



Pathogens Transmission and Cytological Composition of Cow's Milk

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Abstract

The article deals with the data on the quantitative and species composition of somatic cells in milk of cows of Black spotted breed. In the main period of lactation, the number of somatic cells in milk is up to 100 ths/cm³. In cases of subclinical mastitis, the somatic cell count in the udder secretion increases to 30-35 mL/cm³. However, it should be noted that in the case of subclinical mastitis their number increases in thousands times. Thus, studying the species composition of somatic cells and morphological structure of basophils in milk of cows with subclinical mastitis, we did not find any relationship between their number, morphological structure

and period of disease. Results of our study show that pathogenic staphylococci (*Staphylococcus aureus*) were the cause of subclinical mastitis in 67-73% of cases. *Streptococcus agalactiae* caused the disease in about 20% of all cases. The results of the study of bacterial contamination of the udder skin showed that regardless of the animal age, pathogens of subclinical mastitis are always present on the udder skin. The main carrier of the subclinical mastitis pathogens from the sick animal to the healthy one is the rubber of milking cups.

Keywords: Cow, milk, somatic cells

Introduction

Milk and dairy products make up a huge part of the food chain of people of any age. In addition to the main components (fat, protein, carbohydrates), cow milk contains about 150 nutrients (vitamins, micro-, macroelements, etc.), which are important for the vital functions of the human body. In addition to the fact that milk and dairy products are essential for life of people, they are also a good nutritional medium for the development of microorganisms. And in case of violation of the sanitary conditions of milk collecting and storing, milk can become a dangerous source of infections (Jensen and Newburg, 1995; Ma et al., 2000).

According to the World Health Organization (WHO), as well as the statistical results of the Sanitary and Epidemiological Service of Ukraine, milk and dairy products are classified in the first

category of risks that cause food intoxication of microbial etiology. At present, one of the most important conditions for the export of domestic dairy products to European markets is the achieving of European level of quality and safety according to the European Union standards. This is an extremely important and responsible task, since the problem of the dairy products safety in Ukraine has not been resolved. According to the international food standard, it is not enough to control the quality and safety of products at the final stage, since it cannot guarantee its real safety. High quality in physico-chemical composition, milk collected in unsanitary conditions can quickly become unsuitable for human consumption or harmful to health. However, high quality and safe milk can only be collected from healthy animals. To solve such problems, modern world food industry introduces new quality management systems. One of them is HACCP (Lelieveld et al., 2016; Romain et al., 2000).

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The quality of milk and milk products and its epidemiological safety, to a large extent, depends on the sanitary state of the technological equipment, inventory and containers. The reason for the release of inappropriate quality products, as a rule, is their poor quality washing and disinfection. Sanitary treatment of milking equipment and dairy equipment is a mandatory operation in the technological process of obtaining, primary processing, storage and transportation of milk. During its operation, on the surfaces in contact with milk, its residues, protein-fat deposits, milk stones gradually accumulate, which in the future is a favorable environment for the development of microorganisms. Therefore, after each milking, it is necessary to carry out sanitary treatment of the entire set of dairy equipment using highly effective detergents and disinfectants without violating their application regimes (Murphy and Boor, 2000).

Among the diseases of dairy cows, mastitis, especially its subclinical (latent) form, deserves special attention. The main cause of this disease is the violation of housing conditions and milking technologies. Non-compliance with the milking technology (violation of the vacuum condition, old rubber of milking cups, "dry milking", etc.) cause microtrauma of the skin, milk epithelium, and parenchyma of the udder. As a result, there are some negative environmental factors, which are subsequently complemented by the pathogenic microflora (Hussain et al., 2012; Olde et al., 2010).

An important indicator of milk safety is the presence of somatic cells (blood cells and epithelial cells that are rejected from the secretory part of the udder and streak canals). According to the cell theory of inflammation, under the inflammatory process in the mammary gland (mastitis) the number of leukocytes increases and the process of phagocytosis begins. As a result, the total number of somatic cells increases, which is an indicator of the cow's udder condition. At the same time, not only the number of somatic cells changes, but also the ratio of their species composition. Thus, to diagnose subclinical mastitis, cytological examination can be used (Dufour et al., 2011; Schalm et al., 1971; Wilson et al., 1997).

