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SHORT COMMUNICATIONS

Incidence of mastitis and activity of milk neutrophils in Tharparkar cows reared under semi-arid conditions

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Abstract Rearing of indigenous Tharparkar (TP) cows (native of arid Thar deserts) under high humid conditions (>75 % humidity) has increased the incidence of mammary infections in them. A study was undertaken to see the number, activity, and expression of milk neutrophils isolated from healthy and mastitic cows. There was a significant ($P < 0.05$) influx in milk somatic cell counts (SCC) and neutrophils in sub-clinical and clinical mastitis cows. No change was observed in the phagocytic activity (PA) of milk neutrophils between healthy and sub-clinical mastitis (SCM) cows, but these activities decreased significantly ($P < 0.05$) in clinical cases. Chemotactic activity showed a significant difference between all the groups. Lactose varied significantly ($P < 0.05$) between healthy, sub-clinical, and clinical mastitis (CM) cows. Expression of chemokine receptor (CXCR1) was more in mastitis cows and also higher as compared to CXCR2. No change was observed in cluster of differentiation molecule (CD62L) among all the three groups of TP cows. Expression of interleukin (IL-8) and CD11b was low in healthy cows, increased significantly ($P < 0.05$) in both sub-clinical and mastitis cows. This study indicates that low producing TP cows are also prone to mammary infections when reared under semi-arid conditions.

Keywords Tharparkar · Semi-arid · Mastitis · Milk · Neutrophils · Activity · Expression

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Introduction

Tharparkar (TP) is a dual purpose breed known for both its milking and draft potential. The home tract of this breed is in the Tharparkar district of southeast Sindh in Pakistan. The climate of Thar region is characterized by low humidity, meager rainfall (100–450 mm/year; ~90 % during July–September), and extreme temperatures (often >45 °C in the peak of summer and sub-zero in winter). In India, these animals are found along the Indo-Pak border covering western Rajasthan and up to Rann of Kutch in Gujarat. Crossbreeding of indigenous Tharparkar breed with temperate dairy breeds like Holstein was undertaken to combine high milk yield and early maturity of European dairy breeds with hardiness, disease resistance, and adaptability of local cattle (Taneja 1999). Tharparkar cattle are known to be hardy and presumed to be resistant to several tropical diseases. It is widely known that these cows are less affected by mastitis as they are low yielders (1800 to 2600 kg per lactation).

Climate at the experimental area where these TP cows are being kept has been classified into three different zones throughout the year viz October to March with mean THI 56.71–73.21, April to September with mean THI 75.39–81.60, and in the months of May and June with mean THI 80.27–81.60 (Dash et al. 2015). Rearing these cows under more humid conditions (>75 % humidity) has increased the incidence of mammary infections in them. Feeding of micronutrients to dairy cows increases the activity of neutrophils (Dang et al.

2012), but, being low producers, the dairy farmers do not provide these TP cows with sufficient nutrients. What is the effect on the activity and expression of milk neutrophils of TP cows when they are reared under semi-arid regions where annual rainfall is about 704 mm/year is not known? Therefore, this study is an attempt to understand the changes occurring in the activity of milk neutrophils of low producing TP cows so that further management interventions can be incorporated for better production of these cows.

Materials and methods

For the present investigation, a total of thirty (30) purebred multiparous TP cows maintained at Livestock Research Centre of National Dairy Research Institute, Karnal, Haryana, India, were screened for mastitis. Milk was collected hygienically after cleaning the udder, and somatic cell counts (SCC) were measured by somatic cell counter. Cows having SCC up to 1.5×10^5 cells/milliliter of milk were grouped as healthy, cows having somatic cells from 1.5 to 3.5×10^5 cells/milliliter of milk were grouped as suffering from sub-clinical mastitis (SCM), and cows having somatic cells more than 3.5×10^5 cells/milliliter of milk were grouped under the clinical mastitis (CM) group. Differential cell counting of milk was carried out to determine the presence of different cell types like lymphocytes, neutrophils, and macrophages in milk. Milk samples were also analyzed for presence of probable mastitis causing organisms by pour plating method. Mainly the samples were analyzed for the presence of *S. aureus*, *E. coli*, *S. agalactiae*, and total bacterial load. Briefly, the samples were serially diluted up to 10^{-7} dilutions. Nutrient agar was used for total bacterial load, Baird Parker agar used for *S. aureus*, eosin methylene blue (EMB) agar for *E. coli*, and blood agar for *S.agalactiae*. After that, incubation was done at 37 °C for 24–48 h until the appearance of colonies. Samples which were positive only for *S. aureus* were selected for further processing.

