Original article

FLOW CYTOMETRIC ANALYSIS OF SOMATIC CELLS AND OXIDANT/ANTIOXIDANT PROFILE IN DAIRY COWS WITH SUBCLINICAL MASTITIS

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Summary

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Flow cytometric analysis of somatic cells count (SCC) and oxidant/antioxidant status of cows with subclinical mastitis (SCM) were investigated in 75 lactating Holstein-Friesian cows (20 controls; 55 SCM cows). Milk from active quarters (n=280) and blood samples (n=75) were aseptically collected. Milk samples were subjected to California Mastitis Test (CMT), microbiologic examination and flow cytometric analysis. Blood and milk samples were analysed for malondialdehyde (MDA) concentrations and total antioxidant capacity (TAC). Microbiological examination revealed 3 major pathogens in a single or mixed infection: *Streptococcus* species (26.9%), *Staphylococcus* species (57.7%) and *Escherichia coli* (49.23%). Flow cytometric analysis showed significant increase (P<0.05) in inflammatory cells in milk of cows with SCM compared to control cows. Differential SCC was characterised by significant increase (P<0.05) in polymorphonuclear leukocyte (PMNL) counts compared to normal milk. Additionally, *Staphylococcus* infection alone or mixed with other lactopathogens resulted in dramatic increase in PMNL. There was a significant decrease (P<0.01) of TAC in plasma and milk (0.49 ± 0.04, 0.27 ± 0.03 mM/L, respectively) and significantly increased (P<0.01) serum and milk MDA concentrations (13.72 ± 0.3, 7.72 ± 0.17 nmol/mL, respectively) in SCM cows. In conclusion, differential SCC is a reliable index for early detection of SCM. Milk PMNL population can be used as a useful indicator for evaluation of udder infection. Additionally, a combination of MDA and TAC can provide complementary information about the health status of udder in dairy farms.

Key words: antioxidant, flow cytometery, mastitis, somatic cell

INTRODUCTION

Mastitis is well known inflammatory disease of the udder affecting dairy herds, causing devastating economic losses due to reduction of milk production, treatment costs and early culling (Radostits *et al.,* 2007). In dairy cattle, the prevalence of

subclinical mastitis (SCM) may reach up to 75% (Kahn & Line, 2010). Approximately 70 to 80% of financial losses are caused by SCM (Colla *et al.,* 2011).

The International Dairy Federation (IDF, 1981) has adopted the somatic cell count (SCC) and microbiological status of the milk samples for assessment of the udder health and diagnosis of SCM. However, some logistic, financial and technical concerns associated with microbiological examination of all suspected milk samples, have precluded this technique from being widely implemented (Sharma *et al.,* 2010). While SCC is generally accepted for assessment of the health status of the mammary gland, the SCC results may vary with the status of lactation, age, stress of the animals, time and frequency of milking, and season. Additionally, SCC could not give information about the type of involved cells.

The ultimate identification of individual cell populations of milk leukocytes (lymphocytes, macrophages, and polymorphonuclear leukocytes) using flow cytometry was recently introduced and proposed to give more accurate results than other tests of SCC (Rivas *et al.,* 2001; Koess & Hamann, 2008).

The involvement of polymorphonuclear leukocytes (PMNL) and macrophages during inflammatory process triggered by pathogens is well documented. The activities of these cells during the early stages of mastitis play a vital role in defense mechanisms against intramammary infection (Abera *et al.,* 2010). Reactive oxygen species (ROS) generated from resident and newly recruited leukocytes during microbial killing process may damage mammary epithelial cells with resultant oxidative stress (Barbano *et al.,* 2006).

Therefore, the present study was carried out to differentiate somatic cells count in milk using flow cytometry for early detection of subclinical mastitis, to screen for the most commonly incriminated lactopathogens, to define a relationship between milk differential somatic cells count and the type of lactopathogens and finally to evaluate the oxidant/antioxidant status of dairy cows with subclinical mastitis.

MATERIALS AND METHODS

Animals

Seventy five lactating Holstein/Friesian cows were included in the current study, housed in different governmental and private farms within the territory of Assiut Governorate – Egypt. They were apparently healthy after being subjected to general clinical examination according to Rosenberger (1990). Diseased cows, and those affected with clinical mastitis, in early lactation and late pregnancy were excluded from the study.

