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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(9): 3285-3290

www.thepharmajournal.com
Received: 28-07-2022
Accepted: 30-08-2022

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Relationship of indirect diagnostic tests, haematological parameters and milk components with subclinical mastitis in dairy cows

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Abstract
The study aimed to understand the relationship of California Mastitis Test (CMT) score and other indirect diagnostic tests [differential electrical conductivity (DEC) and absolute EC] with milk Somatic Cell Count (SCC) to improve the diagnosis of subclinical mastitis (SCM) in lactating Deoni (N=27) and HF crossbred (N=32) cows. Cows with milk SCC of $\geq 200\times10^3$ cells/ml were considered as SCM, while cows without any abnormality in milk or udder parenchyma, systemic signs and milk SCC of $\leq 200\times10^3$ were considered as healthy. It is observed that healthy and SCM affected Deoni cows had lesser milk SCC than HF crossbred cows. We found that breed had significant ($p<0.001$) effects on blood hemoglobin and packed cell volume (PCV) level but Red Blood Cell (RBC) counts, Total Leucocytes Count (TLC) and Differential Leucocytes Count (DLC) were not altered by breed and udder health status of Deoni and HF crossbred cows. Higher trend of fat: protein ratio was observed in SCM affected than healthy cows. In this study, the relationship of CMT score with SCC has been established and the finding will be useful for diagnosis of SCM using indirect diagnostic methods in Indian dairy cattle.

Keywords: Blood haematology, california mastitis test, dairy cows, milk fat protein ratio, somatic cell count and subclinical mastitis

1. Introduction
Mastitis in dairy animals causes huge economic losses to dairy farmers in India and across the globe. Mastitis occurs in two forms; subclinical mastitis (SCM) and clinical mastitis (CM). The clinical manifestation in CM makes easy for its diagnosis and early treatment. But diagnosis of SCM is difficult due to absence of overt clinical manifestation and requires diagnostic tests. Therefore, SCM often unnoticed and cause huge economic losses than CM due to its longer period of infections and consequent negative impact on milk yield and milk composition. SCM affected animals also become important source of infections for herd mates (Seegers *et al.*, 2003) [8]. Therefore, periodical screening and diagnosis of SCM at herd and individual cow level is very important for early implementation of suitable management strategies. On the other hand, failure to diagnosis and treatment of SCM at an early stage may leads to development of chronic inflammation of udder and become unresponsive to antibiotic treatment or they may develop into CM conditions. Therefore, several indirect diagnostic tests such California Mastitis Test (CMT) and online electrical conductivity tests are used for diagnosis of SCM and they are very less sensitive. Milk Somatic Cell Count (SCC) at cow level and herd level through Bulk Milk Somatic Cell Count (BMSCC) are common practices in dairy developed countries to understand the status of SCM. But estimation of SCC at cow level and herd level at regular intervals is not commonly practiced in Indian dairy farming conditions where CMT is common due to cheap and easy technical procedure. But the relationship of indirect diagnostic methods including CMT with more sensitive SCC methods is not known in Indian dairy animals. The relationship of udder health status with blood haematology and milk fat protein ratio in Indian dairy cattle also not clear. Indian dairy cattle are relatively resistant to infectious diseases and high yielding cows [e.g., Holstein Frisian (HF), Jersey, and their crossbred] are generally considered to be more susceptible to intramammary infection than Zebu cows (Sharma, 2003) [9]. Therefore, we aimed to study the relationship of indirect diagnostic tests, haematological parameters and milk composition with subclinical mastitis in lactating Deoni and HF crossbred cows.

2. Materials and Methods
2.1 Study area and animals
Present study was conducted on 32 lactating crossbred (Holstein Friesian × *Bos indicus*) and 27Deoni (*Bos indicus*) cows maintained at Livestock Research Centre (LRC), Southern Regional Station (SRS) of ICAR-National Dairy Research Institute (NDRI), Bengaluru (Karnataka). The experiment was conducted as per the guidelines and approval of Institute Animal Ethical Committee (IAEC). The climatic condition of the study area is of subtropical where temperature raises up to 36 ºC in summer and comes closer to 15 ºC during the winter season and the average rainfall ranges from 800-1200 mm and the maximum rain is received during July to October.

