MASTITIS PATHOGENS AND SOMATIC CELL COUNT IN EWES MILK

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ABSTRACT

The aim of this study was to determine the occurrence of pathogens in selected group of ewes and the relationship between somatic cell count (SCC) and the presence of pathogens. The experiment was carried out on a dairy farm, where predominantly breed was a Tsigai. Sampling was carried out in monthly intervals as part of the milk recording test day from February to July 2019. A total of 303 ewes were included in the survey, during the milk recording test day. The ewes with SCC $\geq 1000 \times 10^3$ cells.mL^{-1} were selected for further sampling at half udder level. Based on SCC the ewes were divided into five groups: $<200 \times 10^3$; $\geq 200 <400 \times 10^3$; $\geq 400 <600 \times 10^3$; $\geq 600 <1000 \times 10^3$; $\geq 1000 \times 10^3$ cells.mL^{-1}. The first group of SCC contained 33.9% of milk samples, the second 14.1% of samples, the third 5.7% of samples, the fourth 6.2% and the fifth 40.1% of samples. The most common pathogens were coagulase negative staphylococci (CNS). The most frequent CNS was *Staphylococcus (S.) simulans* (24.4%). *S. aureus* was identified in 5.3% of bacteriological positive samples. Almost 70% of ewes with bacteriological positive samples were repeated identified the presence of pathogens during tested period. SCC $\geq 500 \times 10^3$ cells.mL^{-1} were detected in 92.5% bacteriological positive milk samples. The presence of pathogens increased SCC in milk ($p < 0.001$) as compared to samples free of pathogens. In conclusion, the SCC $\geq 500 \times 10^3$ cells.mL^{-1} could be important for detection of subclinical mastitis at half udder level in dairy ewes.

Keywords: ewes; mastitis; somatic cell count; SCC; pathogens

INTRODUCTION

Mastitis is big healthy, economic and welfare problem in dairy animals. The main cause of increase SCC in milk of ewes is intramammary infection (**Souza et al., 2012**). Thus, SCC in milk can be used as indicator for diagnostic of subclinical mastitis (**Tvarožková et al., 2019; Olechnowicz and Jaskowski, 2005; Leitner, Silanikove and Merin, 2008; Zigo et al., 2019**). However, the physiological level of SCC in ewe's milk is still under discussion despite the researches. The results of researches point to the need to set a limit for physiological level of SCC in raw ewe's milk in relation to mastitis (**Persson et al., 2017**). Our preliminary results also support that high SCC are caused by presence of pathogens (**Uhrinčať et al., 2019**). CNS are the most common pathogens isolated from milk samples of ewes (**Holko et al., 2019; Zigo et al., 2014; Zafalon et al., 2018**).

Scientific hypothesis

The hypothesis of this article is that high SCC in milk is caused by presence of mastitis pathogens. The aim of this study was the evaluation of occurrence of pathogens in milk of ewes and the possible relation of pathogens with SCC.

MATERIAL AND METHODOLOGY

The experiment was carried out on farm with Tsigai ewes as dominantly kept breed, together with Lacaune and Improved Valachian (303 dairy ewes in farm). The ewes were on pasture during the day and received concentrates in amounts of 200 g per day during milking.

The milk sampling was performed once a month during morning milking as a part of regular milk recording test day from February to July 2019 (February, March, May, June, July). SCC was determined using a Somacount 150 (Bentley Czech, USA). The ewes with SCC $\geq 1000 \times 10^3$ cells.mL^{-1} at any time during the regular sampling period were selected for further sampling at half udder level three days later. All these ewes were sampled again always on third day after further regular recording test days during whole lactation period even if they had low SCC at regular sampling. The milk samples were collected at half udder level and analysed on SCC and presence of pathogens. Thus 95 ewes (407 milk samples) without symptoms of clinical mastitis were selected into study.

For the bacteriological cultivation and the presence of pathogens the milk samples were colltableected by discarding first squirts of milk and subsequently cleaning of the teat end with 70% alcohol and approximately 5 mL of milk from each udder halves was taken in sterile tube.