Samples

Thirty mL of milk samples (n=280) from each quarter were collected aseptically according to the National Mastitis Council (1990). Collected samples were tested by California Mastitis Test (CMT) and transferred immediately to the laboratory for microbiologic, biochemical and flow cytometric analysis.

In addition, blood samples (5 mL each) were collected by jugular vein puncture using sterile disposable syringes. A whole blood sample was collected into vacutainer tubes coated with heparin to obtain non-haemolysed clear blood plasma for estimation of total antioxidant capacity. The second blood sample was

collected into dry, clean centrifuge tube without anticoagulant to obtain non-haemolysed clear serum for estimation of MDA level.

Clinical, CMT and microbiological examination

All animals in the study were subjected to general clinical examination according to Rosenberger (1990). California Mastitis Test (CMT) results were read and evaluated with 5 scores: −, ±, +, ++, and +++, which are reported as homogenous fluid, very slight flocculation, slight flocculation, flocculation (flaks), and gel formation, respectively (Schalm *et al.,* 1971). Ten mL of milk samples were centrifuged in sterile screw-capped test tubes for 30 min at 3000 rpm. The cream and supernatant milk were discarded. Milk sediment was simultaneously cultured on selective media, isolated and identified for major lactopathogens including *Staphylococcus, Streptococcus* and *E. coli.* The resulting growth was identified on the basis of morphology, colony characteristics, Gram's reaction and biochemical reactions. Animals culturally positive for at least a single quarter were considered SCM positive.

Flow cytometric analysis of somatic cells

Milk samples were centrifuged for 15 min at $200 \times g$ and 4 °C, cream layers and supernatants were discarded and pellets were suspended in phosphate buffered saline (PBS). Cell staining buffer (CSB) up to ~15 mL was added and centrifuged at $350 \times g$ for 5 minutes. Pellets were collected and 3 mL of lysing buffer was added to each tube, kept on ice for 5 min. Cell lysis was stopped by adding 10 mL CSB, centrifuged for 5 minutes at $1500 \times g$ for 3 min at 4 °C, supernatants were discarded and cell pellet was agitated and suspended in 3.5 mL PBS. Viable cells were counted and resuspended in CSB at $5{-}10 \times 10^6$ cells/mL and 100 μL/tube of cell suspension $(5{-}10 \times 10^5$ cells/tube) was distributed into 12×75 mm plastic tubes. Surface monoclonal antibodies (mAb) against CD11a and CD18 were obtained from the Monoclonal Antibody Center at Washington State University (Cat. No. BOV2023 recognising CD11a and Cat.; No. BOV2030 recognising CD18) and dispensed into all appropriately labelled tubes. Tubes were agitated gently to mix and incubated in dark for 20 min at 4 ºC. Secondary coloured conjugated monoclonal antibodies, Goat Anti-Mouse IgG1 Alexa Fluor 488, and Goat Anti-Mouse IgG2a, R-Phycoerythrin, (Cat. No. A21121 and Cat. No. P21139, Thermo Fisher Scientific, Rockford, IL, USA) were added, incubated for 30 min/4 ºC. Samples were analysed by multicolour flow cytometry (FACS-Caliber, Beckton Dikinson, USA). The flow cytometry was adjusted to count up to 10,000 cells per sample. The percentage of specific cell population was calculated as its proportion to the total number of cells counted by flow cytometry.

Biochemical analysis

Total antioxidant capacity (TAC) was determined by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminated a certain amount of the provided H_2O_2. The residual H_2O_2 was determined colorimetrically by an enzymatic reaction, which involves the conversion of 3,5, dichloro-2-hydroxy benzensulphonate to a coloured product (Koracevic *et al.,* 2001). The absorbance of the resultant product was measured at 505 nm wavelength.

Malondialdehyde (MDA) was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea *et al.* (1994). Thiobarbituric acid (TBA) reacts with MDA in acidic medium at 95 ºC for 30 min to form thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured at 534 nm wavelength.

Spectrophotometry (Optizen 3220 UV, Mecasys Co. Ltd, Korea) and standard test kits for TAC and MDA (Biodiagnostica Co-Egypt) were used in both assays.

