balkamy *et al*. 1997, Shoshani *et al.* 2000).

Nowadays acute phase proteins are considered a more rapid and sensitive marker of inflammation than somatic cell count *(*Gronlund *et al*. 2005). Acute phase proteins (APP) are blood proteins primarily synthesized by the hepatocytes as a part of the acute phase response (APR). The APR has been referred to as the 'molecular thermometer' whereby quantification of individual APP can provide an assessment of the response to the triggering event. It was proved that APP was the most reliable indicator in differentiating cattle with acute and chronic inflammation and confirmed that the use of APP

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measurement was better than a haematological test, such as neutrophil count for the presence of infection. Serum amyloid A and haptoglobin are the two major acute-phase proteins and fibrinogen is a minor acute-phase protein in cattle. They are potentially useful as disease markers owing to their low concentration in normal animals, the rapid increase in their concentration during the acute phase of inflammation, and their rapid decrease with the resolution of the disease (Gruys *et al.* 1994, Eckersall 2001). Their results suggested that serum amyloid A was produced locally in the mammary gland, potentially making it an early, specific marker of mastitis, which might prove more sensitive than a bacteriological examination and less influenced by the physiological stage of the cow than the somatic cell count or the electrical conductivity of the milk (Biggadike *et al*. 2002). Thus, the evaluation of these proteins using specific assays has become one of the most promising methods to detect sub-clinical infections. Based on the afore-mentioned economic and public health importance, the present study was conducted with the biomarker of the clinical diagnosis of sub-clinical mastitis with therapeutic management against the diseases.

MATERIALS AND METHODS

A total of 301 (three hundred and one) dairy cows were examined randomly in and around Kolkata, West Bengal, India. The individual udder was first examined visually and then by palpation to detect fibrosis, inflammatory swellings, visible injury, tick infestation, atrophy of the tissue, and swelling of supra mammary lymph nodes. The size and consistency of mammary quarters were inspected for the presence of any abnormalities, such as disproportional symmetry, swelling, firmness, and blindness. Mammary quarters often became blind when exposed to repeated infections and little or no treatment was provided. Information relating to the previous health history of the mammary quarters and causes of blindness was obtained from the owners of the farm. The viscosity and appearance of milk secretion from each quarter were examined for the presence of clots, flakes, blood, or watery secretion. Udders and teats were cleaned and dried before sample collection. Each teat end was scrubbed vigorously with cotton gauze moistened (but not completely wet) with 70% ethyl alcohol, sequentially the teats on the far side of the udder first, then those on the near side. A separate pledge or sponge was used for each teat to avoid contamination of teats during scrubbing. Scrubbing was continued until a new surface of the cotton or sponge remained clean.

Therapeutic trial

The therapeutic management was conducted with 60 (sixty) animals suffering from sub-clinical mastitis. For drug trials, an udder massage cream, Bovimint oil containing peppermint, eucalyptus, Calendula, Marigold, and Tea tree in a water-based micro Remulsion that absorbs quickly into the skin (Mara Healthcare Ltd, Canada), and an intra-mammary infusion, Mastotreet, a Tea-Tree oil at a concentration of 2.5% (Mara Healthcare Ltd, Canada) were procured from Canada, where it is widely used to combat sub-clinical mastitis. Injection Enrofloxacin was selected in this study as an antimicrobial agent as it is found sensitive in-vitro in disk diffusion test for most of the isolates (more than 70% cases). Bovimint was used @ 10 gm/quarter twice daily as a local application for 3 days, Mastotreet was used @ 1 tube of 5 gm/day as an intra-mammary infusion for 3 days and Inj. Enrofloxacin was @ 5mg/kg body wt. once daily for 3 days. Animals selected for the drug trial were divided into 4 groups (8 animals in each Group) i.e. Group A to Group D. Group A animals were treated with only Bovimint, Group B animals with Bovimint + Mastotreet, Group C with Bovimint + Mastotreet + Inj. Enrofloxacin and Group D with Bovimint + Inj. Enrofloxacin. Pre-treatment and post-treatment response was observed after 7 days, 21 days, 49 days, and 70 days and milk and serum were collected accordingly. In this study, the in-vitro efficacy of Klengard (Sodium Dichloro Iso Cyanurate/ NaDCC) was also observed against different organisms causing sub-clinical mastitis in different dilutions (manufacturers recommended dilution, above the recommended dilution and below the recommended dilution) as antimicrobial sanitizer as teat dipping reagent and recommended its use in field condition after every milking to all the lactating animals. In this study cows with sub-clinical mastitis were considered as a pre-treatment group which was further divided into 4 groups according to the type of treatment provided. Group A animals were treated with only Bovimint, Group B animals with Bovimint + Mastotreet, and Group C with Bovimint + Mastotreet + Inj. Enrofloxacin, and Group D with Bovimint + Inj. Enrofloxacin. At the same time strict hygienic measures and dipping of teats with Klengard, a water sanitizer recommended for cattle, after every milking was used to restrict the entry of new infection during the entire period of trial.