Literature data suggest the following changes in the milk composition from quarters definitely positive to mastitis screening tests based on somatic cell counts compared to normal quarters. Although most of the changes in milk composition in high cell count milk can be related to decreased synthesis or increased "leakage" due to damage to udder tissue, these explanations are obviously over simplified and much more complex phenomena are involved in the total changes occurring (Schukken et al., 2003; Schultz, 1977).

The second indicator of milk safety is the bacterial contamination which reflects sanitary conditions of milk production the most accurately. The number of somatic cells depends on the cow's udder condition. But the bacterial contamination depends on many factors: milking conditions, sanitary condition of the

milking equipment, cleanliness of the cow udder and skin covering adjacent to the udder, etc. (Knight-Jones et al., 2016).

So, the determination of the quantitative and species composition of somatic cells in the milk of clinically healthy animals and animals with subclinical mastitis, as well as to find out the main sources and ways of milk contamination by the microflora is relevant and requires more detailed research.

Material and Methods

The research protocol of the current study was approved by the Ethic Committee of the Sumy National Agrarian University (Approval number: 2017/01).

The work was carried out in the Laboratory of Clinical Diagnostics of the Sumy National Agrarian University and in conditions of production at the FH "Vladana" of the Sumy region (North-eastern Ukraine) during May-June of 2017.

Animals

The study was conducted on cows of Black-spotted breed (I-IV lactation).

The experiment involved 780 heads of cows, from which 4 groups of animals with evidence of subclinical mastitis were formed. First (I) group (the first lactation) included 10 heads of cows, the II group (second lactation) - 16 heads of cows, the III group (third lactation) - 16 heads of cows, the IV group (fourth lactation) - 12 heads of cows.

Milking cows runs 2 times a day by means of milking equipment "Delaval".

The animals are kept unconstrained in a typical building. Parameters of the microclimate in the room in the study period were the following: air temperature - $16.0 \pm 0.7^\circ\text{C}$, relative humidity - $56.8 \pm 2.0\%$, carbon dioxide - $0.19 \pm 0.09\%$, hydrogen sulfide - $7.0 \pm 0.7 \text{ mg/m}^3$, ammonia - $15.0 \pm 0.7 \text{ mg/m}^3$, air speed - $1.6 \pm 0.04 \text{ m/s}$, bacterial contamination - 60.2 ± 2.1 thousands of CFU/m³ (colonies forming units).

All experimental procedures were carried out in accordance with the "Regulations for the Use of Animals in Biomedical Research" and in accordance with the recommendations of the European Convention for the Protection of Animals used for experimental purposes (Porter, 1992).

Somatic cell count

To determine healthy and infected udder quarters, was used the Rapid Mastitis Test (Kerbl Shooft, Germany), and test for the SCC (somatic cell count) in milk. After the state of udder quarters was determined, secretion from positively reacting quarters was collected into sterile cups, observing the rules of asepsis. Milk was smeared in the laboratory on Standard Methods for the (Marshall, 1992) and other references. The total somatic cell count was calculated by Prescott and Breed method

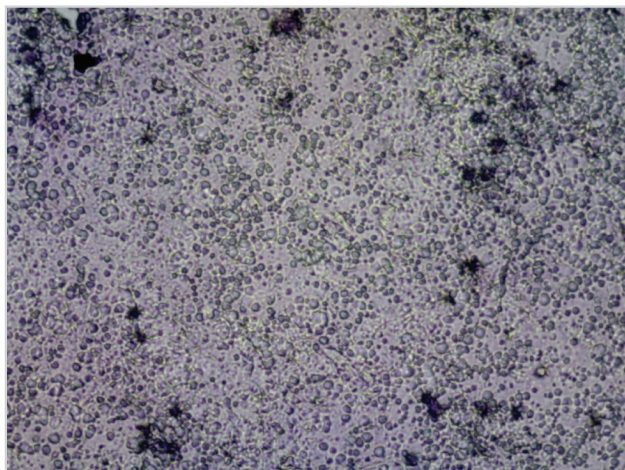


Figure 1. Somatic cells in cow's secretion with subclinical mastitis (magnification: x100)