The inoculum of each sample of milk was inoculated onto blood agar (Oxoid LTD, Hamshire, UK). All plates were incubated aerobically at 37 °C and evaluated after 24 hours. The all plates were re-evaluated after another 24 hours incubation. Colonies were identified on basis of cells morphology, Gram staining, type of hemolysis, the activities of catalase (3% H_2O_2, Merck, Darmstadt, Germany) esculin hydrolysis and cytochrome oxidase C (Bactident Oxidase, Merck). Presumptive *Staphylococcus aureus* were detected with the clumping factor test (DiaMondiaL Staph Plus Kit, Germany). Esculin positive streptococci were cultivated on modified Rambach agar to identification *Streptococcus uberis* or *Enterococcus* sp. according to **Watts, Salmon and Yancey (1993)**. Lancefield serotyping (DiaMondiaL Strept Kit, Germany) was used to characterize esculin negative streptococci. The species of gram-negative rods were identified used by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Contagious pathogens (*Staphylococcus aureus, Streptococcus agalactiae*) were classified as positive if one or more colony-forming unit (CFU) were found. Other pathogens were classified as positive if at least five CFU were found. Samples were classified as contaminated if three and more pathogens were isolated from one milk samples and growth of contagious pathogens were not identified.

The identification to species level by applying MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Briefly, fresh colony material was spotted by direct transfer method on to MALDI-TOF MS target plate, allowed to dry at room temperature and overlaid with 1 μL of matrix solution (saturated solution α-cyano-4-hydrosy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and allowed to dry at room temperature. Before the matrix solution was added 1 μL of 70% formic acid to the bacterial spot and allowed to dry for direct transfer-formic acid method. A loopful of bacterial colony was suspended in 300 μL distilled water and 900 μL ethanol was added for the protein extraction. The supernatant was discarded after centrifugation of cell suspension at 17,000 × g for 2 min. The centrifugation was repeated and the remaining ethanol was discarded. After dried the pellet was resuspended in 5 to 50 μL formic acid-water (70:30) in depending on the size of pellet and an equal volume of acetonitrile was added finally. 1 μL of the supernatant was transferred to the MALDI-TOF MS target plate after centrifugation at 17,000 × g for 2 min and allowed to dry before applying 1 μL of matrix solution. The MALDI Biotyper software version 2.0 (Bruker Daltonics) was used for bacterial identification.

Statistical analysis

Samples on the basis of SCC at half udder level were divided into following five SCC groups for evaluation of the distribution of milk samples: first $<200 \times 10^3$ cells.mL^{-1}; second $\geq200 <400 \times 10^3$ cells.mL^{-1}; third $\geq400 <600 \times 10^3$ cells.mL^{-1}; fourth $\geq600 <1000 \times 10^3$ cells.mL^{-1}; fifth $\geq1000 \times 10^3$ cells.mL^{-1} (Using Excel, Microsoft, USA). The distribution of samples according SCC group was done by Microsoft Excel. Somatic cell

score was used for statistical evaluation (SCS) and SCS was calculated according formula:

$$SCS = LOG_2 (SCC/100000) + 3$$

For statistical evaluation the data were divided according month of sampling where five groups of samples were created: Feburary, March, May, Juni and July. The samples also were divided into 9 pathogens group differed by presence of pathogens (1st group – major (*S. aureus, Str. agalactiae*), 2nd – minor (environmental pathogens other than CNS), 3rd – *S. simulans*, 4th – *S. schleiferi*, 5th – *S. caprae*, 6th – *S. epidermidis*, 7th – *S. chromogenes*, 8th – other CNS. Control group (9th group) consists from samples free of pathogens. Obtained data were processed by Microsoft Excel program and statistically evaluated by SAS/ 8.2 (2002). The model was tested by using Fisher's F-test. Differences between the levels of the effects were tested by Scheffe's multiple range test for studied trait. Data are presented as LSmeans ± standard error for evaluation of somatic cells the following model was used:

$$y = X\beta + Zu + e$$

y – was the measurements for somatic cell counts
β – the fixed effects of months, pathogens
e – random error, assuming $e \sim N (0, I \delta^2_e)$
X, Z – incidence matrices for fixed effects and random cow effect, resp.

