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Identification of *Mycoplasma Bovis* Bacteria by Polymerase Chain Reaction and Sequencing.

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ABSTRACT

Mycoplasmas belong to the class *Mollicutes*, the order *Mycoplasmatales*, the family *Mycoplasmataceae*, which is represented by two genera of importance in animal pathology: *Mycoplasma* (76 species) and *Ureaplasma* (2 species). The sequences of *Mycoplasma bovis* isolates from the GeneBank nucleotide sequence database were analyzed. All sequences were aligned using a software algorithm; as a result, primer pairs were selected from a specific region of the 16s rRNA gene for identification of the microorganism. Optimized amplification and sequencing conditions. Mycoplasmosis of cattle are important diseases worldwide and are of significant economic importance. *Mycoplasma bovis* is an important opportunistic pathogen in young calves, and their infection leads to mortality of calves, loss of weight in surviving calves and reduced milk production. The material for the study was smears and scrapings from the vagina of cows with clinical signs of vulvovaginitis, with impaired reproductive function and with hidden mastitis, as well as mastitis milk, smears from the nose of calves in rhinitis, bronchitis and bronchopneumonia, light aborted calf fetus. According to the results of sequencing, the sequences obtained belong to the microorganism *Mycoplasma bovis*. The analysis of the results of PCR studies showed a high frequency of occurrence of microorganisms of the genus *Mycoplasma bovis* in clinical specimens in 53% of cases. Based on the data obtained, it was concluded that one of the causes of mastitis and reproductive infections of cows, as well as respiratory infections of calves in the conditions of the animal breeding complex - *Mycoplasma bovis*.

Keywords: mycoplasmosis, *Mycoplasma bovis,* cattle, genotyping, polymerase chain reaction, sequencing.

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INTRODUCTION

Mycoplasmosis of animals is widespread in the world and is one of the most difficult problems of infectious pathology of industrial animal husbandry, causing serious economic damage caused by animal mortality, forced slaughter, lack of live weight, offspring, quality of products, costs of treatment, prevention and elimination of the disease. According to the conclusion of many domestic and foreign scientists, this is one of the main obstacles to the development of industrial livestock [1,2,3,7,10,15].

Mycoplasmas belong to the class of *Mollicutes*, the order of *Mycoplasmatales*, the family of *Mycoplasmataceae*, which is represented by two genera of importance in animal pathology: *Mycoplasma* (76 species) and *Ureaplasma* (2 species) [5,6,7].

The prevalence of mycoplasmas and the economic damage caused to beef and dairy cattle breeding is often underestimated. Mycoplasmas increase the susceptibility of cattle to secondary infections, which complicates their course, leading to the death of animals. For some species of mycoplasmas, their primary role in the etiology of mycoplasmosis has been proven. In cattle it is – *M. pneumoniae*, *M. mycoides, M. agalactiae, M. alkalescens, M. arginini , M. bovirhinis, M. bovis* and *M.bovigenitalium*, as well as *Ureaplasma diversum* [2,3,4].

Mycoplasma bovis (M. bovis), first isolated in 1961 in severe mastitis in cattle, it can cause pneumonia, otitis media in young calves, mastitis and arthritis in elderly animals [6, 7, 8, 9]. These diseases were designated as *M. bovis*-associated diseases (MbAD) [9,10,11,12].

Mycoplasma bovis — opportunistic pathogen in cattle. The diseases caused by it affect the respiratory and reproductive systems of animals. At the same time, the carriage of these mycoplasmas is widespread among the cattle population of various countries. In recent years, *M. bovis* is an important pathogen in young calves in Europe and North America [6], and its infection leads to mortality of calves, weight loss in surviving calves and a decrease in milk production [7]. *M. bovis* are resistant to antimicrobial therapy [8,9].

In recent years, molecular genetic methods for the detection and typing of mycoplasmas based on polymerase chain reaction (PCR) have been intensively developed. The value of these methods has increased significantly due to the accumulation of data on the biological diversity of representatives of the family *Mycoplasmataceae* and the fundamental changes in their classification, which is based on the principles of modern genosysymatics.