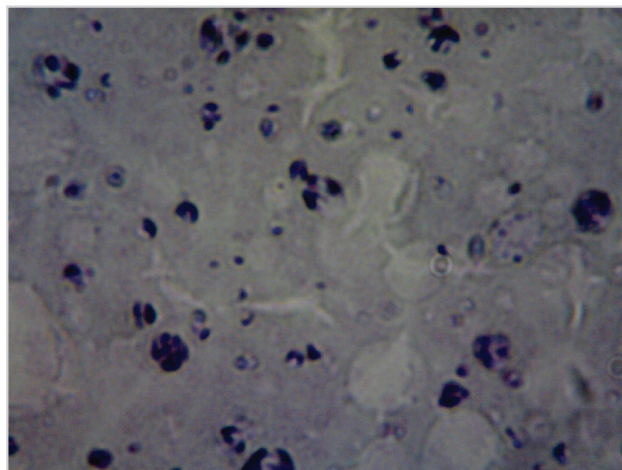


Figure 2. Somatic cells in cow's secretion with subclinical mastitis (magnification: x400)

(Prescott and Breed, 2010) and their species composition was determined.

Milk samples were taken during the morning milking from every quarter of the udder in quantity 50 mL.

To determine the number of somatic cells in cm^3 , we made a smear of 1 cm^2 in a volume of 0.02 mL. After drying, the smear in the air was fixed with alcohol-denaturate for 30 min. Then again dried and stained for Levowitz-Weber (L-W). Number of somatic cells was determined using a microscope "XS 2610 (MICROmed, Poltava, Ukraine)". To convert the number of somatic cells into 1 cm^3 of milk, we used a constant of 120.405, which was determined by us earlier (Andrievskiy et al., 2013; Shkromada et al., 2019).

Microbiological studies

Before the start of milking, disinfection of milking equipment was carried out. The study of bacterial contamination of milk cups was carried out every time when cows were milked.

To determine the microflora composition of milk, skin, udder, teats and milking equipment microbiological methods were used (Arulraj et al., 2015). For microbiological research, R-BIO-PHARM TEST SYSTEMS (Germany) were used, namely RIDA[®] COUNT, RIDA CHECK. LumitesterPD-20; LuciPacPen, RIDASCREEN Verotoxin, RIDASCREEN SET A, B, C, D, RIDASCREEN Salmonella (AFNOR EN/ISO 16140), RIDASCREEN Listeria, Sure-FoodBAC, which enable rapid and qualitative determine not only the presence of microorganisms, but also their number. To determine the conditional pathogenic microflora on the milk cups, the rapid control of the surface and liquid purity using the RIDA[®]ATP set was used, for the rapid control of pathogenic microorganisms RIDA[®]COUNT cards were used.

Used the next time and the incubation mode: to determine the total microbial number – 35°C – 24 h, to identify coliforms – 35°C – 24 h, *Escherichia coli* – 35°C – 24 h, *Salmonella* – 35°C – 24

h, *Staphylococci* – 35°C – 24 h. For microbiological monitoring the computer program "WHONET" was used.

Statistical analysis

The obtained data are statistically processed using the Fisher-Student method, taking into account the arithmetic meanings and their statistical errors, as well as the determination of the probable difference of the indicators that were compared. Significance was declared at $p < 0.05$, $p < 0.01$ and differences between means with $0.05 < p < 0.10$ were accepted as representing tendencies (Mankiewicz, 2004).

Results and Discussion

The microscopic studies of milk smears have determined specific features and number of somatic cells (Figure 1, 2). They are differentiated as lymphocytes, monocytes, neutrophils (Wall et al., 2018).

The studies carried out on the smears of cow's milk from healthy and affected quarters indicate that the somatic cell count in cow's milk is in the range from 50 to 100 ths/cm^3 .

According to the results presented in Table 1, it can be noted that in the secretion of the affected quarter of the udder, the somatic cell count increases by a thousand times. So the average amount has increased about 3 thousand times.

