



Evaluation of indirect diagnostic tests and PBMC expression of innate immune genes in subclinical mastitis in dairy cows

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ABSTRACT

The aim of this study was to estimate the prevalence of subclinical mastitis (SCM), its innate immunity and to compare the efficacy of indirect diagnostic tests [California mastitis test (CMT), differential electrical conductivity (DEC), absolute EC, enzyme based color detection method (Tanucheck kit) and brothymol blue strip (BTB) tests] to milk somatic cell count (SCC) method using 200×10^3 cells/ml as a cut off value in Deoni (N=27) and HF crossbred (N=32) cows. Cumulative prevalence of SCM was 38% and 63% in Deoni and HF crossbred cows, respectively on milk SCC basis (N=215). Breed, udder health status and its interaction had significant effect on SCC level. The overall sensitivity and specificity of above indirect tests were 38 and 99%; 52 and 72%; 52 and 69%; 76 and 31%, and 1 and 100%, respectively. mRNA expression of serum amyloid A (SAA), IL-1 β and TNF- α genes were significantly up-regulated while, TNF- α gene was significantly down-regulated in PBMC of SCM affected HF crossbred and Deoni cows, respectively. It is concluded that, DEC is more suitable indirect method to detect SCM and mRNA expression of SAA and TNF- α was strongly related to SCM in HF crossbred and Deoni cows, respectively.

Keywords: Dairy cows, Indirect diagnostic tests, Innate immune genes expression, Somatic cell count, Subclinical mastitis

Mastitis is the second most costly disease in India next to foot and mouth disease (Varshney and Mukherjee 2002). It causes the huge economic losses to dairy farmers and industry through reduced milk production, milk quality, treatment and culling cost. The estimated annual economic loss due to subclinical mastitis (SCM) is higher (58–72%) than clinical mastitis (CM; 28–42%) in India (Dua 2001, Bansal and Gupta 2009). The importance of SCM is due to its more prevalence, it remains the source of infection for herd mates, its longer duration of infections, it usually proceeds into clinical form, its difficulty of detection and its adverse effect on milk yield and its quality (Seegers *et al.* 2003). However, SCM has been rarely addressed in India due to unorganized, small holder dairy production system, lack of knowledge and infrastructure, and poor economic status of dairy farmers.

Meta-analysis studies from India revealed the prevalence of SCM on cow and individual quarter basis, as 41–46% and 23–27%, respectively (Bangar *et al.* 2015,

Krishnamoorthy *et al.* 2017). They observed heterogeneity between studies could be due to difference in sampling and diagnostic methods, parity, lactation stage and genetic makeup of animals, agroclimatic conditions and farm management practices. The prevalence rate of SCM in India was mostly studied through cow-side tests like CMT and other detergent based diagnostic methods which are simple, less expensive and less time-consuming methods. Although, several researchers compared the efficacy of CMT and other indirect tests to standard SCC or milk culture-based methods (Sharma *et al.* 2010, Badiuzzaman *et al.* 2015, Iraguha *et al.* 2017), they found differential efficacy of CMT compared to standard milk SCC method. But studies on comparative efficacy of CMT, EC based tests, pH- and enzyme-based methods in relation to milk SCC are few in India. Moreover, the herd level prevalence of SCM should be estimated at fortnightly or monthly interval through repeated sampling for better understanding about new intramammary infections (IMIs), which is rarely followed in India (Osteras and Solverod 2009).

In general, SCM is resolved by body's immune system and failure of which, often leads to development of CM. Therefore, appropriate early immune responses to mastitic pathogens are very important and it is mostly mediated neutrophils and cytokines. Upon detecting pathogens, the

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inflammatory cells secrete pro-inflammatory cytokines such as IL-1 β and TNF- α , which initiate the recruitment of neutrophils to mammary gland and also enhance its functionality for clearance of udder infections (Oviedo-Boyso *et al.* 2007). We hypothesized that the prevalence and innate immune response could vary between crossbred and indigenous (Deoni) cows and hence the present study was aimed to estimate the prevalence of SCM, its innate immunity and to compare the efficacy of indirect diagnostic tests to milk SCC method in Deoni and HF crossbred cows. We also studied the influence of breed, stage of lactation, parity and diurnal sampling on milk SCC level in these cows.