Statistical analysis

The SPSS package for Windows version 10.0 was used for statistical analysis (SPSS, 1999). Data were expressed as mean ± SEM. Differences between groups are determined by means of Student t-test or Duncan's new multiple range test whenever needed. Significance level was set at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Analysis of animals' data revealed that the average age, parity and milking period of cows under investigation were 3.9 years, 2.25 parturitions and 3.7 months, respectively. There were no significant differences ($P>0.05$) in the age, parity and milking period between control and subclinical mastitis groups. The majority of cows with subclinical mastitis were at the third (42%) and fourth (45%) months of lactation.

A total number of 280 quarter milk samples were classified according to CMT into negative (n=80) from 20 healthy cows, one positive (n=85), two positives (n=50), and three positives (n=65).

Table 1. Number and frequency of isolated pathogens from milk samples in cows with subclinical mastitis (n =130)

| Microorganism	SCM milk samples	
	Number	Frequency, %
Staphylococcus sp.	43	33.0
Streptococcus sp.	16	12.3
E. coli	32	24.7
Mixed infection		
Staphylococcus + E. coli	20	15.4
Streptococcus + E. coli	7	5.4
Staphylococcus + Streptococcus	7	5.4
Staphylococcus + Streptococcus + E. coli	5	3.8
Total isolates	130	100.0

Table 2. Total antioxidant capacity and malondialdehyde in blood and milk in control cows and cows affected with subclinical mastitis. Data are presented as mean ±SEM

		Control	Subclinical mastitis
Total antioxidants (mM/L)	plasma (n=75)	0.88 ± 0.10	0.49 ± 0.04*
	milk (n=280)	0.40 ± 0.04	0.27 ± 0.03*
Malondialdehyde (nmol/mL)	serum (n= 75)	4.53 ± 0.38	13.72 ± 0.30*
	milk (n=280)	5.27 ± 0.27	7.72 ± 0.17*

* statistically significant difference at $P<0.01$.
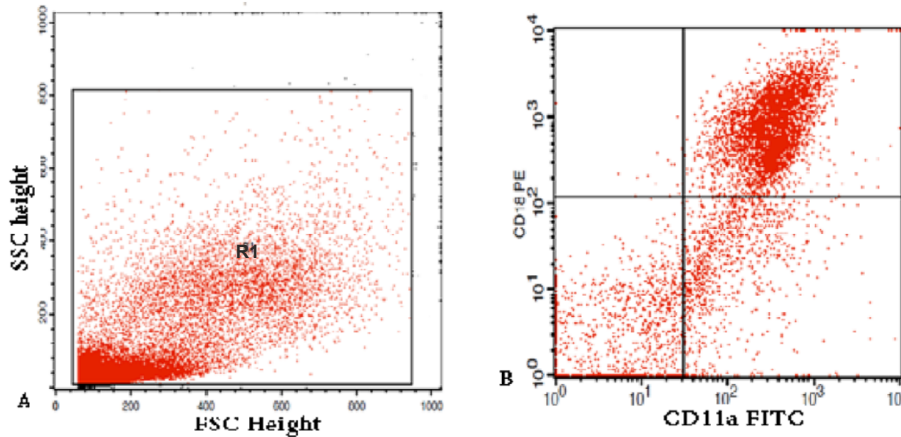
Fig. 1. Flow cytometric analysis of milk leukocytes. Forward (FSC) and side scatter (SSC) histogram was used to define the total somatic cells in milk (R1). Events defined as somatic cells (R1) were then examined for their CD11a and CD18 binding, to detect total leukocyte (the cells that co-express CD18 and CD11a) count.
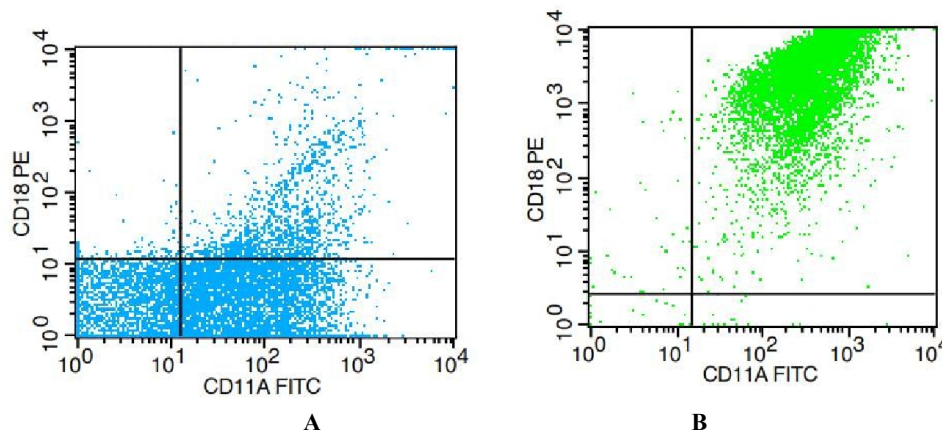
Fig. 2. Flow cytometric analysis of control (**A**) and SCM milk sample (**B**) with CD11a and CD18 mAb showing low and high number of inflammatory cells, respectively.