Determination of the species composition of somatic cells was carried out in the same smears using the immersion lens x100.

Determination of the species composition of somatic cells shows (Table 2), that both, in the milk of a healthy quarters and in the affected, species composition remains the same, but the ratio changes. So, the number of epithelial cells and lymphocytes in the milk of the affected particle decreased by 4.4 and 6.2 times, respectively. However, the number of neutrophils increased by 5.25 times.

Table 1. The content of somatic cells in cow's milk from healthy quarters and affected by subclinical mastitis (M±S.E., SCC - ths/cm³)

Group of animals (lactation)	Healthy particle	Affected particle
I (n=10)	76.1±1.9	29012.4±275.0*
II (n=16)	77.8±1.4	33396.2±265.3*
III (n=16)	78.1±1.2	27567.1±248.1*
IV (n=12)	80.4±1.3	24832.8±279.0*
Average	78.1±1.3	228702.1±286.5*

*p<0.001; SCC: somatic cell count (compared to healthy samples)

Table 2. Species composition of somatic cells in the milk of healthy and affected quarters (M±S.E., n=16, %)

Lactation	Somatic cells	Quarter	
		Healthy	Affected
		Somatic cell count	
II	Epithelial	44.8±1.7	10.1±1.0*
	Lymphocytes	38.6±1.4	6.2±0.7*
	Neutrophils	16.6±1.4	84.9±5.8*

*p<0.001; SCC: somatic cell count (compared to healthy samples)

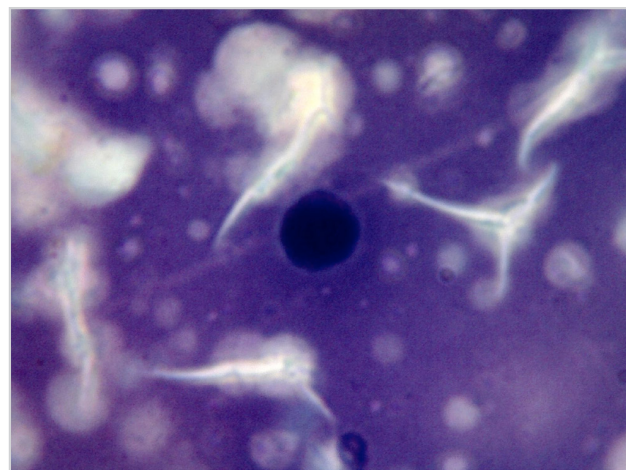


Figure 3. Lymphocyte in cow's secretion with subclinical mastitis (magnification: x1000)

Studies on determining the number of somatic cells showed that in the main period of lactation of clinically healthy animal, the SCC is up to 100 ths/cm³. In the case of subclinical mastitis, SCC increases in tens and even thousands times.

Thus, in Figure 3, the milk lymphocyte is shown in cow's secretion with subclinical mastitis. Typically, it is rounded when colored by Levowitz-Weber (LW), its nucleus of a dense consistency is intensively stained in a dark purple color; a small circle of bluish cytoplasm is clearly visible around the nucleus.

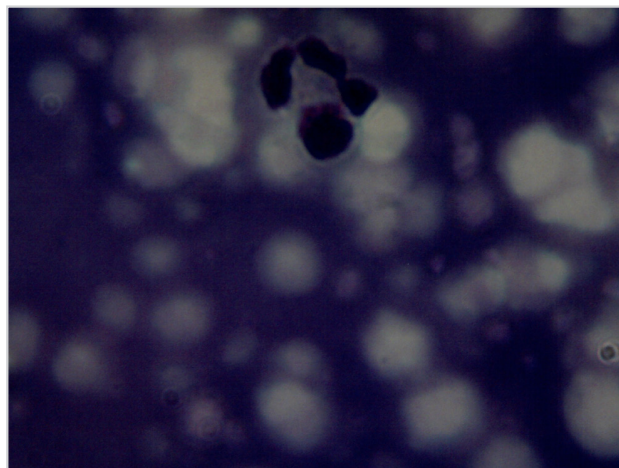


Figure 4. Segmented neutrophil in cow's secretion with subclinical mastitis (magnification: x1000)

