Bacteriophage-mediated sensibilization of mammary gland

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Abstract. In the scientific and practical fields of dairy farming a lot of attention is given to pathologies and diseases of cow’s mammary gland. Mastitis, as a nosological unit, is an actual targeted direction for many researchers. The development of preventive and therapeutic medicines based on bacteriophages is a promising and demanded direction in dairy farming. In the course of work was shown reactogenicity of mammary gland after the intracisternally administration of viral antigen. It was manifested by vividly expressed clinical signs of sero-fibrinous inflammation. The described reaction is very similar to the manifestation of a non-specific antiviral immune response. It was noted the dose-dependent intensity of the somatic reaction when inactivated bacteriophages were administrated. The clinical manifestation of a non-specific protective antiviral mechanism of the mammary gland was shown too. Method of phage-mediated sensitization of the mammary gland was proposed for the first time.

1. Introduction
The mammary gland is a unique organ, the composition of its secret is still not reproducible in laboratory conditions. Milk provides nutrition and protection of progeny in the early postnatal period of ontogenesis.

Protective factors of mammary gland secretions are various and include as cellular components: T- and B-lymphocytes, macrophages, nonspecific soluble factors – lysozyme, lactoferrin and others, so and useful microflora: lactic streptococci (St. lactis, St. cremoris, St. citrovorum, St. Paracitrovormn, St. Terraophilius), lactobacilli (L.bifidum, L. casei, L. acidophilus, L. Bulgaricu), lactococcus (Lactococcus lactis) and others [1, 2, 3, 4, 5].

Non-specific antibacterial protection of progeny is carried out with milk by lactoferrin, lactoperoxidase, antagonistic strains of lactic bacteria, by macrophages. The key factors of specific protection are immunoglobulins of classes A and G [6, 7, 8].

In the last decade, researchers have noticed the essential role of intestinal microflora in the immune status of mammary gland [9, 10].

In research of the possibility of preventive and therapeutic using of bacteriophages in mastitis in cows [11] was recorded somatic reaction in response to the administration of products with bacteriophages. Thus, arose the task of studying this reaction.

The purpose of this study was to explore occurrences of somatic reaction to intracisternal administration of bacteriophages.
Research objectives:

- determine the sensitization factor of the non-specific immune response of mammary gland;
- to study the strength of the somatic reaction to bacteriophages;
- determine whether phag-mediated immunization can be used.

2. Materials and methods

We used bacteriophages from the collection of SPC "MicroMir" (a mixture of phagolysates of medication «Phagoderm»), and bacteriophages from the medication "bacteriophage Typhimurium". The experiments were carried out on the base of CJSC "Plemreproductor Vasilievskoe", on a dairy farm "Alferievo" on cows of black-motley (holstein) breed.

For intracisternal administration of fagolizates were used sterile single-use syringes, firstly, before injection we got rid from "bacterial plug" through short milking, the solution of filtered fagolizates was injected into one lobe of udder. Samples of funds were administrated immediately after milking. The number of somatic cells in milk was measured using the viscometric apparate "Somatos-mini" and the California test (DeLaval). Samples for microbiological studies were transported using Hiculture Transport Swabs w/Cary-Blair Medium with Cary-Blair medium. Seeding was carried out on media: MPA, MPB, BHI, Endo medium, chromodistris medium, Levin agar, streptococcal broth, Schedler medium, chromogenic medium "Uriselect-4", mannitol-salt agar, FMH-agar, Saburo agar. Identification of microorganisms was performed using cultural, morphological, and biochemical characteristics, and the definitive species identification was determined using the Bruker Daltonik Biotyper mass spectrometer.

Groups were formed on the principle of analogues. 5 experiments were performed:

- Single administration of complex of bacteriophages into a healthy udder in a dose of 10 ml with concentration of bacteriophages of 5,6±3,7x10^6 PFU/cm^3;
- Single administration of phages into a healthy udder lobe, identification of persistent bacterial species, administration of non-specific bacteriophage to isolated bacteria, using the medication "bacteriophage Typhimurium" in a dose of 10 ml with bacteriophage titer of 9,1±4,2x10^8 PFU/cm^3;
- Single administration of an inactivated bacteriophage specific to Staphylococcus aureus;
- Single administration of cocktail of bacteriophages into a healthy udder in a dose of 10 ml with concentration of 2,1±0,5x10^3 PFU/cm^3;
- Double administration of cocktail of bacteriophages in the udder lobes of cows with subclinical mastitis at an interval of 24 hours in a dose of 10 ml with concentration of 3,4±0,3x10^6 PFU/cm^3.

