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SCREENING OF DAIRY COWS' HERD FOR PRESENCE IN MILK OF HEMOLYTIC MICROORGANISMS IN RELATION TO SOMATIC CELL CONTENT

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Abstract

Inflammation of mammary glands of cows or mastitis is one of the most common infectious diseases of dairy cattle, which causes significant economic losses to livestock and affects the quality of dairy products. This work was aimed at monitoring the presence of hemolytic streptococci and Staphylococcus aureus as the main causative agents of mastitis in the high producing dairy cows and identifying relationship between the milk somatic cell count (SCC) and the infection with S. aureus. Whole herd of clinically healthy Holstein cows of the Central region of Russia from first to seventh lactations (n = 345) were used for the milk sampling. Depending on the presence of microorganisms in the milk, the cows were distributed between four groups: I - hemolytic streptococci and S. aureus are not found; II - only hemolytic streptococci are detected; III - only S. aureus are detected; IV — both hemolytic streptococci and S. aureus are detected. Determination of antibiotic susceptibility profile of S. aureus isolates was carried out on a special medium using disco-diffusion method. Determination of methicillin-resistant staphylococcus (MRSA) was carried out using the MRSA Alert w/swabs test («HiMedia Laboratories Pvt. Ltd», India). Staphylococcus aureus MRSA ATCC 43300, Staphylococcus aureus ATCC 25923 were used as reference strains. Data processing was performed using MS Excel software. The significance of differences was evaluated by Student's t test. It was found that only 22.0 % of clinically healthy cows did not contain studied forms of microorganisms in their milk, while in 78.0 % of them the presence of hemolytic streptococci and/or S. aureus in milk was detected. The rate of S. aureus infected animals increased with increasing number of lactations (from 14.7-19.2 % in the first and second lactations to 32.0-45.2 % in animals of the third and more lactations). The higher levels of SCC in the milk of cows in group III with S. aureus (+1191.1×10³, +1124.3×10³ and 1099.6×10³ cells/cm³ comparing to groups I, II and IV, respectively; p < 0.001) was established. The content of *S. aureus* in the milk of cows of groups III and IV varied from < 1.0 to 7.5 lg CFU/cm³, and from 1.0 to 7.8 lg CFU/cm³, respectively, and averaged 3.62±0.36 and 3.47±0.23 lg CFU/cm³, respectively. The associations between titers of S. aureus in the milk of cows and SCC were not identified. More than 95 % of S. aureus isolates were resistant to lincomycin and more than 50 % were resistant to erythromycin and fuzidin. Above 95 % of isolated S. aureus strains were sensitive to oxacillin, gentamicin and vancomycin. Some strains were resistant to oxacillin (1.3 %), so the further study and elucidation of the mechanisms of action using molecular methods are required.

Keywords: high-producing dairy cows, hemolytic microorganisms, *Staphylococcus aureus*, somatic cells, mastitis.

Inflammation of mammary glands, or mastitis, is a widely spread infectious disease of dairy cattle [1, 2]. According to the FAO (Food and Agriculture Organization of the United Nations, Italy), mastitis is the most devastating disease, which leads to huge economic losses [3]. Mastitis affects cows at any time during lactation, with heavy yielders more commonly develop the disease. Major milk failure falls on the illness and subsequent recovery periods. In some cows, long-term treatment results in the irreversible changes in mammary gland tissues, and the previous levels of milk yield cannot be restored. For example, in the Netherlands, a country with high culture of farming, the losses per cow attributable to mastitis range from 17 to 198 EUR per year [4]. In Russia, clinical form of mastitis is diagnosed in 20-25 % or more cows, with subclinical form being in more than 50 %, and the infection may persist for several lactations. More than 20 % of cows are rejected after the disease due to atrophy of one or more udder quarters [5]. Due to mastitis, yearly herd milk production is 12.8 % reduced [6, 7].