Out of 200 CMT positive milk samples, 130 samples were randomly selected and tested microbiologically. Three microbial pathogens were isolated either alone or in association with other pathogens: *Streptococcus* species (n=35, 26.9%), *Staphylococcus* species (n=75, 57.7%) and *Escherichia coli* (n=64, 49.23%). The frequency of isolation of each pathogen is illustrated in Table 1.

The levels of MDA and TAC in blood and milk of control and subclinical mastitis cows are illustrated in Table 2. There was a significant ($P \leq 0.01$) decrease in the milk and plasma TAC and a marked ($P \leq 0.01$) increase in the milk and serum level of MDA in cows with subclinical mastitis compared to healthy cows.

A **B**
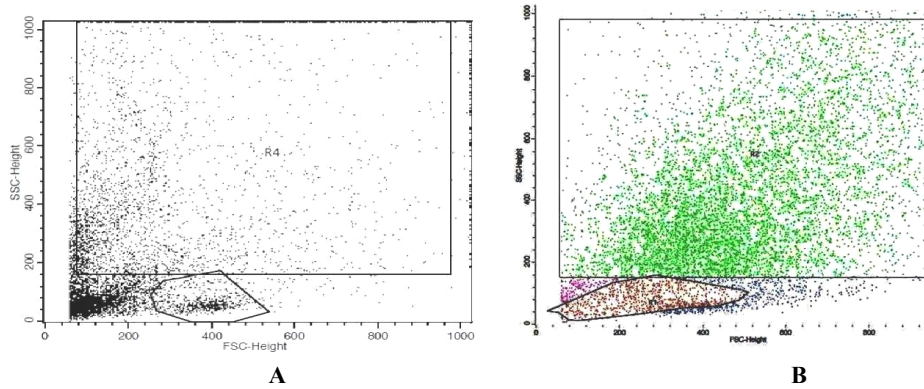
Fig. 3. Flow cytometric analysis of control and subclinical mastitis samples with CD11a and CD18 mAb showing predominant fields of lymphocytes (**A**) and neutrophils (**B**).

Table 3. Inflammatory and non-inflammatory cells in milk of control cows and cows affected with subclinical mastitis (mean ± SEM and percentage)

	Control (n=5)	Subclinical mastitis (n=45)
Inflammatory cells	1045 ± 312.8 (14.5 ± 4.1%)	5337.6 ± 422.5* (71.4 ± 4.2%)
Neutrophils	786.33 ± 176.50 (8.66 ± 0.96%)	3696.4 ± 1502.12* (44.2 ± 17.43%)
Lymphocytes	2252.67 ± 1694.9 (22.84 ± 16.45%)	1285.7 ± 1098.13* (14.69 ± 10.1%)
Non-inflammatory cells	6106.7 ± 1167.1 (85.5 ± 4.1%)	2180.9 ± 409.4* (28.2 ± 4.2%)

* statistically significant difference at $P<0.05$

Table 4. Inflammatory and non-inflammatory cells in milk of control cows and cows with subclinical mastitis in relation to lactopathogens (mean ±SEM and percentage)