2.2 Feeding and milking management
All experimental cows were maintained under loose housing system. Animals were fed as per requirement through institute grown seasonal green fodders, dry fodder (2-3 Kg of ragi straw) and commercially available concentrates feed (Nandini gold-cow feed containing 16-18% crude protein, 70-72% TDN, 2.5-3.5% fat, 5.5-6% crude fiber, 1-1.5% acid insoluble ash and 10-11% moisture, M/s Karnataka Milk Federation, Bengaluru). About 3-5 kg of concentrate mixture was divided in equal proportion and fed at the time of morning and evening milking.

Deoni cows were milked by the milkers after partial suckling by their calves (1 min) for letdown of milk. In crossbred cows, bucket type machine milking was practiced. At the end of machine milking the residual milk was removed by hand milking to ensure complete removal of milk from udder. The milk yield (Kg) was recorded by electronic weighing balance. The average milk yield of Deoni and HF crossbred cows during the study period was 4 (range 3.4 to 4.6) and 12 (range 10.7 to 14.3) kg per day, respectively.

2.3 Collection of milk samples
All the four quarters were washed and dried with clean cotton towel before sampling of milk in each cow. Before sampling, initial two to three strips of milk were discarded from each quarter. Quarter level samples from these cows were screened with indirect diagnostic methods including, California Mastitis Test (CMT), differential Electrical Conductivity (DEC) and absolute EC (i.e. EC) methods.

2.4 Diagnostic tests
The milk SCC was estimated using a DeLaval cell counter (DCC; M/s DeLaval, Tumba, Sweden).Cows with a cow composite milk SCC of ≥200,000cells/ml was considered as having SCM, while cows without any abnormality in milk or udder parenchyma, systemic signs and milk SCC of ≤ 200,000 cells/ml was considered as healthy. The CMT was performed as per manufacturer's recommendation (M/s Immucell Corporation, Portland, USA, M/s DeLaval Pvt. Ltd, Pune).The EC of milk samples was detected through mastitis detector (Draminski™ 4Q; DEC) and EC-meter (M/s Oanki portable EC meter; absolute EC).

2.5 Estimation of milk fat and protein
Total 53 milk samples were collected from healthy (N=23) and subclinical (N=30) mastitis affected Deoni (N=28) and HF crossbred cows (N=25) during morning milking period. The milk samples were collected in 50 ml sterilized centrifuge tubes from individual animals at weekly interval for milk fat and protein estimation using Gerber's method (IDF 1991) and micro Kjeldhal's method using official methods of analysis of AOAC international (2000), respectively.

2.6 Blood sample collection
Total 60 blood samples (5 ml/animal) were collected from the jugular vein from 39 cows (14 Deoni and 25 HF crossbred cows), two times at weekly intervals for estimation of blood haematological parameters.

2.7 Statistical analysis
All the data were analyzed by linear mixed random effect model. The milk SCC data were log-transformed before the analysis and presented as untransformed data. Values are expressed as mean±SE and $p<0.05$ was considered as significant. All the analysis was done by using statistical software package SPSS version 16 (SPSS for windows, V16.0; M/s SPPS Inc., Chigago, IL, USA).

3. Results and Discussion
3.1 CMT, DEC and EC in healthy and SCM affected Deoni and HF crossbred cows diagnosed based on milk SCC
The CMT, DEC and EC levels in healthy and SCM affected Deoni and HF crossbred cows which are diagnosed based on milk SCC is presented in Table 1. We found that CMT and DEC were significantly differed between healthy and SCM affected cows in both the breeds. While, EC value was not differed between healthy and SCM affected cows in both the breeds. It indicated that DEC is better than direct EC method to differentiate the healthy from SCM affected cows (Ruegg, 2003) [7]. We also reported better efficacy of DEC than direct EC method based on the statistically significant agreement higher value between SCC and DEC than SCC and EC in these animals (Hallolli *et al.,* 2020) [3]. We also reported earlier in these animals that unlike SCC and CMT score, the DEC is not increased with severity of infections in HF crossbred cows (Hallolli *et al.,* 2020) [3] and cows with CMT score 2 may not reflect the actual ionic changes in terms of DEC and direct EC.

