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BIOMARKERS OF SUBCLINICAL MASTITIS IN THE MAMMARY GLAND OF COWS

V. R. Mazurenko[1, 2]
O. V. Manchulyak[3]

[1]"Institute of Biology and Medicine" NSC of Taras Shevchenko Kyiv National University
[2]"Center for Veterinary Diagnostics"LLC, Kyiv
[3]"Krasnosilske" DE, "Nadiya" ALLC, "Milkiland Agro"LLC, Ukraine, Chernihiv region

E-mail: mazurenkovictoriaa@gmail.com

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The aim of the study was to create an algorithm for controlling subclinical forms of mastitis of cows on the basis of determining the activity of lactate dehydrogenase and the number of somatic cells in milk. Milk samples were taken from conditionally positive cows according to the results of the California test; the activity of lactate dehydrogenase was determined and compared with the content of somatic cells in milk.

According to the results of the analyzes, 2 out of 20 milk samples had low values of lactate dehydrogenase activity, an increased number of somatic cells (more than 250 000 in 1 ml) and negative results of bacteriological examination, which may indicate on the absence of intra-infection and a physiological increase in the number of secreted somatic cells. With increased lactate dehydrogenase activity and a somatic cell level of no more than 250 000 in 1 ml, *Streptococcus agalactiae* or *Staphylococcus aureus* bacteria were isolated, indicating on a mono-infection. At the level of somatic cells from 250 000 to 500 000 in 1 ml (4 of 20 milk samples) bacteria *Streptococcus agalactiae* and *Staphylococcus aureus* were isolated, indicative on of mix infections.

Thus, the determination of lactate dehydrogenase activity makes it possible to more accurately determine the presence of inflammatory processes in the udder, since the number of somatic cells can also increase with physiological changes (e. g., stress, etc.). The results obtained can be used to determine the subclinical forms of mastitis in the infected herd. Recommendations developed on the basis of this study were implemented in practice in the economy of the Chernihiv region.

Key words: subclinical mastitis, lactate dehydroginase, the mammary gland of cows.

The lowered breast resistance and penetration of pathogenic microorganisms into the parenchyma of the organ is the trigger mechanism in the mastitis development. Mastitis is a multifactorial disease, which causes a number of interrelated issues relevant not only to the animal welfare, but also to the food safety. Economic damage caused by mastitis is the reduction in milk yields, deterioration in the quality of milk leading to its rejection, as well as the costs of animal treatment [1]. Milk contamination by pathogenic bacteria from infected cows can cause a wide range of human diseases (tuberculosis, streptococcosis, staphylococcosis, brucellosis, leptospirosis, bacterial food poisoning, etc.) [2, 3].

In most herds, subclinical mastitis is more common than clinical, so that it is difficult to diagnose due to the lack of visual signs [4]. The inflammation of the mammary gland lobes is characterized by the increased content of somatic cells in its secretion, among which leukocytes (neutrophils, macrophages, lymphocytes) account for 75%, red blood cells and epithelial cells — for 25%. Inflammatory processes are also accompanied by the release of a number of enzymes, including lactate dehydrogenase (LDH) into the milk [5, 6].

Some authors admit that the content of somatic cells up to 150,000 in 1 ml is normal [7–8]. Somatic cells (SC) in milk are one of the infection status determinants, since blood monocytes enter the milk as macrophages. During the lactation and dead period in cows, the percentage of macrophages in milk is the highest up to 68%, and up to 21% in the postpartum period.

Similar to neutrophils, somatic cells perform nonspecific functions such asphagolysisof bacteria and their destruction

by proteases and reactive oxygen species (ROS) [5, 9]. Polymorphonuclear neutrophils (PMNs) absorb and lyses bacteria; however in cows the phagocytic activity of polymorphonuclear lymphocytes (PMNLs) in milk can be inhibited by dairy fat globules and casein that leads to decay. Mastitis with a high content of somatic cells in milk is characterized by the increased proteolytic activity of enzymes [10, 11].

Bacterial toxins, enzymes, cell wall components directly affect the functions of epithelial cells, and stimulate the production of multiple inflammatory mediators. Epithelial cells of the mammary gland can produce various inflammatory mediators: cytokines, chemokines, protective peptides and arachidonic acid metabolites, which play a protective role in the infectious process prevention, enhancing the absorption and digestion of phagocytised microbes [12]. Normally, mammary epithelial cells regenerate, but during the infectious process, their number in milk will increase significantly due to the release of neutrophils into the mammary secretion [13].