Multiple investigations have shown that staphylococci are the main causative agents of mastitis in cows. They are identified in acute and subacute forms of mastitis in 75-80 % and 90-98 % of cases, respectively [8-11]. The most common staphylococcus is *Staphylococcus aureus*, with some of its strains being opportunistic ones, which express virulence factors (enterotoxins), a set of small proteins that are resistant to high temperatures, acids, alkalis and proteolytic enzymes [12-14]. *S. aureus* has been found in the milk in 8.7 % of clinically healthy cows, 59.3 % of cows with subclinical mastitis and 28.8 % of cows with clinical form, with 25.0 %, 42.2 % and 33.3 % of staphylococci, respectively, being enterotoxigenic. Bulk milk contained staphylococci in 18 % of samples, 36.4 % of which were enterotoxigenic [15].

Although under the conditions adopted industrially for milk pasteurization (i.e. 75-76 °C for 15-20 sec.) staphylococci generally die, toxins produced by them essentially retain their properties. Therefore, it is necessary to monitor herds for the presence of mastitis and develop methods for their treatment.

One of the factors indicating the presence of subclinical mastitis is the increased somatic cell (SC) counts in milk, mostly white blood cells (neutrophils, macrophages, lymphocytes), erythrocytes and epithelial cells. Somatic cell counts (SCC) in the milk of healthy cows are usually less than 100 thousand cells per cm³ [16], while bacterial infection may lead to an increase in SCC \geq 1000 thousand cells per cm³ (17). The increase in SCC in mastitis occurs mainly due to the influx of neutrophils into the area of infection [18]. Most countries have established local standards for the allowable somatic cell count. In the EU, maximum limit is no more than 400 thousand cells/cm³, being 500 thousand cells/cm³ in Canada, and 750 thousand cells/cm³ in the United States.

The lowest mean number of somatic cells in cow's milk is recorded in Sweden, about 100 thousand cells/cm³, in New Zealand this value is 200 thousand cells/cm³, and in Canada it reaches 267 thousand cells/cm³ [19, 20]. Under the Technical Regulations of the Customs Union, TRCU, the standards for somatic cell counts in Russia are 500-750 thousand cells/cm³ [21]. Increased SSC (\geq 500 thousand cells/cm³) suggests the presence of pathogens in the milk, even without clinical signs of disease in the animal [22].

The main treatment methods for mastitis in cows involve the use of antibiotics. However, this method is non-effective in some cases because some strains of *S. aureus* contain specific factors of antimicrobial resistance [23-26].

In the animal survey of the Central region of Russia reported herein, we revealed a relatively high percentage of cows, the milk of which contained opportunistic pathogens, i.e. hemolytic streptococci and *Staphylococcus aureus*, and established susceptibility of the isolates to a number of antibiotic agents. A higher mean somatic cell count (SCC) has been detected in the milk of cows with *S. aureus*, however, correlation analysis has not found a relationship between the SCC values and titers of the microorganism.

This work was aimed at evaluating the presence of hemolytic pathogens, i.e. streptococci and staphylococci, in the milk of high producing dairy cows and its association with somatic cell count (SCC), as well as the antibiotic susceptibility profile of *Staphylococcus aureus* strains isolated from the milk.

Technique. Samples of milk collected individually from clinically healthy Holstein cows in a herd (n = 345) in the Central region of Russia in April 2015 were used as test materials. Cows were kept loose. Feeding was carried out in accordance with the standards established by the All-Russian Research Institute of Animal Husbandry [27]. Distribution of cows from the lactation 1 to 7 was 41.4 %, 30.1 %, 9.0 %, 7.5 %, 7.2 %, 3.2 % and 1.4 %, respectively.

Somatic cells in raw milk were counted using laser flow cytometry on a Somacount 150 instrument (Bentley Instruments, USA). For isolating and quantifying coagulase positive staphylococci, Baird Parker Agar (HiMedia Laboratories Pvt. Ltd, India) was used. Azide Blood Agar Pronadisa (Conda, Spain), with the addition of defibrinated sheep blood, was used to examine the hemolytic properties of the isolated bacteria. Identification of bacteria species was carried out using microbiological media (The State Science Centre for Applied Microbiology&Biotechnology, Moscow Province) and the kit for biochemical identification of staphylococci KB004R (HiStaph, HiMedia Laboratories Pvt. Ltd, India). The coagulase positive status of staphylococci was determined with dry citrated lyophilized rabbit plasma (CJSC ECOlab, Russia).