Collection of milk samples

Procedures for collecting milk samples were followed according to Schalm *et al.* (1971) and Quinn *et al.* (1994). The first 3-4 strips of milk were discarded. The collecting

vial was as near horizontal as possible and by turning the teat to a nearly horizontal position; 5 ml of milk was collected into each vial.

Somatic cell count

The somatic cell count was estimated as per the standard protocol described by Reneau (1986). In brief, 10 (ten) µl of milk was placed exactly in the center of the one sq. cm template and was spread evenly to cover all the area delineated by the template. From each sample, two films were prepared using successive areas of the slide. The films were dried at room temperature and then stained by Newman– Lampert staining technique. The slides with milk smears were placed on the slide rack and were flooded with modified Newman-Lampert stain (Himedia) for 2 min. The excess stain was drained off by keeping the slides in a standing position on absorbent paper and then air dried. The slides were rinsed in three changes of tap water at 42-45°C and air-dried. The counting of cells in stained films was performed under the oil immersion objective and the number of cells in 30 fields was counted. The fields were selected by moving the slide horizontally from one edge of the film through the center to the opposite edge and then, repeated in a vertical direction. The average number of cells per field was multiplied by the microscopic factor. The diameter of the microscopic field seen through the oil immersion objective was measured using a stage micrometer slide ruled in 0.1 and 0.01 mm. The diameter of the field was measured up to two decimal points and the area of the field was calculated using the formula ðr2. The area of the smear was taken in mm^2. The diameter (d) of the field was 0.018, and then the radius (r) was 0.009 (using a stage micrometer slide ruled in 0.01 mm).

So, the microscopic factor (MF) =

$$\frac{100 \times 0.009 \times 0.009}{3.14}$$

Modified California Mastitis Test (MCMT)

The test was performed as per the method described by Devi (1989) since the original Schalm reagent (Tri-ethanolamine sulfate and bromocresol purple) was not available in India; a 3% sodium lauryl sulfate solution was used with the same accuracy.

Collection of blood samples

Blood samples were taken from the jugular vein into a 10 ml disposable syringe, 4 ml of blood was transferred to 0.5% oxalated collection tube for separation of plasma for estimation of fibrinogen, and the remaining was left to clot for 18 to 20 hours and then centrifuged at 2500 g for 15 minutes; serum was separated and stored at -80°C until analyzed for estimation of total protein, serum amyloid A, and haptoglobin.

Serum amyloid A (SAA) assay

The concentrations of serum amyloid A were determined with a commercially available ELISA (Bovine Serum Amyloid A (SAA) Kit; Cusabio Biotech Co. Ltd., USA) and the test was performed according to the instructions as described by Nielsen *et al.* (2004). The serum and milk samples including the standards were diluted accordingly as per standard protocol. The optical densities were determined on an automatic plate reader (model 550; Bio-Rad) with a reference at 595 nm. Milk and serum samples from healthy animals, sub-clinical cases, and post-treatment cases were tested in this assay.

Haptoglobin (Hp) assay

The concentration of haptoglobin in the serum and milk samples was determined using a commercially available ELISA (Immunology Consultants Laboratory, Inc., USA) Kit, as described by Nielsen *et al.* (2004), following the manufacturer's instructions. The serum and milk samples including the standards were diluted accordingly as per standard protocol. The optical densities were determined on an automatic plate reader (model 550; Bio-Rad) with a reference at 595 nm. Milk and serum samples from healthy animals, sub-clinical cases, and post-treatment cases were tested in this assay.

Fibrinogen estimation

Fibrinogen from plasma was detected as per the colorimetric method described by Sutton and Hobman (1975).

