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Prevention of mastitis and hyperkeratosis in cows

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Abstract

Background and Aim. Mastitis in cows is widespread, causing great economic damage to agriculture. Treatment of the udder before and after milking is an important stage in the process of caring for the skin of the teats, aimed on preventing of infection of the udder and the development of hyperkeratosis, mastitis; to protect the sphincters of the teat after milking, specialized products are used for treatment by dipping the teat into a cup. In addition to antiseptic properties, these products should include caring components; they are necessary to soften and moisturize the skin of the nipple and accelerate the restoration of the epidermis.

Materials and Methods. The study of the preventive effectiveness of LACTIC DIP PRO against mastitis and therapeutic effectiveness of nipple hyperkeratosis was carried out in the Zhigar agricultural production complex of the Gergebil region of Dagestan Republic, on 45 Holstein cows, divided into two groups (experiment - control). For cows of the experimental group (n=30), all teats were treated with LACTIC DIP PRO, by dipping the teats into a glass during 40 days after each milking. Animals of the control (n=15) did not have their teats treated after milking and served as controls. The qualitative composition of milk was studied in the cows of the experimental group on days 15, 30 and 40 of the experiment.

Results. The hygienic product provided protection of the udder teats and mammary glands of the experimental cows from contamination by pathogenic microflora and the preventive effectiveness during the experiment was 100%.

Conclusion. No significant biochemical changes were observed in the milk of cows, therefore, the use of a hygiene product after milking does not impair the quality of milk.

Keywords: cow; hyperkeratosis; hygienic treatment; mastitis; mammary gland; prevention.

Introduction

Dairy farming plays an important role in agriculture, providing people with food. The main problem, facing dairy producers, is mastitis, an inflammatory disease of the udder in cows that can lead to serious consequences, for both animals and milk producers. Currently, this problem occurs in all countries of the world with developed cattle breeding. The extent of disease spread varies from 12 to 60% [1, 2, 3]. The disease is registered during the period of lactation, start-up and dry wood [4, 5].

The course and form of mastitis depend on the degree of virulence of the microflora, the state of the animal's local and general defense systems, the influence of unfavorable factors, the effectiveness and timeliness of treatment and preventive measures.

According to a number of authors, mastitis is mainly isolated from staphylococcus (Staphylococcus), streptococcus (Streptococcus) and, less commonly, Coliforme microbes [6]. Microorganisms can penetrate in to the mammary gland through the nipple canal, by lymphogenous, or hematogenous routes.

Most often, pathogen penetration occurs through the sphincter of the nipple [7]. After milking, the teat canal remains open during 30 minutes, however, if milking technology is violated, the time to restore of the teat canal may increase.

Inflammation of the mammary gland in highly productive cows is widespread. The greatest economic problem is represented by latent subclinical mastitis, which occurs in 4...5 times more often than clinically expressed ones. Jin mastitis, there is a decrease of milk production, deterioration of milk quality, impaired of reproductive function, premature culling of animals and treatment costs. In such milk there was a decrease of the content of fat, protein, lactose and an increase of the number of somatic cells [8].

The etiological causes of mastitis include a complex of reasons, including unsatisfactory sanitary and hygienic conditions of detention, violations of the milking routine, untimely identification of sick animals, as well as unbalanced feeding of dairy livestock [9]. Violation of the technology of machine milking can lead to injury to the udder tissue, the manifestation of hyperkeratosis on the nipples, which, in turn, provokes the appearance of mammary gland diseases, in particular, the development of mastitis [10].

Hyperkeratosis is excessive keratinization of the epidermis, expressed in thickening of the skin surrounding the opening of the teat canal in cattle. Changes in the structure of the teat tissue, especially in the area of the sphincter and the teat canal, due to disruption of machine milking, increase the risk of mastitis, since the effectiveness of the barrier function of the teat canal to infections decreases [11, 12]. In places where the epidermis of the nipples grows, conditions are created for the development of pathogenic microorganisms that can penetrate into the udder during and after milking, causing inflammation of the mammary gland.

In the conditions of modern livestock farming, positive dynamics in the fight against latent mastitis is provided by the use of pharmacological drugs for treatment and preventive measures to prevent the development of diseases of the udder and teats in cows during lactation.

Subclinical mastitis often develops into clinically pronounced inflammation of the mammary gland, and often causes atrophy of the affected quarters of the udder. Animals that have suffered from subclinical mastitis lose milk production by average 10-15% [13].

Treating of the udder before and after milking is an important step in the process of teat skin care, aimed on preventing infection of the udder and the development of mastitis [14].

The main goals of treating of teats before milking include caring for the skin of the udder, removing dirt and preventing it from getting into the teat rubber and into the milk line, and reducing of bacterial contamination of milk. To protect the nipple sphincters after milking, specialized means are used to treat the nipple by dipping it into a cup. In addition to antiseptic properties, the composition includes products to soften and moisturize of the skin of the nipples and accelerate the restoration of the epidermis. The protective film, formed after application of the product helps to reduce the time when the nipple is open to the penetration of pathogenic microflora.

A product for treating, the udder after milking based on lactic acid LAKTIK DIP PRO (developed by the VIC group) fits these criteria. The product has a complex composition, including: surfactants, softening, moisturizing and functional additives, allantoin, D-panthenol, which promotes nipple skin regeneration. The product forms a film, protecting the nipple canal from the penetration of microorganisms. LAKTIK DIP PRO has a high-quality color indication that allows you to control the quality of treatment of the udder teats after milking.

The purpose of the work is to study the preventive effectiveness of the hygiene product LAKTIK DIP PRO against mastitis and nipple hyperkeratosis.

Materials and methods

A study of the preventive effectiveness of LAKTIK DIP PRO against mastitis and therapeutic effectiveness in nipple hyperkeratosis was carried out in the Zhigar agricultural complex of the Gergebil region of Dagestan Republic.

For the first series of experiments, 45 heads of Holstein cattle were selected with an average productivity 6 tons per head per year. The animals were divided into two groups (experiment - control).

Cows of the experimental group (n=30) had all teats treated with LACTIC DIP PRO during 40 days after each milking by dipping the teats into a glass for "udder treatment after milking", filled with at least 3⁄4 volume of the product. The size of the dipping container should ensure that at least 3⁄4 of the surface of the nipple skin is treated.

Animals in the control (n=15) did not have their teats treated after milking. Served as control.

The qualitative composition of milk was studied in the cows of the experimental group on days 15, 30 and 40 of the experiment.

In the second series of experiments, 31 heads were selected and divided into two groups (experiment - control). The experimental group included 16 heads with hyperkeratosis, in which, during 40 days after each milking, all teats were treated with LACTIC DIP PRO, by dipping the teats into a glass for "udder treatment after milking", filled with at least 3⁄4 volume of the product. The size of the dipping container should ensure that at least 3⁄4 of the surface of the nipple skin is treated.

Animals of the control group (n=15) did not have their teats treated after milking and served as controls.

Results

Milk is a complete, universal food product; it contains almost all the nutrients necessary for humans and, therefore, is a highly valuable food product. It contains proteins, fats, carbohydrates (milk sugar), mineral salts, vitamins, and in a very easily digestible form, which is why milk is considered indispensable in the diet of adults and especially children.

The widespread spread of mastitis in cows has a serious negative impact on the development of dairy production due to the deterioration of the reproductive capacity of the livestock, a decrease in milk yield, culling of animals, and deterioration in the sanitary and technological qualities of milk.

The structural and mechanical properties of acid and acid-rennet curds also change; they have higher viscosity, lower density and separate whey worse.

Results of studying some physicochemical parameters of milk from experimental cows during 15, 30 and 40 days of the experiment.