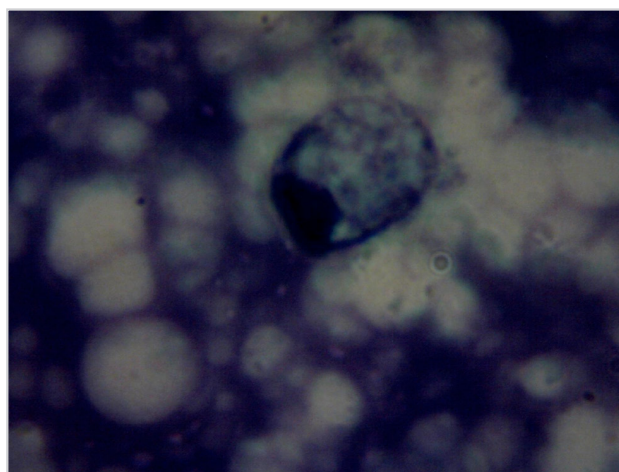


Figure 5. Basophil in cow's secretion with subclinical mastitis (magnification: x1000)

In Figure 4 segmented neutrophil is shown. Our studies have shown that neutrophils can be found in both milk of clinically healthy cows and in milk of cows with subclinical mastitis. However, it should be noted that in the case of subclinical mastitis their number increases in thousands times. In the case of disease, the number of neutrophils can amount up to 90% of all cells. Along with segmental neutrophils, stab and immature neutrophils appear in milk.

In the udder secretion of cows with subclinical mastitis, monocytes appear (Figure 5). Macrophages accumulate in large quantities in the areas of inflammation. They have a strong capacity for phagocytosis.

Basophils are granulocytes that are clearly visible on the Figure 6. They have an incorrectly rounded shape, with the nucleus of a dense consistency pushed to the periphery.

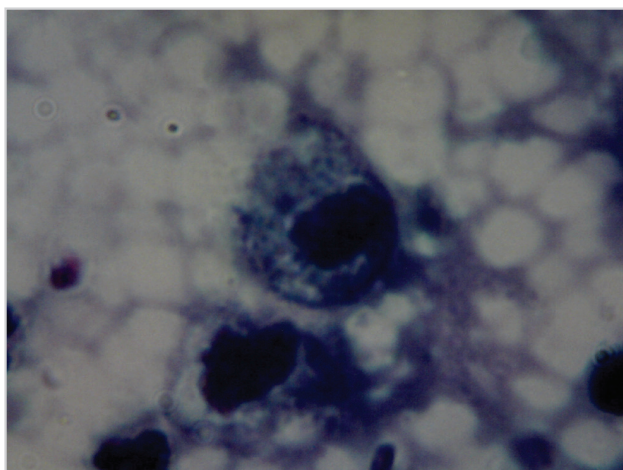


Figure 6. Monocyte in cow's secretion with subclinical mastitis (magnification: x1000)

According to the results of the study (Table 3), it can be noted that the total bacterial contamination of the milk cups was within the limits 2.1 ± 0.1 - 2.3 ± 0.3 CFU/cm² in the beginning of milking. The total bacterial contamination of the milk cups after milking five cows of I group increased by 254.6 times and after ten cows – by 636.5 times.

The same tendency was observed in relation to the general bacterial contamination of the milk cups, which were exposed to the skin of the cow's teats from other experimental groups.

Studies have shown, that on the udder skin of cows I group (Table 4) *S. aureus* forms 29%, *S. agalactiae* – 60% and associated microflora – 11% of the total number of colony-forming units.

However, the percentage of pathogenic microorganisms varied depending on the age of the animals. So, on the udder skin of cows IV group rate of *S. aureus* increased to 48% and rate of *S. agalactiae* decreased to 43% of the total number of colony-forming units.

Thus, it can be stated that even after careful cleaning and disinfection of milk cups, microorganisms still remain on it and the general microbial contamination is dynamic in the direction of increase. So, it can be assumed that the pathogens of the subclinical mastitis from the skin of the affected cow through the milk cups affect the tissues of healthy animals, thus causing transfer infection from animal to animal.