Estimation of biochemical indice

Serum total protein, Alkaline Phosphatase (ALP), Aspartate aminotransferase (AST), and Alanine aminotransferase (ALT) levels were determined by the commercially available kit of Span Diagnostics Ltd., Surat, India as per the manufacturer's instruction. Estimation of serum total protein Serum total protein was estimated according to the manufacturer's instruction of the commercially available kit of Span Diagnostics Ltd., Surat, India. The result was taken in a UV-Vis spectrophotometer (Systronics).

Estimation of serum alkaline phophatase (ALP)

The test was performed according to the manufacturer's instructions for the commercially available

kit of Span Diagnostics Ltd., Surat, India. By the method using 4-aminophenazone, the serum alkaline phosphatase activity was measured. At pH 10.0, alkaline phosphatase from serum converted the phenyl phosphate into inorganic phosphate and phenol. In the alkaline medium and the presence of oxidizing agent 4- aminophenazone reacted with phenol and an orange-red colored complex was formed and was measured in spectrophotometer at 510 nm and the value was expressed in IU/L.

Estimation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The tests were performed according to the manufacturer's instructions for the commercially available kit of Span Diagnostics Ltd., Surat, India. Serum AST and ALT activities were measured by using 2, 4-Dinitrophenyl hydrazine (DNPH). A standard curve was prepared by using dilutions of pyruvate solution in a UV-VIS Spectrophotometer at 505 nm.

The enzyme activity was expressed in terms of ìg of pyruvic acid liberated per mg of protein in a serum sample incubated for one hour at 37ºC in the case of AST and for 30 minutes at 37ºC in the case of ALT.

Statistical analysis

The results obtained in the pre-treatment and post-treatment groups of cows were presented by means and standard error and analyzed statistically using Duncan multiple range test.

RESULTS AND DISCUSSION

The single most important factor affecting somatic cell count in milk is mammary gland infection status. The Somatic Cell Count (SCC) is the main indicator of milk quality. From table 1 it was observed that the calculated mean somatic cell count (SCC) in milk samples differed significantly ($p < 0.01$) between the pre-treatment and healthy group which indicated the increase in mean SCC in the pre-treatment group than the healthy group. Among different treatment groups, the mean of the SCC decreased significantly ($p < 0.01$) in groups C and D than the group's A and B of post-treatment groups (that is 7, 21, 49, and 70 day post-treatment groups). There was also a significant difference among the post-treatment groups (21st, 49th, and 70th day) of cows with sub-clinical mastitis. But there was no significant difference between pre-treatment and 7th-day post-treatment groups A and group B, but this differed significantly among all the treatment groups C and D and the pre-treatment group. There was also no significant difference among 70 days post-treatment group-C, group-D, and the healthy group which indicated after 70 days of treatment the SCC remained almost the same as healthy animals in group C and group D but a significant difference persisted in groups, A and B. Pitkala *et al.* (2004) studied 6 years on the effect of antimicrobials on mitogenic pathogens and observed no bacterial growth on cultural examination of milk samples after the treatment but enhanced CMT scores and SCC persisted for a prolonged period. Suojala (2010) also in their study reported similar observations, where they observed that even after 21st day of post-treatment, there was a significant difference in SCC in sub-clinical mastitis although the samples became culturally negative. Sharma *et al*. (2009) mentioned that SAA in milk was believed to be a more sensitive indicator of mastitis than SCC, because there was a gradual decrease in SCC even after physical recovery, but in case of acute phase protein the curve of mean concentration changed sharply. Furthermore, increased SAA level was found to occur much earlier in serum and preceded increased somatic cell count (Petersen *et al.* 2004). From Table 1 it was also observed that there was

Table 1. Somatic cell count (SCC) (10^3) in milk in different treatment groups (n=8) (Mean±SE).

Parameter	Pre-treatment	Post treatment groups			
		A	B	C	D
Healthy	147.44 ± 6.27	249.01 ± 5.36			
Day 7		241.45^{A}±4.57	241.26^{A}±4.78	$235.90^{B,1}$±4.35	$236.90^{B,1}$±4.75
Day 21		$226.07^{A,1}$±3.28	$225.08^{A,1}$±3.37	$209.69^{2B,2}$±5.34	$211.71^{B,2}$±5.13
Day 49		$203.14^{A,2}$±3.78	$203.25^{A,2}$±3.39	$185.03^{B,3}$±3.93	$184.21^{B,3}$±3.41
Day 70		$189.03^{A,3}$±3.79	$188.27^{A,3}$±3.61	$157.05^{A,4,@}$±6.27	$156.37^{A,4,@}$±5.35

