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Rapid determination of the activity of bacteriolytic factors of milk against bacteria of three different families

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Abstract. This study proposes a method for determining the total content of bacteriolytic factors using cow's milk samples. Bacteriolytic factors (lysozymes, lactoferrins, cationic antimicrobial peptides) play a crucial role in the mammalian immune system. Measuring bacteriolytic activity by turbidimetry, based on the rate of bacterial suspension clarification, is simple and rapid. Three bacteria types from different families were used in the study: gram-positive *Micrococcus luteus* (a representative of human symbiotic flora), gram-negative *Escherichia coli* (a representative of human symbiotic flora and an analogue of dangerous pathogens from the same family), and spore-forming gram-negative bacilli, *Priestia megaterium* (a nonpathogenic analogue of the anthrax pathogen). Activity values for 50 cow's milk samples against each of the bacteria were obtained. The ratios of the obtained values characterize the parameters of milk varieties obtained from 50 animals of the same species and breed. Some types of milk have been identified that exhibit antimicrobial activity against different groups of bacteria. The rapid nature of the method allows formation of a large database and, with its help, the development of a rapid and accessible diagnostic system for pathologies in dairy cattle. The same groups of antimicrobial factors are present in human milk, so further application of the proposed method holds great promise in veterinary and medical diagnostics.

1 Introduction

Infectious diseases of dairy cattle, including cows, significantly reduce the productivity of dairy farming. The search for new methods for diagnosing infections and determining immune status remains relevant. Given the enormous diversity of pathogenic bacteria and viruses, identifying not only the pathogens themselves but also markers of the immune response, including antimicrobial bacteriolytic factors [1-3], is a winning strategy. Bacteriolytic factors are substances that attack bacteria and cause rapid destruction of the bacterial cell membrane. Screening for levels of antibacterial bacteriolytic factors in

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biological fluids also has the potential to identify animal resistant to certain types of infections. It is also logical to assume that the immune response to bacterial infection in mammals may increase the expression of certain proteins and peptides with antibacterial properties. This means that antibacterial factors are an important target for diagnosing infectious diseases. In the case of dairy cows, it is advisable to use milk for analysis.

Among the antibacterial bacteriolytic factors present in milk, lysozyme is of primary interest [1, 2]. Lysozyme is an enzyme that catalyzes the hydrolysis of bacterial cell wall copolymers. Lysozyme is also a cationic protein capable of damaging the negatively charged lipid bilayer membrane of bacteria. It has been relatively recently discovered that transferrins [3], which include milk lactoferrin, also possess bacteriolytic antibacterial activity. Furthermore, antibacterial peptides—defensins and cathelicidins [4]—can potentially exhibit bacteriolytic activity.

Lysozyme is the most widely known antibacterial agent, present in various biological fluids, including milk of mammals [5]. Despite the long history of lysozyme research, much new information about its properties has recently emerged. In particular, there is information about the antiviral and anticancer activity of lysozyme [1, 2, 6]. The phenomenon of lysozyme activation by free amino acids potentially present in biological fluids has been discovered [7]. Lysozymes present in mammalian organisms can belong to two types, called “c-type” and “g-type” [8]. "C-type" lysozyme is the main one and is usually expressed in greater quantities. “G-type” lysozyme can also make a significant contribution to the overall antibacterial activity of milk. “C-type” lysozymes are effective against many gram-positive and gram-negative bacteria, while “g-type” lysozymes are highly specific to certain families of gram-negative bacteria [8, 9]. Lysozyme concentrations in cow's milk were previously assessed using a single substrate, *Micrococcus luteus* [10].

Another milk protein with a wide range of functions is lactoferrin, a member of the transferrin family. In addition to its iron transport function, it has a diverse protective potential [11]. Its antiviral, antibacterial, anticancer, and immunomodulatory properties are known [11-13]. Direct bacteriolytic activity of transferrins has been demonstrated [3]; however, the mechanism by which these proteins influence bacterial cells requires further study. Lactoferrin has cationic properties [11-13], which suggests the presence of a cationic mechanism for bacterial lysis, where a positively charged protein damages the negatively charged lipid bilayer membrane of a bacterial cell. In addition to lactoferrin itself, its fragments lactoferricin and lactoferrampin [14] have antibacterial activity.