3.2 Relationship of CMT score with DEC, EC and SCC in healthy and SCM affected lactating dairy cows
Although SCC is most sensitive and specific method to diagnose SCM affected cows, it is not used very commonly in India due to cost and other production related conditions. But CMT is used commonly due to rapid, easy technique and lesser cost involvement with reagent. However, the relationship of CMT score with other methods are not clear and thus we explored relationship of CMT score with DEC, EC and SCC in Deoni and HF crossbred cows (Table 2 and Table 3 respectively). Irrespective of confirmed udder health status by milk SCC, the increasing CMT score have correspondingly increased the SCC level in cows. In Deoni cows, true healthy (by CMT and SCC methods) animals had lesser SCC (59,840 cells/ml), but the CMT score in Deoni cows may show as negative (i.e., false negative) when the animals having SCC up to 424.61x10^3 cells/ml. The average milk SCC of true positive Deoni cows (by CMT and SCC methods) was 571x10^3 cells/ml. The corresponding SCC value in true healthy HF crossbred cows was 98,169 cells/ ml but HF crossbred cows may show CMT negative when these animals having SCC up to632.73x10^3 cells/ml. Further, the CMT score of 1 and 2 in HF crossbred cows had 932.65 and

1004.80x10^3 cells/ml, respectively. Although, this relationship of CMT score with SCC range was known fact in exotic animals (Leslie *et al*., 2002), the same has not been established in Indian dairy cattle. Although, Leslie *et al*., 2002 reported higher sensitivity (88.5%) and specificity (86.2%) of CMT, other studies (Dingwell *et al.*, 2003; Roy *et al*., 2008) [3, 14] reported that CMT is qualitative and subjective and thus CMT has lesser sensitivity (61-69%) and specificity (65-68%). Therefore, the observed false negative results in this study substantiate the possible error by CMT. Incorrect ratio between CMT reagent and milk, inadequate mixing of reagent and milk by proper swirling and subjective variation in assigning CMT score based on gel formation are some of the most common reasons for error and consequent reduced sensitivity and specificity of CMT. Physiological variations during early lactation and dry off period makes CMT is not ideal during this period, though it can be used as screening tool for further testing such as microbial culturing, etc., (Dingwell *et al*., 2003) [3]

3.3 Relationship of individual cow SCC with Bulk Milk SCC (BMSCC) in lactating dairy cows

The relationship of individual cow SCC (ICSCC) with BMSCC is presented in Fig. 1. Although ICSCC from healthy cows was different from SCM affected cows, when they pooled together (i.e., healthy and SCM affected milk mixed together), the higher SCC from SCM affected cows were significantly reduced in bulk milk due to dilution effects. The BMSCC was observed below accepted threshold value (i.e., 400000 cells/ml) of human consumption as per EU standards. Further, the BMSCC of 377,166 cells/ml was associated with 25-30% prevalence of SCM in this study. BMSCC is an overall indicator of milk quality (Norman *et al*., 2000) [5] and increased level of BMSCC is associated with increased mastitis incidence while, low level BMSCC at farm level is associated with low level prevalence of infection caused by major mastitis pathogen (Schukken *et al*., 2012) [10]. BMSCC depends on SCM prevalence and new infections rateamong lactating animals in a herd and BMSCC is highly correlated with SCM prevalence and thus it can be used for estimation SCM status in dairy farms (Lukas *et al*., 2005) [11]. Kelly *et al.* (2009) [8] reported that dry cow therapy, post-milking teat disinfection and frequent cleaning were associated with low BMSCC.

3.4 Blood haematology and milk Fat: Protein ratioin healthy and SCM affected lactating dairy cows

Hematological parameters in healthy and SCM affected lactating Deoni and HF crossbred cows are presented in Table 4. We found that Red Blood Cell (RBC) counts, Total Leucocytes Count (TLC) and Differential Leucocytes Count (DLC) were not altered by breed and udder health status of Deoni and HF crossbred cows. We found that breed had significant (P<0.001) effects on blood hemoglobin and PCV level. Garba *et al*., (2019) [4] reported no significant difference in hematological parameters between mastitic and non-mastitic goats. Similarly, Siddiqe *et al.*, (2015) [20] reported no significant difference in haematological parameters between healthy and SCM affected cows. In contrast, other few studies (Sedek *et al.*, 2017; Choudhary *et al.*, 2019) [16, 2] reported significantly (p<0.05) higher TLC with neutrophilia and lymphopenia conditions in SCM affected dairy cows. Significantly lesser Hb, PCV and TEC and higher TLC and neutrophils along with lesser lymphocytes count were observed in SCM affected buffaloes (Krishnappa *et al.*, 2016) [9].