3. Research results

As a result of the 1st experiment, the appearance of a somatic reaction was confirmed, findings are presented in table 1.

Table 1. Dynamics of the number of somatic cells with a single administration of a cocktail of bacteriophages into a healthy udder lobe.

| # Cow number | Day of monitoring |
|--------------|------------------|
|              | 1  | 2  | 3  | 5  | 7  | 9  | 10 | 11 | 12 | 14 | 16 | 18 | 20 |
| 1  1689  | 390 | 592 | 890 | 1002 | 945 | 543 | 392 | 376 | 355 | 352 | 327 | 349 | 402 |
| 2  9541  | 405 | 856 | 987 | 995 | 876 | 451 | 493 | 409 | 378 | 376 | 403 | 409 | 379 |
| 3  4291  | 419 | 778 | 897 | 786 | 659 | 721 | 550 | 532 | 437 | 391 | 340 | 320 | 347 |
The analysis of the data presented in the table 1 shows that after a single administration of a cocktail of bacteriophages in a dose of 10 ml with concentration of 5,6±3,7х10^6 PFU/cm^3 causes vividly expressed increase of somatic cells number in the milk, abundant releasing is accompanied by excretion of fibrin into the cistern and, as a result, blockage of the teat meatus. This experiment confirmed the reaction obtained during the therapeutic use of a cocktail of bacteriophages in clinical forms of mastitis in cows.

The following experiment was conducted to increase the understanding of the process of sensibilization, whether this reaction is the result of abundant lysis of ubiquitous species of bacteria. The use of "bacteriophage Typhimurium" containing active against S. typhimurium bacteriophages showed similar results, despite the absence of these types of bacteria in mammary gland, established by microbiological studies, the occurrence of the inflammatory process was similar to that shown in table 1. The group was formed from 12 cows. The dynamics of increasing and decreasing the number of somatic cells in intensity and time points was, within the statistical error, similar to the previously conducted experiment.

Intracisternal administration of inactivated bacteriophage vB-SaP-Z1/3.1 specific to S. aureus yielded similar results. Inactivation was performed by heating to 70 C for 1 hour, followed by control on bacterial cultures. Thus, the hypothesis of mucosa of the mammary gland sensibilization by components of bacteriophages acting as antigen was put forward.

A single administration of a cocktail of bacteriophages with reduced titer of 2,1±0,5х10^6 PFU/cm^3 showed a similar result in a group of 7 cows. However, the peculiarity of using a reduced phage concentration was a smaller number of somatic cells at the peak of the response, so, on average, the values were kept for 2 days at the level of 634±49 thsd, while returning to original values was longer than when using high doses, on average for 11±3 days.

**Figure 1.** Dynamics of the number of somatic cells in milk after phage-mediated sensibilization.
Analysis of the research results suggested the possible using of bacteriophages as initiators of an inflammatory reaction in subclinical mastitis with a chronic course in order to exacerbate the process and try to increase the grade of milk. For this purpose, health examination of the dairy herd during the peak lactation period was carried out and 7 cows were selected. Based on the results of previous experiments, a dose of 3,4±0,3x10⁶ PFU/cm³ of cocktail containing 54 bacteriophages specific to opportunistic and pathogenic types of bacteria was selected for preliminary trials on phage-mediated sensibilization. At the same time, the number of administrations was increased to 2 times with an interval of 24 hours. The results of the experiment are shown in figure 1.

The graphs show, that after 2-fold administration the number of somatic cells in milk increased to a limit value which viscosimetrically analyzer "Somatos mini" can recognise -1500*10⁳/ml, which was sharply reduced by 5-7 days after first injection. In 4 animals, the number of somatic cells on the 18th day after the first administration of bacteriophage cocktail decreased in comparison with the number of cells before administration.