MATERIALS AND METHODS

Study area and animals: Present study was conducted on 32 lactating crossbred (Holstein Friesian $\times$ *Bos indicus*) and 27 Deoni (*Bos indicus*) cows maintained at Livestock Research Centre, Southern Regional Station of ICAR-National Dairy Research Institute, 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°C in summer and comes closer to 15°C during the winter season and the average rainfall ranges from 800 to 1,200 mm and the maximum rain is received during July to October.

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 fibre, 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.

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. Total 60 ml of milk from all four quarters and pooled milk of individual cows were collected aseptically, separately. Total 215 cow level milk samples were collected at weekly intervals (3–5 samples/cow) from apparently healthy Deoni (27 cows; 72 samples) and HF crossbred (32 cows; 143

samples) cows from different stage of lactation (early: 59, mid: 61, late: 95 samples) and parity (one: 61, two-four: 110, >4: 44 samples) during morning (n=90) and evening (n=125) milking times for milk SCC estimation. Quarter level samples from these cows were screened also with indirect diagnostic methods including, California mastitis test (CMT), differential electrical conductivity (DEC) and absolute EC (i.e. EC) methods, Tanucheck kits and brothymol blue (BTB) strip methods.

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 $\geq 200,000$ cells/ml were considered as having SCM, while cows without any abnormality in milk or udder parenchyma, systemic signs and milk SCC of $\leq 200,000$ cells/ml were 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). If DEC is ≤ 50 units (i.e. the difference between highest unit quarter and rest of any three-quarter units), it was considered as healthy; whereas the difference of ≥ 50 units were considered as SCM affected quarters. EC values of ≥ 4.5 (mS/cm) was considered as SCM affected quarters. The substrate present in the strip of Tanuchek kit changes to blue colour by the membrane bound enzymes released from somatic cells, which was compared with colour card provided with kit. BTB strip test was done as per manufacturers recommendations.

Estimation of serum amyloid A (SAA) concentration and relative expression of SAA and cytokines genes in PBMC of healthy and subclinical mastitic cows

Blood collection and assays: Blood samples were collected from jugular vein of healthy and SCM affected Deoni and HF crossbred cows (n=6 from each groups) using vacutainer (Vacurette®, Greiner Bio-one GmbH, Austria) containing EDTA as an anticoagulant. Immediately after collection, blood samples were centrifuged at 4°C (1,500 g for 20 min), plasma was separated, and stored in cryovials at –20°C till ELISA. The concentration of SAA was estimated using commercially available bovine specific ELISA kit as per manufacturer's recommendations (M/s Uscn Life Science Inc., Wuhan, Hubei, China). For gene expression study, peripheral blood mononuclear cells (PBMC) were harvested using ice cold 1 $\times$ RBC lysis buffer through centrifugation at 3,500 rpm for 25 min at 4°C (twice).

Sample preparation for RNA isolation: The total RNA was isolated from PBMC pellet using GeneJET Whole Blood RNA Purification Mini Kit (M/s Thermo Scientific, Lithuania) as per manufacturer's protocol with slight modifications.

cDNA synthesis: The total RNA was reverse transcribed into cDNA using Maxima first strand cDNA synthesis kit for Real time quantitative polymerase chain reaction (RT-

qPCR) (M/s Thermo Scientific, Lithuania) as per manufacturer's protocol with slight modifications. The product of the first strand cDNA synthesis was diluted to a final concentration of 25 ng/ μ L with nuclease free water and 2 μ L of diluted cDNA was used for each reaction in qPCR.

Quantitative RT-PCR analysis: The relative expression of selected genes was studied using SYBR green chemistry (M/s Maxima SYBR green qPCR master mix, Fermentas, USA). The 20 μ L reaction was carried out in duplicates using 50 ng of template and 0.5 μ M primer concentrations. The real time qPCR reaction conditions were: enzyme activation at 95°C for 10 min and amplification cycle (40 cycles; initial denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec). The beta actin gene was used as an internal control and the relative gene expression was calculated using the formula Δ CT (Livak and Schmittgen 2001). The results were expressed in fold changes (control=1 fold) in SCM infected cows as compared to apparently healthy cows.