A large number of antibacterial drugs used in the treatment of mastitis in cows do not meet modern requirements of veterinary medicine for reasons of insufficient therapeutic effectiveness, milk rejection for long periods, the emergence of resistance in pathogenic microorganisms, and inhibition of natural neurohumoral mechanisms of local and general defense of the body.

Udder treatment before and after milking is an integral part of cattle udder care, and is important for the prevention of mastitis. When choosing products for treating the udder, pay attention to the physical characteristics, of which the most important are:

Film formation – after applying the product, a uniform film should form, covering the sphincter of the udder nipple;

Minimal drip after application;

Retention time on the nipple after application is at least 40 minutes while maintaining the integrity of the film;

Color indication of the treated udder teat, providing the possibility of visual control;

Consumption of product when treating udder teats.

The product for treating of the udder after milking "LAKTIK DIP PRO" has shown high preventive effectiveness against mastitis infection; its use in nipple hyperkeratosis also shows the therapeutic effectiveness. The product has high adhesive film properties, which allows you to protect the open nipple channel during 30 minutes. To assess the economic efficiency, the rate of fall of the product was calculated, which was 2 drops and high preservation of the film on the nipple, which allows reducing the amount of product consumed (Table 1).

Table 1 – Evaluation of the effectiveness of using of the product for treating of the udder after milking LAKTIK DIP PRO

Product viscosity/drop point	2 drops
Adhesive property of the film	High
Duration of fixation of the product on the nipple/minutes	30
Color intensity	Greenish
Drug consumption, ml/head	8,3
Allergic reactions	No
Incidence of mastitis, %	0
Manifestation of hyperkeratosis on the beginning of the experiment, %	16
Manifestation of hyperkeratosis on the end of the experiment, %	4

The data in Table 1 shows that the use of LACTIC DIP PRO in cows does not cause an allergic reaction; in the experimental group (n=30), within 40 days after birth, the incidence of mastitis was prevented on 100%, and the manifestation of hyperkeratosis decreased on 12%.

The results of studying of the physicochemical parameters of milk from in cows of the experimental group during 15, 30 and 40 days are shown in Table 2.

LACTIC DIP PRO has strong regenerative properties of the skin epidermis due to the presence of caring components in its composition, which had a therapeutic effect when used in animals with hyperkeratosis.

Correct and regular processing helps to maintain animal health, maintains of production performance and provides consumers with high-quality and safe dairy products.

Milk is a universal food product, it contains almost all the essential nutrients for humans and, therefore, is a highly valuable food product.

One of the significant issues in the fight against latent (subclinical) mastitis in lactating cows is their early diagnosis, which consists in determining of the sanitary indicators of the resulting milk: somatic cells, titometric and active acidity and bacterial contamination. The admixture of mastitis milk leads to changes in the chemical composition of the collected milk, as a result of which the biochemical and microbiological processes during its processing are disrupted. Such milk does not coagulate well with rennet, is less heat-sensitive, and production-valuable lactic acid bacteria do not develop well in it. The structural and mechanical properties of acid and acid-rennet curds also change; they have higher viscosity, lower density and separate whey worse.

The admixture of mastitis milk leads to changes of the chemical composition of the collected milk, as a result of which the biochemical and microbiological processes during its processing are disrupted. Such milk does not coagulate well with rennet, is less heat-sensitive, and production-valuable lactic acid bacteria do not develop well in it. The structural and mechanical properties of acid and acid-rennet curds also change; they have higher viscosity, lower density and separate whey worse.

Results of studying of some physicochemical parameters of milk of experimental cows during 15, 30 and 40 days of the experiment.

As follows from Table 2, when studying milk in experimental cows after 15, 30 and 40 days, the number of somatic cells, pH, titratable acidity and density of milk during the study period corresponded to the physiological norm.

Table 2 – Milk parameters of cows of the experimental group (n=30)

Indicators	Research days		
	15	30	40
	$\bar{x} \pm m_x$	$\bar{x} \pm m_x$	$\bar{x} \pm m_x$
Content of somatic cells, 1sm³, no more	347693.17±4621.33***	387349.02±3977.21	372764.07±9752.14

Continuation of table 2

pH	6.64±0.01	6.63±0.01	6.64±0.01
Titrimetric acidity, °T	17.99±0.08	18.01±0.06*	17.94±0.08*
Density, kg/m³	1028.17±0.03	1028.31±0.08	1028.42±0.11
Note: * – P <0.05, ** – P <0.01, *** – P <0.001			

As follows from Table 2, when examining of milk from experimental cows after 15, 30 and 40 days, all milk parameters studied corresponded to physiological parameters.

Discussion and conclusion

Studies have established that when treating of teats after milking in cows, the preventive effectiveness of the hygiene product LAKTIK DIP PRO was 100%, the therapeutic effectiveness in hyperkeratosis was 75.0%. The quality of milk in experimental cows remained within the physiological norm during the experimental period.

It was determined that the number of somatic cells, pH, acidity and density of milk remained within the physiological norm.

Thus, LACTIC DIP PRO for the prevention of mastitis and treatment of nipple hyperkeratosis is highly effective and can be recommended for wide practical use.

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Development of a real-time PCR for the identification of *Anaplasma marginale* in cattle

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Abstract

Anaplasma marginale is a gram-negative, obligate intracellular bacterium that infects cattle, buffalo, deer, and antelope, causing significant economic losses worldwide. In recent years, due to an increase in the area of uncultivated land and uncontrolled use of pastures, there has been a tendency for a sharp increase in the population of blood-sucking ticks - carriers of blood-parasitic animal diseases. In this regard, the development of molecular genetics methods for the diagnosis of blood parasitic diseases is relevant for taking effective measures to prevent the spread of tick-borne infections. The lack of sensitivity and specificity of the microscopic and immunological diagnostic methods used, as well as the tendency for a sharp increase in the population of blood-sucking ticks, aggravates the epizootological situation. As part of this research, a real-time PCR test system for the identification of *Anaplasma marginale* was developed. Species-specific primers and a fluorescence-labelled TaqMan probe were developed for the highly conserved gene of the heat shock family protein *groEL.* The developed real-time PCR test system showed high specificity and sensitivity, allowing the detection of the *groEL* gene of *A. marginale* at the level of 8 copies in the reaction. The developed real-time PCR test system can be used for early diagnosis of anaplasmosis in cattle and conducting monitoring studies.

Keywords: *Anaplasma marginale*; cattle; real time PCR; qPCR; *groEl.*

Introduction

Anaplasmosis an infectious disease of animals caused by rickettsias of the genus *Anaplasma.* The disease is caused by gram-negative obligate intraerythrocytic parasites of the family *Anaplasmataceae* of the genus *Anaplasma* of the following species: *A. ovis, A. bovis, A. capra, A. marginale, A. centrale, A. phagocytophilum, A. platys*. Various members of the *Anaplasma spp.* can infect large and small livestock, horses, deer, antelope, moose, dogs, cats, as well as humans [1, 2].

Anaplasmosis is one of the tick-borne infections of economic importance to agriculture, leading to loss of meat and dairy productivity, and in severe cases lead to death [3]. In addition, weakened animals are more susceptible to other infectious or parasitic diseases, which increases the mortality of livestock. The most acute pathological process is characteristic of the species A. marginale, during which about

70% or more of red blood cells are affected, which can lead to the death of 30-50% of cattle [4, 5].

A. marginale is widespread all over the world, especially in tropical and subtropical regions. The disease is accompanied by hemolytic anemia, fever, disorders of the gastrointestinal tract and respiratory organs, and weight loss [6]. The main vectors of anaplasmosis are ticks of the species *Ixodes, Rhipicephalus, Haemaphysalis, Dermacentor,* etc., as well as mosquitoes, horseflies and other blood-sucking insects, which means that the disease is naturally localized and can quickly spread to neighboring farms [7, 8].