^{A,B}- significant changes appeared among different treatment groups in row ($p < .01$), $^{1,2.3}$- significant changes appeared within different post-treatment groups in column ($p < .01$), $^{@}$ -no significant changes among different post-treatment groups and healthy group, *-significant changes appeared between healthy and post-treatment groups ($p < .01$), $^{\#}$- significant changes appeared between pre and post-treatment groups ($p < .01$).

a change in SCC but not at significant level between pre-treatment and 7th day post-treatment group of A and B but significant change occurred in group C and D. There was also significant change between group A and B with group C and D, in each post-treatment days, which was a clear indication of better treatment response of group C and D than group A and B in respect of SCC. From Table 2 it was observed that in pre-treatment group, the score of MCMT was 2+ and after 7th day of treatment, group A and group B remained unchanged but in group C and group D the MCMT scores settled down to 1+. Similarly, 21st day post-treatment group A and group B differed from group C and group D. On 49th and 70th post-treatment day all the animals became MCMT negative. Gerardi (2009) mentioned that sub-clinical mastitis was frequently diagnosed by california mastitis test (CMT), which may suffer from a lack of reproducibility, and the cell count (as CMT gives an indirect measurement of the number of somatic cells present) was not useful in discriminating between the clinical and sub-clinical form of mastitis. Suojala (2010) also in their study reported similar observation, where they observed that even after 21st day of post-treatment, there was a significant difference in SCC and MCMT in sub-clinical mastitis although the samples became culturally negative. Analysis of this result indicated that group C and group D provided better response with their treatment. From Table 3 it was observed that mean serum amyloid A (SAA) concentration in milk samples of pre-treatment group was significantly ($p < 0.01$) higher than that of the healthy group. Among the different post-treatment groups, the concentrations of SAA of the samples of group A and B were significantly higher ($p < 0.01$) than that of the samples of group C and D. There was significant ($p < 0.01$) difference in mean SAA concentration in milk samples in the treated groups compared to the pre-treatment group on 7th days post-treatment. There was no significant difference on 21, 49, 70 days post-treatment. There was no significant difference among 70th days post treatment group-C, group-D and healthy group. Suojala (2010) also found the activity of SAA in milk up to 7 and 21 days and above. Gerardi *et al.* (2009) and Kovac *et al*. (2007) also found similar activity of SAA in milk in their study. From the above discussion it was observed that post-treatment group C and D expressed better recovery than group A and B to the given treatment. In the present study serum amyloid A (SAA) concentration in serum samples was shown in Table 3 and the mean concentration of SAA from serum was significantly ($p < 0.01$) higher in pre-treatment group than that of the healthy group (4.54±0.71 µg/ml). Among different post-treatment groups, the mean SAA concentrations of the serum samples of group A and group B were significantly higher ($p < 0.01$) than that of the serum samples of group C and group D. Among the different days of post-treatment, the 7th day post- treatment group varied significantly ($p < 0.01$) with the other three (21st, 49th and 70th day post-treatment) groups. But there was no significant difference among 21st, 49th and 70th day post-treatment groups in case of SAA from serum. There was no significant difference among 70th day post-treatment group D and healthy group. Suojala (2008) also found the activity of SAA in serum up to 7 and 21 days and above. Gerardi *et al.* (2009) and Kovac *et al.* (2007) also found similar activity of SAA in serum in their study. From the above discussion it was observed that post- treatment group C and D expressed better recovery than group A and B to the given treatment. From table 4 it was observed that the mean haptoglobin concentrations in milk samples (Hp-M) differed significantly ($p < 0.01$) between pre-treatment and healthy group . Among the post-treatment groups, the haptoglobin concentrations of the samples of group A and group B were significantly higher ($p < 0.01$) than that of the samples of group C and group D. Cows with sub-clinical mastitis being significantly different ($p < 0.01$) among the 7th day and other three that is, the group of 21st, 49th, 70th day post- treatment. There was no significant difference between 7 and 21 days post-treatment but there was significant difference among 7th day and 49th day post treatment groups in group A, B, C. There was no significant difference among 70th days post treatment group-C, group- D and healthy group. Nielsen *et. al.* (2004) estimated haptoglobin in milk in different intervals and reported similar observation. Suojala (2008) detected that the activity of Hp in milk up to 7 days and above. Gerardi *et al.* (2009) also found similar activity of Hp in milk in his study. From the above discussion it was