Milk fat, protein and its ratio in healthy and SCM affected lactating Deoni and HF crossbred cows are presented in Fig. 2. We found that fat percentage was significantly differed between breeds (P=0.02) and udder health status (P=0.03). Though the fat: protein ratio was statistically non-significant, we observed higher trend of fat: protein ratio in SCM affected than healthy HF crossbred (1.55 Vs 0.95 respectively) and Deoni cows (1.78 Vs 1.46 respectively). Jamrozik and Schaefer (2012) [4] reported that cows with mastitis had larger F:P. They suggested that the difference of F:P between healthy and infected cows was most profound during early lactation due to more energy deficiency. They also found that the heritability of F: P ratio was larger and suggested as a potential trait for selection against mastitis. Toni *et al*., (2011) observed no significant effect of F:P on the incidence of CMin dairy herds.We observed more fat percentage in SCM affected cows. Ogola *et al*. (2007) [6] observed no difference in the Kjeldahl values for total protein percentage in between healthy and SCM affected cows as observed in our study. Bruckmaier *et al*. (2004) [1] and Gonçalves *et al*. (2016) [2] also find no significant changes on protein level in between normal and SCM affected cows.

Table 1: CMT, DEC and EC in healthy and SCM affected Deoni and HF crossbred cows diagnosed based on milk SCC

Tests	Deoni		HF Crossbred	
	Healthy	SCM	Healthy	SCM
CMT	1.02^a±0.04	1.22^b±0.05	1.00^a±0.07	1.44^b±0.05
DEC	36.00^a±5.24	68.33^b±6.72	27.45^a±4.98	59.00^b±3.82
EC	3.78±0.12	4.59±0.16	4.08±0.13	4.60±0.10
*SCC (x100 cells/ml)	624.66^a±177.78	4571.48^b±22951	981.69^a±48520	7391.22^b±372.34

Values are mean±SE. Mean values bearing different superscript within breed differ significantly. In Deoni, the P value for DEC, EC, SCC is 0.00, while for CMT is 0.05. In HF crossbred cows, the P value for CMT, DEC, and SCC is 0.01; while, P value for EC is 0.03 by general linear model- univariate method. *SCC cut-off value of 200x10^{3}cells/ml used to differentiate healthy from SCM affected cows.

Table 2: Relationship of CMT score with DEC, EC and SCC in Healthy and subclinical mastitis affected Deoni cows

CMT score	Healthy			SCM*		
	DEC	EC	**SCC (×10^{2}cells/ml)	DEC	EC	SCC (×10^{3}cells/ml)
0	35.23^a±5.14 (44)	3.77±0.12 (44)	598.40^a±171.69 (44)	60.24^b±7.4 (21)	4.52±0.18 (21)	424.61^b±24.85 (21)
***1				96.64±13.93 (6)	4.83±0.34 (6)	571.00±46.49 (6)

*False negative samples by CMT (i.e., SCC was >200x10^3 cells/ml, but CMT score was 0). Values are mean±SE. **SCC of true negative (i.e., healthy) by CMT differ significantly with false negative SCC (P<0.05). ***true positive SCM cases and their corresponding DEC, EC and SCC.

Table 3: Relationship of CMT with DEC, EC and SCC in Healthy and subclinical mastitis affected HF crossbred cows

CMT score	Healthy			SCM*		
	DEC	EC	**SCC ($\times 10^2$cells/ml)	DEC	EC	SCC($\times 10^3$ cells/ml)
0	27.45^{a}±4.48	4.06±0.14	981.69^{a}±458.80	52.25^{b}±4.55 (59)	4.55±0.12 (59)	632.73^{b}±43.12 (59)
***1				81.00±7.48 (20)	4.85±0.22 (20)	932.65±74.68 (20)
***2				55.85±11.10 (11)	4.50±0.32 (11)	1004.80±105.62 (11)

Values are mean±SE. *False negative samples by CMT (i.e., SCC was >200x10^3/ml, but CMT score was 0). **SCC and DEC of true negative (i.e., healthy) by CMT, differ significantly with false negative DEC and SCC (p<0.001), respectively. ***True positive SCM cases and their corresponding DEC, EC and SCC.