In the southern regions of Kazakhstan, *Anaplasma marginale* predominates among tick-borne infections of cattle, accounting for 48.9% among tested animals [9]. At the same time, effective measures to control the spread of infection are not taken, mainly due to the complexity of differential diagnosis and the lack of highly sensitive and specific test systems, which contribute to the spread and chronic course of the disease with periodic relapses.

Currently, the most commonly used methods for diagnosing anaplasmosis are Giemsa staining of smears and serological methods. Accuracy of diagnosis is often hampered by insufficient numbers of circulating infected cells, especially during the prodromal period or in cases of latent animal carriers. False-positive results are also possible with Giemsa staining due to staining artifacts and Heinz and Howell-Jolly bodies similar to anaplasma-like structures [10].

In the early acute phase of infection, serological tests are of limited value due to the lack of detectable antibodies. In addition, the difficulty of differential diagnosis in serological studies is determined by similar symptoms and possible cross-reactions not only between similar types of anaplasmas, but also with other tick-borne infections: babesiosis, borreliosis, ehrlichiosis, Lyme disease, rickettsiosis [11]. A seronegative course of anaplasmosis is possible, which prevents timely detection and contributes to the further spread of infection through common pastures and grazing areas.

Despite the fact that a number of different diagnostic methods have been developed for intraspecific identification of anaplasmosis (complement-enzyme linked immunosorbent assay, card agglutination test, complement fixation test, loop-mediated isothermal amplification, reverse line blot, PCR, nested PCR, quantitative PCR), most of them have insufficient sensitivity and specificity [12]. The best results today are shown by PCR; however, universal and generally accepted methods are not suitable for intraspecific diagnosis of anaplasmosis. PCR methods based on amplification of the 16S rRNA gene have already been used in the analysis of the *Anaplasma* genus. Due to the high degree of similarity between the species *A. marginale, A. centrale* and *A. ovis*, this method does not allow them to be differentiated, which is due to the low rate of evolutionary changes in the 16S rRNA gene [13].

As part of these studies, the groEL gene was selected as the most suitable for single locus genotyping. It is highly conservative, but contains variable regions that allow it to be used to differentiate closely related species [14].

The purpose of this work was to develop a PCR protocol for the detection and species identification of *Anaplasma marginale*.

Materials and methods

Ethical approval

This study was approved by the local ethics committee in the National Center for Biotechnology (Protocol № 2 dated 04 April, 2022). The respective cattle owners gave their approval for sampling. No animal was harmed during the sampling.

Sampling and sample preparation

To screen for anaplasmosis among cattle, 962 whole blood samples were collected from the Turkestan region. Blood was collected in EDTA vacutainers and transported to the laboratory at 4 °C for 48 hours. For lysis of erythrocytes, a lysing buffer (1.5 M NH_4Cl (PanReac AppliChem, Darmstadt, Germany), 100 mM $NaHCO_3$ (Thermo Fisher Scientific, Fair Lawn, USA), 10 mM EDTA (BioRad, Richmond, USA), H_2O) was used in a ratio of 1:3, and after stirring and 5 minutes' incubation at room temperature, centrifugation was carried out for 5 minutes at 13,000 rpm, followed by removal of the supernatant. The pellet was stored at minus 80 °C.

DNA isolation

DNA isolation was carried out on the basis of sorbent DNA binding. The precipitate was dissolved in 150 µl of lysing solution 1: 0.4 M NaCl (Titan Biotech Ltd., Rajasthan, India), 10 mM Tris-HCl pH 8.0 (BioRad, Richmond, USA), 2 mM EDTA, 5% SDS (Sigma-Aldrich, Darmstadt, Germany), 1 mg/ml of proteinase K (Magen, Guangzhou, China). Samples were incubated at 37 °C for 4 hours and at 50 °C for 50 minutes. 500 µl of lysis buffer 2 (3.2 mM GuaSCN (PanReac AppliChem, Darmstadt, Germany), 20 mM Tris-HCl (pH 7.4), 50 mM EDTA, 4% Triton X-100 (Amresco, Solon, USA) and 30% isopropanol (Sigma-Aldrich, St. Louis, USA) was added and incubated at 60 °C for 10 minutes. At the next stage, a sorbent (3:7 SiO_2 (Alfa Aesar, Kandel, Germany) and Celitre 545 AW (Sigma-Aldrich, St. Louis, USA) respectively) in an amount of 70 µl was added to the test tube, the samples were incubated for 5 minutes at 60 °C and centrifuged for 1 minute at 2 000 rpm. Purification of sorbent-bound DNA was carried out at 5 000 rpm for 1 minute, first in 300 µl of wash buffer 1 (3.2 M GuaSCN, 0.1 M Tris-HCl), then twice in 500 µl of wash buffer 2 (75% C_2H_6O (DOSFARM, Almaty, Kazakhstan), 10 mM Tris-HCl). The sorbent was dried for 10 minutes at 60 °C, and DNA was eluted in 200 µl of 0.1xTE buffer (PanReac AppliChem, Darmstadt, Germany) for 10 minutes at 60 °C. Concentration measurements were carried out spectrophotometrically using Nanodrop-1000 (Thermo Fisher Scientific, Wilmington, USA).

Identification and species identification of Anaplasma spp.

The identification and species identification of *Anaplasma spp.* was carried out by amplification and sequencing of the *groEL* fragment with the following primers: anapl_F-1393 5'-aaggatggatayaaggtmatgaa-3' and anapl_R1852 5'-cgcggwcaaactgcatac-3'. The reaction mixture was prepared in 30 µl and contained 10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 300 nM of each primer, 2.5 mM $MgCl_2$, 200 nM of each dNTP, 2 units of Taq DNA Polymerase (Biolabmix, Novosibirsk, Russia) and 5 µl of DNA. The PCR cycling program was carried out on the MasterCycler ProS (Eppendorf, Hamburg, Germany): 1 denaturation cycle for 5 minutes at 95 °C, 35 amplification cycles (95 °C - 30 s, 60 °C - 40 s, 72 °C - 50 s), 1 final extension cycle for 5 minutes at 72 °C. The products were detected on a 1,5% agarose gel with ethidium bromide as intercalating agent. Visualization was performed on the GelDoc system (BioRad, Hercules, USA) using Image Lab Software (BioRad).

Species identity was determined by Sanger sequencing. Magnetic particles were used as previously described [15] for purification of PCR products. The BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Vilnius, Lithuania) was used for sequencing according to the manufacturer's instructions. Fragments were separated using a capillary genetic analyzer 3730xl (Applied Biosystems, Carlsbad, USA). The resulting contigs of the forward and reverse primers were analyzed in the SeqMan program (Lasergene, DNASTAR) and identified using the Nucleotide BLAST tool in the GenBank NCBI database.

Preparation of plasmids

Plasmid DNA was used to determine PCR sensitivity. A 445 bp fragment of the *groEL* gene was cloned into the pGEM-T plasmid using the pGEM-T Easy Vector Systems I kit (Promega, Madison, USA) according to the manufacturer's instructions and transformed into chemocompetent E. coli DH5α cells. Plasmid DNA was isolated using the Wizard SV 96 Plasmid DNA Purification System Kit (Promega, Madison, USA) according to the manufacturer's instructions. DNA concentration was measured on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA) using Qubit dsDNA HS Assay Kits (Invitrogen, Eugene, USA).