Table 2. Modified California mastitis test (MCMT) in milk in different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment (Score)	Post treatment groups (Score)			
		A	B	C	D
Healthy	0	2+			
Day 7		2^+	2^+	1^+	1+
Day 21		1+	1+	T	T
Day 49		0	o	0	0
Day 70		ND	ND	ND	ND

ND=Not done

Table 3. Serum amyloid A (SAA) activity (µg/ml) in milk and serum with different treatment groups (n = 8) (Mean ± SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy (MIlk)	$5.167 \pm 1.09^{@,O}$	$54.80 \pm 1.53^{\#}$			
Day 7		$12.65 \pm 0.89^{a,1}$	$12.70 \pm 0.93^{a,1}$	$10.93 \pm 0.81^{b,1}$	$10.56 \pm 0.93^{b,1}$
Day 21		$10.56 \pm 0.77^{a,1,2}$	$10.59 \pm 0.78^{a,1,2}$	$7.59 \pm 0.71^{b,1,2}$	$7.43 \pm 0.84^{b,1,2}$
Day 49		$8.47 \pm 0.69^{a,2}$	$8.48 \pm 0.58^{a,2}$	$6.39 \pm 0.56^{b,2}$	$6.16 \pm 0.51^{b,2}$
Day 70		$6.91 \pm 0.56^{a,3}$	$6.94 \pm 0.52^{a,3}$	$5.38 \pm 0.46^{b,2@}$	$5.19 \pm 0.54^{b,2@}$
Healthy (Serum)	$4.54 \pm 0.71^{@,O}$	$14.21 \pm 1.516^{\#}$			
Day 7		$8.91.65 \pm 0.49^{a,1}$	$8.73 \pm 0.41^{a.1}$	$7.14 \pm 0.36^{b.1}$	6.98 ± 0.45^{b}
Day 21		$8.38 \pm 0.34^{a,2}$	$81.6 \pm 0.34^{a,2}$	$7.02 \pm 0.33^{b,1}$	$6.35 \pm 0.37^{b,1,}$
Day 49		$7.88 \pm 0.35^{a,2}$	$7.81 \pm 0.31^{a,2}$	$6.72 \pm 0.36^{b,1}$	$5.55 \pm 0.41^{b,2}$
Day 70		$7.03 \pm 0.37^{a,2}$	$6.82 \pm 0.34^{a,2}$	$6.07 \pm 0.31^{a,b.2}$	$5.14 \pm 0.32^{b,2@}$

^{a,b}- significant changes appeared among different treatment groups in row ($p < .01$), $^{1,2.3}$- significant changes appeared within different post-treatment groups in column ($p < .01$), $^{@}$ -no significant changes among different post-treatment groups and healthy group, ^{O}-significant changes appeared between healthy and post-treatment groups ($p < .01$), $^{\#}$- significant changes appeared between pre and post-treatment groups ($p < .01$).

observed that post-treatment group C and D expressed better recovery than group A and B to the given treatment. From Table 4 it was observed that the mean haptoglobin concentration in serum samples (Hp-S) differed significantly ($p < 0.01$) between pre-treatment and healthy group. Among the post-treatment groups, the Haptoglobin (Hp-M) concentrations of the samples of group A and group B were significantly higher ($p < 0.01$) than that of the samples of group C and group D. From the table it was evident that there was significant ($p < 0.01$) difference in 7 and 21 day post- treatment groups but there was no significant difference in 49 and 70 day post-treatment groups. Haptoglobin concentrations in serum samples in cows with sub- clinical mastitis after treatment were significantly different ($p < 0.01$) among the 7th day post-treatment and other three that was the 21st, 49 and 70 days post-treatment. There was no significant difference among 70 days post-treatment group-C, group-D and