In laboratory practice, test systems are used to detect the DNA of the pathogens of mycoplasmosis Mycoplasma spp. with the use of electrophoretic detection and in real time are made by FBUN "Central Research Institute of Epidemiology" Rospotrebnadzor, LLC Biofractal, LLC "VET Factor" and others, which are actively used both for diagnostic purposes and epizootological studies.

However, one of the priority directions for the development of laboratory diagnostics of urogenital infections is the introduction of new molecular genetic methods and the improvement of test kits into practice, which will allow species and strain identification and differentiation of mycoplasmal pathogens of animals, increasing sensitivity, specificity and speed of execution.

An important clinical significance in the epidemiological surveillance of infection caused by the pathogens of mycoplasmosis, in determining the tactics of preventing and treating a disease, is the identification of the type of pathogen.

Molecular genetic methods are used both for diagnostic purposes to solve the main clinical task - setting an etiological diagnosis and the main task of epizootological surveillance of mycoplasma pathogens, etiological interpretation of cases, and for carrying out genotyping of the pathogens. The advantages of diagnostic methods based on PCR lie, first of all, in high sensitivity and specificity of detection of specified DNA fragments, which exceeds 85% and 97%, respectively. An important advantage of using PCR for making an etiological diagnosis is the absence of the need to work with traditional microbiological methods, which reduces the time research and conduct simultaneous analysis of more clinical material. The 16 S rRNA gene is used as genetic targets for PCR [9, 10, 11, 12, 13].

In the system of epidemiological surveillance of infectious diseases in the Russian Federation, a significant role is assigned to the operational determination of the specificity of pathogens, ensuring timely management decisions on the necessary complex and scope of preventive and antiepizootic measures. The effectiveness of the diagnostic subsystem of epizootic surveillance is determined by the integration of modern molecular technologies for the indication and identification of pathogens into the laboratory diagnostic system. The use of new high-tech approaches significantly reduces the analysis time, ensures its high sensitivity and specificity with a significant increase in information content.

Epizootological surveillance of mycoplasmosis, ureaplasmosis and chlamydia should be based on the etiological interpretation of pathogens released during invasive (generalized) forms of the corresponding infections, and taking into account cases of disease in certain groups of animals in the observed territory. Identification of the type of pathogen is the basis of epidemiological surveillance and determines the tactics of the planned antiepizootic measures.

Genetic identification of microorganisms consists in the detection of specific bacterial DNA sequences, and can be carried out using a wide range of molecular-biological methods, of which PCR has been most prevalent in recent years.

Therefore, the need to study the genome and genetic variability of microorganisms for several genes circulating in the territory of each region and conducting phylogenetic analysis in each case to improve molecular genetic monitoring of reproductive infections and develop and improve methods for their prevention and treatment is relevant.

Genetic identification of microorganisms consists in the detection of specific bacterial DNA sequences, and can be carried out using a wide range of molecular-biological methods, of which real-time PCR (PCR) and various DNA sequencing techniques are most common in recent years. [14,15,18,19].

The result of sequencing is the characterization of the strain based on certain nucleotide or corresponding amino acid sequences [18,19].

The development of automatic genetic analyzers and databases has led to the fact that the modern taxonomy of bacteria is based on the nucleotide sequence of the 16S rRNA gene. The length of this gene is approximately 1540 bp, the nucleotide sequence alternates between conservative for all types of bacteria and variable regions, which allows to select universal primers for PCR amplification of almost complete sequence [18,19,20].

Objective: genetic identification of bacteria based on sequencing using the analysis of the nucleotide sequence of the 16S rRNA gene to improve molecular genetic monitoring of pathogens circulating in Russia.

MATERIAL AND METHODS

The material for the study was smears and scrapings from the vagina of cows with clinical signs of vulvovaginitis, with impaired reproductive function and with hidden mastitis, as well as mastitis milk, smears from the nose of calves in rhinitis, bronchitis and bronchopneumonia, light aborted calf fetus.