Table 4: Hematological parameters in healthy and subclinical mastitis affected Deoni and HF crossbred cows

Parameters	Deoni		HF crossbred cows		Average	
	Healthy	SCM	Healthy	SCM	Healthy	SCM
RBC (10^6 cells/µL)	6.00±0.19 (12)	5.46±0.19 (13)	4.63±0.19 (8)	4.78±0.12 (27)	5.45±0.19 (20)	5.00±0.15 (40)
Hemoglobin (%)	7.58±0.21 (12)	7.53±0.20 (13)	6.62± 0.26 (8)	6.46±0.13 (27)	7.10±0.23 (20)	6.99±0.16 (40)
MCV (fL)	47.41±1.21 (12)	50.38±1.10 (13)	51±1.4 (8)	50±0.79 (27)	49.20±1.30 (20)	50.19±0.94 (40)
MCH (pg)	13.00±0.31 (12)	13.69±0.20 (13)	13.87±0.3 (8)	13.67±0.20 (27)	13.43±0.35 (20)	13.53±0.20 (40)
MCHC (g/dL)	27.33±0.23 (12)	27.30±0.22 (13)	27.37±0.28 (8)	27.39±0.15 (27)	27.33±0.25 (20)	27.34±0.18 (40)
PCV (%)	27.41±0.70 (12)	27.39±0.68 (13)	23.75±0.86 (8)	23.78±0.46 (27)	25.58±0.57 (20)	25.58±0.58 (40)
TLC (x10^3 cells/mm^3)	9.44±0.43 (12)	8.06±0.42 (13)	8.66±0.64 (8)	10.14±0.5 (27)	9.13±0.53 (20)	9.48±0.44 (40)
Neutrophils (%)	42.42±2.41 (12)	43.15±1.8 (13)	50.0±2.09 (8)	46.52±1.4 (27)	45.45±2.3 (20)	45.13±1.6 (40)
Lymphocytes (%)	45.25±2.23 (12)	44.38±1.9 (13)	39.88±2.14 (8)	40.04±1.9 (27)	43.10±2.8 (20)	41.45±1.5 (40)
Monocytes (%)	8.92±0.49 (12)	9.38±0.53 (13)	9.25±0.54 (8)	10.75±0.4 (27)	9.05±0.51 (20)	10.38±0.5 (40)
Eosinophils (%)	3.4±0.69 (12)	3.27±0.66 (13)	2.12±0.84 (8)	3.03±0.45 (27)	2.76±1.11 (20)	3.12±0.55 (40)
Platelets (x 10^3 cells/mm^3)	410.92±34.18 (12)	456.31±32.842(13)	390.63±41.87 (8)	437.41±22.38 (27)	433.61±28.28 (20)	414.02±32.12 (40)

Values are mean±SE. figures in parenthesis are number of samples. Where SCM means subclinical mastitis. Breed had significant effects on haemoglobin and PCV (*p*<0.001)

