The Study of Antiphagocytic Activity of Streptococcus Isolated from Cows During Mastitis and Endometritis

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Abstract. Streptococcal mastitis causes enormous economic damage in dairy farming. Milk loses its nutritional value due to the appearance of pathogenic bacteria in it that causes food toxicosis. In this study we used 28 isolates of streptococci isolated from cows with mastitis and endometritis. Of these, 38.9% belonged to S. agalactae, 33.3% to S. dysgalactae, and S. equi subsp. zooepidemicus - 22.2%. A small number of isolates - 5.6% belonged to the species S. equi subsp. equi and S. uberis. In the test with mouse peritoneal macrophages shows that the antiphagocytic activity was 75% for isolates of S. zooepidemicus, 57% for S. agalactiae, and 64% for S. dysgalactiae. At the same time, the phagocytic number and phagocytic index were higher in the phagocytosis of the most strains featured with trypsin compared to the initial ones indicating to the antiphagocytic role of the pathogenic structures presented in them at the stage of bacteria capture. The numbers of factors of streptococci pathogenicity including M-proteins make them able to resist opsonization and phagocytosis.

1. Introduction
Currently, mastitis and endometritis of infectious etiology remain a significant problem for dairy farming. Despite the efforts of the last 30 years for the eradication of mastitis and endometritis caused by these microorganisms these diseases remain very common (60-70%) and cause the greatest economic damage to livestock.

It is known that more than 140 types of bacteria can be the causative agents of the mastitis however more often streptococci and staphylococci are isolated [1,6]. According to a number of authors [7,8,16], the causative agents of clinical mastitis were streptococci (36.0%), staphylococci (28.0%), Escherichia (14.0%), enterococci (11.0%), enterobacteria (4.0%), Pseudomonas aeruginosa (4.0%) and Proteus (3.0%). While the examination of the udder secretion samples of cows with mastitis from the Vologda Region 608 cultures of microorganisms were isolated, including pathogenic staphylococci (Staphylococcus aureus) in 20.2%, conditionally pathogenic staphylococci in 32.8%, streptococci in 18.0%, enterobacteria in 13.4% and in 15.6% of cases mixed microflora [1]. According to the V.V. Kryukova. et al. [10] in the farms of the Leningrad region Staphylococcus spp. compose 43%; Streptococcus groups A, B, C, E - 29%; Escherichia coli - 22%; Streptococcus group D - 0.1%; genus Bacillus - 3%; yeast-like funguses - 2%. Streptococci that cause mastitis often belong to the hemolytic species: S. agalactiae - 32%; S. facialis - 19%; S. dysgalactiae –10%; S. uberis - 7%; S. pyogenes - 3% [10,19], which belong to serogroups A, B, C, D, E according to R.C. Lancefield [11,15].
Streptococci penetrated into milk and dairy products are able to cause food toxicosis in humans. This is due to the fact that these bacteria produce exotoxins, which accumulate in food and cause intoxication [3,20]. Streptococcal toxins are pyrogenic enterotoxins (A, B, C, D), which are sympathicotrophic toxins inducing synthesis of tissue necrotic factor and interleukins. As a result of their action, fever and shock are developed [13]. In addition, streptococci have other equally important virulence factors: a capsule, adhesins, enzymes (hyaluronidase, plasminogen-binding protease, streptokinase, enolase, deoxyribonuclease) [14,17], etc. The capsule of streptococci serogroups A and C consists of polymers, hyaluronic acid and therefore, it does not induce an immune response in infected animals, which ensures that bacterial antigens are not recognized by phagocytes, complement and antibodies. The capsule of streptococci consists of three layers, the main component of which is peptidoglycan, consisted of polysaccharide-C and lipoteichoic acid, which ensures the attachment of streptococci to the epithelial cells of the macroorganism. In S. agalactiae, the surface antigens C and Rib, showing resistance to trypsin and are of the greatest importance. S. equi subsp. zooepidemicus, protein M and M-like proteins SzP and SeM are important virulence factors [17]. M-proteins make streptococci be able to resist opsonization and phagocytosis. Protein M is one of the virulence factors: mutants lacking fimbriae are not virulent. It was believed that knowledge of the structure and function of M-proteins can help in the development of vaccines against streptococcal infections. However, it turned out that not all streptococci express the fimbrial antigen M. Moreover, during cultivation, many isolates lose the ability to synthesize it. It has been found that there are about 100 M serotypes. At the same time, antibodies against one serotype do not provide protection against others, as at the N-terminus of M-proteins there is also a hypervariable region of 11 amino acid residues, the sequence of which is different for different serotypes of M-proteins [4,5,9,17,18]...