The scrapings of the mucous membranes were diluted in 0.5-1.0 ml of physiological saline, precipitated in a microcentrifuge at 11-12 thousand rpm for 5-10 minutes. The supernatant was removed, and the precipitate was resuspended in 100 µl of saline and used for the extraction of DNA set "AmpliPrime Sorb-V" (FBUN Central Research Institute of Epidemiology Rospotrebnadzor, Moscow) in accordance with the manufacturer's instructions.

Milk samples (30-50 ml) were taken in sterile plastic containers after careful treatment of the udder with soapy water, disinfection of the nipples with 70% ethyl alcohol and returning the first portion of milk to a separate container.

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For the extraction of DNA from the samples, 1 ml of milk was collected and centrifuged at 13000 × g for 5 minutes, followed by the removal of fat and supernatant. The remaining precipitate was resuspended in 100 µl of buffer solution.

The selection of nucleic acids from samples of mastitis milk was carried out by the sorption method using the RIBO-prep reagent kit (FBUN TsNII Epidemiology) in accordance with the manufacturer's instructions.

In general, it was shown that the use of the sorbent method of DNA extraction is less effective than the use of the method, which includes the stage of alcoholic precipitation of nucleic acids. Because of the ease of use associated, including, with the reduction of the total extraction time, the Ribot-prep set was chosen for further work.

The MIK-KOM test systems were used to amplify the DNA of various mycoplasma species of the genus Mycoplasma with electrophoretic detection in agarose gel Amplisens (Russia).

For identification of Mycoplasma bovis, primers were used representing the 16s rRNA gene region and were synthesized using the amidophosphite method on ASM-2000 DNA / RNA synthesizer (Biosset, RF). We evaluated the complementarity and specificity of the selected primers by comparing with the NCBI nucleotide sequence base using the BLAST algorithm for searching for related sequences. The homology of the selected primers with the sequences of cattle strains and isolates was established.

A number of experiments were carried out to determine the working concentrations of primers that provide the necessary sensitivity of the analysis, and the conditions for performing PCR were optimized. The oligo calculator (Oligo Calc: Oligonucleotide Properties Calculator) was used, then the data were compared with the result of calculations using the formula: Tm (° C) = 2 x (A + T) + 4 x (G + C), choosing the best option.

PCR was performed in a final volume of 50 µl. The reaction mixture contained 20 µl of the isolated DNA, 10 pmol of each primer, 0.25 mM of each nucleotide, 2.5 units. Taq polymerase, 1.5 mM MgCl, 1HPCR buffer. The following temperature parameters were used: 95 ° C-5 min. -1 cycles subsequent 35 cycles of 95 ° C for 10 seconds, 55 ° C - 20 seconds, 72 ° C - 10 seconds, and final elongation at 72 ° C for 3 minutes.

The PCR was set up on a Terzik amplifier (DNA-Technology, RF). PCR products were visualized by electrophoresis in a 1.8% agarose gel containing ethidium bromide. Recording of PCR results is carried out by the presence or absence of a specific band of amplified DNA on an electrophoregram (Figure 1). The size of the 16s rRNA genome fragment was 400 p.n.

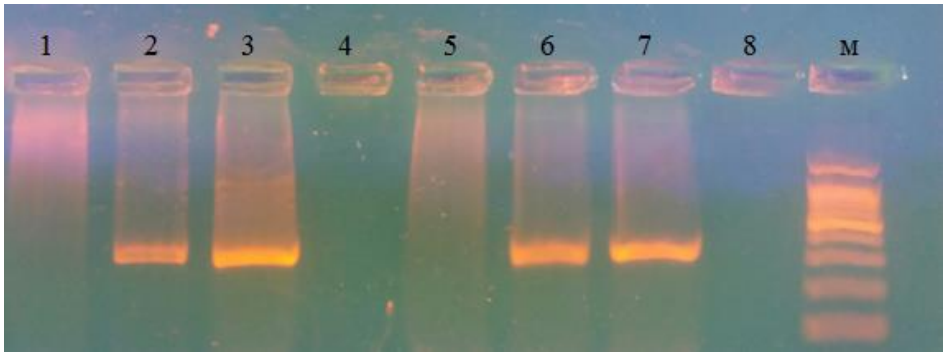

Figure 1: Results of Mycoplasma bovis electrophoregram

M - molecular DNA markers "GelPilot 100bp Plus Ladder (100)" (QIAGEN, Germany), 1.5 –negative clinical samples, 2,3,6,7-positive clinical samples, 4-control negative, 8- negative control sample (NCS).