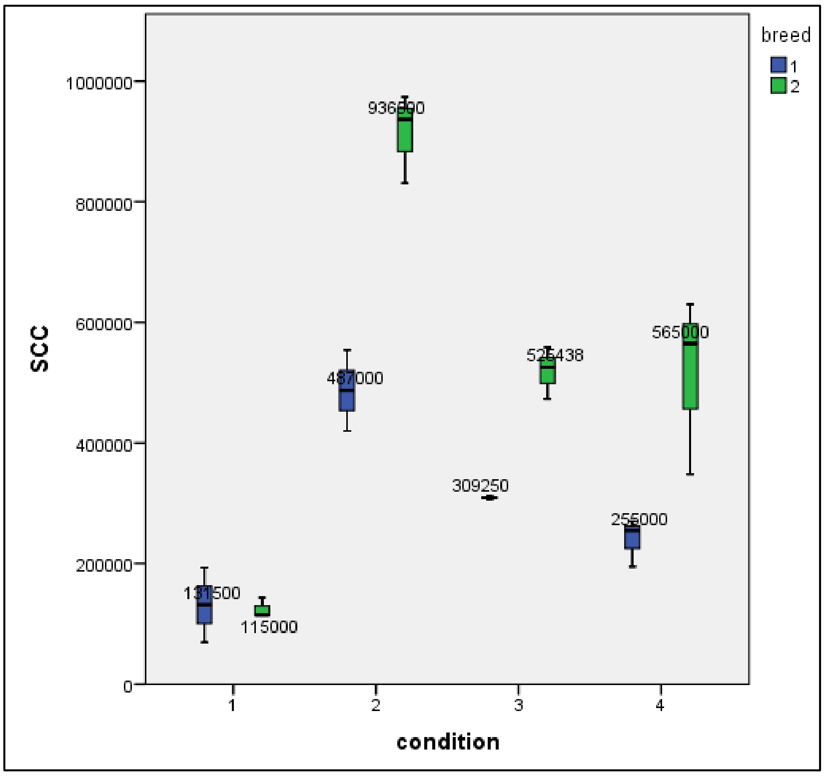

Fig 1: Relationship of individual cow SCC with Bulk milk SCC. Values are mean±SE. Where Condition 1: Healthy, 2: subclinical mastitis, 3: individual cow SCC, 4: bulk milk SCC; breed 1: Deoni, 2: HF crossbred cows

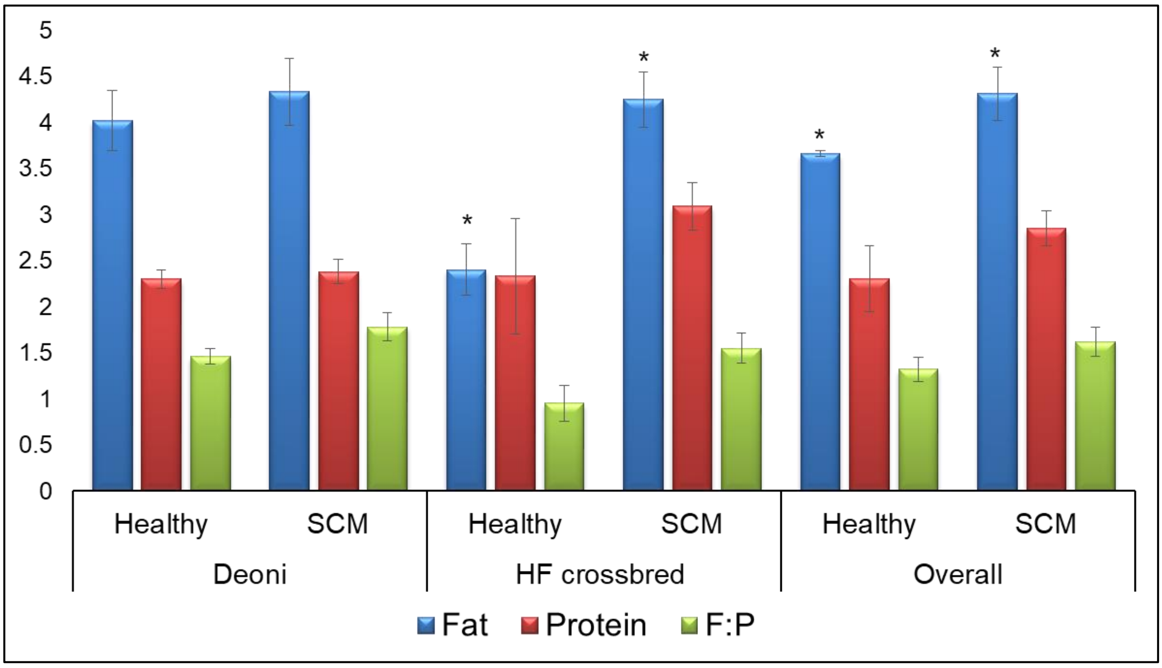

Fig 2: Milk fat, protein and its ratio in healthy and subclinical mastitis affected Deoni and HF crossbred cows. Values are mean±SE. *fat percentage differ significantly between breeds (*P*=0.02) and udder health status (*P*=0.03). SCM: subclinical mastitis

4. Conclusion

Although the relationship between SCC and CMT was established in exotic dairy cattle, this study established the relationship of CMT score and other indirect diagnostic tests with SCC for lactating Indian dairy cattle. This finding will be useful for diagnosis of subclinical mastitis using indirect diagnostic methods in Indian dairy cattle.

5. Acknowledgments

The authors are thankful to Director, ICAR-NDRI, and Head, SRS of ICAR-NDRI for providing needful facilities. Authors are also thankful to in-charge, veterinarian and staffs of LRC, SRS of ICAR-NDRI for their support and assistance.

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