Statistical analysis: Influence of breed, stage of lactation, parity and diurnal sampling on milk SCC in healthy and SCM was analyzed by linear mixed random effect model. The milk SCC data were log-transformed before the analysis and presented as untransformed data. Sensitivity (Se), specificity (Sp) and accuracy of various indirect diagnostic methods compared to milk SCC were calculated as per Sharma *et al.* (2010). The efficacy of EC and DEC methods and their threshold values were calculated by receiver-operating-characteristic (ROC) curve analysis considering SCC as reference test. Though, ROC analysis produces range of potential threshold values, the values with highest corresponding combined Se and Sp was considered as critical threshold value of diagnostic tests. Kappa analysis and Pearson correlations were done to determine the level of agreement and correlation, respectively between CMT, DEC, and EC with milk SCC. Values are expressed as mean $\pm$ 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 SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Prevalence of subclinical mastitis in cows: The repeated screening of milk samples through DCC method revealed

the cumulative prevalence of SCM as 38 and 63% in Deoni and HF crossbred cows, respectively. The overall cumulative prevalence of SCM in these cows was 50%. Several researchers reported the observed prevalence of SCM in India (Mukherjee and Dash 2003, Patel and Tripathi 2018) and in other countries (Mdegela *et al.* 2009, Rahman *et al.* 2010).

Influence of breed and other physio-environmental factors on milk SCC in healthy and subclinical mastitis affected cows: We found significant influence of breed ($P=0.001$) and udder health status ($P=0.001$), but not its interaction ($P=0.40$) on milk SCC (Fig. 1a). Several researchers found lesser milk SCC in indigenous cow as compared to HF crossbred cows as also found in our study (Alhussien *et al.* 2016a; 2016b). Lesser prevalence of SCM in Deoni compared to HF crossbred cows could be the reason for this effect (Kathiriyia *et al.* 2014, Hoque *et al.* 2015). The observed variations of milk SCC in these cows could be the possible reason for lack of interactive effects between breed and udder health status.

We found no significant influence of stage of lactation (Fig. 1b), parity (Fig. 1c) and diurnal sampling (Fig. 1d) on milk SCC in healthy and SCM affected Deoni and HF crossbred cows. Likewise, several researchers found no influence of these factors on milk SCC in Indian dairy cattle (Syridion *et al.* 2012, Fahim *et al.* 2017, Alhussien and Dang 2018).

Comparative efficacy of indirect diagnostic methods for subclinical mastitis: Comparison of CMT, DEC, EC, Tanucheck kits and BTB tests with DCC method revealed the Se and Sp as 38% and 99%, 52 and 72%, 52 and 69%, 76 and 31%, 1 and 100%, respectively (Supplementary Fig. 1a). The observed variation of Se and Sp with other studies could be due to species, breed, parity and stage of lactation, sampling method, type of pathogens, etc. (Bansal *et al.* 2007, Sharma *et al.* 2010, Tiwari *et al.* 2018). The lesser Se and higher Sp of CMT is also reported by other researchers (Ruegg 2003, Aarsharaj *et al.* 2017). Subjective interpretation of CMT results and difference in reference methods could be the possible reasons for such variations. Though it is limited number of samples, we observed 53% of Se in crossbred cow and 22% in Deoni cows. Therefore, influence of breed on this differential Se cannot be ruled out and it could be due to lesser trend of SCC in healthy udder secretion of Deoni than crossbred cows. Although,

Table 1. Primers for gene-specific Real Time-PCR

Primer name	Primer sequence	T _m	Size (bp)	A.C. No
BovTNF α -F	CTCTTCTGCCTGCTGCACTTC	66.9	205	NM_173966.3
BovTNF α -R	CCATGAGGGCATTGGCATAACG	71.6		
BovIL-1 β -F	AGCATCCTTTCATTCATCTTTGAAG	65.6	78	NM_174093.1
BovIL-1 β -R	GGGTGCGTCACACAGAAACTC	67.4		
BovSAA3-F	GGTGCCTGGGCTGCTAA	65.2	62	NM_181016.3
BovSAA3-R	GGGTCTGTGATTCCCTGAATAGTCT	66.3		
BovACTB-F	AGGCATCCTGACCCTCAAGTA	65.2	145	NM_173979.3
BovACTB-R	GCTCGTTGTAGAAGGTGTGGT	63.5		