RESULTS AND DISCUSSION

When evaluating the entire observation period tested ewes on udder half level the first group of SCC contained 33.9% of samples, the second 14.1% of samples, the third 5.7% of samples, the fourth 6.2% and the fifth 40.1% of samples (Figure 1). In our previous works we presented higher percentage (from 58.9% to 71.8%) of ewes in group $<200 \times 10^3$ cells.mL^{-1} under usual test day sampling for determination of physiological levels of SCC in healthy udder **(Tančin et al., 2017a; Tvarožková et al., 2018)**. The proposed physiological threshold of SCC for diagnosis of mastitis in Sarda sheep was determined by **Caboni et al. (2017)** at 265×10^3 cells.mL^{-1}. **Zafalon et al. (2016)** detected the value of SCC $>400 \times 10^3$ cells.mL^{-1} for diagnose of subclinical mastitis in flocks. Earlier proposed value of SCC for the diagnosis of mastitis was 500×10^3 cells.mL^{-1} **(Nunes et al., 2008)**. Low percentage of samples in group $<200 \times 10^3$ cells.mL^{-1} in present work could be explained by sampling schedule, where these samples were colected from the ewes with high SCC at udder level three days before. Thus some health problems of udder should be expected as it is presented later in article by cultivation of milk sample on pathogens presence. In ewes with SCC above 400×10^3 cells.mL^{-1} three or more months during lactation there were 5.6 to 7.5-fold higher probability of a subclinical mastitis in compared with ewes with SCC below above limit **(Spanu et al., 2011)**. **Berthelot et al. (2006)** reported 15% occurrence of subclinical mastitis if SCC in flock was over 650×10^3 cells.mL^{-1}.

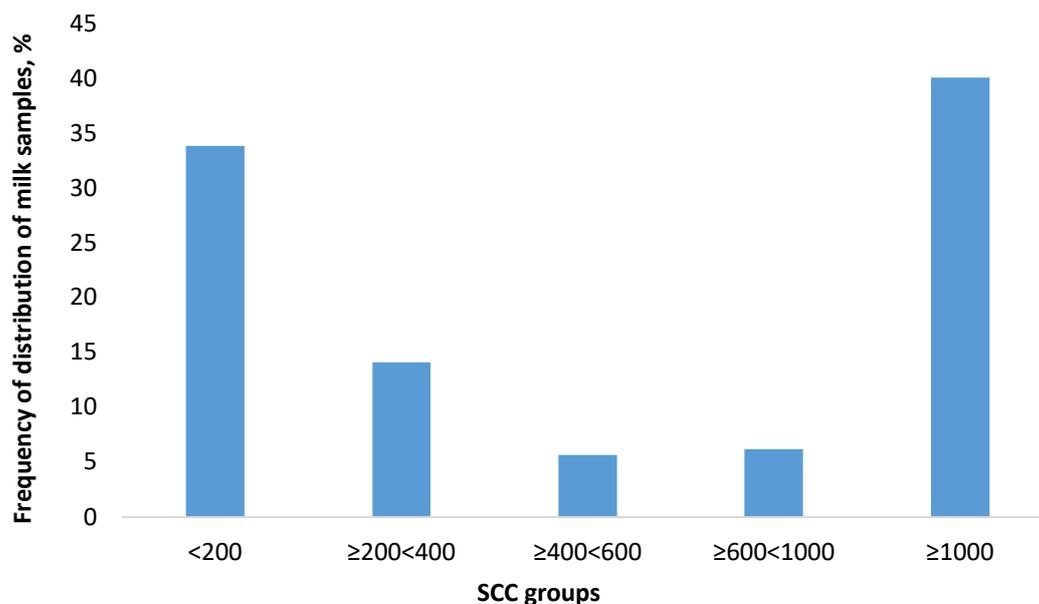**Figure 1** Frequency of distribution of milk samples in SCC groups.

Table 1 The occurrence of pathogens in months of observation.

Pathogen	February	March	May	Jun	July
Enterococcus faecalis	2	1	3	-	-
Micrococcus luteus	-	-	-	-	1
S. aureus	1	2	4	1	-
S. capitis	1	2	-	-	-
S. caprae	2	3	2	1	20
S. cohni	-	-	-	-	1
S. epidermidis	3	7	3	3	5
S. felis	-	-	1	-	-
S. haemolyticus	1	-	-	-	-
S. chromogenes	5	4	1	-	-
S. lentus	-	-	-	-	1
S. piscifermentans	-	-	-	4	-
S. sciuri	1	-	-	-	-
S. schleiferi	-	-	8	21	-
S. simulans	7	6	15	3	2
S. warneri	-	-	1	-	-
Str. pluranimalium	1	1	-	-	-

Total 407 examined milk samples tested on presence of mastitis pathogens were as negative classified 63.1% of milk samples, out of these samples 75.9% had SCC below 500×10^3 cells.mL^{-1}. 36.1% of samples were classified as bacteriological positive and 0.7% of milk samples were classified as contaminated. Only 7.5% of bacteriological positive samples had SCC below 500×10^3 cells.mL^{-1}. Two pathogens were identified in 2.7% of bacteriological positive samples. In 67.9% of ewes with bacteriological positive samples there were repeated detected the presence of pathogens during tested period. Thus pathogens could persist in udder throughout whole lactation. Also up to 21.1% from these ewes had infected both udder halves pathogens repeatedly.