There have been numerous attempts to create vaccines against streptococcal mastitis, which have been ineffective. At the same time, the research results indicate that each of the surface antigens, taken in purified form, has a low immunogenic activity. Possibly in this case these or those combinations of antigen surface can have a protective effect [2,6,]. In our opinion, current vaccines do not take into account such factors of bacterial resistance to immunity as protection from phagocytosis. From the fundamental studies, it is known that the treatment of streptococci of group A with trypsin inhibits their antiphagocytic activity [11]. We decided to use this methodological technique to study strains of streptococci isolated from cows in the test of phagocytic activity with peritoneal macrophages of the mouse. In the future, it was supposed to select the strains with the highest antiphagocytic activity in order to study and obtain an immunogen from them for the subsequent stages of vaccine development.

2. Materials and methods
Scientific work was carried out in the period 2018-2019 in the laboratory of microbiology with the museum of typical cultures of the Federal State Budgetary Institution "Federal Scientific Center VIEV" (FGBNU FSC VIEV RAS) in conjunction with the Vologda branch.

In the studies 28 isolates of streptococci species S. equi subsp. zooepidemicus, S. dysgalactiae, S. agalactiae, S. equi subsp equi, S. uberis isolated from cows from regions of five farms of Vologda, two of Yaroslavl, one of Kostroma and one of Moscow were used. As pathological materials, samples of milk and vaginal from cows with mastitis and endometritis were used. For the determination of serological group of the isolated strains, a kit for the identification of streptococci Patho DxtraTM was used.

Comprehensive bacteriological diagnostics was carried out using differential diagnostic and selective culture solid nutrient media: bile esculin agar with sodium azide, selective agar for streptococci, blood 5% agar, nutrient agar with 0.5% glucose, as well as liquid nutrient media: trypsinase - soy broth, broth for streptococci (according to Todd-Hewitt), glucose-whey broth, Hottinger's broth Himedia (India). We also used a selective additive for the isolation of streptococci Strepto Supplement manufactured by Himedia (India) and staphylococcus S. aureus (for SAMP - test). To assess the phagocytic activity, consumables were used: phosphate buffer, pH 7.2 (PBS), medium 199, fetal bovine serum (FS),
streptococcal cells S. equi subsp. zooepidemicus, S. dysgalactiae, S. agalactiae, grown for 18-24 hours, methyl alcohol, azure B-eosin solution, 0.25% trypsin solution.

In addition, to study the saccharolytic and proteolytic properties of isolated bacterial isolates and for the purpose of their generic and species identification, biochemical tests "Strepto test 16" "Lachema", (Czech Republic) with additional reagents Phosphatase and Hippurat were used.

Cultivation of the strains was carried out according to standard microbiological methods, including the study of colony morphology, biochemical properties, and Gram staining of smears.

Cultures were inoculated in a AirStream ESCO Class 2 BBC microbiological safety box (USA). A Zeiss Axio Vision microscope (Germany) was used to study smears and count cells. The inoculations were incubated in a Sanyo incubator MIR 262 dry air thermostat (Japan), and peritoneal macrophages in a Sanyo CO₂ incubator (Japan). To determine the concentration of microbial cells by the McFarland method, a DEN-1B densitometer (Latvia) was used, and the cells were centrifuged using an MPW-380R centrifuge (Poland). After the end of the research work, all the equipment was treated with 70% ethyl alcohol, the air in the room was disinfected with an ultraviolet bactericidal mobile irradiator-rectifier ORUBp-3-5- "KRONT" (Dezar-7) (RF) for 60 minutes.

To assess the phagocytic activity of peritoneal macrophages, peritoneal exudate cells were taken from mice, which were centrifuged twice at 300g for 5 min. After washing, it was resuspended in medium 199 containing 5% PS and added to polystyrene Petri dishes (40 mm). The cells were incubated for 1 hour at 37 °C, then washed with a monolayer with phosphate buffer (pH 7.2).

Phagocytic activity of peritoneal macrophages (PM) was determined using streptococcal cells in the stationary growth phase with a concentration of 106 cells / ml in an amount of 1 ml in medium 199 supplemented with 10% fetal serum without antibiotics. Co-incubation of bacteria with peritoneal cells of mice was carried out for 30 min at T 37 °C in a CO₂ incubator with 5% CO₂ in a ratio of 1:30 (macrophages / bacteria). After washing the bacteria five times with PBS, the cells were fixed with methyl alcohol for 10 min, and stained with a solution of azure B-eosin for 20 min.

When treated with 0.25% trypsin solution, the cell suspension in 0.9% NaCl solution was combined in a 1: 2 ratio (cells / trypsin) and left for 10-15 min at room temperature. Then it was centrifuged at 300g for 10 min, and the pellet was resuspended in PBS to the concentration required for phagocytosis.

The experimental results were evaluated microscopically by two parameters: phagocytic number (PN) and phagocytic index (PI) per 100 macrophages [12].

3. Results and discussion
Totally, 28 streptococcal isolates were isolated, which, according to their biochemical properties, were assigned to the following species: Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus equi subsp. zooepidemicus, S. equi subsp equi, S. uberis.