Selection of qPCR primers

The selection of primers and fluorescent probe for A. marginale was based on the alignment of the nucleotide sequence of the groEL gene in BioEdit software (Hall T. A). The groEL gene sequences for alignment were obtained from the NCBI GenBank database and included members of the following species: *Anaplasma marginale, Anaplasma centrale, Anaplasma phagocytophilum, Anaplasma ovis, Anaplasma bovis, Anaplasma platys, Anaplasma capra.*

qPCR temperature optimization

qPCR was carried out using a temperature gradient from 58 to 64 °C to optimize amplification efficiency. The reaction was performed in 3 samples of each species: *A. marginale, A. centrale, A. ovis,* and an *E. coli* sample as a negative control. Amplification was carried out in a CFX96 instrument (BioRad,

Singapore) using a BioMaster UDG HS-qPCR kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer's instructions. The PCR mix in a volume of 25 µl is included: 1x ready-to-use BioMaster UDG HS-qPCR (Biolabmix, Novosibirsk, Russia), 300 nM of each primer, 300 nM fluorescent probe and 5 µl of DNA. qPCR protocol program: 1 cycle of anti-contamination treatment for 2 minutes at 50 °C, 1 cycle of pre-denaturation for 5 minutes at 95 °C, 45 cycles of amplification at 95 °C - 15 s and 58-64 °C - 60 s. Fluorescence results were considered at the annealing/elongation stage, excluding the first 10 amplification cycles. Analysis was performed using BioRad CFX Manager software.

Determination of qPCR sensitivity and specificity

Intraspecific specificity was assessed on 32 samples of *A. marginale*, 24 samples of *A. centrale* and 24 samples of *A. ovis* obtained by screening PCR in cattle and identified by Sanger sequencing. In order to determine the interspecies specificity, 3 samples of *A. marginale* were used as positive controls, 3 samples were used as negative controls and 90 species of bacteria were selected from the internal collection, including the following species: *Acinetobacter junii, Aerococcus urinaeequi, Alcaligenes (A. aqualitis, A. faecalis), Arthrobacter polychromogenes, Atopobium vaginae, Bacillus (B. aerius, B. amyloliquefaciens, B. cereus, B. licheniformis, B. pumilus, B. sonorensis, B. velezensis, B. wiedmannii), Bacterium spp., Bordetella bronchiseptica, Brevibacillus borstelensis, Brucella (Br. abortus, Br. Melitensis), Campylobacter (C. coli, C. jejuni, C. rectus, C. showae), Clostridium (Cl. chauvoei, Cl. Haemolyticum), Delfitia tsuruhatensis, Enterobacter cloacae, Enterococcus (E. durans, E. faecalis), Erwinia (Er. endophytica, Er. Tasmaniensis), Exiguobacterium (Ex. alkaliphilum, Ex. aurantiacum, Ex. profundum, Flavobacterium sp, Halomonas nitritophilus, Klebsiella (Kl. michiganensis, Kl. oxytoca, Kl. Pneumonia), Lactobacillus (L. crispatus, L. gasseri, L. iners, L. jensenii, L. paracasei, L. paraplantarum, L. pontis), lactococcus garvieae, Lysinibacillus (L. alkalisoli, L. xylanilyticus), Macrococcus (M. canis, M. caseolyticus, M. equipercicus), Mannheimia (M. granulomatis, M. varigena), Massilia putida, Moraxella bovoculi, Ochrobactrum (O. anthropi, O. thiophenivorans), Paenibacillus (P. mucilaginosus, P. sordellii), Pantoea agglomerans, Pasteurella multocida, Pediococcus acidilactici, Propionivibrio limicola, Pseudomonas (Ps. mandelii, Ps. mucidolens, Ps. peli, Ps. putida, Ps.silesiensis, Ps. syringae), Rhizobium (R. nepotum, R. pusense), Rhodococcus (Rh. corynebacterioides, Rh. kroppensteti, Rh. opacus), Salmonela enteretidis, Serratia (S. liquefaciens, S. marcescens, S. proteamaculans), Shigella (Sh. flexneri, Sh. sonnei), Solibacillus isronensis, Staphylococcus (S. chromogenes, S. epidermidis, S. haemolyticus, S. intermedius), Streptococcus Str. criceti, Str. pluranimalium, Str. salivarius, Xenophilus arseniciresistens.*

The sensitivity of the reaction was determined by diluting plasmid DNA with an initial concentration of 1.845 ng/µl containing 4.96×10^8 copies of the target gene. The copy number was calculated using the online calculator «https://www.technologynetworks.com/tn/tools/copynumbercalculator». A qPCR reaction was set up using 5 µl of DNA, first diluted to 4.19×10^6 copies for the first row of wells in 3 replicates, followed by 4-fold dilutions to 16 copies and then 2-fold dilutions to 1 copy.

Results

Primer selection

The results of the *groEL* sequence alignment revealed a highly conserved region for *A. marginale.* The following primers and probe were selected A. marg-U 5'-gatgagattgcacaggttgct-3', A. marg-R 5'-tcctcaaccgttattaccccg-3', An. marg-PF FAM-tgccaacttcccttacgcactgtgc-BHQ1.

As shown in Figure 1, the selected forward and reverse primers have variable nucleotides on the 5' sequences to other *Anaplasma* species, which will not allow them to anneal.

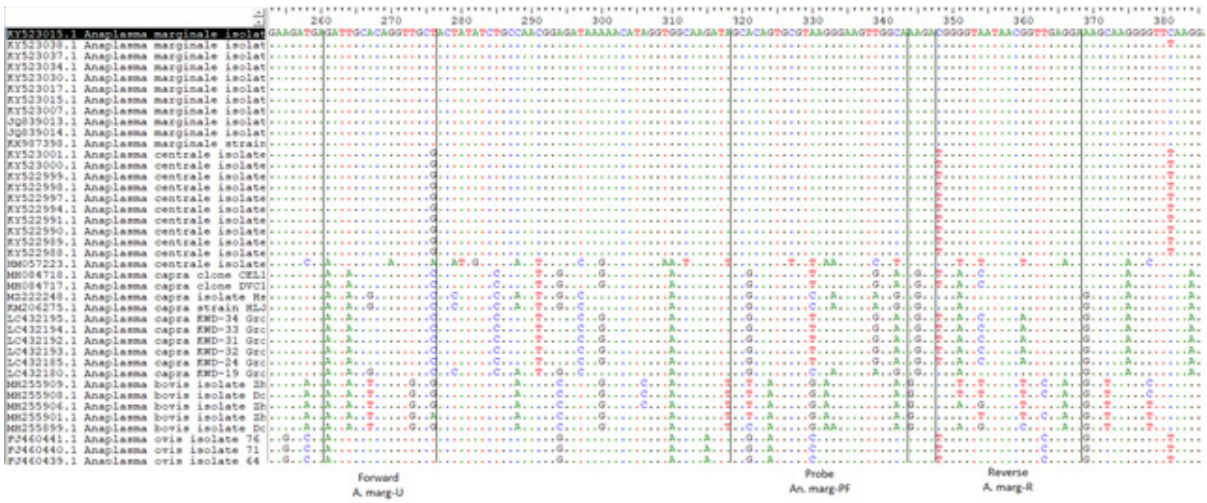

Figure 1 – Selection of primers and fluorescent probe

Testing with PrimerSelect (Lasergene, DNASTAR) did not reveal the presence of refractory dimers at the 3' ends. The annealing temperature in NCBI Primerblast was 58.91 °C and 59.52 °C for the forward and reverse primers, respectively.

qPCR optimization

According to the qPCR results shown in Figure 2, there was no non-specific annealing of primers for other *Anaplasma* species and E. coli, and the optimal temperature was 60 °C. This temperature showed the maximum level of fluorescence when reaching a plateau and a comparable value of the threshold cycle (Ct) with the results at temperatures of 61-64 °C for all three *A. marginale* samples. Lowering the temperature below 60 °C increased Ct by 1 value and decreased the fluorescence intensity. At higher temperatures, no significant differences in Ct were detected, but a decrease in fluorescence level was recorded when the curve reached a plateau.