Purification of the PCR product

Before sequencing the amplified fragment of the genome, the PCR product was purified from unincorporated primers and dNTPs. For purification, 5 mm^3 of PCR product was taken, 2 mm^3 of reagent was added to purify PCR products and mixed by pipetting.

The resulting solution was incubated at 37 ° C for 15 minutes. Then it was shaken and precipitated by short-term centrifugation and again incubated at 80 ºC for 15 minutes.

Sequence reaction

The PCR products were sequenced from the forward and reverse PCR primers. For carrying out the reaction, 1 mm^3 of purified PCR product, 0.8 mm^3 of a molar concentration of 1 µmol / dm^3 and 1 mm^3 of sequencing reagent mixture and 2.2 mm^3 of deionized water were added to thin-walled microtubes with a capacity of 0.2 cm^3. The total volume of the reaction mixture should be 5 mm^3.

Thermal cycling was carried out on a 2720 Thermal Cycler Thermostatted Lid Thermostat (Applied Biosystem, United States) using the Big Dye® Terminator v1.1.Cycle Sequencing Kit: initial denaturation - 1 min at 96 ° C, then 25 cycles: 96 ° denaturation C - 10 s, primer annealing - 5 s, elongation at 60 ° C - 4 min. Annealing for all primers used in the sequencing reaction is optimized for 55 ° C.

The sequencing products obtained after amplification — the reaction mixture with a volume of 5 mm^3 — were used for further testing.

Purification of the reaction mixture after sequencing

The reaction mixture was purified from an excess of dNTPs, fluorescently labeled ddNTPs, a sequencing primer, and salts by precipitation of polynucleotides with isopropyl alcohol. To this end, 30 mm^3 of 75% isopropyl alcohol was added to the reaction mixture with a volume of 5 mm^3 in a microvial and incubated for 20 min at room temperature.

Then the microtubes with the mixture were centrifuged at 13000 rpm for 20 minutes and the supernatant was removed.

100 mm^3 of 75% isopropyl alcohol was added to the resulting precipitate with a single-channel dispenser, centrifuged at 13000 rpm for two minutes and the supernatant was removed.

Dry the obtained precipitate in a thermostat at 65 ° C for 10-15 minutes and dissolve in 20 mm^3 of high purity formamide Hi-Di ™ Formamide

The obtained analyzed samples in formamide were transferred to a 96-well plate, covered with a compactor and subjected to thermal denaturation at 95 ° C for 3 min, and then at 4 ° C for 3 min.

Determination of nucleotide sequence by capillary electrophoresis

For DNA sequencing using the Sanger method, an automatic ABI PRISM 3130 Genetic Analyzer Genetic Analyzer (Applied Biosystems, USA) was used to reliably determine a sequence of up to 700-800 base pairs in a single reading. All stages of the study and interpretation of the results were performed in accordance with the instructions of the manufacturer of the genetic analyzer.

Prepared analyzed samples containing sequencing products were separated by capillary electrophoresis with detection of the fluorescence signal.

The software of the genetic analyzer automatically analyzes the received signals (chromatograms), evaluates the quality of reading (QV) and determines the sequence of nucleotides. The result of the analysis is the decoded nucleotide sequence shown above the chromatogram peaks in the abi file format.

The correctness of the reading of nucleotides was checked additionally manually in order to eliminate the error of automatic analysis. The nucleotides at the beginning of the chromatogram and the reverse primer region at the end of the PCR product sequenced were removed poorly readable due to background bursts.

The resulting sequences were leveled using the AlignX program from the Vector NTI Suite 6.0 package and analyzed using the MEGA4 program.

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RESULTS AND DISCUSSION

As a result of the work, specific primers were selected for the 16 s rRNA region of *Mycoplasma bovis*; the conditions of their thermal cycling for the PCR reaction were optimized.