**Isolates of the genus Streptococcus isolated, %**

![Figure 1](image)

**Figure 1.** Isolation of isolates of the genus Streptococcus from cows with mastitis and endometritis, %.
As follows from Figure 1, the largest number of isolates from cows with clinical signs of mastitis and endometritis belonged to the species S. agalactiae - 38.9%, S. dysgalactae - 33.3%, as well as S. equi subsp. zooepidemicus - 22.2%, and only a small number of isolates 5.6% belonged to the species S. equi subsp. equi and S. uberis. When determining the serogroup according to Lancefield R. in S. agalactiae, 72% of isolates belonged to group B and 28% to group C. 100% of isolates from group C were in S. equi subsp. zooepidemicus, S. dysgalactae and S. equi subsp. equi. According to serological identification by the group polysaccharide antigen, S. agalactiae species were assigned to groups C and B, which is probably due to the most immunologically active rhamnose that is part of the cell wall, which causes a cross-reaction between streptococci of groups B and C.

The next stage of our research was to study the effect of the obtained strains of Streptococcus on the functional activity of macrophages during their co-cultivation. For this purpose, we took the species S. equi subsp. zooepidemicus, S. dysgalactae, and S. agalactiae.

As can be seen from Figure 2, majority of the strains had the ability to significantly reduce phagocytosis upon contact with the peritoneal macrophages of mice, which indirectly indicated the presence of antiphagocytic structures in them. Treatment of living bacterial cells with trypsin at a final concentration of 0.53 PU/ml in order to destroy these structures significantly (2.6 times on average) reduced the antiphagocytic activity of the treated streptococci as compared to intact ones.

![Figure 2. Phagocytic index and phagocytic number of murine peritoneal macrophages against S. equi subsp. zooepidemicus, S. dysgalactae and S. agalactiae treated with 0.25% trypsin solution and controls (no treatment).](image-url)

For many pathogens the function of protection against phagocytosis is the most important in the mechanism immunity resistance. At the same time, overcoming the killer mechanisms developed by the phagocyte can occur at various stages and are often duplicated. Despite the fact that the mechanisms allowing pathogens to escape from the action of phagocytes are largely unclear, one of the key roles is played by the capsule and capsule-like structures of microorganisms. At the same time, the capsule, which forms gel-like structures due to its hydrophilicity, remains sufficiently permeable for many active molecules - immunoglobulins, complement, etc. In this regard, a second "echelon" of protection has formed in pathogenic microorganisms, which resists the attack from the immune system. The molecules of staphylococcal protein A or M-protein of streptococci can be as an example of such protection. A whole set of pathogenicity factors is associated with the mechanisms of disturbance of killing of microorganisms inside phagocytes. Perhaps, there are other not yet studied mechanisms that prevent the adhesion and phagocytosis of pathogens. Another group of pathogenicity factors are molecules that...
inhibit or, conversely, mimic the action of certain cytokines. It should be noted that many bacterial antigens are capable of altering the production of cytokines in the host organism. This brief information confirms a huge variety of pathogenicity factors and their unique, often duplicated functions.

As it is already mentioned above, it seemed relevant to us to investigate the antiphagocytic activity of strains of streptococci isolated from cows upon contact with peritoneal macrophages of the mouse in order to choose among them those that actively resist phagocytosis.

Of the studied streptococci of different species, antiphagocytic activity was shown by: S. zooepidemicus - 75% of isolates; S. agalactiae - 57% and S. dysgalactiae - 64%. The phagocytic number and phagocytic index were higher in the phagocytosis of most of the isolated strains treated with the enzyme compared to the initial ones, which indicates the antiphagocytic role of the pathogenic structures present in them at the stage of bacterial capture.

In addition to the control treatment of bacteria with trypsin, which destroys surface pathogenic structures, we took into account that the presence of a capsule can also enhance the antiphagocytic effect of streptococci upon contact with macrophages [6,15]. To exclude the effect of the capsule antigens on phagocytosis, which was present in some isolated strains, cultures were used after 9 hours of growth, when the capsule collapses and loses its pathogenic properties. In our experiments, the absence of a capsule in "old" cultures increased phagocytosis compared with "young" cultures - 6-9 hours.

Since the study was carried out with the addition of inactivated fetal serum, the blockade of complement activation and antibody binding, apparently did not play a role here. It is possible that surface pathogenic structures linked other humoral factors present in fetal serum or blocked the recognition of streptococci by pattern-recognizing macrophage receptors [12].

4. Conclusion
The most widespread streptococcal species isolated from cows with clinical signs of mastitis and endometritis from 9 farms of four regions of the Russian Federation were: Streptococcus agalactiae, S. dysgalactiae, S. equi subs. zooepidemicus belonging to groups B and C. The increase in phagocytic activity (phagocytic index - 2.6 times and phagocytic number - 3 times) processed with the enzyme streptococci were evaluated as a sign of the possible presence of M-like structures. Thus, numerous virulence factors allow streptococci to resist the immune system, penetrate into the tissues of macroorganisms and survive after phagocytosis.

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