The in vitro phagocytic activity (PA) of milk neutrophils was estimated by nitro blue tetrazolium (NBT) assay. For this, neutrophils were isolated through density gradient centrifugation using Histopaque 1119 and Histopaque 1077. Neutrophils were collected at the interface of the Histopaque 1119 and Histopaque 1077 layers. These neutrophils were then washed 3 times in PBS (300 × g, 10 min, 4 °C) and suspended in Roswell Park Memorial Institute (RPMI) media for further analysis. Viability of isolated cells was checked by trypan blue exclusion assay. The cell suspension (neutrophils) was adjusted to 5×10^6 live cells/milliliter by the culture media (RPMI 1640). About 100 μl of the diluted cell suspension per well in triplicate was placed in a 96-well flat-bottomed tissue culture plate. The cells were allowed to proliferate with Zymosan (650 μg/ml) and NBT (250 μg/ml) concentrations that had been determined previously to provide maximal stimulation of bovine phagocytes (Dang et al. 2012). In all the cases, final culture volume was 200 μl. The blank wells consisted of 200 μl of culture media along with same concentrations of NBT and zymosan. All cultures were allowed to incubate at 37 °C in a humidified CO_2 incubator (95 % air and 5 % CO_2) for 2 h. After that, OD was taken at 540 nm multiwell-scanning spectrophotometer (Microscan MS-5608A).

Chemotactic activity of milk neutrophils was carried out using Dunn chemotaxis chamber (DCC 100) as per method given by Zicha (1997). Isolated neutrophils were diluted with 500 μl RPMI medium. Cells were seeded onto a sterile cover slip and allowed to settle prior to assembling the chemotaxis chamber. Outer annular well was filled with chemoattractant (LPS 50 μg/ml of RPMI) medium and inner well filled with control medium (RPMI media). The cover slip was inverted onto the chamber. The chamber was checked microscopically by adjusting the field having bridge shows movement of cells. Time-lapse option was selected to capture images after fixed time interval. Milk composition of all the three group of cows was estimated by Lactoscan milk analyzer.

Total RNA extraction from isolated milk neutrophils were performed using TRIzols Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Integrity of the RNA was checked by the agarose gel electrophoresis (2.5 % agarose), and quantity and quality of RNA was examined by nanodrop. Complementary DNA (cDNA) was prepared from 1 μg of RNA using the Novagen first strand cDNA synthesis kit (La Jolla, CA, USA) according to the manufacturer's protocol. Synthesized cDNA was kept at −20 °C (or −70 °C for long-term use) till use.

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed by Roche's Lightcycler 480 instrument as per the methods of Pfaffl (2001) with some modifications. Primers for specific bovine CXCR1, CXCR2, IL-8, CD62L, and CD11b genes shown in Table 1 (Sigma Chemicals Co., St. Louis, Missouri, USA). For normalization of qPCR data, GAPDH was used as a reference gene. Reaction mix for qRT-PCR was prepared as follows: 1 μL template; 5 μL (2×) SYBR green mixes, 0.5 μL each of reverse and forward primer, and 3 μL nuclease free PCR grade water. The reaction was continued for 45 cycles at 95 °C for 15 s, annealing at 59 °C for 20 s, and performed the denaturation kinetics to assess the reaction produced a single product.

All analysis was done using one-way ANOVA considering group as factor by the SYSTAT software package. The relative expression ratio of the target genes was tested and analyzed for significance by the Relative Expression Software Tool REST version 2009 V2.0.13 (Pfaffl et al. 2002).

Table 1 Details of primers used in the experiments

Genes	Sequence (5′→3′)	Account no.	Size (bp)	Basic Temp (°C)
CXCR1	F AGTCCCCGTGAGATAAGCAC R CCAGGTTCAGCAGGTAGACA	EF597244.2	163	59
CXCR2	F CAACACTGACCTGCCCTCTA R CCAGGTTCAGCAGGTAGACA	DQ328664.1	197	59
IL-8	F TGCTCTCTGCAGCTCTGTGT R CAGACCTCGTTTCCATTGGT	EU276073.1	190	59
CD11b	F CAAACTGGCAGAAAGCAACA R TCCAGGAAGACTCTGGAGGA	NM_175781.1	183	59
CD62L	F CCGATTGCTGGACTTACCAT R CCAAGTCCACACCCCTTCTA	NM_174182.1	194	59
GAPDH	F GGGTCATCATCTCTGCACCT R GGTCATAAGTCCCTCCACGA	NM_001034034	176	59

