

Genetic markers of antibiotic resistance of pathogenic bacteria in the milk of cows and goats

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Abstract— This article represents the results of studies of the microorganism resistance to antimicrobial agents detected by PCR in the milk of cows and goats. In 94.1% of cow milk and in 80.7% of goat milk samples specific DNA regions of microorganisms were detected: *Staphylococcus* spp., *E. coli*, *S. aureus*, *S. agalactiae*. The more common pathogenic agents were the bacteria of the *Staphylococcus* spp. group (*S. epidermidis*, *S. saprophyticum*, *S. haemolyticus*) and *E. coli*. The opportunistic bacteria of the *Staphylococcus* spp. group most often were carriers of the *ErmB* gene, which is responsible for resistance to macrolides of the 1st generation. *E. coli* had a high percentage of resistance to inhibitor-protected penicillin and cephalosporin of the 3rd and 4th generations. Acquired resistance of *E. coli* and bacteria of the *Staphylococcus* spp. group (*S. epidermidis*, *S. saprophyticum*, *S. haemolyticus*) is associated with the frequent use of antimicrobials in the agricultural enterprises, such as penicillin, 3rd and 4th generation cephalosporin and 1st generation macrolides. Also the poly-resistant *E. coli* isolates (2.2%) resistant to several groups of antimicrobial drugs were detected in the cow milk samples.

Keywords— resistance, gene, DNA, antimicrobial drugs, bacterial pathogens, genetic determinants, milk.

I. INTRODUCTION

A significant increase in the resistance of opportunistic and pathogenic microorganisms to antimicrobial drugs has been observed worldwide in the recent years. When carrying out incorrect therapeutic measures with the use of antibiotics, two problems will arise: an increase in the frequency of isolation of microorganism strains with resistance genes and a lack of antimicrobial drugs that can suppress their development and spread [12, 17, 24].

The resistance of microorganisms to antimicrobial drugs is a worldwide problem that is found in many national and international programs (UN, WHO, EU, EMA) [12, 25, 29]. Antibiotic resistance has acquired particular relevance in the Russian Federation in connection with the approval of the Strategy for the Prevention of the Spread of Antimicrobial Resistance in the Russian Federation for the Period up to 2030 (Government Decree of September 25, 2017 No. 2045-p) [4].

The development of antibiotic resistance of microorganisms is genetically determined: the acquisition of new genetic information or a change in the expression level of their own genes. Genes that encode the resistance to antibiotics, emerge as a result of mutations in the

chromosomes of bacteria; or they get inside the microbes from the outside, using plasmids and bacteriophages; or they are captured by microbes from the environment (free DNA of dead bacteria) [1, 8, 30]. The main interspecific transfer of antibiotic resistance factors is the R-plasmid, which determines the resistance of microorganisms to many antibiotics. It has been established that *E. coli*, as a donor, is capable of transmitting resistance genes to *Salmonella* and *Shigella*. In addition, the fact of the circulation of plasmids of opportunistic microorganisms from animals to humans and from humans to animals is now proven, which contributes to the rapid spread of antibiotic resistance of microorganisms throughout the world [13, 15, 17].

Farm animals and animal products are considered as the main carriers and sources of resistant strains of *E. coli* and *Salmonella*, producing extended-spectrum β -lactamase (ESBL) [6, 20, 28]. The most common ESBL groups found in farm animals have the CTX-M resistance gene. CTX-type β -lactamases are found in various representatives of Enterobacteriaceae (mainly in *E. coli* and *Salmonella enterica*) [16, 18, 19]. To date, more than 90 allotypes of bla CTX-M genes are known, subdivided into six subtypes based on the analysis of their nucleotide sequences. The CTX-M genes evolved due to the accumulation of point mutations [21]. Gram-positive bacteria are resistant to β -lactamase antibiotics due to modification of penicillin-binding proteins (PBP2a). In *Staphylococcus aureus*, the production this protein is encoded by the *mecA* gene, which is transmitted in the population of the mobile staphylococcal cassette chromosome (SCCmec) [2, 11, 22, 23]. The blaDHA gene encoded on plasmids determines the resistance of *E. coli* to inhibitor-protected penicillin and cephalosporin of the 3rd and 4th generations. The blaDHA is found among Enterobacteriaceae in many parts of the world and causes growing concern in the world of medicine as it leads to unsuccessful therapeutic measures. Currently, 24 types of DHA genes are known. The regulation of this expression of β -lactamase is closely related to the recirculation of the cell wall [5, 13]. The *ErmB* gene determines the resistance of *Staphylococcus* spp. and *Streptococcus* spp. to the macrolides of 1st generation [4].