	Non-inflammatory cells	Inflammatory cells	
	Count	Count	%
Control	6106.70 ± 1146.88	1045.00 ± 312.80	14.50 ± 4.10
Subclinical mastitis			
Staphylococcus sp.	2756.83 ± 892.10	4949.80 ± 745.50	62.27 ± 8.50
Streptococcus sp.	2649.50 ± 201.50	3160.50 ± 211.80	52.39 ± 6.90
E. coli	2832.33 ± 345.60	3828.70 ± 645.77	56.81 ± 10.86
Mixed infection			
Staphylococcus + E. coli	1391.56 ± 390.47	6365.44 ± 457.95	82.06 ± 4.02
Streptococcus + E. coli	5040.33 ± 1032.50	4330.00 ± 732.87	45.21 ± 14.62
Staphylococcus + Streptococcus + E. coli	117.50 ± 47.98	8409.00 ± 587.14	97.67 ± 6.41

Leukocyte population were determined by flow cytometry and calculated as positive cells (labelled) by the CD11a/ CD18 mAb. Epithelial cells were calculated as the negative cells (not labelled) by CD11a/ CD18 mAb. The location of positive cells labelled with mAb used on the dot plot [Forward-angle light scatter (FSC) by logarithm side-light scatter (SSC)] map is presented on Fig. 1–3.

Quarters free of intramammary infection had significantly increased CD11a/ CD18 negative cells and were reported as non-inflammatory cells. Additionally, healthy udder showed a higher population of lymphocyte than that reported in SCM based on the location of predominant cells on the flow cytometric scatter (low FSC and SSC) (Fig. 2A). On the other hand, flow cytometric analysis of somatic cells revealed significant ($P \leq 0.05$) increase in leukocytes (inflammatory) cells in milk of cows with SCM compared to control ones (Fig. 2B, Table 3). A dramatic increase in leukocyte population was reported in SCM cases due to *Staphylococcus* infection either alone or in mixed infection (Table 4).

DISCUSSION

Somatic cells count in milk has been adopted in mastitis control programmes and milk quality control; however, some physiological factors such as status of lactation, age, stress, time and frequency of milking, and season may negatively affect the SCC. Moreover, SCC could not give information about the type of involved cells (inflammatory and non-inflammatory). Differential somatic cell count (DSCC) provides more detailed evaluation of the udder health status (Schwarz *et al.,* 2011) and allows for early detection of mastitis.

The receptors of CD11a and CD18 are distributed on various immune cells including T, B and NK cells, macrophages and granulocytes (Larson & Springer, 1990). In this study, immune and non-immune cells (epithelial cells) were identified by flow cytometry using specific monoclonal antibody against CD11a/ CD18 receptors. Epithelial cells were identified as the negative cells to the mAb anti-CD11a/CD18 receptor whereas positive cells on dot plot of FC scatter were identified as lymphocytes and neutrophils. Fig. 1–2A showed forward (FSC) and side scatter (SSC) histogram that used to define SCC and differentiate milk leukocytes (lymphocytes and granulocytes) according to their size and complexity. Lymphocytes were characterised by their small size (low forward scatter) and lower complexity and granulations (low side scatter) while granulocytes – by their large size (high forward scatter) and higher complexity and granulations (high side scatter) at the total somatic cells in milk.

Our results showed that milk samples from culture-negative udders revealed high epithelial cells and lymphocyte percentages of 85.5±4.1% and 22.84±16.45%, respectively while PMNL were 8.66± 0.96%. Similar observations were reported in previous studies (Rivas et *al.,* 2001; Schwarz *et al.,* 2011).

On the other hand, inflammation is generally defined as increased leukocytes, especially PMNL in tissues or body fluids in response to pathogens. An increased transfer of PMNL from blood into the area of infection at the beginning of an inflammation is well documented and could be detected (Kehrli & Shuster, 1994; Paape *et al.,* 2002). Therefore, a high PMNL percentage in milk is accepted as an important indicator of inflammatory reaction (Pillai *et al.,* 2001;