Results and discussion

Tharparkar breed is well-known for its adaptation to arid and semi-arid regions. This breed is an efficient converter of roughage and resistant to many diseases. A number of transcriptome level studies have been carried out for the selection of high milk producing cows against mastitis (Tiezzi et al. 2015), but there are no studies on the expression of mastitis resistance genes and activity of neutrophils in low producing cows (TP). Researchers also suggest that when indigenous cows are selected for high milk production or crossbreeding programs, they lose their main traits (Santana et al. 2015). Response of indigenous TP cows against mammary infections in a new environment have been studied and presented in this study. Results obtained in milk samples collected from healthy, sub-clinical, and clinical mastitis TP cows have been presented in Table 2. There was a significant ($P<0.05$) difference in the milk SCC of healthy, SCM, and CM cows. The lowest milk SCC values were recorded in healthy group and the highest in the CM group of TP cows. This is because the milk yield of TP cows is significantly lesser (5–7 kg/day) than exotic cows (15–20 kg/day) and also SCC is positively correlated with milk yield. Humoral and cellular defenses of the mammary gland in response to mammary infections affect milk composition (Le Marechal et al. 2011). In this study, mastitis did not alter the composition of fat; it significantly ($P<0.05$) decreased the milk lactose percentage but overall there was no change in the SNF% and pH in the three groups of cows. There was no change in the milk protein and electrical conductivity between healthy and sub-clinical cows. But these parameters increased significantly ($P<0.05$) in CM cows.

Table 2 Milk SCC and composition of TP cows

	Healthy	Sub-clinical mastitis	Clinical mastitis
SCC ($\times 10^3$)	126.7 ± 19.91^a	314.80 ± 30.67^b	504.30 ± 45.55^c
Fat (%)	4.32 ± 0.09	4.31 ± 0.04	4.08 ± 0.08
Protein (%)	3.30 ± 0.05^a	3.34 ± 0.04^a	3.70 ± 0.05^b
Lactose (%)	4.84 ± 0.02^a	4.71 ± 0.03^b	4.41 ± 0.02^c
SNF (%)	9.73 ± 0.15	9.61 ± 0.18	9.35 ± 0.21
pH	6.61 ± 0.07	6.63 ± 0.03	6.80 ± 0.06
EC	5.90 ± 0.17^a	6.01 ± 0.04^a	7.21 ± 0.06^b

Values are expressed as mean ± SE. Values lacking a common letter within a row differs significantly ($P<0.05$)

Of all the cells observed in the milk samples, maximum influx was seen in the neutrophils (Table 3). Neutrophils are the first cells to migrate from blood into an inflamed area after initiation of inflammation. There was a significant ($P<0.05$) increase in milk neutrophils in sub-clinical and clinical mastitis cows but this influx of neutrophils was lesser than 90 % as reported in exotic cows (Barbano et al. 2006). Both segmented and band neutrophils showed a significant ($P<0.05$) change between healthy and clinical cases of mastitis. However, milk macrophages decreased significantly ($P<0.05$) with the increase in the severity of infection in these cows. The number of lymphocytes did not differ between healthy and sub-clinical groups but showed a significant ($P<0.05$) decrease in mastitis cows. Viability of milk neutrophils was always higher in healthy samples (about 94 %) but decreased subsequently in mastitis samples (83 %).

The main function of neutrophils is phagocytosis and intracellular killing in the mammary gland. Neutrophils move toward bacteria under the influence of a chemoattractant and engulf bacteria by two distinct mechanisms, the respiratory burst and digestion by the lysosomal enzymes. On estimating the PA of milk neutrophils, it was found that there is no change in the PA between healthy and sub-clinical mastitis cows. But

Table 3 Milk DLC, chemotactic, and phagocytic activity of TP cows

	Healthy	Sub-clinical mastitis	Clinical mastitis
Milk neutrophils (%)	19.25 ± 0.26^{a}	35.78 ± 1.66^{b}	60.83 ± 1.00^{c}
Segmented neutrophils (%)	97.00 ± 0.36^{a}	96.50 ± 0.56^{ab}	95.00 ± 0.44^{b}
Band neutrophils (%)	3.00 ± 0.36^{a}	3.50 ± 0.56^{ab}	5.00 ± 0.44^{b}
Milk lymphocytes (%)	27.05 ± 0.81^{a}	25.77 ± 0.91^{a}	18.22 ± 0.52^{b}
Milk macrophages (%)	54.53 ± 1.00^{a}	40.45 ± 1.00^{b}	22.78 ± 0.63^{c}
Viability of neutrophils (%)	94.01 ± 0.36^{a}	89.33 ± 0.55^{b}	83.50 ± 0.76^{c}
Phagocytic activity	1.00 ± 0.02^{a}	1.00 ± 0.01^{a}	0.73 ± 0.06^{b}
Chemotactic activity	3.62 ± 0.04^{a}	2.74 ± 0.14^{b}	1.66 ± 0.08^{c}

Values are expressed as mean ± SE. Values lacking a common letter within a row differs significantly ($P < 0.05$)

the PA decreased significantly ($P < 0.05$) in clinical cases. However, chemotactic activity differed significantly ($P < 0.05$) between all the three groups of cows (Table 3).