Table 4. Haptoglobin (Hp) activity (µg/ml) in milk and serum with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy (MIlk)	$0.826 \pm 0.058^{@,O}$	$3.51 \pm 0.110^{\#}$			
Day 7		$2.38 \pm 0.074^{a,1}$	$2.23 \pm 0.065^{a.1}$	$1.73 \pm 0.073^{b.1}$	$1.68 \pm 0.061^{b,1}$
Day 21		$1.61 \pm 0.08^{a,1,2}$	$1.56 \pm 0.079^{a,1,2}$	$1.13 \pm 0.079^{b,2}$	$1.01 \pm 0.67^{b,2}$
Day 49		$1,45 \pm 0.055^{a,2}$	$1.37 \pm 0.057^{a,2}$	$0.989 \pm 0.063^{b,2}$	$0.978 \pm 0.054^{b,2}$
Day 70		$1.39 \pm 0.057^{a,2}$	$1.32 \pm 0.053^{a,2}$	$0.961 \pm 0.042^{b,2@}$	$0.943 \pm 0.051^{b,2@}$
Healthy (Serum)	$0.127 \pm 0.018^{@,O}$	$0.523 \pm 0.026^{\#}$			
Day 7		$0.340 \pm 0.012^{a,1}$	$0.335 \pm 0.011^{a.1}$	$0.298 \pm 0.019^{b.1}$	$0.285 \pm 0.008^{b,1}$
Day 21		$0.278 \pm 0.026^{b,2}$	$0.269 \pm 0.024^{b,2}$	$0.226 \pm 0.021^{b,2}$	$0.221 \pm 0.019^{b,2}$
Day 49		0.213 ± 0.016^{2}	0.215 ± 0.015^{2}	0.198 ± 0.008^{2}	0.192 ± 0.009^{2}
Day 70		0.187 ± 0.013^{2}	0.181 ± 0.014^{2}	$0.150 \pm 0.015^{2@.2}$	$0.146 \pm 0.013^{3@}$

^{a,b}- significant changes appeared among different treatment groups in row ($p<.01$),. $^{1,2.3}$- significant changes appeared within different post-treatment groups in column ($p<.01$), $^{@}$ -no significant changes among different post-treatment groups and healthy group, ^{O}-significant changes appeared between healthy and post-treatment groups ($p<.01$), $^{\#}$- significant changes appeared between pre and post-treatment groups ($p<.01$).

Table 5. Fibrinogen (Fb) activity (mg/dl) in plasma with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy	291.27±27.13	327.32±18.81			
Day 7		311.13±16.81	304.45±10.28	294.37±5.91	297.24±6.33
Day 21		309.54±6.83	299.31±3.22	292.72±4.92	293.87±2.73
Day 49		305.39±5.72	294.16±2.25	292.57±1.93	290.28±2.71
Day 70		327.32±18.81	323.56±24.22	313.47±10.92	319.73±16.34

healthy group. Eskersall *et al.* (2001) reported the activity of haptoglobin in serum in similar pattern. Nielsen *et al.* (2004) estimated haptoglobin in serum in different intervals and reported similar observation. Suojala (2010) detected that the activity of Hp in milk up to 7 days and above. Gerardi *et al.* (2009) also found similar activity of Hp in milk in his study. From the above discussion it was observed that post-treatment group C and D expressed better recovery than group A and B. In the present study plasma concentration of fibrinogen (Fb) obtained was presented in Table 5. From the table it was observed that the fibrinogen concentrations in plasma samples did not differ significantly ($p< 0.01$) between pre-treatment and healthy group though there was increase of plasma fibrinogen (Fb) concentration in pre-treatment group than the healthy group. Among the treatment groups, the concentrations of the Fibrinogen (fb) of group A, B, C and D were also not significantly different ($p< 0.01$). The results of the present study indicated that there was no significant change within the different days of post-treatment as well as no significant change between the groups. Feldman *et al*. (2000) and Latimer *et al*. (2003) mentioned that fibrinogen may be treated as better indicator of inflammation in cows. Murata *et al.* (2004) reported that the mean concentration of plasma Fb in sub-clinical cases with healthy cows showed significant difference ($p<0.1$). But in the present study it was revealed that there was an increase in mean concentration of fibrinogen in sub-clinical mastitis animals than the healthy animals, but the change was not significant statistically. Treatment group C and D responded little better than treatment group A and B in respect of fibrinogen in the present study. From Table 6 it was observed that concentration of total protein from serum samples did not differ significantly ($p< 0.01$) between pre-treatment and healthy group though there was increase in concentration of total serum protein in pre-treatment group than the healthy group. Among different treatment groups, the concentrations of the total protein in group A, B, C and D did not differ significantly ($p < 0.01$). There was also no significant difference among the post-treatment groups (7, 21, 49 and 70 days) of cows with sub-clinical mastitis which indicated that the increase of mean concentration of total protein was singly not enough to differentiate among the different treatment group as well as within different days of treatment. Trolldenie (1995) mentioned that the proteins mainly serum albumin and immunoglobulins were implicated in udder defence mechanisms. According to Pandey (2005), immunoglobulin plays an important role in host immunity and inflammation, and there is a correlation between total serum protein (albumin and globulin) and somatic cells count in milk. Matei *et al.* (2010) also found elevated level of total protein in sub-clinical mastitis than healthy animals and they also mentioned that the increased proteins and globulin in the blood of cows indicated an activation of immune response following infection of the mammary gland. In the present study it was observed the increased level of total protein, which could be due to the initiation of immune response in face of sub-clinical mastitis. From the above

