

The Sensitivity of the Determination of Inhibitory Substances Using a Test Culture of Thermophilic Streptococcus and Indicator Resazurin to Various Antimicrobial Agents

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ABSTRACT

The presence of inhibitory substances in the dairy industry is an urgent problem. The article presents data from experiments on expanding the spectrum of determination of various groups of antibiotics and the sensitivity of the method for determining inhibitory substances using a test culture of thermophilic streptococcus and indicator resazurin. Various groups of antibacterial substances in the form of veterinary drugs and State standard samples were artificially introduced into milk. In the laboratory series of experiments, the following sensitivity of the method to antibiotics, found in the form of residual concentrations in milk: cephalosporins – 0.01 mg/l; aminoglycosides, fluoroquinolones – 0.001 mg/l; lincosamides, phenols – 0.0001 mg/l. This rapid method remains optimal for the analysis of antibiotics and other inhibitory substances.

Keywords: *inhibitory substances, thermophilic streptococcus, indicator resazurin, antibacterial substances*

1. INTRODUCTION

In our country, milk and dairy products have historically been an important food product. The biological and nutritional value of milk determines its perfect quality. It contains all the organic substances necessary for the human and animal body (proteins, fats, carbohydrates), minerals and vitamins in a balanced and easily digestible form.

The production of high quality milk must meet sanitary and hygienic standards and the requirements of processing enterprises. Large milk processing enterprises pay special attention to safety indicators and accept milk, considering both traditional indicators and a number of other requirements. To ensure the safety of milk and dairy products, TRCU 021/2011 "On Food Safety", approved by a decision of the Customs Union Commission on December 9, 2011, and TRCU 033/2013 Technical Regulation of the Customs Union "On the Safety of Milk and Dairy Products", approved by a Council Decision, are used. The Eurasian Economic Commission of October 9, 2013, which sets the rules, forms for assessing and confirming the conformity of milk and dairy products with the requirements of the law [1, 2].

Of particular danger to milk are various inhibitory substances, especially residual amounts of antibiotics. According to GOST 23454-2016, inhibitory substances are "any substances in milk that, regardless of their nature,

inhibit the development of microorganisms" [3]. When such products are consumed in food, various allergic reactions, dysbiosis, and even resistance to their effects, develop in the human body. Also, the presence of antibiotics in milk is a danger to the dairy industry and leads to serious economic losses, since a decrease in the vitality of starter microflora leads to a violation of technological processes for the production of dairy products.

The main antibiotic inhibitors most commonly found in the dairy industry during monitoring studies are beta-lactams (these are penicillin, amoxicillin, ampicillin, ceftifur, cefapirin, cloxacillin, dicloxacillin, oxacillin); tetracyclines (tetracycline, oxytetracycline); sulfamides (sulfadiazine, sulfamethazine); microlides (tylosin, spiramycin, erythromycin); aminoglycosides (gentamicin, neomycin); as well as trimethoprim, dupzone, etc.

Inhibiting substances enter milk in various ways. For example, in case of improper antibiotic treatment, in case of non-compliance with the terms of rejection of milk after treatment of cows, the use of poor-quality feed and feed additives containing antimicrobial agents as growth stimulants, etc. It is forbidden to feed lactating cows in the herd with compound feed intended for other types of animals and production groups of cattle. It is also very dangerous in this regard to administer intravenously antimastitis medicines containing antibiotics, sulfonamides and nitrofurans as active substances, since after treatment is stopped, these drugs are stored in the animal's body for

several days and excreted along with milk (monomycin 7 days, oxytetracycline 5 days, etc.). Therefore, it is necessary to ensure that such milk does not enter the total milk yield. And the rejection period is long, which is unprofitable for private households [4].

If any inhibitory substances are found in milk, it is classified as non-sorted which leads to large economic losses. At dairy farms, the producer is obliged to ensure the safety of raw milk and to ensure the complete absence of residual quantities of any veterinary drugs, in particular antibiotics, in it.

There are a huge number of modern tests ("Beta-starR", etc.) and instrumental methods for determining the residual quantities of drugs in milk, such as immunochip technology, temple mass spectrometry, delvotest, etc. But they are quite expensive at high sensitivity and require expensive equipment. Microbiological methods remain available for assessing the quality of milk for the presence of impurities [5, 6, 8, 9].

Until recently, GOST 23454-79 "Milk. Methods for the determination of inhibitory substances." It used two methods for the determination of inhibitory substances with resazurine or methylene blue. Both methods are based on the restoration of indicators during the development of *Streptococcus thermophilus* microorganisms sensitive to inhibitory substances in milk. The second method allowed the determination of penicillin with a sensitivity of 0.01–0.1 IU/cm³, streptomycin 30–50 µg/cm³, tetracycline and oxytetracycline – 1 IU/cm³, as well as oleandomycin 10 IU/cm³. The authors of [7] in 2014 showed that the spectrum of determination of antibiotic groups using this method can be expanded. It is possible to determine the presence of antibiotics belonging to groups such as cephalosporins, phenicols, ansamycins, lincosamides and nitrofurans. However, not all antibiotics from the quinolone group were determined. In the subsequent revision of GOST, the method using the methylene blue indicator was excluded.