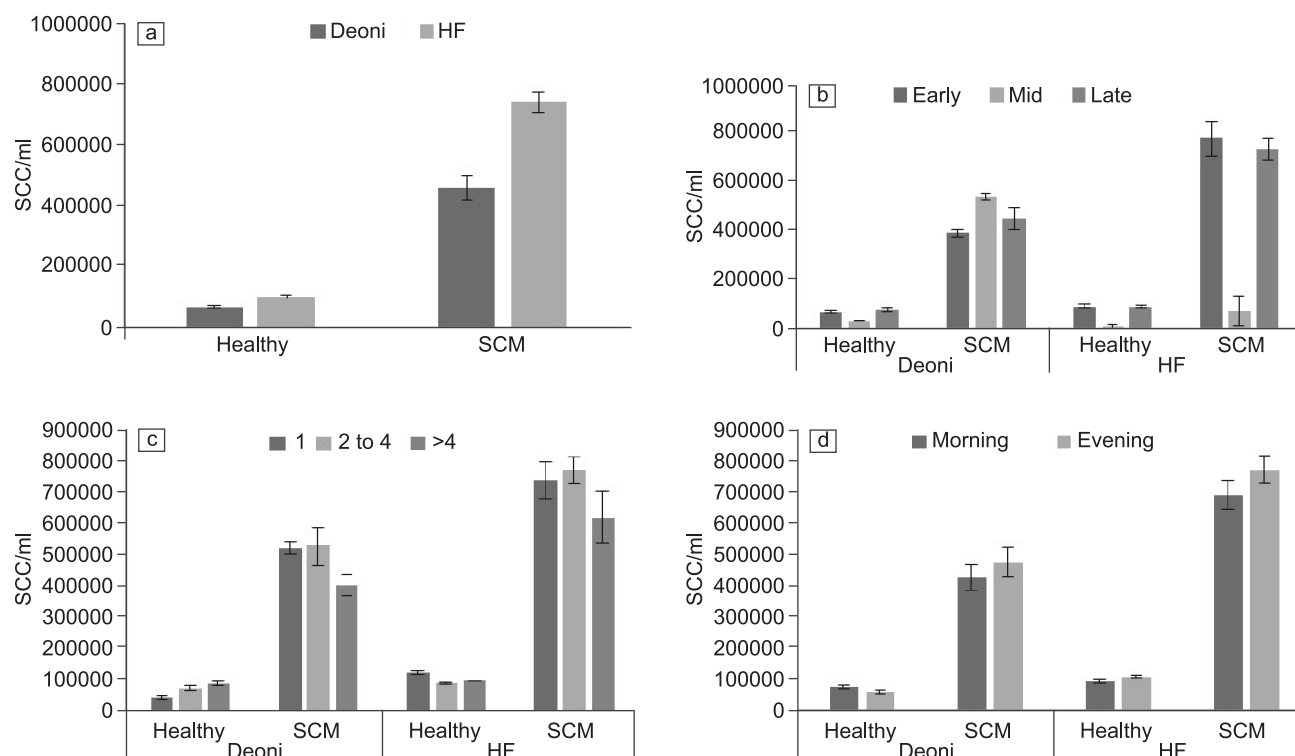


Fig. 1. Influence of breed (1a), stage of lactation (1b), parity (1c) and diurnal sampling (1d) on milk SCC in healthy and subclinical mastitis (SCM) cows. Breed ($P=0.001$) and udder health status ($P=0.001$), but not its interaction ($P=0.40$) had significant effects. Values are mean $\pm$ SE of 98 healthy and 117 SCM milk samples from Deoni ($n=45$ and 27 respectively) and HF crossbred cows ($n=53$ and 90 respectively).

high-producing HF crossbred cows are expected to have higher trends of milk SCC due to their more sensitivity to IMIs, the selected cutoff value of milk SCC (i.e. 200000/ml) may not be an accurate threshold level for HF crossbred cows. Although we considered that observations made in this study at each week were independent of time, further studies is warranted in larger population at given period of time for estimation of true prevalence rate and accuracy of various indirect tests. For instance, Jadhav *et al.* (2018) reported SCC of 310,000 cells/ml of milk as threshold value with 92.6% Se and 91.5% Sp for the diagnosis of SCM in HF crossbred cows.