4. Result discussion
Bacteriophages, like any antigen, can cause a response from the immune system. However, this phenomenon is extremely rare, and it is difficult to obtain a stable and long-lasting immune response to parenteral administration of bacteriophages in the laboratory. The response to the intracysternal administration of bacteriophages is a feature of the mammary gland, currently it is not established what exactly causes such reaction. Conducted clinical trials of "Vetagin", consisting of bacteriophages intended for the prevention and treatment of postpartum endometritis in cows, did not reveal a correlation between the number of somatic cells in milk and repeated use of the drug by uterine sanitation. Additional studies are conducted to determine the type of inflammatory reaction, hematological, histological, and cytological, as well as immunological studies. The conducted clinical trials show that it is possible to exclude the version of associated with abundant lysis of bacterial cells reason of an inflammatory reaction in the mammary gland. Clinical manifestations of an inflammatory reaction and an increase in the number of somatic cells are detected when inactivated bacteriophages are introduced. Phagolysates may contain residues of bacterial debris, total protein and lipopolysaccharides, however, the samples of bacteriophages used in research are strictly controlled for the content of incidental structures, their content in the medications is extremely low, LPS - 10±2 mcg/ml, total protein 5±1 mcg/ml.

Studies of phage-mediated sensibilization have shown that it was possible to achieve a positive result in 4 out of 5 experimental animals. It was possible to reduce the number of somatic cells in milk by 2-fold administrations of cocktail in a dose 10 ml with bacteriophage titer of 3,4±0,3x10⁶ PFU/cm³. The duration of the effect is not shown in these studies, observations continue.

5. Conclusion
Bacteriophages have a tremendous perspective in terapeutical and preventive measures in veterinary medicine and agriculture. The experience of their usage is likely to have more weight than detailed selection of bacteriophages using molecular genetic studies. Studies have shown the possibility of nonstandard use of bacteriophages for phage-mediated sensibilization of mammary gland in order to achieve a therapeutic effect. It is worth noting that further research is needed to track the duration of the effect, increase the ability to control sensibilization by selecting doses, the frequency of use and the time of administration.

References
[1] Bonsaglia E C, Gomes M S, Canisso I F et al. 2017 Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland Scientific Reports 7 8067
[2] Tong J, Zhang H, Zhang Y et al. 2019 Microbiome and Metabolome Analyses of Milk From Dairy Cows With Subclinical Streptococcus agalactiae Mastitis-Potential Biomarkers Front
Microbiol 10 2547

[3] Andrews T, Neher D A, Weicht T R et al. 2019 Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status PLoS ONE 11(7) 1-16

[4] Oikonomou G, Addis M F, Chassard C et al 2020 Milk Microbiota: What Are We Exactly Talking About? Front Microbiol (2020)00060

[5] Addis M F, Tanca A and Uzzau S 2016 The bovine milk microbiota: insights and perspectives from -omics studies Molecular BioSystems 12(8) 2359-72

[6] van Hooijdonk A, Kussendrager K and Steijns J 2000 In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence British Journal of Nutrition 84(S1) 127-34

[7] Paape M, Mehrzad J and Zhao X 2002 Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes Journal of Mammary Gland Biology and Neoplasia 7 109-21

[8] Reinhardt T, Sacco R, Nonnecke B et al. 2013 Bovine milk proteome: Quantitative changes in normal milk exosomes, milk fat globule membranes and whey proteomes resulting from Staphylococcus aureus mastitis Journal of Proteomics 82 141-54

[9] Ma C, Sun Z, Zeng B et al. 2018 Cow-to-mouse fecal transplantations suggest intestinal microbiome as one cause of mastitis Microbiome 6 200

[10] McGuire M K and McGuire M A 2017 Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk Curr. Opin. Biotechnol 44 63-8

[11] Pimenov N V, Glazunov E A and Sotnikova L F 2016 Studying of preventive and medical effectiveness of bacteriophages’ preparation at mastitis of cows in the conditions of lactic and commodity farm RJOAS 5(53) 83-8