Table 6. Total protein (TP) activity (g/dl) in serum with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy	7.61 ±0 .66	9.02 ±0.57			
Day 7		8.52 ±.58	8.29 ±.65	8.41 ±.54	8.51 ±.57
Day 21		7.93 ±.52	7.88 ±.46	7.70 ±.32	7.58 ±.42
Day 49		7.69 ±.42	7.64 ±.46	7.65 ±.33	7.60 ±.32
Day 70		7.71 ±.48	7.61 ±.43	7.5 ±.37	7.59 ±.45

Table 7. Serum alkaline phospatase (ALP) activity (U/L) in serum with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy	28.58±2.89[@]	46.83±3.51			
Day 7		42.27[a]±2.43	41.45[a]±2.78	36.81[1b]±1.69	36.47[1b]±1.72
Day 21		34.30[1]±1.51	34.81[1]±1.28	32.43[1,2]±1.18	32.03[1,2]±1.04
Day 49		30.13[2]±1.18	30.01[2]±1.13	29.13[2]±0.89	29.01[2]±1.03
Day 70		29.90[2]±1.34	29.41[2]±1.19	28.13[2]±1.18	28.67[2]±1.09

[a,b]- significant changes appeared among different treatment groups in row (p<.01), [1,2,3]- significant changes appeared within different post-treatment groups in column (p<.01), no significant changes among different post-treatment groups and healthy group, *-significant changes appeared between healthy and post-treatment groups (p<.01). [#]- significant changes appeared between pre and post-treatment groups (p<.01).

Table 8. Serum alanine aminotransferase (AST) activity (U/L) in serum with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy	101.29± 3.04[@]	113.14±2.89			
Day 7		111.88±2.89	111.67±3.05	109.73±3.09	109.35±3.01
Day 21		106.97[1]±2.61	106.64[1]±2.49	105.27[1]±2.79	105.49[1]±2.81
Day 49		104.51[1]±2.63	104.89[1]±2.69	104.27[1]±2.93	104.11[1]±2.84
Day 70		102.78[1,@]±2.53	102.63[1,@]±2.59	101.78[1,@]±2.73	101.69[1,@]±2.87

[a,b]- significant changes appeared among different treatment groups in row (p<.01), [1,2,3]- significant changes appeared within different post-treatment groups in column (p<.01), no significant changes among different post-treatment groups and healthy group, *-significant changes appeared between healthy and post-treatment groups (p<.01), [#]- significant changes appeared between pre and post-treatment groups (p<.01).

discussion it revealed that, although there was no statistical significant differences among the groups, but group C and group D showed better response than group A and group B. From Table 7 it was observed that the mean alkaline phosphatase (ALP) activity in serum differed significantly (p< 0.01) between pre-treatment and healthy group. Among post- treatment groups, the concentrations of ALP in group A and group B were significantly higher (p < 0.01) than that of group C and group D in 7 day post-treatment groups but there was no significant difference in 21, 49 and 70 day post-treatment groups. ALP concentrations in serum samples in cows with sub-clinical mastitis after treatment were significantly different (p < 0.01) among the 7 day and other three post-treatment groups that was the group of 21, 49 and 70 day post-treatment. There was no significant difference among 70 day post-treatment groups with healthy group. Matei *et al.* (2010) also observed significant increase in the serum ALP level in sub-clinical mastitis affected cows. From the table it was evident that there was no significant change of 7 day post-treatment results with pre-treatment group. The significant change (p < 0.01) was observed at 21 day post-treatment result. There was no significant change within 21, 49, and 70 day post-treatment groups irrespective of treatment group, but gradual decrease in concentration was observed. From the above discussion it was evident that post-treatment group C and D expressed better response than group A and B to the given treatment. In the present study the mean activity of aspartate aminotransferase (AST) was estimated from serum and result was presented in Table 8. From the table it was observed that the activity of AST in serum differed significantly (p < 0.01) between pre-treatment and healthy group which indicated the increase in concentration of AST in the pre-treatment group than healthy group. Among different treatment groups, the activity of the AST did not differ significantly. There was also no significant difference among the post-treatment groups (21, 49 and 70 day) of cows with sub-clinical mastitis and there was also no significant difference between pre-treatment and 7 day post-treatment group. The significant change was observed among healthy and

Table 9. Serum alanine aminotransferase (ALT) activity (U/L) in serum with different treatment groups (n = 8) (Mean±SE).