The effective marker for the destruction of secretory tissues in the udder is the LDH concentration in milk, which determines the infectious process in the mammary gland. LDG is a glycolytic enzyme involved in the last stage of glycolysis process — the pyruvic acid conversionto lactic acid. The enzyme consists of four subunits of two different types H and M, respectively; therefore there are five LDH isoenzymes. The LDH concentration is interrelated with the level of somatic cells in milk, which depends on the effects of such conditions as stress, nutrition, lactation stage, etc. [14].

The enzyme presence in the milk of cows with mastitis is associated with an increased number of leukocytes and epithelial cells in the udder. Studies suggest that LDH activity often rises earlier than the content of somatic cells, which makes it an excellent biomarker for early detection of intra-venous infection [15]. Measurements of LDH activity using modern automated milk systems (e.g.:Delavale, etc.) are included in the breast health monitoring program and used as an early indicator of mastitis [16,17].

The purpose of study was to create the subclinical mastitis monitoring algorithm to determine the activity of lactate dehydrogenase and the content of somatic cells in the milk of cows.

For this purpose, it was necessary to collect milk samples from conditionally- positive animals based on the California test results, and determine the ratios of lactate dehydrogenase activity and somatic cell content in milk.

Materials and Methods

The subjects of the study were milk samples taken from cows suspected of subclinical mastitis based on the California test. 22 cow milk samples collected at the Ukrainian farm in 2017 were examined.

Milk samples to be studied were taken to sterile bottles of 30 ml and stored at +8 °C for no longer than 8 hours. [17] Containers with samples selected for testing were marked with the date of sampling. Before the study, milk samples were heated up to +20 °C and mixed until a uniform consistency was obtained.

The lactate dehydrogenase activity in milk was determined using the UdderChecktm kit (PortaChek^{tm}, USA). The test strip was extracted with tweezers and lowered into a pre-selectedsample (from one quarter of the udder) for 10 s. Then the strip was removed, and the milk remainder was shaken off, after 2 min, it was compared with the colour scale on the manufacturer's jar.

The test strip pad contains an immobilized substrate — L-lactate. After a series of enzymatic reactions, this substrate isoxidized by milk LDH, while the Nitrotetrazolium Blueindicator is converted to violet formazan. The colour intensity of the final formazan is proportional to the lactate dehydrogenase activity in milk. The results were evaluated according to Table 1.

Table 1. **Determination of LDH activity level on the basis of visual evaluation of test results UdderChecktm**

Result UdderChecktm	Probability of infection	LDH activity level
−	Low	< 100 U/L
+	Middle	100–200 U/L
++	High	200–500 U/L
+++	Very high	> 500 U/L

The number of somatic cells was determined using the PortaSCC Quick Test^{tm} kit (PortaChek^{tm}, USA). Milk drops were added to the test strip hole, without touching the strip with the pipette tip. After the milk was absorbed, 4 drops (150 µl) of the activating solution were added to the same hole. In 5–6 min, the number of somatic cells was evaluated by comparing the strip with the colour scale.

This method is based on the principle of enzymatic reaction of esterase. Enzymes located on the white blood cell wall have esterolytic activity. White blood cells in milk samples are retained in a special reagent layer on a test strip, which also contains a substrate represented by an immobilized dye. Enzyme esterase from white blood cells catalyses the reaction of substrate staining to blue, the intensity of which is proportional to SC in the sample (for more details visit www.portacheck.com).

The result is considered negative or zero if the substrate colour has not changed (< 100,000). If a light blue colour is observed, the result was considered as "one plus" (200,000 in 1 ml). If the test area is coloured sea-green, the result was considered as "two pluses" (200,000–500,000 in 1 ml). The blue colour of test strip was counted as "three pluses" (1,000,000 cells/ml, respectively). Any change in the colour of test strip, corresponding to a cell count above 250,000 per ml, is considered positive.

For a bacteriological study, the milk sample was aseptically distributed over the burner flame with a bacterial loop on the agar surface. Colombia's CNA agar + 5% sheep blood was used to extractthe pure culture (Biomerieux, France). Then it was incubated in a thermostat at +37 °C for 18–24 hours. After that, the grown colonies were subject to examination using Petri dishes and microscopic examination. After confirming the culture purity, the oxidase and catalase test was carried out, and the haemolysin response in the grown colonies was also evaluated. Colonies suspicious for *Staphylococcus aureus* were subject to a coagulase test with rabbit plasma. Gram-positive coccoid bacteria with negative response to catalase activity were identified by biochemical tests Strepto-test 16 (Lachema, Czech Republic).