At the moment, GOST 23454-2016 requires methods for the determination of inhibitory substances, in which the

main method is the determination of inhibitory substances using a test culture of thermophilic streptococcus and indicator resazurin. The method is based on the registration of the growth process in milk of the sensitive test culture *Streptococcus thermophilus* B19 and the restoration of resazurin by redox enzymes secreted into the milk by microorganisms. This method determines the following antibiotics in milk: penicillin in a mass concentration of 0.004 mg/dm³, streptomycin – 10 mg/dm³, tetracycline – 1 mg/dm³, levomycetin – 5 mg/dm³. But at the present stage, when using more complex and modern medicines in animal husbandry, it is necessary to expand the capabilities of this method and determine its sensitivity in determining antibiotic preparations of other groups. Thus, this method has not been sufficiently studied and this has become the subject of our experiments.

2. MATERIALS AND METHODS

The study was carried out according to the method of GOST 23454-2016 [3]. There was *Streptococcus thermophilus* B19 used for skim milk.

To simulate the experimental antibiotic content, 1 g of the antibiotic was added to 9 ml of sterile 3.2 % milk and a decimal series of dilutions was prepared to obtain samples with different concentrations. The experiment was performed in duplicate.

The samples were heated to the required temperatures in a laboratory PE-4300 water bath (Ekohim LLC, Russia).

In the absence of various inhibitory substances in the analyzed milk, including the control sample, or their presence in an amount equal to or less than detection limit, the contents of the tubes will turn pink with a lilac, pink or white. If milk contains inhibitory substances, including antibacterial ones, the contents of the tubes will have a color from gray-lilac to lilac.

Table 1 The results of an experiment to determine the sensitivity of the method for determining inhibitory substances using a test culture of thermophilic streptococcus and indicator resazurin when introduced into milk of various concentrations of antibiotics

| № | Antibiotic name | The concentration of the introduced antibiotic in milk g/ml | | | | |
|---|---------------------|---|------|-------|--------|---------|
| | | 0.1 | 0.01 | 0.001 | 0.0001 | 0.00001 |
| 1 | Gentamicin sulfate | + | + | - | - | - |
| 2 | Kanamycin | + | + | +/- | - | - |
| 3 | Enroflon | + | + | - | - | - |
| 4 | Doxycycline hyclate | + | + | + | + | - |
| 5 | Dioxidine | + | + | + | + | - |
| 6 | Tiocefur | + | +/- | - | - | - |
| 7 | Lincomycin | + | + | + | +/- | - |
| 8 | Pneumostop | + | + | + | +/- | - |

Note: "+" – the presence of inhibitory substances, "-" – the absence of inhibitory substances, "+/-" – in one test tube of two there is a plus.

Table 2 The experimentally determined sensitivity of the method for the determination of inhibitory substances using a test culture of thermophilic streptococcus and the resazurin indicator to various groups of antibiotics

| № | Antibiotic name | Antibiotic group | The experimentally determined sensitivity of the method, mg/l of milk |
|---|--|--------------------------------------|---|
| 1 | Gentamicin sulfate | Aminoglycosides | 0.001 |
| 2 | Kanamycin | Aminoglycosides 1st generation | 0.001 |
| 3 | Enroflon (AD enrofloxacin hydrochloride) | Fluoroquinolones | 0.001 |
| 4 | Doxycycline hyclate | Tetracyclines | 0.00001 |
| 5 | Dioxidine (AD Hydroxymethylquinoxalindioxide) | Other synthetic antibacterial agents | 0.00001 |
| 6 | Tiocefur (AD Ceftiofur Sodium) | Cephalosporins 3 generations | 0.01 |
| 7 | Lincomycin | Lincosamides | 0.0001 |
| 8 | Pneumostop (AD florfenicol) | Phenicols | 0.0001 |

Samples of sterilized milk were taken from the turnover of the distribution network; antimicrobial agents were purchased in a veterinary pharmacy. We used antibiotics from various manufacturers used to treat cows for endometritis, mastitis, infectious diseases of the gastrointestinal tract, and respiratory organs.

3. RESULTS

When controlling samples of 0.05 and 3.2 % of milk into which antibiotics were added and a culture of thermophilic streptococcus was seeded, there was no inhibitory substance detected. Control with SKIV was also normal. The data obtained with the introduction of various antibiotics in a concentration of from 0.1 to 0.00001 g/ml are shown in table 1.

According to the results obtained, the method for the determination of inhibitory substances using a test culture of thermophilic streptococcus and a resazurin indicator determines a wider range of antibacterial drugs than indicated in GOST 23454-2016 p 7. The sensitivity obtained experimentally is shown in *table 2*.

4. CONCLUSION

In recent years, in conditions of an increase in the number of cattle, there many cases of the presence of inhibitory substances in milk. The income of milk producers directly depends on losses during the sale, and in the case of the presence of antibiotics, this is a violation of grade and quality. Inhibitors pose a particular problem to small farms producing craft cheeses; these enterprises often cannot

afford modern research methods such as high performance liquid chromatography or competitive enzyme immunoassay. The presence of inhibitory substances is one such factor affecting production processes. Milk is a perishable product and it is very important to quickly identify a wide range of toxicants, including antibacterial inhibitory drugs. The method of determining inhibitory substances using the test culture of thermophilic streptococcus and the resazurin indicator is available, simple, has a low cost with high productivity, and can also be used in everyday screening studies. According to our data, the method highly determines not only the antibiotics declared in GOST 23454–2016, but also other groups – such as aminoglycosides, phenols, lincosamides, etc. with a sensitivity of up to 0.00001 mg/l milk, which is very important in modern the stage of frequent use for the treatment of animal antibiotics of 3–5 generations.

Of course, this method does not allow them to be differentiated, it is not quantitative, but at the same time it can be used in the future for a preliminary assessment of the presence of residual amounts of antibacterial drugs in milk. In our opinion, this method is ideally suited to small farmers for daily control of inhibitory substances in milk.

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