Laboratory studies of microorganisms and their resistance to antibiotics are currently carried out by the microbiological and the molecular genetic methods (PCR). PCR diagnostics makes it possible to determine the presence of microbial pathogens in biological samples and their genes of resistance to important classes of antibiotics; and

significantly reduce the time taken by the veterinarian to decide about the antibiotic choice. Also, this research helps to study the mechanisms of antibiotic resistance of bacterial agents and to carry out typing of microbial pathogens with the subsequent prediction of the resistance spread [4, 14, 26].

II. METHODS

Research was conducted in the Ural Federal Agrarian Scientific Research Center. The object of research was 170 cow milk samples and 21 goat milk samples from 17 farms from 5 regions of Russia (Sverdlovsk, Tyumen, Chelyabinsk and Kirov regions and the Republic of Udmurtia).

The milk samples were studied by the real-time polymer chain reaction (PCR) method on the Rotor Gene-3000 (Corbett Research, Australia). We used the reagent kit of test systems “VETSKRIN. Streptopol-V”, “VETSKRIN. Stafipol”, “VETSKRIN. Kolipol” and “VETSKRIN. Streptopol” (Russia) in order to determine the specific DNA region of bacterial pathogens (Staphylococcus aureus, Streptococcus agalactiae, E. coli, Staphylococcus spp.)

We used the reagent kits by “Lytech” (Russia) in order to determine antibiotic resistance: resistance of Staphylococcus aureus to 2nd generation cephalosporin (detection of MecA gene) - “Vetskrin. Resistance to cephalosporin – 2”; resistance of Enterobacteriaceae bacteria to inhibitor-protected penicillin and 3rd and 4th generation cephalosporin (blaDHA gene detection) - “Vetskrin. Resistance to inhibitor-protected penicillin and cephalosporin”; resistance of Staphylococcus spp. and Streptococcus spp. to macrolides of the 1st generation (detection of the ErmB gene) - “Resistance to macrolides - 1”; resistance of Enterobacteriaceae family bacteria to 1st generation cephalosporin and fluoroquinolones (detection of STX-M gene) - “Vetskrin. Resistance to cephalosporin-1”.

III. RESEARCH RESULTS

As a result of PCR studies, in 160 (out of 170) cow milk samples (94.1%) the DNA of pathogenic and opportunistic microorganisms were detected. Specific DNA regions of microorganisms such as Staphylococcus spp. (S. epidermidis, S. saprophyticum, S. haemolyticus) were identified in 75.0% of samples, E. coli in 35.6% of samples, S. aureus in 23.1% of samples, S. agalactiae in 15.6% of samples (Tab. 1).

TABLE I. THE FREQUENCY OF DNA EXTRACTION OF PATHOGENIC AND OPPORTUNISTIC MICROORGANISMS IN COW MILK (N = 160)

Microorganism type	Quantity of samples (n)	%
<i>Staphylococcus spp.</i>	120	75,0
<i>S. aureus</i>	37	23,1
<i>E. coli</i>	57	35,6
<i>S. agalactiae</i>	25	15,6

*Staphylococcus spp.** – *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*

The obtained data on the regional distribution of bacterial pathogens suggest that in all the incoming milk samples from cows from 5 regions of the Russian Federation, DNA of Staphylococcus spp., As well as E. coli, was most often detected.