Important group of samples are those in fifth group – with very high SCC (Figure 1). In fifth SCC group there were almost 80% bacteriological positive samples, which indicated the reason of high SCC. Also even 92.5% bacteriological positive samples had SCC $\geq 500 \times 10^3$ cells.mL^{-1}. We detected significant lower SCS in milk of ewes without (4.03 ±0.12) than with the presence of any pathogens ($p < 0.001$). There was no effect of different pathogens on SCS which ranged from 6.68 ±0.41 to 8.11 ±0.63. Also no effect of month of sampling on SCS was found out. Different pathogens could differently influence SCC (**Abu Baker Idriss et al., 2013; Bagnicka et al., 2011**). **Kiossis et al. (2007)** used level of SCC of $\geq 500 \times 10^3$ cells.mL^{-1} and bacteriological positive milk samples for the diagnose of subclinical mastitis. Significantly higher SCC in bacteriological positive samples as compared with bacteriological negative samples found out **Ozenc et al. (2011)** in their study. **Świderek et al. (2016)** reported that milk samples without bacteria had the lowest average SCC. Also, **Persson et al. (2017)** detected significantly higher SCC for udder halves with intramammary infection compared to udder halves without bacterial findings.

From major pathogens only *S. aureus* was identified in 5.3% of bacteriological positive samples (Table 1). Other contagious pathogens were not found out in tested group of ewes. Low frequency of contagious pathogens in milk was also presented in our previous work (**Tančin et al. 2017b; Holko et al. 2018**) or abroad works (**Ergűn et al. 2009; Kern et al. 2013**). **Zigo et al. (2011)** detected *S. aureus* in 9.3% of positive samples. **Moroni et al. (2007)** isolated *S. aureus* in 8.4% of infected milk samples. *S. aureus* was determined in 6.2% of subclinical mastitis cases (**Queiroga, 2017**). The most frequent pathogens isolated from the milk samples were CNS. Also high occurrence of CNS was reported in study **Zigo et al. (2014)** and **Vasileiou et al. (2018)**. The most frequent CNS was *S. simulans* (24.6%) followed by *S. schleiferi* (21.6%), *S. caprae* (20.9%) (Table 1). From CNS found in farm had the highest occurrence *S. epidermidis* (36.3%) and *S. caprae* (21.3%) (**Pilipčincová et al., 2010**). **Rahman et al. (2016)** showed that the most dominant CNS were *S. epidermidis*, *S. xylosus* and *S. chromogenes.* **Vasiľ et al. (2018)** investigated that the most frequent CNS were *S. epidermidis* (24.3%), *S. schleiferi* (16.6%) and *S. chromogenes* (15.3%). *Enterococcus faecalis* and *Streptococcus (Str.) pluranimalium* was determined also (Table 1). **Zigo et al. (2017)** determined the incidence of *Enterococcus faecalis* in 6.1% of positive samples. **Grazia**

Puggioni et al. (2019) detected 29.4% the occurrence of *Enterococcus faecalis* in their study.

CONCLUSION

CNS were the most common group of pathogens in milk followed by increased SCC in milk. *Staphylococcus (S.) simulans* (24.4%) was the most frequent CNS in milk samples. From contagious pathogens was identified *S. aureus* in 5.3% of bacteriological positive samples. The presence of mastitis pathogens during tested period were repeated detected in 67.9% of ewes with bacteriological positive samples. 92.5% bacteriological positive milk samples had SCC $\geq 500 \times 10^3$ cells.mL^{-1}. The high SCC $\geq 500 \times 10^3$ cells.mL^{-1} and bacteriological positive milk samples from udder halves may be useful criterion for detection of subclinical mastitis and poosible use for selecting ewes for dry treatment or culling.

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