The results of the study of microbial contamination of teat and udder skin show that it always contains microorganisms that can cause subclinical mastitis (Busato et al., 2000). There is only difference in the ratio of pathogens.

Cows of 1st lactation have the smallest number of *S. aureus* and at the same time, the largest number of *S. agalactiae*. However, this ratio changes somewhat with animal aging. So, the amount of *S. aureus* slightly increases and the amount of *S. agalactiae* conversely decreases. Moreover, we have not detected any changes in the amount of associated microflora. One might assume that microorganisms on the udder skin are antagonists among themselves, especially *S. aureus* and *S. agalactiae* (Joshi and Gokhale, 2006; Schwarz et al., 2011).

Thus, pathogens of subclinical mastitis are always present on the udder skin of animals. Therefore, it is mostly impossible to treat the herd of cows completely of subclinical mastitis. But it possible to control it and keep the rate of animals infected by mastitis pathogens within 5-6%.

The main stages of disease prevention are strict compliance of the milking technology, systematic examination of cows by "cow side" tests, such as the California Mastitis Test, and measuring the electrical conductivity of milk. The separation of infected animals from healthy ones can also be used in order

Table 3. The dynamics of bacterial contamination of milk cups (M±S.E.)

Group of animals (samples)	Before milking CFU/cm ²	The fifth cow thousand CFU/cm ²	The tenth cow thousand CFU/cm ²
I (n=60)	2.1±0.1	534.7±29.9*	1336.7±45.1*
II (n=60)	2.3±0.2	601.4±23.0*	1486.3±34.6*
III (n=60)	2.3±0.3	843.6±45.0*	1568.4±67.6*
IV (n=60)	2.1±0.2	811.3±48.1*	1489.9±59.0*

*p<0.05 compared to the start of milking; CFU: colony-forming units

Table 4. Microbial contamination of the udder skin of cows (M±S.E.)

Group of animals (lactation)	Skin microflora of teats (%)		
	<i>S. aureus</i>	<i>S. agalactiae</i>	Associated
I (n=10)	29±1.4	60±3.7	11±2.0
II (n=16)	38±2.0	56±2.3	6±1.4
III (n=16)	49±3.8	44±2.9	7±1.3
IV (n=12)	48±3.7	43±4.2	9±1.2

It is known that subclinical mastitis is an infectious disease; therefore, the disease of animals can be transmitted from one animal to another. Since the transmission path is pin and the greatest contact occurs through the milk cups.

In accordance with the research objectives, we have studied the dynamics of bacterial contamination of milk cups. Before the start of milking, milk cups was thoroughly mechanically cleaned, washed with water, and disinfected. After the disinfection, milk cups was thoroughly washed with distilled water, and dried. The study of bacterial contamination of milk cups was carried out at the beginning of milking (before connecting to cows) and then every five cows. The results of the study are presented in Table 3.

to break the epizootic chain. One of the reasons for the rapid spread of subclinical mastitis in the herd is the transfer of pathogens during milking, especially from the diseased animal with increased pathogenicity to the healthy one. The main mechanical carrier of pathogens is the milk cup, since it directly contacts with the udder skin both of affected and healthy.

Therefore, if a cow affected by subclinical mastitis is present on the first stage of milking, there is a high probability that the mastitis pathogens will be transmitted to the udder skin of other animals.

Conclusion

1. In the case of subclinical mastitis the number of somatic cells in the secretion of the affected quarter of udder increases and its species composition changes.
2. Subclinical mastitis pathogens are always present on the skin of a cow's udder, but only their ratio changes with aging of the animal.
3. The main carrier of pathogens of subclinical mastitis from the infected cow to healthy one is the milk cup.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Sumy National Agrarian University with approval number 2017/01.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – O.Sk., O.Sh.; Design – O.Sk., A.P.; Supervision – O.Sh., G.I.; Resources – O.Sk., O.Sh., A.P., G.I.; Materials – O.Sk., G.I.; Data Collection and/or Processing – O.Sk., O.Sh.; Analysis and/or Interpretation – O.Sh., A.P.; Literature Search – O.Sh., G.I.; Writing Manuscript – O.Sk., A.P.; Critical Review – O.Sh.

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