The greatest distribution of monocultures of microorganisms had the: Staphylococcus spp. * - 51.8%, S. agalactiae - 5.0%, E.coli - 5.6%, S.aureus - 2.5%, and associations of two microorganisms — Staphylococcus spp., E. coli — 21.2%; Staphylococcus spp., S.aureus - 11.2%, Staphylococcus spp., S.agalactiae - 3.7%; less often S.agalactiae, S.aureus / E. coli - 1.8%. Associations of three and four microorganisms were found in 12.5% of the samples, most often among them Staphylococcus spp., S.aureus, E.coli - 6.2% of the samples (Tab. 2).

TABLE II. THE FREQUENCY OF DETECTION OF MONOCULTURES AND ASSOCIATIONS OF OPPORTUNISTIC AND PATHOGENIC MICROORGANISMS IN COW MILK (N = 160)

Types of monocultures and associations of microorganisms	Quantity of samples (n)	%
<i>Monocultures</i>		
<i>Staphylococcus spp.</i>	55	51,8
<i>S.aureus</i>	4	2,5
<i>E.coli</i>	9	5,6
<i>S. agalactiae</i>	8	5,0
<i>Associations</i>		
<i>Staphylococcus spp.+ S.aureus</i>	18	11,2
<i>Staphylococcus spp.+ E.coli</i>	34	21,2
<i>Staphylococcus spp.+ S.agalactiae</i>	6	3,7
<i>S.agalactiae + S.aureus</i>	3	1,8
<i>E. coli + S. agalactiae</i>	3	1,8
<i>Staphylococcus spp.+ S.aureus+ E.coli</i>	10	6,2
<i>Staphylococcus spp.+ S.aureus+ S. agalactiae</i>	4	2,5
<i>Staphylococcus spp.+ E.coli + S. agalactiae</i>	3	1,8
<i>S.aureus+ S. agalactiae+ E.coli</i>	2	0,25
<i>Staphylococcus spp.+ S.aureus+ E.coli + S. agalactiae</i>	1	0,6

*Staphylococcus spp.** – *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*

When conducting PCR studies of microorganisms isolated from cow milk for the presence of resistance genes, it was found that 54.3% of bacterial agents are carriers of mutation genes encoding resistance to various antimicrobial agents. The blaDHA gene, which is responsible for Enterobacteriaceae resistance to inhibitor-protected penicillin