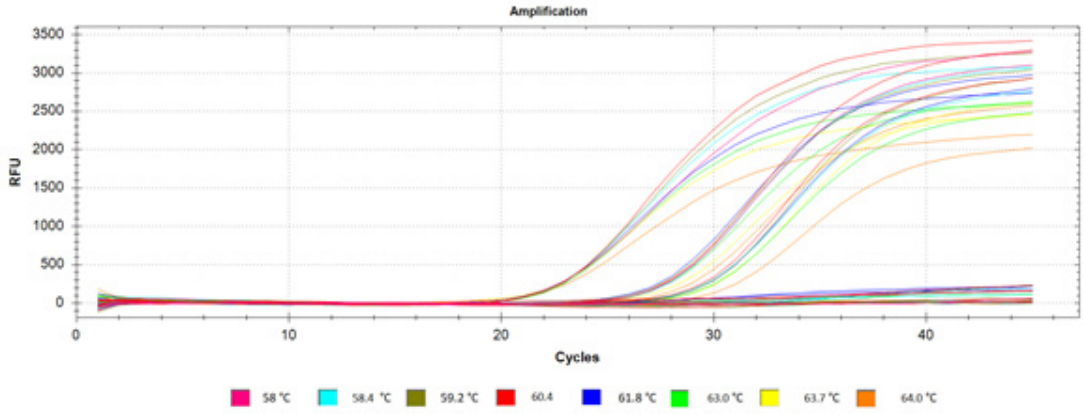

Figure 2 – Graphs of amplification curves in a temperature gradient of 58-64 °C

Testing qPCR specificity and sensitivity

Specificity and sensitivity were assessed at the optimal selected temperature of 60 °C. As a result of qPCR, specific annealing and amplification were only detected in samples containing *A. marginale*, as shown in Figure 3. The negative control in the form of *E. coli, A. ovis* and *A. centrale* species did not show any increase in the amplification curves.

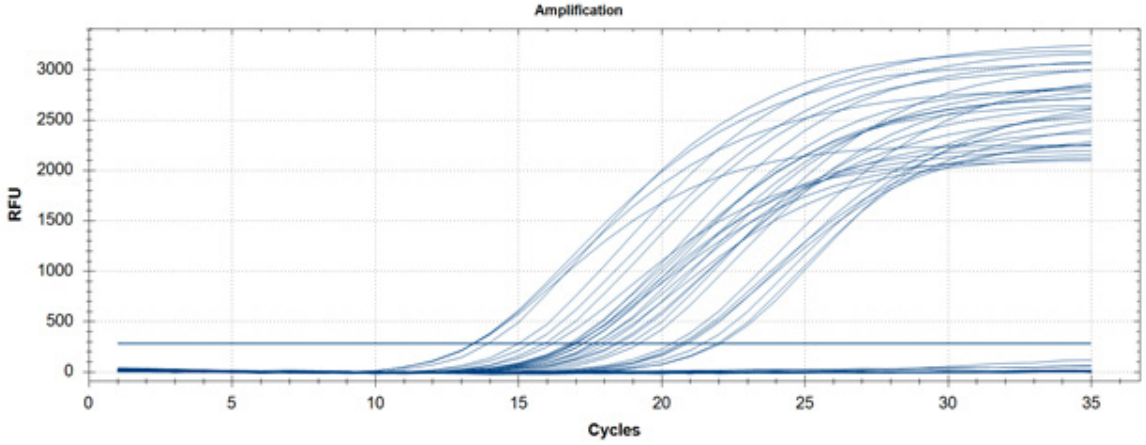

Figure 3 – Assessment of primer species specificity

The qPCR results shown in Figure 4 confirmed the absence of specific annealing to 90 other bacterial species and the presence of fluorescence curves only on the target organism.

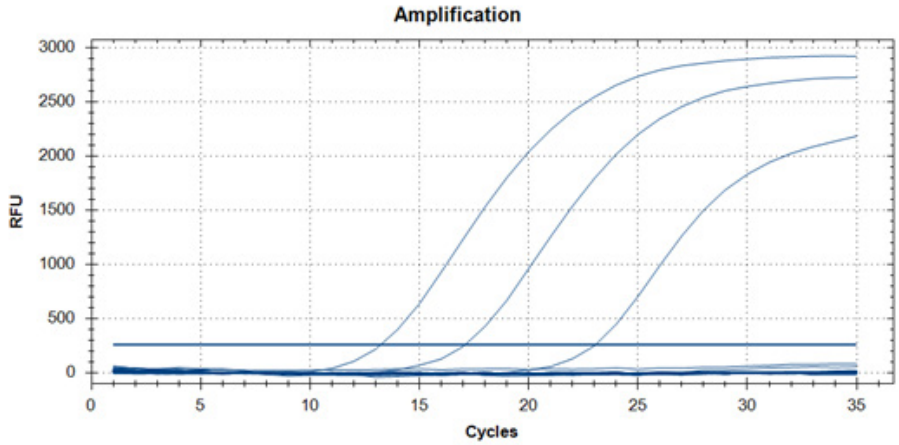

Figure 4 – Testing non-specific annealing of primers for other types of bacteria

Sensitivity testing was carried out on a plasmid containing the *A. marginale groEL* gene. The minimum sensitivity threshold for qPCR was found to be 8 copies per reaction; there was no increase in the fluorescence curves at lower copy numbers. The results are shown in Figure 5.

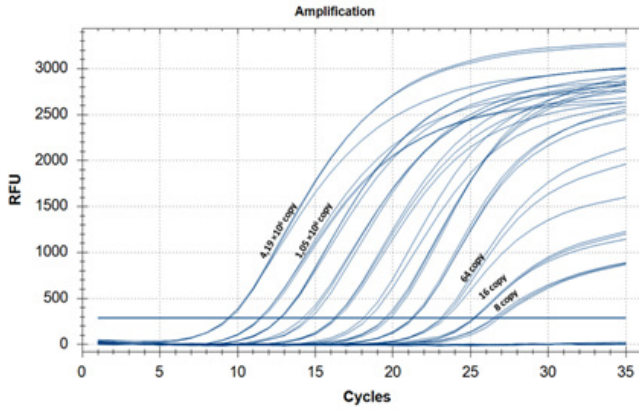

Figure 5 – Amplification curves for sensitivity assessment

Discussion

As a result of this study, a real-time PCR (qPCR) test system was developed for the identification of *A. marginale* with high specificity, allowing differentiation from other species of *Anaplasma spp.* The sensitivity of the method was 8 copies per reaction.

In molecular diagnostics, *Anaplasma spp.* are targeted for species identification. are genes for heat shock protein (*groEL*), disulfide oxidoreductase (*dsd*), citrate synthase (*gltA*) and basic surface proteins (*msp1, msp1a, msp2, msp4, msp5*) [16].

Previously, qPCR was used for the identification of *A. marginale* in the work of G. Carelli et al. where a comparison was made with reverse line blot (RLB) and nested PCR methods. The two-step nPCR method and the faster qPCR method for the multilocus *msp1β* gene showed the same sensitivity of 10 DNA copies and allowed the identification of positive samples not detected by the RLB method [17]. M. Chaisi's results confirmed that the sensitivity of qPCR was superior to RLB, 25 DNA copies versus 2500 copies. They also found that there was variability in the target region of the *msp1β* gene, leading to false negatives when using nPCR [18]. R. Giglioti compared the loop-mediated isothermal amplification (LAMP) they developed with qPCR on a region of the *msp1β* gene. The sensitivity of qPCR was 21 copies per 1 μL, which is 10 times higher than the LAMP [19].