To resolve an intrammamary infection, the blood neutrophils have to rapidly migrate into the mammary gland. When a pathogen invades the mammary gland, the neutrophils start transmigrating from the blood vessels by rolling, tethering, and adhering to endothelial cells and are extravasated toward the source of infection. This cascade of events is dependent on the expression of cell adhesion molecules such as selectins (CD62L), integrins (CD11b) and their counter receptors or ligands. Selectin mediates the rolling action, whereas, integrin allows the adherence of neutrophils to the endothelial cells. No change was observed in CD62L among all the three groups of TP cows. Expression of CD11b was low in healthy cows, increased significantly ($P < 0.05$) in sub-clinical and mastitis cows. However, Della Libera et al. (2015) did not observe any difference in the expression of selectin and integrin by the milk neutrophils in bovine leukemia virus infected and non-infected mammary quarters.

Recruitment of neutrophils to the site of infection is promoted by IL-8 which is considered as the most potent neutrophil chemoattractant. We found that expression of IL-8 increased significantly ($P < 0.05$) with infection of cows. Baggiolini and Clark-Lewis (1992) reported that when IL-8 bounds to neutrophil, it leads to conformational changes in the neutrophils which allow its adherence to endothelial cells. This causes exocytosis of soluble storage proteins from secretory vesicles and granules causing increased expression of adhesion molecules such as CD11b and CD18 that are essential for adhesion to endothelial cell. Galvao et al. (2011) reported a positive association between the IL-8 gene expression level and the incidence and severity of mastitis. IL-8 activates two receptors—CXCR1 and CXCR2.The receptor CXCR1 has been seen to activate the respiratory burst (Jones et al. 1996), whereas, CXCR2 primarily induces survival from spontaneous apoptosis (Glynn et al. 2002). Youngerman et al. (2004) also detected significant association between CXCR2 and percentages of sub-clinical mastitis in Holsteins. Buitenhius et al. (2011) also showed a significant increase in IL-8 gene transcription in experimentally induced mastitis compared to non-challenged mammary glands. Interleukin-8 has the capacity to alter the integrity of the blood—mammary barrier allowing increased concentrations of somatic cells and serum proteins to enter the milk and dramatically increase the neutrophil population within the mammary gland (Watanale et al. 2008).

Chemokine receptors like CXCR1 and CXCR2 present on the neutrophil surface (Del Rio et al. 2001) and the receptor-ligand interaction between CXCR1 and IL-8 permits their chemotaxis to the site of infection where they release their antimicrobial components (Olson and Ley 2002). Expression of CXCR1 was more in mastitis cows and also higher as compared to CXCR2. Expression of CXCR1 increased significantly ($P < 0.05$) in all the groups, whereas, CXCR2 increased significantly ($P < 0.05$) only in the mastitis cows (Fig. 1). Verbeke et al. (2015) also reported that there is an upregulation of CXCR1 when the quarters of Holstein cows are inoculated with *S. chromogenes*.

In conclusion, this is the first report that shows mastitis also occurs in low producing TP cows reared under regions of higher humidity. As these cows are being used under

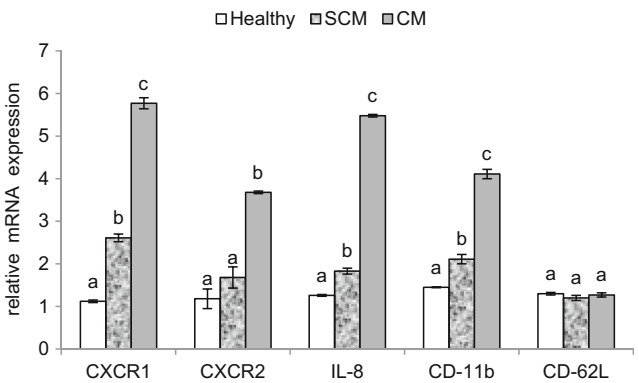

Fig. 1 Relative mRNA expression of CXCR1, CXCR2, IL-8, CD11b, and CD62L in milk neutrophils of healthy, sub-clinical, and clinical mastitis Tharparkar cows. *a*, *b*, and *c* indicate significant differences ($P < 0.05$) between the groups for each gene

crossbreeding program with exotic cows due to their inherited disease resistance ability, therefore, providing better management conditions as done for high milk producers may decrease the incidence of mastitis in TP cows and also increase their milk productivity.

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Compliance with ethical standards

Statement of animal rights All animal experimental procedures were approved by the Animal Ethics Committee of the National Dairy Research Institute, Karnal, Haryana, India.

Conflict of interest The authors declare that they have no conflict of interest.

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