Parameter	Pre-Treatment	Post treatment groups			
		A	B	C	D
Healthy	13.31± .45[@]	17.31±.56			
Day 7		16.61[a]±.44	16.57[a]±.45	15.68[b,1]±.43	15.59[b,1]±.47
Day 21		14.97[1]±.43	14.83[1]±.34	14.43[1,2]±.49	13.35[1,2]±.37
Day 49		14.23[1]±.43	14.27[1]±.48	13.98[2]±.49	13.89[2]±.43
Day 70		14.07[1]±.33	14.11[1]±.38	13.77[2@]±.43	13.71[2@]±.31

[a,b]- significant changes appeared among different treatment groups in row (p<.01), [1,2,3]- significant changes appeared within different post-treatment groups in column (p<.01), no significant changes among different post-treatment groups and healthy group, *-significant changes appeared between healthy and post-treatment groups (p<.01), [#]- significant changes appeared between pre and post-treatment groups (p<.01).

21 day post-treatment group. There was no significant difference among 70 day post treatment group-A, group-B, group-C and group-D with healthy group which indicated that, after 70 days the AST level of post-treatment animals remained almost same as that of healthy animals. Sharma (2003), in his study on correlation of minerals and enzymes in blood serum and milk from sub-clinical mastitis showed evidence of direct passage of blood into the milk as indicated by the changes of some blood enzymes level and he also observed the high activity AST level in his study during episode of mastitis. The result of the present study did not corroborate with the observation of Matei *et al.* (2010) who observed significant decrease in the serum AST level in sub-clinical mastitis affected cows. From the above discussion it evident that post-treatment group C and D expressed better responds than group A and B to the given treatment. From Table 9 it was observed that the mean concentration of alanine aminotransferase (ALT) in serum samples differed significantly ($p< 0.01$) between pre-treatment and healthy group which indicated the increase in activity of ALT in the pre-treatment group than the healthy group. Among different treatment groups, the activity of the ALT decreased significantly ($p < 0.01$) in group C and D than the group of A and B only in 7 day post-treatment group, but in other 3 groups (21,49 and 70 day post treatment) there no significant difference among the groups A,B, C, and D. There was no significant difference among treatment group C and D on 70 day post treatment with healthy group. From the above discussion it was evident that post-treatment group C and D expressed better response than group A and B. There was also no significant difference among the post-treatment groups (21, 49 and 70 day) of cows with sub-clinical mastitis. There was no significance difference between pre-treatment and 7 day post- treatment groups A and B, but differed significantly with 7 day post- treatment group C and D and pre-treatment groups. But in group C and D, there was significant difference among 7, 21, 49 day of post- treatment. There was no significant difference among 70 day post treatment group-C, group-D and healthy group. The findings were in agreement with the observations of Zahid (2004) who also observed increase in mean concentration of ALT level in sub-clinical mastitis infected animals caused by most of the pathogens as compared with healthy animals. From the above discussion it was evident that treatment group C and D expressed better responses than group A and B to the given treatment in respect of ALT.

CONCLUSION

In the present study therapeutic management was performed in field level dividing the SCM infected animal into four treatment groups and results were recorded in 7, 21, 49 and 70 days of post-treatment. The collected samples were analyzed on different post- treatment days with diagnostic parameters (SSA-M, SSA-S, Hp-M, Hp-S, Fb, TP, AST, ALT, and ALP). From the study, it was revealed that treatment with Bovimint and Inj Enrofloxacin showed the best recovery than the other treatment groups. Effective antimicrobial coverage along with local application on the udder would come out as a suitable regimen of treatment against sub-clinical mastitis. Strict hygienic measures and the practice of regular teat dipping with appropriate sanitizer after every milking may be adopted to reduce the occurrence of SCM.

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