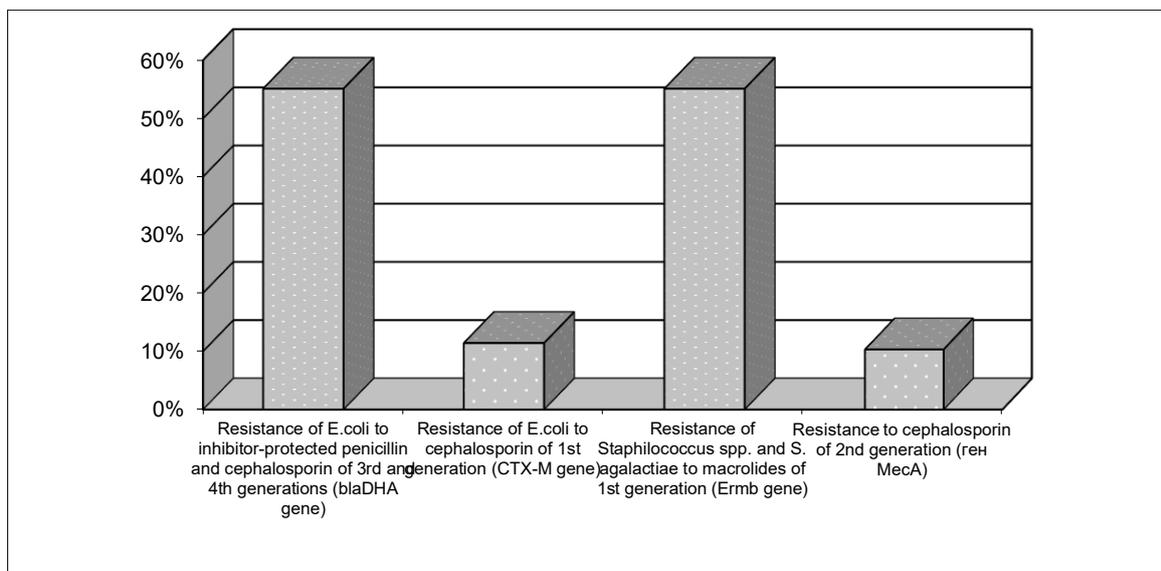


Fig. 1. Distribution of genetic determinants of antibiotic resistance of microorganisms isolated from cow milk

and cephalosporin of the 3rd and 4th generations, was detected in 55.1% of E.coli. The gene of cephalosporin resistance of the 1st generation (CTX-M) was present in 11.4% of E. coli. In 2.2% of the E. coli DNA samples, two CTX-M and blaDHA mutations were detected simultaneously. Resistance to macrolides of the 1st generation (ErmB gene) was found in 55.1% of samples of the Staphylococcus spp. and Streptococcus spp. Among them, the ErmB gene was more commonly detected in Staphylococcus spp. (*S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*) - 44.8% and much less frequently in *S. agalactiae* - 10.3%. Resistance of *S. aureus* to 2nd generation cephalosporin was found in 10.3% of microorganisms (Fig. 1).

During PCR studies of goat milk, DNA of pathogenic bacteria was found in 80.7% of the samples (*E. coli* - 66.6%, *S. aureus* - 33.3%, *Staphylococcus spp.* - 52.3%, *S. agalactiae* - 14.2%) (Tab. 3).

TABLE III. THE FREQUENCY OF DNA EXTRACTION OF PATHOGENIC AND OPPORTUNISTIC MICROORGANISMS IN GOAT MILK (N = 21)

Types of microorganisms	Quantity of samples (n)	%
<i>Staphylococcus spp.</i>	11	52,3
<i>S. aureus</i>	7	33,3
<i>E. coli</i>	14	66,6
<i>S. agalactiae</i>	3	14,2

*Staphylococcus spp.** – *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*

Monocultures in biological samples were represented by the following microorganisms: *E. coli* - 19.0%, *Staphylococcus spp.* - 14.2% and *S. aureus* -4.6%. *S. agalactiae* was found only in associations with *E. coli* (9.5%). Associations of two microorganisms were more often detected: *E. coli* + *Staphylococcus spp.* - 19.0%; *Staphylococcus spp.* + *S.aureus*, *E.coli* + *S. aureus*, *S. agalactiae* + *E.coli* - 9.5%.

Associations of three and four microorganisms were isolated less frequently: *E. coli*, *Staphylococcus spp.*, *S.*

aureus - 4.6%; *S. aureus* + *S. agalactiae* + *E. Coli* + *Staphylococcus spp.* - 4.6% (Tab. 4).

TABLE IV. THE FREQUENCY OF DETECTION OF MONOCULTURES AND ASSOCIATIONS OF OPPORTUNISTIC AND PATHOGENIC MICROORGANISMS IN GOAT MILK (N = 21)