Depending on the presence of microorganisms in the milk, the cows were distributed between four groups as follows: hemolytic streptococci and *S. aureus* not found (I); only hemolytic streptococci detected (II); only *S. aureus* detected (III); both hemolytic streptococci and *S. aureus* detected (IV).

Determination of antibiotic susceptibility profile of *S. aureus* isolates was carried out using the disc diffusion method [28, 29] on a special medium prepared in accordance with the instructions (The State Science Centre for Applied Microbiology&Biotechnology, Moscow Province). Antibiotic susceptibility or resistance of microorganisms was assessed by the diameters of the inhibition zones in accordance with the instructions of Research Centre of Pharmacotherapy (St. Petersburg). Determination of methicillin-resistant staphylococci (MRSA) was carried out using the MRSA Alert w/swabs test (HiMedia Laboratories Pvt. Ltd, India). *Staphylococcus aureus* MRSA ATCC 43300 and *Staphylococcus aureus* ATCC 25923 were used as reference strains (The Federal Culture Collection of Pathogen Microorganisms, Russia).

Data processing was performed using MS Excel software. The significance of differences was evaluated by Student's *t*-test.

Results. It was found that only 22.0 % of clinically healthy cows did not contain studied forms of microorganisms in their milk, while in 78.0 % of cows the presence of hemolytic streptococci and/or *S. aureus* in milk was confirmed (Table 1).

Cows that are free from hemolytic streptococci and *S. aureus* were identified in the cohorts from the lactation 1 to 5, while all cows of the lactation 6 were infected with hemolytic microorganisms. The proportion of cows having hemolytic streptococci detected in their milk (group II) was not significantly different between animals of different lactations and ranged from 42.3 % to 60.6 %. It should be noted a tendency to an increase in number of *S. aureus*-infected animals with increasing number of lactations (i.e., from 14.7 % and 19.2 % in the first and second lactations, respectively, to 32.0-45.2 % in animals of the third and up to the sixth lactation (Fig.).

1. Categorization of the surveyed Holstein cows depending on the presence of studied forms of microorganisms in the milk (n = 345, Moscow Province, 2015)

Group	Hemolytic	Star byla ag agus guraus	Number of cows			
	streptococci	Staphylococcus auteus	п	proportion from total population, %		
Ι	N/D	N/D	76	22.0		
II	+	N/D	190	55.0		
III	N/D	+	25	7.0		
IV	+	+	54	16.0		
3.7	1.11/17					

N o t e. «+» and N/D means that microorganisms are detected and not detected, respectively.



Categorization of the surveyed Holstein cows depending on the lactation number and the presence of studied forms of microorganisms in the milk: a — hemolytic streptococci and *Staphylococcus aureus* not detected (group I); b — only hemolytic streptococci identified (group II); c — only *S. aureus* identified (group III); d — both hemolytic streptococci and *S. aureus* detected (group IV) (n = 345, Moscow Province, 2015).

SCC values ranged from 8000 to 6099, and averaged to 509.8 ± 46.4 thousand cells/cm³. Distribution of cows based on the SCC value according to the TRCU standard (< 500000, 500000-750000 or > 750000 SC/cm³) was 73.3 %, 14.8 % and 11.9 %, respectively.

2. Somatic cell counts (SCC) in the milk of surveyed Holstein cows with different microbiological status (Moscow Province, 2015)

Group	SCC, thousand SC/cm ³			and SC/cm ³	Distribution by class, %			
	min	med	max	mean	< 500 thousand SC/cm ³	500-700 thousand SC/cm ³	> 750 thousand SC/cm ³	
Ι	11	149	2016	391.1±44.9 ^a	81.6	9.2	9.2	
II	8	212	6099	457.9±59.2 ^b	74.7	13.2	12.1	
III	60	1275	4642	1582.2±284.0 ^{a, b, c}	28.0	8.0	64.0	
IV	8	227	5380	482.6±119.7°	77.8	13.0	9.3	
Note.	$\overline{SC} - s$	somatic	cells; n	nin, med, max mean	the minimum value,	median, and the max	imum value, respec-	
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tively. Group description is given in the Technique section. Distribution by class is based on the SCC value (thousand SC/cm³) according to the Technical Regulations of the Customs Union (TRCU) standard 033/2013 [22]. ^{a, b, c} Differences between groups marked with the same letter are significant at p < 0.001.