We investigated clinical samples taken from animals in farms of the North-West region. *Mycoplasma bovis* was identified by sequencing and the level of homology was 100%, which confirms the correctness of the selected sequences (Figure 2).

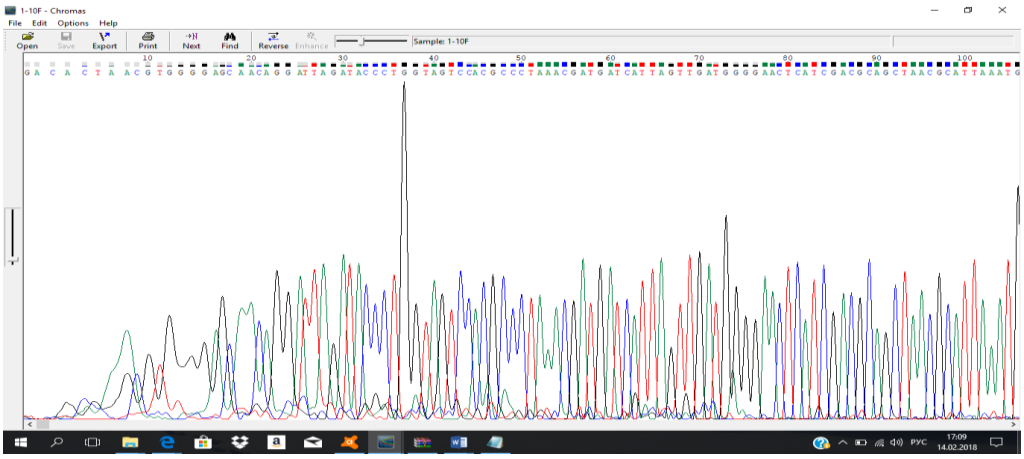
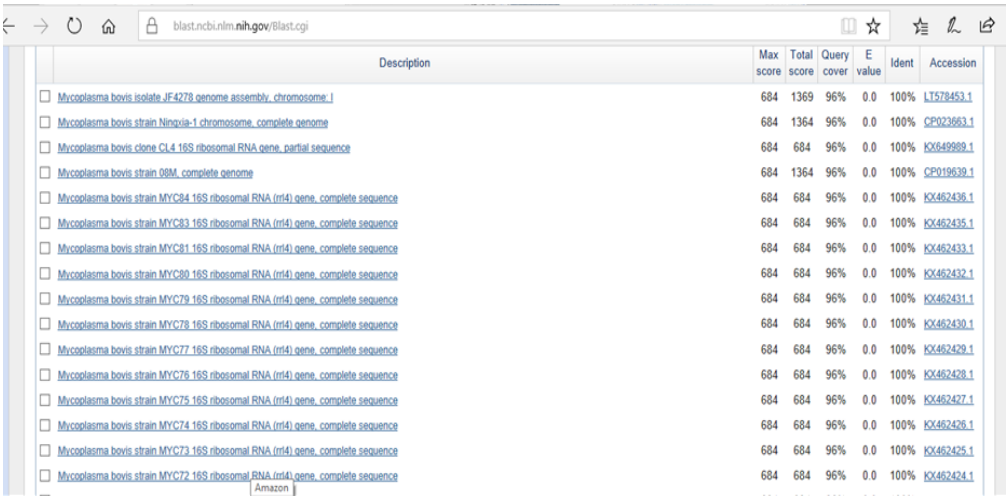


Figure 2: Fragment of the chromatogram in the identification of Mycoplasma bovis

To search for homologous sequences in NCBI databases, the BLAST algorithm was used on the Internet search resource www.ncbi.nlm.nih.gov. The search report is presented as a list of sequences with which the resulting nucleotide sequence has the greatest homology (Figure 3).



Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycoplasma bovis isolate JF4278 genome assembly, chromosome: I	684	1369	96%	0.0	100%	LT578453.1
Mycoplasma bovis strain Ningxia-1 chromosome, complete genome	684	1364	96%	0.0	100%	CP023663.1
Mycoplasma bovis clone CL4 16S ribosomal RNA gene, partial sequence	684	684	96%	0.0	100%	KX649989.1
Mycoplasma bovis strain 08M, complete genome	684	1364	96%	0.0	100%	CP019639.1
Mycoplasma bovis strain MYC84 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462436.1
Mycoplasma bovis strain MYC83 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462435.1
Mycoplasma bovis strain MYC81 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462433.1
Mycoplasma bovis strain MYC80 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462432.1
Mycoplasma bovis strain MYC79 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462431.1
Mycoplasma bovis strain MYC78 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462430.1
Mycoplasma bovis strain MYC77 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462429.1
Mycoplasma bovis strain MYC76 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462428.1
Mycoplasma bovis strain MYC75 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462427.1
Mycoplasma bovis strain MYC74 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462426.1
Mycoplasma bovis strain MYC73 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462425.1
Mycoplasma bovis strain MYC72 16S ribosomal RNA (rrl4) gene, complete sequence	684	684	96%	0.0	100%	KX462424.1

Figure 3: BLAST search report form

In a molecular study of clinical material from cows with impaired reproductive function and calves with respiratory infections, Mycoplasma spp. Was isolated (47% of cases) and My*coplasma bovis* was identified (53% of cases) (Figure 4).

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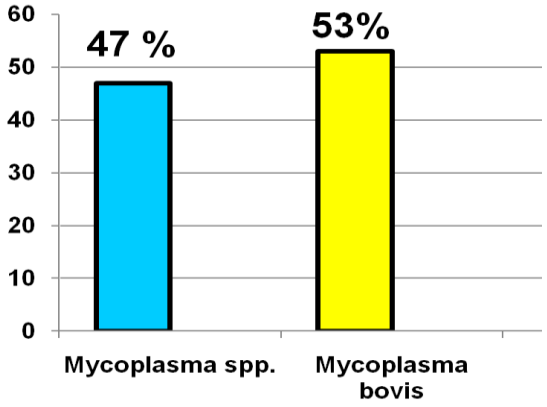

Figure 4: Species and percentage composition of microorganisms of the genus Mycoplasma, isolated by molecular genetic methods

CONCLUSION

According to the results of sequencing, the sequences obtained belong to the microorganism *Mycoplasma bovis*.

The analysis of the results of PCR studies showed a high frequency of occurrence of microorganisms of the genus *Mycoplasma bovis* in clinical specimens in 53% of cases.

Based on the data obtained, it was concluded that one of the causes of mastitis and reproductive infections of cows, as well as respiratory infections of calves in the conditions of the animal-breeding complex is *Mycoplasma bovis*.

Since the PCR study does not indicate the viability of the microorganism, it is necessary to create a scheme for laboratory control of mycoplasma infection, which will include both the PCR method and bacteriological studies using modern media for growing difficult-to-cultivate microorganisms.

Thus, it is more expedient to carry out genetic identification on the basis of the analysis of the nucleotide sequence of the 16S rRNA gene, since it allows the identification of cultures to species and reduces the time of research.

In veterinary laboratory practice, various types of PCR are increasingly being used to quickly identify pathogens, which are too long and difficult to cultivate, or difficult because of the widespread use of antibiotics.

It is necessary to constantly improve and create new methods and reagents for laboratory diagnosis of mycoplasma infections in order to increase the information content, specificity and sensitivity of molecular genetic methods for laboratory diagnosis, indication and identification of pathogens.

Currently, PCR and sequence analysis can be optimized using appropriate software with validated databases, such as MicroSeq (Applied Biosystems Inc.) or SmartGene IDNS.

PCR allows in a short time to diagnose and apply preventive and therapeutic measures, which reduces and prevents economic damage.

The diagnostic capabilities of molecular genetic methods in epizootology are not limited to using them to identify sources of infection and transmission factors of the pathogen during epizootological outbreaks. The main purpose of molecular genetic methods in epizootology in the near future should be tracking the population structure of infectious disease pathogens in order to assess, predict the epizootic situation and justify timely intervention in the course of the epizootological process - that is, molecular genetic monitoring.

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