Types of monocultures and associations of microorganisms	Quantity of samples (n)	%
Monocultures		
<i>Staphylococcus spp.</i>	3	14,2
<i>S.aureus</i>	1	4,6
<i>E.coli</i>	4	19,0
<i>S. agalactiae</i>	0	0
Associations		
<i>Staphylococcus spp.</i>	2	9,5
<i>S.aureus</i>		
<i>E. coli</i>	4	19,0
<i>Staphylococcus spp.*</i>		
<i>E.coli</i>	2	9,5
<i>S. aureus</i>		
<i>S. agalactiae</i>	2	9,5
<i>E.coli</i>		
<i>E. coli</i>	1	4,6
<i>Staphylococcus spp.</i>		
<i>S. aureus</i>		
<i>Staphylococcus aureus</i>	1	4,6
<i>S. agalactiae</i>		
<i>E. coli</i>		
<i>Staphylococcus spp.*</i>		

The results of the detection of antibiotic resistance genes of microorganisms isolated from goat milk showed that 80.9% of the samples contained antimicrobial resistance genes. The ErmB gene was identified in 35.2% of the *Staphylococcus spp.* and *S. agalactiae*, which is responsible for resistance to macrolides of the 1st generation. The gene of resistance to cephalosporin of the 2nd generation was found in 11.7% of *S.aureus*. 76.4% of *E.coli* was resistant to inhibitor-protected penicillin and cephalosporin of the 3rd and 4th generations due to the regulation of the blaDHA gene (Fig. 2).

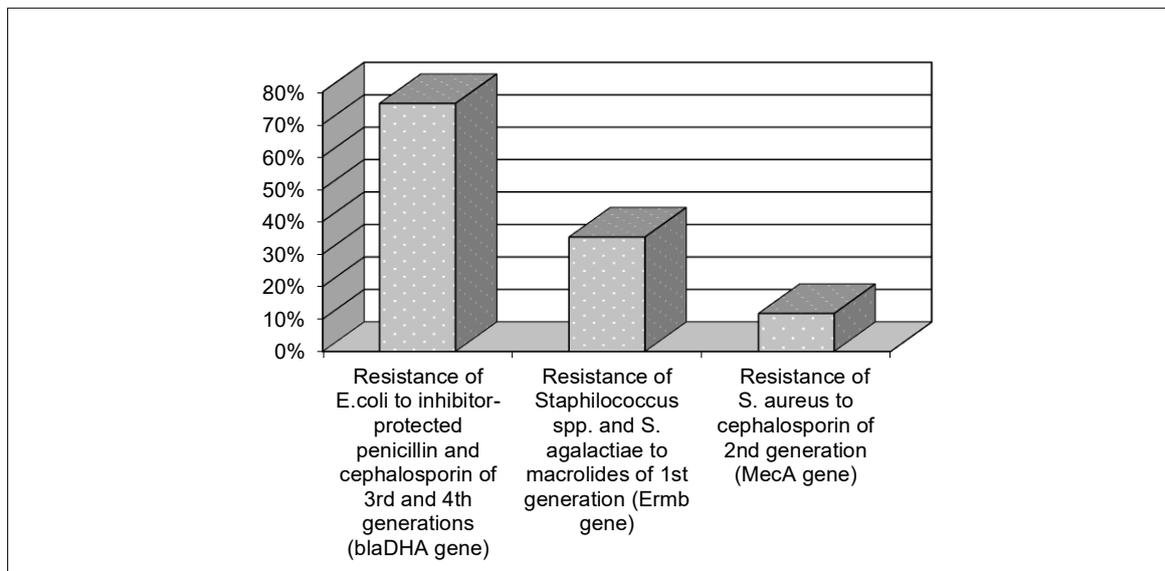


Fig. 2. Distribution of genetic determinants of antibiotic resistance of microorganisms isolated from goat milk

As a result of the research, a widespread antibiotic resistance of pathogenic agents isolated from the milk of cows and goats has been proven. A large percentage of the *E. coli* had the blaDHA gene and *Staphylococcus* spp. and *S. agalactiae* – the ErmB gene, which is associated with the frequent use of penicillin and cephalosporin of the 3rd and 4th generation, as well as the 1st generation macrolides, in agricultural enterprises.