Milk contains a number of peptides belonging to the defensin and cathelicidin families, which are capable of destroying the bacterial cell membrane [3-4]. The charge of the molecules of all types of defensins and cathelicidins is positive [3-4], which suggests a cationic mechanism of action.

The listed factors (lysozyme, lactoferrin, defensins, cathelicidins) have immunomodulatory properties [1, 2, 4-6, 8-14], and their expression largely depends on the state of the animal's immune system. A sharp increase in the concentration of any of the bacteriolytic factors can occur in response to infection.

In this paper, a turbidimetric method is proposed for determining bacteriolytic activity. This method allows for the spectrophotometric measurement of the rate of bacterial cell destruction in real time. The method is distinguished primarily by its simplicity and rapidity [7]. The simplicity of the turbidimetric method allows its use in virtually any laboratory equipped with a photometer.

Different bacteriolytic factors vary in their potency against different bacteria, so the total bacteriolytic activity measured on several bacterial substrates will characterize the ratio of different bacteriolytic factors for a given sample. In this study, three bacterial species with significantly different cell surface structures were selected: the gram-positive coccus

Micrococcus luteus, the gram-negative *Escherichia coli*, and the spore-forming bacillus *Priestia megaterium*.

2 Materials and methods

2.1 Materials and equipment

The following reagents were used in the work: Bactotryptone (BD Difco, USA), yeast extract (Biospringer, France), D-glucose (RokettFrer, France), NaCl (Panreac, Spain), HCl (Komponent-Reaktiv, Russia), Tris, sodium citrate monohydrate, EDTA (Amresco, USA), $FeCl_3$, $MgCl_2$, $MnSO_4$ (Khimmed, Russia), chicken egg lysozyme (c-type), and human recombinant lysozyme (c-type) (Sigma-Aldrich, USA). The microorganisms used in the study were *Priestia megaterium* ATCC 14581 (B-9869) and *Escherichia coli* K-12 (B-3254) from the All-Russian Collection of Industrial Microorganisms (Kurchatov Institute, Russia), and *Micrococcus luteus* (*Micrococcus lysodeikticus*) ATCC 4698, which was cultured from lyophilized cells (Sigma-Aldrich, USA). Fifty samples of morning milk from different healthy black-and-white cows (Holstein) were obtained from various farms in the Moscow and Nizhny Novgorod regions of Russia.

An Explorer Pro EP114C analytical balance (Ohaus, Switzerland) and an Orion 120 pH meter (Thermo, USA) with an ESLC-01.7 electrode were used in the study. A New Brunswick Innova 44 incubator (Eppendorf, Germany) was used for cell cultivation. Centrifugation of 50 ml tubes during cell cultivation was performed on a 5804R centrifuge, rotor F-34-6-38 (Eppendorf, Germany). Centrifugation of tubes with a volume of up to 2 mL was performed on a MiniSpin centrifuge (Eppendorf, Germany). Bacteriolytic activity was measured on a Microplate Photometer Uniplan (Pikon, Russia) with a 540-590 nm light filter.

2.2 Growing (culturing) bacterial cells

LB (Lysogeny broth) medium, pH 7.6 (containing bactotryptone, yeast extract, D-glucose, NaCl, Fe^{2+}, Mg^{2+}, and Mn^{2+} salts), was used as a liquid nutrient medium for bacterial growth. *P. megaterium*, *M. luteus*, and *E. coli* were cultivated in two stages: overnight (14-16 hours) and daytime (4-8 hours). The incubator temperature was 30°C for *P. megaterium*, and 37°C for *E. coli* and *M. luteus*. The shaker speed for all three cultures was 120 rpm.

After cultivation, the medium containing the bacteria was transferred to 50 mL test tubes and centrifuged for 30 minutes at 5000 rpm at +4°C. The cell pellet was resuspended in a buffer solution: 0.02 M Tris-HCl, 55 mM NaCl, pH 8.0. Portions of 1 mL of the bacterial suspension in the buffer solution were frozen to a temperature of -70°C. The frozen bacteria were stored at a temperature of -20°C for no more than 2 weeks and thawed immediately before the experiment.