The counts of SC in the milk of cows in group III were significantly higher when compared to groups I, II and IV (+1191.1 thousand, +1124.3 thousand and +1099.6 thousand SC/cm³, respectively; p < 0.001). Group III was characterized by a minimum proportion of animals with SCC below the threshold (< 500 thousand SC/cm³) and the maximum number of cows with SCC > 750 thousand SC/cm³. The presence of hemolytic staphylococci (group II) had no considerable impact on the SCC, therefore, there was another reason of infection. Since there were no apparent differences in the SCC value between cows in groups I and IV, further investigations on a larger sample of animals were required (Table 2).

The *S. aureus* number in the milk of cows ranged in group III from < 1.0 to 7.48 lg CFU/cm³, and in group IV from 1.0 to 7.78 lg CFU/cm³, and averaged to 3.62 \pm 0.36 and 3.47 \pm 0.23 lg CFU/cm³, respectively. The associations between titers of *S. aureus* and SCC were not identified.

Investigation of the *S. aureus* strains isolated in the milk confirmed their species status. Inoculation of Azide Blood Agar with stock cultures resulted in a clear visualization of large areas of hemolysis around isolated typical colonies of

the microorganism due to the effect of produced hemotoxin possessing pronounced hemolytic activity. *S. aureus* isolates possessed lecithinase activity, produced coagulase, which, when binding with prothrombin, converted fibrinogen to fibrin, thereby leading to a clot formation, and also fermented carbohydrates (sucrose, lactose, trehalose, maltose) with release of acid without gas, and mannitol in aerobic and anaerobic conditions.

Measurement of the inhibition zone size showed that 97.5 % of *S. aureus isolates* identified are resistant to lincomycin, 59.5 % to erythromycin, and 50.6 % to fucidin. Some strains (1.3 %) that could be classified as MRSA were resistant to oxacillin.

Antimicrobial	Categorization of isolates based on the diameter (D) of inhibition zones						
Antimicrobian	resistant		intermediate		susceptible		
agent, dosage	D, mm	%	D, mm	%	D, mm	%	
Benzylpenicillin, 10 ED	20	12.7	21-28	3.8	29	83.5	
Oxacillin, 10 µg	15	1.3	16-19	0	20	98.7	
Gentamicin, 10 µg	13	3.8		0	14	96.2	
Erythromycin, 15 µg	14	59.5	15-20	32.9	21	7.6	
Lincomycin, 15 µg	19	97.5	20-23	0	24	2.5	
Rifampicin, 5 µg	12	16.5	13-15	2.5	16	81.0	
Ciprofloxacin, 5 µg	15	11.4	16-20	12.7	21	75.9	
Vancomycin, 30 µg	11	1.3		0	12	98.7	
Fucidin, 10 µg	16	50.6	17-20	45.6	21	3.8	
Novobiocin, 30 µg	16	26.6		0	17	73.4	

3. Distribution of the identified *Staphylococcus aureus* isolates from the surveyed Holstein cows' milk based on their susceptibility (resistance) to antimicrobial agents (Moscow Province, 2015)

Thus, screening of a dairy herd in the Central region of Russia revealed a relatively high percentage of cows, the milk of which contained hemolytic streptococci and *Staphylococcus aureus*. i.e., conditionally pathogenic microorganisms. A higher mean somatic cell count (SCC) has been detected in the milk of cows with *S. aureus*, however, correlation analysis has not found a relationship between the SCC values and titers of the microorganism. Comparison of antibiotic susceptibility showed that over 95 % of the *S. aureus* strains were resistant to lincomycin, and 50 % strains were resistant to erythromycin and fucidin. The identified oxacillin-resistant strains can be classified as MRSA (methicillin-resistant staphylococcus), therefore further investigations of their genetic structure using molecular techniques are necessitated.

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