A rather low percentage of the CTX-M gene in *E. coli* was also identified, which is most likely due to the rare use of drugs belonging to the 1st generation cephalosporin. These results make it possible to predict the high effectiveness of the use of antibacterial drugs of the above-described group in order to carry out therapeutic measures in mammary gland diseases of cattle caused by pathogenic agents of *E. coli* bacteria.

IV. CONCLUSION

As a result of the studies performed by the PCR method, the presence of DNA of pathogenic microorganisms was detected in 94.1% of milk samples. Specific DNA regions belonged to such bacterial agents as *Staphylococcus* spp. (*S. epidermidis*, *S. saprophyticum*, *S. haemolyticus*) – in 75.0% of the samples, *E. coli* – 35.6%, *S. aureus* – 23.1%, *S. agalactiae* – 15.6%. In this case, microorganisms were more often present in the form of associations. The blaDHA gene, which determines resistance to protected penicillin and cephalosporin of the 3rd and 4th generations, was found in *E. coli* in 55.1% of the samples. The gene (CTX-M), which is resistant to the 1st generation cephalosporin in *E. coli*, was detected in 11.4% of samples. In 2.2% of the *E. coli* DNA samples, two CTX-M and blaDHA mutations were detected simultaneously. Resistance of *Staphylococcus aureus* to the 2nd generation cephalosporin (MecA gene) was identified in 10.3% of the samples. The gene of resistance to macrolides of the 1st generation (ErmB) was found in *Staphylococcus* spp. and *S. agalactiae* in 55.1% of the samples.

In 80.7% of goat milk samples, specific microbial DNA regions were detected: *E. coli* – 66.6%, *S. aureus* – 33.3%, *Staphylococcus* spp. – 52.3%, *S. agalactiae* – 14.2%. Most of the bacteria in the milk were present in the form of associations. The antimicrobial resistance genes were found

in 80.9% of the samples: the ErmB gene in bacteria of the *Staphylococcus* spp. and *S. agalactiae* group – 35.2%; the MecA gene in *S. aureus* – 11.7%; the blaDHA gene in *E. coli* – 76.4%.

The data showed that the main pathogens in the milk samples of cows and goats are the bacteria of *Staphylococcus* spp. (*S. epidermidis*, *S. saprophyticum*, *S. haemolyticus*) and *E. coli*. At the same time, *E. coli* had a high percentage of resistance to protected penicillin and cephalosporin of the 3rd and 4th generations; and the opportunistic bacteria of *Staphylococcus* spp. most often were the carriers of the ErmB gene, which regulates resistance to macrolides of the 1st generation. This is due to the frequent use of antimicrobials such as penicillin, 3rd and 4th generation cephalosporin and 1st generation macrolides in agricultural enterprises.

The multiple resistance of *E. coli* (CTX-M and blaDHA genes) can create difficulties for the treatment of the mammary gland diseases in cattle, which is why determination of the antibiotic resistance of Enterobacteriace bacteria in cow and goat milk samples is rational.

Given the high frequency of contamination of cow and goat milk with bacterial agents and the presence of a large percentage of antimicrobial resistance, monitoring the antibiotic resistance of these microorganisms is an urgent task in order to take practical measures on the use of antibiotics in agriculture.

To prevent and control the spread of antibiotic resistance, it is necessary to prescribe antibiotics to animals only under veterinary control; to not to use antibiotics to stimulate growth or prevent disease in healthy animals; to vaccinate animals and use alternatives to antibiotics when it is available; to periodically replace widely used drugs with newly created or reserve ones; to combine a number of drugs; to assess the type of antibacterial drug (approximately once a year), which was most often used for therapeutic purposes, with an analysis of the results of treatment. As a way to reduce the resistance of microorganisms, the use of narrow-spectrum antibiotics is recommended, and the sanitary and hygienic standards in agricultural enterprises must be obeyed [3, 6, 7, 8, 9, 10, 12].

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