Results and Discussion

Cow milk samples were selected based on conditionally positive response in the California test. The results of analysis of 20 milk samples obtained from dairy farms for determination of LDH activity with simultaneous SC detection and the results of bacteriological examination of mammary secretion are presented in the Table 2.

The findings showed that 2 out of 20 milk samples had low values of LDH activity, an increased SC number (250,000 in 1 ml), and negative bacteriological test results, which may indicate the absence of intra-infection and a physiological increase in the SC number in the secretion. With increased LDH activity and a SC level not exceeding 250,000 per ml, *Streptococcus agalactiae* or *Staphylococcus aureus* bacteria were extracted, indicating a mono-infection. At a SC level of 250,000 to 500,000 in 1 ml (4 of 20 milk samples), *Streptococcus agalactiae* and *Staphylococcus aureus* bacteria were extracted, indicative of mix infections.

Based on the findings, it could be concluded that the enzyme activity is interrelated with the increase in the SM number in the mammary gland secretion. Thus, the tests for LDH activity enables to determine the presence of inflammatory processes in the udder more accurately, since SC can also increase due to physiological changes (stress, etc.). There are no data that the level of LDH activity raises earlier than the SC number.

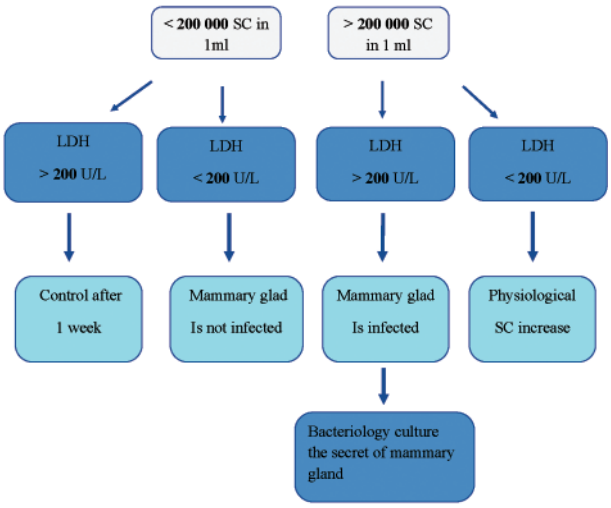
Based on the conducted studies, the authors propose the following milk analysis algorithm for the detection of subclinical mastitis: the California test, the subsequent analysis of conditionally positive samples (+/–) for LDH activity and the SM number in milk, and, if necessary, bacteriological studies (Figure).

Thus, the control of main markers of the infectious process (the level of LDH activity and the presence of somatic cells in milk) allows for the more precise determination of subclinical mastitis in the infected herd. The analysis is necessary to identify risk groups and to improve the milk quality monitoring, as well as to monitor the effectiveness of preventive measures (vaccination, udder treatment with special preparations before and after milking, etc.)

Test systems for the LDH and SC determination were provided by PortaChek^{tm}, USA, free of charge.

Table 2. **Results of detection of biomarkers of infection and carrying out microbiological investigation in secretion of the mammary gland in lactating cows**

№ milk samples	LDH (U/L)	SC (in 1 ml)	Bacteriological culture
1	< 100	< 100 000	Negative
2	200–500	250 000	*Streptococcus agalactiae*
3	100–200	250 000	Negative
4	< 100	< 100 000	Negative
5	200–500	500 000	*Streptococcus agalactiae; Staphylococcus aureus*
6	200–500	250 000	*Staphylococcus aureus*
7	< 100	< 100 000	Negative
8	200–500	500 000	*Streptococcus agalactiae; Staphylococcus aureus*
9	< 100	< 100 000	Negative
10	< 100	< 100 000	Negative
11	< 100	250 000	Negative
12	100–200	250 000	*Staphylococcus aureus*
13	200–500	250 000	*Streptococcus agalactiae*
14	200–500	500 000	*Streptococcus agalactiae; Staphylococcus aureus*
15	500	500 000	*Streptococcus agalactiae; Staphylococcus aureus*
16	500	250 000	*Streptococcus agalactiae; Staphylococcus aureus*
17	< 100	250 000	Negative
18	200–500	250 000	*Streptococcus agalactiae*
19	< 100	< 100 000	Negative
20	200–500	250 000	*Streptococcus agalactiae*
21	< 100	< 100 000	Negative control
22	> 500	1 000 000	Positive control



Milk analysis algorithm to detect subclinical mastitis:
SC — number of somatic cells in a sample of milk from one lobe of the breast; LDH — lactate dehydrogenase activity measured in secretions of milk taken from one lobe of the breast

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БІОМАРКЕРИ ІНФЕКЦІЙНОГО ПРОЦЕСУ У МОЛОЧНІЙ ЗАЛОЗІ КОРІВ

В. Р. Мазуренко[1, 2], *О. В. Манчуляк*[3]

[1]ННЦ «Інститут біології та медицини» Київського національного університету ім. Тараса Шевченка, Україна
[2]ТОВ «Центр ветеринарної діагностики», Київ, Україна
[3]МТФ «Красносільська» СТОВ «Надія», ТОВ Мілкіленд Агро, Україна, Чернігівська обл.