Among two EC based methods, DEC had more efficacy than absolute EC as also reported by Ruegg (2003). The observed higher values of AUC in these cows (irrespective of breed) for DEC than absolute EC, further substantiate the better accuracy of DEC to discriminate the SCM affected quarters from healthy quarters (Supplementary Fig. 1b). DEC was also found to be in better agreement and correlation with SCC, though the level of agreement was low (Table 2). Altogether, it indicated that DEC is better than absolute EC method to differentiate the healthy from SCM affected cows (Ruegg 2003). Nielen *et al.* (1992) also reported that the DEC would reduce the intrinsic variation and thus improve the Se and Sp. Although, EC based methods could differentiate the healthy from infected quarters, their magnitude of changes between severe (CMT score 2) and less severe cases (CMT score 1) is not different,

Table 2. Statistical agreement between CMT, DEC and EC with SCC

Screening test	Kappa value	Pearson correlation
CMT	0.15**	0.50**
DEC	0.32**	0.39**
EC	0.25**	0.23**
TC	0.09	0.09
BTB	0.003	0.04

**Correlation is significant at the 0.01 level (2-tailed).

particularly in HF crossbred cows (data not presented). It revealed that, ionic changes in milk reach some saturation points and thus DEC or EC based methods may not be useful in very severe cases of SCM. The EC depends on the number of ions in milk, which increase much later than SCC (Spakauskas *et al.* 2006) and hence EC based methods should not be used as the sole method of diagnosis in cows with high milk SCC (Pyorala 2003). Under this situation, combined methods may be useful for diagnosis of SCM. We also reported earlier that combinations of two diagnostic tests increased the predictive ability of IMIs (Syridion *et al.* 2012).

The established critical threshold values of EC and DEC based on maximum combined Se and Sp was 4.01 mS/cm and 25 units, respectively (Table 3). Kamal *et al.* (2014) also reported EC value of 4–5.5 mS/cm in normal milk and more than 5.3 mS/cm in SCM affected cows.

Table 3. Threshold values of milk EC and DEC by ROC analysis

Threshold values		EC		DEC	
EC	DEC	Sensitivity	Spfecity	Sensitivity	Spfecity
1.03	9.0	1.00	0.00	1.00	0.00
2.05	15.0	1.00	0.01	0.93	0.23
2.51	25.0	1.00	0.07	0.76	0.62
3.03	32.5	0.94	0.19	0.65	0.70
3.52	45.0	0.90	0.33	0.60	0.70
4.01	52.5	0.77	0.52	0.48	0.73
4.51		0.54	0.72		0.82

Threshold value that has the highest corresponding combined Se and Sp is critical threshold value.

Level of serum amyloid A (SAA) and its mRNA expression in healthy and subclinical mastitis affected HF crossbred cows: SCM affected cows had significantly ($P<0.05$) higher plasma concentration of SAA than healthy HF crossbred cows (24.31 ± 3.41 Vs 34.17 ± 3.88 ng/ml). SAA is a major acute phase proteins in cattle and effective marker of mastitis (Murata *et al.* 2004, Petersen *et al.* 2004). Several researchers found higher SAA concentration in mastitis affected cattle (Fathi and Farahzadi 2011, Hussein *et al.* 2018) as observed in our study. The significantly higher expression of SAA in PBMC of SCM affected crossbred cows further substantiates the role of SAA in mastitis (Supplementary Fig. 2a).

Relative expression of cytokines genes in PBMC of healthy and subclinical mastitis affected cows: The expression of TNF- α gene was significantly ($P<0.05$) down-regulated in SCM affected Deoni cows. In contrast, TNF- α and IL-1b genes (Supplementary Fig. 2b and 2c) were significantly ($P<0.05$) up-regulated in SCM affected HF crossbred cows. The lower expression of cytokine genes could be due to lesser load of intra mammary infections as evidenced by lower level of milk SCC and lesser prevalence of SCM in Deoni cows. Under this circumstance, the immune gene might have down-regulated (Verma *et al.* 2018). Several studies reported up-regulation of cytokines and SAA genes in mastitis affected crossbred cows as observed in this study (Rainard and Riollot 2006, Molenaar *et al.* 2009).

Based on the findings of this study, it is concluded that the prevalence of subclinical mastitis is lesser in indigenous cows (Deoni) compared to HF crossbred cows. Among the various indirect diagnostic methods, DEC is found to be more suitable method to detect SCM in these cows and mRNA expression of SAA and TNF- α genes are strongly related to SCM in HF crossbred and Deoni cows, respectively.

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