Paape *et al.,* 2002). The obtained results of flow cytometric analysis of somatic cells revealed a significant increase (P<0.05) in inflammatory cells in milk of cows with SCM (71.4±4.2%) compared with apparently healthy cows (Fig. 2b). Most of these positive cells (labelled CD11a/CD18) were defined as PMNL on the dot plot flow cytometric position (PMNL gate, high FSC and SSC) (Fig. 3). Milk of cows with SCM showed a significant increase (P<0.05) in neutrophils counts and percentage compared to control milk (negative CMT). PMNL were the dominant cell population with significant average share (44.2±17.43%) among tested SCM samples compared with control milk. Similar findings have been previously reported in cattle and buffaloes (Zaman *et al.,* 2009; Hussein *et al.,* 2012). Abera *et al.* (2010) stated that neutrophils are the predominant cell type found in mammary tissues and secretions during inflammation and might constitute more than 90% of total mammary gland leukocytes. The increase in SCC is mainly due to the migration of PMNL that are essential components of the innate immunity (Boutet *et al.,* 2004) as they form the first line of defense against bacterial infections. The essential role of the CD11a/CD18 receptors as adherence molecules is well documented (Tizard, 1996). Normally, neutrophils float freely in the blood and do not bind to endothelial cells. As a result of challenge with a lactopathogen, resident macrophages ingest bacteria and secrete unidentified mediators. In the next step, surface expression of the integrin CD11a/CD18 increased, inflammatory cells adhere to endothelial cells and transmigrate to the area of infection. Therefore, DSCC enables one to detect mastitis in its initial phase and could be used successfully to monitor udder health.

A significantly decrease in the proportion of lymphocytes and non-inflammatory cells in SCM was recorded. Similarly, lymphocyte, monocyte and macrophage population were significantly decreased in the milk of infected animals (Gargouri *et al.,* 2008; Hussein *et al.,* 2012).

Data of flow cytometry demonstrated an interesting relationship between the DSCC and type of infection. It seems that the proportion of the cell types, or subtypes, rather than their total number, was a factor defining the type of the lactopathogen. Flow cytometric data screening showed various degree of significant increase in neutrophils in relation to type of lactopathogens. The highest proportion of inflammatory cells was reported in milk from quarters infected by *Staphylococcus* alone or in association with other lactopathogens. The results of the present study strongly suggested the existence of a correlation between the immune response and different mastitic pathogens. It also emphasized the importance of studying the particular leukocyte population patterns for each pathogen. Similar observations were reported in previous studies (Soltys & Quinn, 1999; Fitzpatrick, 2001).

The influx of inflammatory cells, primarily PMNL, into the udder tissue in response to infection helps destroying the invading bacteria by oxygen-dependent and oxygen-independent systems. Concurrently, they potentially damage the secretory epithelium of mammary gland by reactive oxygen species (ROS) and proteolytic enzymes (Knaapen *et al.,* 1999; Hamed *et al.,* 2008). The determination of lipid peroxidation status via assessment of MDA is among the most widely used methods for determination of the oxidative stress (Kohen & Nyskaa, 2002). The present study revealed a significant increase (P<0.01) in the plasma and milk

concentration of MDA in cows with SCM compared to normal cows. Similar results have been reported in other studies on dairy cows (Suriyasathaporna *et al.,* 2006; Andrei *et al.,* 2010; Yang *et al.,* 2011). In contrast, Ranjan *et al.* (2005) and Kizil *et al.* (2007) reported an enhanced erythrocyte and plasma lipid peroxide content in clinical, but not in subclinical mastitis cases. On the other hand, concentration of TAC showed a significant decrease (P<0.01) in milk and blood samples of subclinically mastitic compared to normal cows. The high production of MDA and low levels of TAC in SCM milk reported in this study confirmed the occurrence of a process of auto-oxidation and oxidative stress associated with SCM in dairy cows. Similar results have been reported in previous studies (Suriyasathaporna *et al.* 2006; Atakisi *et al.,* 2010).

Total antioxidant capacity identifies a wide range of all activities of the measurable exogenous and endogenous antioxidants (Somogyi *et al.,* 2007). TAC consumption by generated ROS leads to oxidative stress and resultant reduction in milk production. There is a significant negative correlation between the TAC and total oxidant capacity (TOC) values (Erel, 2005).

It can be concluded that differential somatic cell count was the most reliable index for early detection of SCM next to bacteriological examination. Neutrophil population in milk can be used a useful indicator in the evaluation of mammary gland infection. Additionally, a combination of MDA and TAC can provide complementary information about the health status of udder in dairy farms.

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