A. Arkhipova et al. used the msp1α gene as a target gene for the development of qPCR; the sensitivity of the method they developed was one copy of the gene, and the specificity was sufficient for differentiation from other *Anaplasma species* [20]. Surface proteins such as *msp1β* and *msp1α* are often used to create PCR test systems, but the disadvantage of these tests is the rapid variability of surface proteins, which requires careful selection of primers for a specific geographic region [21].

In the studies by S. Kovalchuk et al, a qPCR based on the single locus *msp4* gene was developed for the identification of A. marginale, which allows differentiation of *A. marginale* from *A. ovis* with a sensitivity of 10^2 DNA copies [22]. Although the difference in identity of the msp4 gene is sufficient to differentiate A. marginale from A. centrale at 83%, the lack of data on the testing of this assay for *A. centrale* may lead to false positive results [23].

In studies by G. Picoloto et al. qPCR and standard PCR targeting the *msp5* gene showed earlier detection of *A. marginale* in calves, while smear analysis confirmed the presence of infection only 5 and 26 days after tick exposure. This study also showed better sensitivity of qPCR, identifying 7 positive reactions from 43 deer samples versus 1 positive reaction by standard PCR [24]. Similar results were obtained by G. Bacanelli, who detected 83.3% of infected animals on day 7 using qPCR for the msp5 gene and 16.7% using standard PCR [25]. The sensitivity of qPCR was also shown to be better than the indirect enzyme immunoassay indirect ELISA in a comparative analysis by A. Ali Turi et al. where qPCR detected 34.8% of infected animals versus 28.7% detected by iELISA [26].

Real-time PCR for the identification of *Anaplasma spp.* has not been used in Kazakhstan before, and its advantage is the speed of analysis, the elimination of additional detection steps, and the ability to determine the carrier status of an infected animal. qPCR requires less labour and greatly simplifies the process compared to traditional methods for identifying *Anaplasma marginale*, and simultaneous detection during thermal cycling significantly speeds up the process compared to standard PCR. The qPCR test system we developed, based on the *groEL* gene, showed high sensitivity and specificity, not inferior to similar developments, and may in the future replace the immunological analysis methods used today.

Conclusion

A highly sensitive and specific real-time PCR test system for species identification of *A. marginale* has been developed. Highly specific primers and a probe for the *groEL* gene were developed to differentiate *A. marginale* from the closely related species *A. centrale* and *A. ovis*. The PCR protocol was optimized and the optimal primer annealing temperature was established. The sensitivity of the test system was 8 copies per reaction. This PCR test system can be used for effective monitoring in epizootically disadvantaged regions of Kazakhstan; identification of carriers will ensure control of the spread of *A. marginale* and timely implementation of preventive measures.

Authors' Contributions

Conceptualization, K.M. and A.Sh.; methodology K.M. and A.Sh.; validation, A.O., A.K. and A.D.; formal analysis, K.M., A.O. and N.T.; investigation, K.M.; resources, K.M.; data curation, A.Sh. and K.M.; writing–original draft preparation, A.O., M.K., N.T., A.D., A.K., A.Sh., M.F., E.Sh. and M.K.; writing–review and editing, K.M and A.Sh.; visualization, A.O. and M.K.; project administration, K.M.; funding acquisition, K.M. All authors have read and agreed to the published version of the manuscript.

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The instrumental method of pregnancy and infertility diagnosis in cattle: Age-wise efficiency

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Abstract

Background and Aim. This article shows the results of the effectiveness of an instrumental method for diagnosing pregnancy and infertility of cows, taking into account the age of the animals. Due to changes in the body of animals of different ages, it is necessary to pay attention to the specifics of the work on the diagnosis of pregnancy and infertility.

Materials and Methods. Research work was carried out in 3 farms. The determination of pregnancy in animals is carried out using clinical and biophysical studies. The device "Pregnancy diagnosis" was used for the instrumental diagnostic method.

Results. The effectiveness of an instrumental diagnostic method has been revealed, which allows to diagnose pregnancy and infertility of cows with high accuracy in the conditions of livestock farms in veterinary medicine, paying attention to the results in cows of different ages. The effectiveness of the instrumental method in the diagnosis of cows under the age of 2 years, 3-5 years of pregnancy is 83%, infertility is 84.6%; and in cows older than 5 years of pregnancy is 79.6%, infertility is 63.6%. The determination of pregnancy of cows showed an error of 7%, and the determination of infertility-3.3%.

Conclusion. There are many methods for early detection of pregnancy in cows, but the most accurate and common are ultrasound and rectal palpation. These methods are safe and effective for diagnosing pregnancy in cattle. Although ultrasound may be more expensive, in this regard, our research shows the results of an instrumental method for diagnosing pregnancy.

Key words: age; cow; infertility; instrumental method; pregnancy diagnosis.

Introduction

Age-related variation in the animal organism is one of the main issues in cattle breeding. In addition, physiological criteria can be considered as one of the most important criterion for this variation, which varies not only during sexual development, but throughout the entire reproductive period of the animal. The value of the animal's age during the birth period is distinguished by the animal's body's resistance to external factors and diseases, that is, this indicator has an impact on the quality and maturity of the young cattle in utero.

Pregnancy detection in cattle is of great practical and economic importance and is one of the most important veterinary and zootechnical measures. This allows controlling the fertilization of animals [1].

The age of the herd may vary from year to year, proper herd management must include an understanding of the impact of age on birth rates, calving intervals, and birth weight [2].

The age of the female during pregnancy has an important influence on the nutritional environment for the growth of the embryo and fetus. The potential influence of the age of the heifer and cow on the

initial period of gestation, fetal growth and development, as well as on the period after calving has been determined [3].

Currently, there is very little data on the influence of the age of an animal on pregnancy and on the young cattle, the specifics of diagnosing pregnancy and infertility in connection with variations in the body of animals of different ages.

In this regard, the effectiveness of the instrumental method for diagnosing pregnancy and infertility of cows, taking into account the age of the animals, was studied.

Materials and methods

The conducted scientific research was approved by the ethical commission of the university "Minutes of the meeting of the local ethical commission on biological and medical ethics for research, the object of which is an animal S. Seifullin Kazakh Agrotechnical University No. 3 dated 03.11. 2022.

Research work was carried out by S. Seifullin Kazakh Agrotechnical University in the Akmola region, Production cooperative "Izhevskoye", "Zhaksylyk-Agro" LLP, "Salut" North Kazakhstan Oblast LLP.

Research materials: obstetric equipment, ultrasound scanner "EMP V-9" (China), ultrasound scanner "Easi-Scan" (UK), device for detecting pregnancy and infertility "Pregnancy Diagnosis", animal registration log, cows of different age, disposable rubber gloves, gown, rubber boots, Vaseline, etc. [4].

Determining the impact of cow age on instrumental diagnostic studies of pregnancy and infertility is directly related to age-related variations in the genital organs that occur in the animal's body.

Pregnancy detection in animals is carried out using clinical and biophysical studies. Clinical methods include both external (inspection, palpation, auscultation) and internal examinations (rectal).

External examination - the cow is checked outdoors or in a well-lit room. During the examination the protrusion of the right abdominal wall from the second half of the uterus and filling of the right hungry fossa are revealed. By the end of the seventh month of gestation period, the lower part of the right abdominal wall goes down, swelling of the abdominal wall and limbs, breast enlargement, and swelling of the vulva are observed [5].

The presence of the fetus and its movement can determined by palpation. To palpate the fetus, the examiner stands to the right of the cow, placing his right hand on the abdominal wall in the lower leg area. Palm pressure pushes the abdominal wall inward and then quickly releases the pressure. When the fetus is present, a push from the solid body is felt. This method is used no earlier than 5 months of pregnancy. Absence of hard body sensation does not exclude pregnancy [6].