2.3 Sample preparation (preparation of whey samples)

Milk was separated into whey and water-insoluble components. The milk was centrifuged for 10 minutes at 13,000 rpm in a MiniSpin centrifuge (Eppendorf, Germany). Three fractions were obtained after centrifugation: a sediment (caseins and somatic milk cells), a supernatant (fat fraction), and a middle fraction (whey). The middle fraction (whey) was used for the study. The whey was additionally passed through nylon filters with pore sizes of 0.4, 0.2, and 0.1 μm. Whey samples were stored in 200 μL tubes at -20°C for no more than 1 month. Samples were thawed immediately before activity measurement.

2.4 Measurement of bacteriolytic activity of whey. Measurement of total protein

A turbidimetric method was used to measure bacteriolytic activity [7]. *M. luteus*, *E. coli*, and *P. megaterium* cell suspensions were used as bacterial substrates. Measurements were performed using the buffer solution: 20 mM Tris, 50 mM NaCl, 1 mM citrate, 1 mM EDTA; pH 8.2. The temperature during measurements was 20 °C. After thawing, the cell suspension was centrifuged for 5 minutes at 4500 rpm in a MiniSpin centrifuge (Eppendorf, Germany). The cell precipitate was resuspended in the buffer solution used for activity measurement.

First, the buffer solution and the standard solution (or serum sample) were added to the wells of a 96-well plate. An equal amount of bacterial suspension was then added to all wells, and changes in optical absorbance were measured. The initial absorbance of the cell suspension in the wells was 0.21-0.22. The optical path length was 0.6 cm. The solution volume in each well was 0.25 mL. The kinetic curves were approximated by a linear equation to calculate the rate of cell lysis.

Total protein was measured using the micro-biuret method [15].

Each measurement was repeated at least six times, and errors were calculated using the Student's t-test at a confidence level of 0.95. If errors are not listed in the table, it is assumed by default that the error did not exceed 20% of the measured value.

3 Results and discussion

In this study, we calculate relative concentrations of chicken lysozyme (using a calibration curve) as units of activity corresponding to the experimentally measured bacterial lysis rate. Chicken lysozyme is used as the primary standard. Figure 1 shows the dependence of cell lysis rate on the concentration of chicken and human c-type lysozyme. If we measure activity in a solution containing only human or chicken c-type lysozyme and then calculate the lysozyme concentration using these calibration curves, we will obtain three identical or similar lysozyme concentration values using calibration curves for different bacteria. If this c-type lysozyme has slightly different activities on three bacteria than chicken and human lysozymes, we will obtain three values that are close in order of magnitude, but slightly different. If active lactoferrin is present in the solution, we can expect additional activity against *E. coli* and *P. megaterium*, since, according to early data, transferrins exhibit greater activity against these bacteria [3].

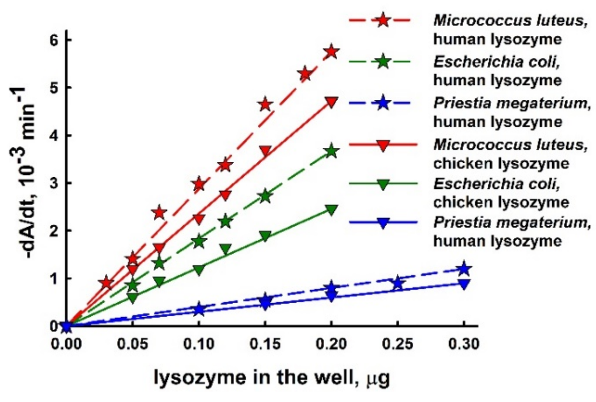

Fig. 1. Dependence of the rate of bacterial lysis on the amount of lysozyme in the well.

Table 1. Milk sample analysis results. Total serum protein. Bacteriolytic activity in units of chicken lysozyme concentration equivalence.