E-mail: mazurenkovictoriaa@gmail.com

Метою дослідження було створення алгоритму контролю субклінічних форм маститу корів на підставі визначення активності лактатдегідрогенази і кількості соматичних клітин в молоці. Зразки молока відбирали від умовно-позитивних корів за результатами каліфорнійського тесту, визначали активність лактатдегідрогенази і порівнювали зі вмістом соматичних клітин у молоці.

За результатами аналізів 2 з 20 зразків молока мали низькі значення активності лактатдегідрогенази, підвищену кількість соматичних клітин (більше 250 000 в 1 мл) і негативні результати бактеріологічного дослідження, що може свідчити про відсутність внутрішньовим'яної інфекції і фізіологічне збільшення кількості соматичних клітин у секреті. За підвищеної активності лактатдегідрогенази і рівня соматичних клітин не вище 250 000 в 1 мл виділялися бактерії *Streptococcus agalactiae* або *Staphylococcus aureus*, що свідчить про моноінфекції. За рівня соматичних клітин від 250 000 до 500 000 в 1 мл (4 з 20 зразків молока) виділялися бактерії *Streptococcus agalactiae* і *Staphylococcus aureus*, які свідчать про мікс-інфекції.

Таким чином, визначення активності лактатдегідрогенази дозволяє більш точно визначити наявність запальних процесів у вимені, оскільки кількість соматичних клітин може підвищуватись і в разі фізіологічних змін (наприклад, стресу і т. д.). Отримані результати можуть бути застосовані для визначення субклінічних форм маститу в інфікованому стаді. Рекомендації, розроблені на підставі цього дослідження, були реалізовані на практиці у господарстві Чернігівської області.

Ключові слова: субклінічний мастит, лактатдегідрогіназа, молочна залоза корів.

БИОМАРКЕРЫ ИНФЕКЦИОННОГО ПРОЦЕССА В МОЛОЧНОЙ ЖЕЛЕЗЕ КОРОВ

В. Р. Мазуренко[1, 2], *О. В. Манчуляк*[3]

[1]ННЦ «Институт биологии и медицины» Киевского национального университета им. Тараса Шевченко, Украина
[2]ООО «Центр ветеринарной диагностики», Киев, Украина
[3]МТФ «Красносельская» СООО «Надежда»,ООО «Милкиленд Агро», Украина,Черниговская обл.

E-mail: mazurenkovictoriaa@gmail.com

Целью исследования было создание алгоритма контроля субклинических форм мастита коров на основании определения активности лактатдегидрогеназы и количества соматических клеток в молоке. Образцы молока отбирали от условно-позитивных коров по результатам калифорнийского теста, определяли активность лактатдегидрогеназы и сравнивали с содержанием соматических клеток в молоке.

По результатам анализов 2 из 20 образцов молока имели низкие значения активности лактатдегидрогеназы, повышенное число соматических клеток (более 250 000 в 1 мл) и негативные результаты бактериологического исследования, что может свидетельствовать об отсутствии внутривыменной инфекции и физиологическом увеличении количества соматических клеток в секрете. При повышенном содержании лактатдегидрогеназы и уровне соматических клеток не выше 250 000 в 1 мл выделялись бактерии *Streptococcus agalactiae* или *Staphylococcus aureus*, что свидетельствует о моноинфекции. При уровне соматических клеток от 250 000 до 500 000 в 1 мл (4 из 20 образцов молока) выделялись бактерии *Streptococcus agalactiae* и *Staphylococcus aureus*, свидетельствующие о микс-инфекциях.

Таким образом, определение активности лактатдегидрогеназы позволяет более точно определить наличие воспалительных процессов в вымени, поскольку количество соматических клеток может повышаться и при физиологических изменениях (например, стрессе и т. д.). Полученные результаты могут быть применены для определения субклинических форм мастита в инфицированном стаде. Рекомендации, разработанные на основании этого исследования, были реализованы на практике в хозяйстве Черниговской области.

Ключевые слова: субклинический мастит, лактатдегидрогиназа, молочная железа коров.