Auscultation is mainly carried out using a phonendoscope; on the right, in the hungry lateral fossa, you can hear the fetal heartbeat, which occurs twice as often as in an adult animal (120-130 beats per 1 min) [7].

The method of rectal diagnostics determines pregnancy, its timing, identifies pathologies of the genital organs, and the causes of infertility [8].

Using ultrasound diagnostics, an embryo can be seen within a month after successful fertilization, and gynecological diseases can also be identified.

The method of instrumental diagnostics using the device "Pregnancy Diagnosis" determines the anatomical and topographical changes in the genital organs of animals during pregnancy [9].

The device for diagnosing pregnancy and infertility of cows consists of three zones of red, green and yellow, which are graduated with a division scale to measure the position of the uterus in the pelvic cavity relative to the external genitalia. At the end of the device body there is a receiver with the help of which mucus is extracted from the genital tract. The head of the device has a handle that helps secure the device and is easy to grip. When working with animals, the handle protrudes from the top of the rod by 1-1.5 cm, so as not to contaminate your hands with mucus [10].

The technological scheme for using the "Pregnancy diagnosis" device for diagnosing infertility and infertility in cows includes the selection of animals for diagnostics of infertility 50-90 days after fertilization of animals. After registration and anamnesis of the animal, the genitals are sanitized, the device is inserted into the vagina, vaginal mucus enters a rubber receiver, the position of the uterus in

the pelvic cavity relative to the external genital organs is determined in accordance with the degree of immersion of the rod with a three-color graduated discharge scale, attention is paid to the consistency, color, smell of mucus.

The method of using the "Pregnancy diagnosis" device for diagnosing pregnancy and infertility is explained by the following example (Table 1).

Table 1 – Parameters for diagnosing pregnancy and infertility in cows 50–90 days after fertilization

Indicators	Infertile	Doubtable	Calf-bearing
Anatomical and topographic location of the uterus relative to the external genitalia	Up to 27 cm	28-30	31 and more
Muscus type	liquid, transparent	viscous, sometimes liquid	sticky, viscous,
Mucosal color	pink	pink	pale

In infertile animals, the uterus is located in the pelvic cavity, the immersion depth of the device for diagnosing pregnancy and infertility of a cow is up to 26 cm. After 50-90 days of pregnancy, the penetration depth of the device will be from 31 to 41.5 cm or more. The uterus and ovaries enter the abdominal cavity, as a result of which the distance to the external genitalia increases by 14.9-16.5 cm compared to the topography of the genitals in infertile animals.

In infertile cows, the cervix, the uterus itself, is located in the pelvic cavity at a distance of 27 cm from the external genitalia, the mucous membrane of the genital tract is pink and moist.

If on the 50-90th day after insemination the immersion depth of the device for diagnosing pregnancy and infertility of a cow exceeds 30 cm (green), the cow is considered pregnant. In case of infertility, accordingly, the immersion depth of the device is less than 26 cm (red color) [11, 12].

Results

Based on data from the artificial insemination log, insemination dates and gestational ages were determined. Depending on the timing of insemination, animals can be divided into the following groups: Group I (28-60 days) – 66 cows, Group II (61-120 days) – 101 cows, Group III (121-150 days) – 33 cows, Group IV (151-180 days) - 42 cows, group V (181 days) - 59 cows.

The results of diagnosing pregnancy and infertility of cows in three farms are shown in Table 2:

Table 2 – Results of diagnostics of pregnancy and infertility using instrumental method

Gestational age	Cattle number	Instrumental method						Ultrasound				Error	
		Calf-bearing		Doubtable		Infertile		Calf-bearing		Calf-bearing		Calf-bearing	Infertile
		n	%	n	%	n	%	n	%	n	%		
28-60	66	23	34.8	11	16.7	32	48.5	29	43.9	37	56	9.1%	7.5%
61-120	101	63	62.4	25	24.8	13	12.9	86	85.1	15	14.9	22.7%	2%
121-150	33	26	78.8	4	12.1	3	9.1	29	87.9	4	12.1	9.1%	3%
151-180	42	35	83.3	6	14.3	1	2.4	40	95.2	2	4.8	11.9%	2.4%
181-285	59	49	83	5	8.5	5	8.5	51	86.4	8	13.6	3.4%	5.1%

It has been established that diagnosing pregnancy and infertility of cows using the instrumental method "Pregnancy Diagnosis" makes it possible to identify on average 85.9% of pregnant and 80.3% of infertile cows. Diagnostic error ranged from 3.4% to 22.7%.

The results of the effectiveness indicator of the instrumental method "Pregnancy Diagnosis" in diagnosing pregnancy and infertility according to Table 2 are presented in the diagram below (Fig. 1):

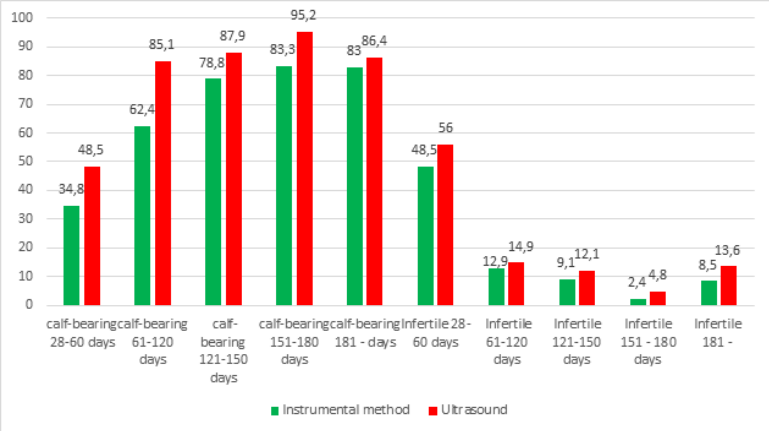

Figure 1– The effeciency of the instrumental method “Pregnancy Diagnosis”

The high efficiency rate of instrumental method of diagnosis “Pregnancy Diagnosis” was 28-60 and 121-150 days after insemination. The diagnostic error was more on days 61-120 after insemination

Studies on the effect of animal age on the reliability of diagnosis were conducted on heifers inseminated under the age of 2 years (n=110), cows aged 3 to 5 years (n=126) and cows over the age of 5 years (n=65). The diagnostic results are presented in Table 3.

Table 3 – The influence of animal age on the effectiveness of determining pregnancy and infertility of cows using the instrumental method

Animal age	Number	Instrumental method						Rectal method				Error	
		Calf-bearing		Doubtable		Infertile		Calf-bearing		Infertile		Calf-bearing	Infertile
		n	%	n	%	n	%	n	%	n	%		
2	110	70	636	14	12.7	26	23.6	81	73.6	29	26.4	10	2.8
3-5	126	83	65.9	21	16.7	22	17.5	100	79.4	26	20.6	13,5	3.1
Above 5	65	43	66.1	15	23.1	7	10.8	54	83.1	11	169	17	6.1

The influence of the animal’s age on the efficiency of diagnosing pregnancy and infertility was revealed; at the age of 2 years, the diagnostic error for pregnancy for the instrumental method is 10%; with age, the error for diagnosing pregnancy increases by 3.5%, and for diagnosing infertility by 0.3-3%.

The efficiency of the instrumental method in diagnosing pregnancy in cows under 2 years of age was, respectively, 86.4%, infertility 89.7%; pregnancy between the ages of 3 and 5 years 83%, infertility 84.6%; and in cows over 5 years of age, pregnancy is 79.6%, infertility is 63.6% (Fig. 2).