	Total protein, g/L	anti *M. luteus*, mg/L	anti *E. coli*, mg/L	anti *P. megaterium*, mg/L
1	12.2	0	0	0
2	9.9	0	0.99	0
3	11.1	0	0	0
4	13.3	0	0	0
5	16.2	0	0	0
6	10.7	0	0	0
7	9.2	0	0	0
8	10.7	0	0	0
9	15.2	0	0	0
10	14.3	0	0	0
11	13.1	0.25	0	0
12	10.6	**0.23**	**0.32**	**5.03**
13	36.6	0.24	0.02	0
14	20.8	**1.59**	**10.92**	**14.03**
15	12.5	0	0	0
16	11.6	0	0	0
17	10.3	0	0	0
18	12.3	0.11	0	0
19	8.2	0.04	0.04	0
20	13.6	0	**3.14**	**1.28**
21	12.2	0.33	0	0
22	11.5	0	0	0
23	12.2	0.17	0	0
24	15.5	**1.33**	**5.18**	**1.58**
25	12.7	0.41	0	0
26	8.6	0	0.02	0
27	12.1	0.63	0.29	0
28	16.7	0.41	0.02	0
29	19.2	**1.01**	**4.83**	**1.18**
30	11.4	**0.52**	**0**	**10.03**
31	16.7	**1.02**	**3.23**	**3.13**
32	40.7	**0.03**	**0.04**	**9.49**
33	12.9	0.43	0	0
34	8.7	0.09	0	0
35	13.7	**1.19**	**1.42**	0
36	8.5	**0.09**	**0.04**	**1.82**
37	13.1	**0.07**	**0.03**	**5.65**
38	7.5	0	0	0.61
39	8.3	0.05	0	0.23
40	9.1	**0.03**	**0.03**	**2.76**
41	13.1	**0.26**	**0.27**	0
42	19.2	0.12	0.02	0.79
43	12.8	**0.8**	**3.68**	0
44	14.2	**1.14**	**2.64**	0
45	14.8	**1.12**	**1.77**	0
46	9.7	0.04	0.02	0.23
47	13.9	0.64	0	0
48	14.6	0.91	0	0
49	12.2	0.41	0	0
50	6.8	**0.08**	**0**	**6.58**

Table 1 shows the expected ratio of activity values for a mixture of c-type lysozyme and lactoferrin for samples 14 and 31 (these results are highlighted in red). For samples 24 and 29 (highlighted in green), we see increased activity against *E. coli*, possibly due, not to lactoferrin, but to additional g-type lysozyme or cationic peptides. Sample 21 (highlighted in light blue) is inactive against *M. luteus*, but is active against *E. coli* and *P. megaterium*; it may contain some type of lactoferrin, but does not contain lysozyme. Samples 12, 30, 32, 36, 37, 40, and 50 (highlighted in purple) have increased activity against *P. megaterium* and may contain a previously unstudied unknown factor. Samples 35, 41, 43, 44, and 45 (highlighted in gold) are active against *M. luteus* and *E. coli*, but are inactive against P. megaterium and may contain a special type of lysozyme. Sample 21 (highlighted in light green) is active only against *E. coli*. A number of other samples (1, 2-10, 15-17, 22, and 34) do not have any antibacterial activity; these are probably cows with latent immunodeficiency.

The results presented here clearly demonstrate that analyzing bacteriolytic activity against a single bacterial substrate does not provide a comprehensive understanding of the diversity of bacteriolytic factors in milk. The ratio of activities on the three bacterial substrates examined is much more informative for determining an animal's immune status. Compared to the antibacterial activity studies conducted, the total protein concentration parameter has limited diagnostic value: 90% of samples had a total protein of 13.4±2.2 g/L, which does not reveal differences between cows.

The cows used in this experiment had similar living conditions and health status. However, the ratio of milk bacteriolytic activity against the three bacterial species was significantly different, possibly due to genetic differences affecting protein and peptide expression. For farm animals, data on the state of bacteriolytic factors in the body can be applied in selection technologies.

The rapid nature of turbidimetric determination of bacteriolytic activity can allow for the rapid collection of a large dataset of healthy and infected cows. Such a database could significantly reduce the time and labor required for dairy cattle diagnostics.

In fact, the same set of bacteriolytic factors (c- and g-type lysozyme, lactoferrin, defensins, and cathelicidins) is present in human milk. The "bacteriolytic activity profile" is a promising diagnostic tool. A deficiency or excess of any of these factors can predispose to certain pathologies. This is a promising area in the field of personalized medicine.

4 Conclusion

Proteins and peptides with bacteriolytic activity are important markers of the immune system of farm animals and humans. This paper proposes a method for analyzing their bacteriolytic activity in milk. The described method is rapid and simple to perform, yet highly informative. It holds great promise for veterinary and medical diagnostics.

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