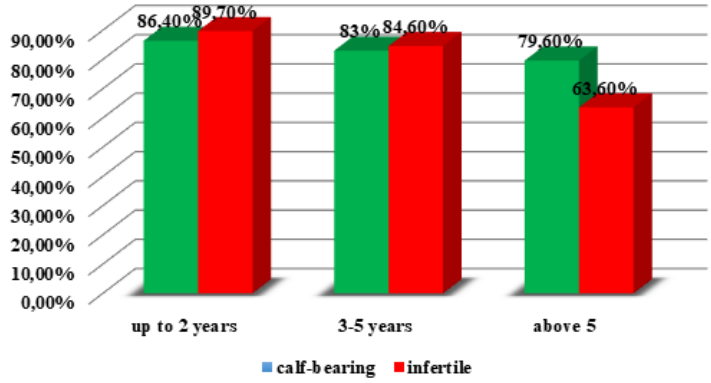

Figure 2 – Efficiency of determining pregnancy and infertility of cows depending on age

Discussion

Pregnancy diagnosis in cows is the decisive point for reducing the calving interval and increasing their reproductive efficiency [12, 13].

A perfect pregnancy test should provide accurate results in early stage of pregnancy (high sensitivity and specificity), be inexpensive and user-friendly [13].

There are many methods for the early diagnosis of pregnancy in cows, but basic and highly accurate detection is often made using ultrasound and rectal palpation. Transrectal ultrasound and rectal palpation are accurate and safe methods for assessing pregnancy in cattle. Although ultrasound may be more expensive, it provides the ability to detect pregnancy early in the breeding cycle, helping to control operating costs [14].

Studies by Hameed O. A. reported the results of using the BioPRYN Visual Pregnancy Test, which had a sensitivity of 99% compared to the BioPRYN ELISA Pregnancy Test Kit. In their work, the authors recommend to use the Bio-RPD visual test as a rapid and accurate method for diagnosing pregnancy on farms where laboratory equipment for enzyme immunoassay or transrectal ultrasound is not available [15].

Rahla Meziane et al [16] conducted a comparative analysis of two methods for diagnosing pregnancy in cows after artificial insemination: analysis of proteins associated with pregnancy (PAG) and ultrasound. The study found that the sensitivity and specificity of the PAG assay were 100% and 93.75%, respectively, and the specificity of ultrasound was 100%. A comparative analysis of these two methods for diagnosing pregnancy confirmed the reliability and earlier availability of biochemical methods.

In our studies we conducted a comparative assessment of the effectiveness of the instrumental method for detecting pregnancy and infertility in cows depending on their age. The results showed that in cows under the age of 2 years, the diagnostic efficiency was 86.4% pregnancy and 89.7% infertility, while in cows aged 3 to 5 years these figures were 83% and 84.6%, respectively. In cows over 5 years the diagnostic efficiency was 79.6% for pregnancy and 63.6% for infertility. The error in pregnancy diagnosis was 7%, and for infertility - 3.3%. It is recommended to use the Pregnancy Diagnosis device to diagnose pregnancy and infertility in cows no earlier than 30 days after insemination. The efficiency of the experimental instrumental method “Pregnancy diagnosis” was 34.8-43.9% for 28-60 days after insemination, 62.4-85% for 61-120 days, and 78.8 – 88% for 121-150 days, 83.3-95.2% for 151-180 days respectively. It has been established that with the help of the instrumental method 85.9% of pregnant and 80.3% of infertile cows are diagnosed. The error in determining pregnancy was 3.4-22.7%, infertility - 2-7.5%.

Conclusion

The efficiency of the instrumental method in detecting pregnancy and infertility of cows, depending on age, was 86.4% pregnancy, 89.7% infertility when diagnosing cows under 2 years of age, 3-5 years of pregnancy 83%, 84.6% infertility; and in cows over 5 years of age, pregnancy is 79.6%, infertility is 63.6%. Determination of cow pregnancy showed an error of 7%, and determination of infertility - 3.3%.

The use of the “Pregnancy Diagnosis” device in diagnosing pregnancy and infertility of cows is recommended to be determined 30 days after fertilization.

Authors' Contributions

IT and EE: Conducted animal research, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. IT, EE and ZhZ: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript”.

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The main biological features and resistance to antibacterial drugs of *Streptococcus agalactiae* isolated from the cow's milk

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Abstract

Background and Aim. The main goal of our research, as stated in the article, was to study the phenotypic characteristics of strains of *S. agalactiae* isolated from milk, identify and evaluate the effectiveness of the main groups of antibiotics recommended for use in veterinary medicine, including those provided by EUCAST.

Materials and Methods. The study of the phenotypic characteristics of *S. agalactiae* and the research on antibiotic resistance were carried out at the base of the Microbiology Laboratory of the Biotechnology Research Institute of Kostanay State University named after A. Baitursynov in 2023, using mastitis milk samples brought to the microbiology laboratory.

The biological characteristics of streptococci in the milk samples were identified using classical microbiological methods. The antibiotic susceptibility testing was performed using the disk-diffusion method in Mueller-Hinton agar.

Results. The results of the study indicate that out of 631 milk samples from animals in the region's dairy farms, 35 of them were found to be *S. agalactiae* isolates.

The text you provided seems to report the results of a study that investigated 631 samples of raw milk for mastitis, identifying 35 *S. agalactiae isolates.* It details the antibiotic susceptibility of these isolates, indicating a high susceptibility to benzylpenicillin (90.4%), amoxicillin (80.9%), kanamycin (76.1%), and moderate susceptibility to ampicillin and neomycin. However, the isolates demonstrated lower susceptibility to doxycycline (47.6%), tylosin (42.8%), gentamicin, and erythromycin (33.3%).

Conclusion. Thus, various mechanisms of antibiotic resistance and ways of their acquisition by bacteria significantly complicate the process of selecting effective antibiotic therapy both in agro-industrial organizations and in medical institutions. The mechanisms of acquired and natural antibiotic resistance are inherently complex and vary from species to species, from strain to strain of microorganisms. Basically, intraspecific and interspecific acquisition of antibiotic resistance genes is carried out through horizontal transfer - conjugation, transformation, and transduction.

Key words: antibiotic; microbiology; sensitive; strain; streptococcus; resistance.

Introduction

When cows are sick with mastitis, streptococci, staphylococci, enterococci are most often isolated. Of etiological importance are contagious, highly pathogenic *Str. agalactiae* and *S. aureus*, which cause severe mastitis and provoke chronic inflammation with a high index of somatic cells, which makes milk unusable [1, 2].

Due to the concerns of the potential impact of *S. agalactiae* on the safety of milk and the issues of antibiotic use in animals, many researchers are worried about the adverse effects on public health. Uncontrolled use of antibiotics against pathogenic microorganisms can lead to various forms of food infections, which are very difficult to treat [3].

Many researchers note that Staphylococcus aureus and agalactylus streptococcus, which affects the mammary gland of cows, pose a threat to public health due to problems with the safety of dairy products and the use of antibiotics. When infected with antibiotic-resistant forms of pathogenic microorganisms, severe forms of food infections are observed, which are very difficult to treat [4, 5].

Streptococci are one of the widely studied pathogens in clinical microbiology. The morphological structure of streptococci is described in detail, their virulence factors and dominant clinical manifestations of diseases are studied [6].

Among the infectious agents involved in mixed infections of domestic animals, streptococci occupy a special position for certain reasons [7].

Uncontrolled use of antibiotics leads to the accumulation of low sub-inhibitory concentrations in the tissues and intestines of treated animals and in the environment, which contributes to the selection of antibiotic-resistant bacteria and enhances their growth. In addition, the presence of antibiotics can stimulate biofilm formation and horizontal gene transfer in some bacteria.

There are a number of mechanisms that contribute to the development of resistance of a bacterial cell to one or more antimicrobial drugs: a decrease in the accumulation of an antimicrobial drug inside the cell through a decrease in the permeability of the antimicrobial drug wall from the bacterial cell; enzymatic modification or degradation of an antimicrobial agent.

One of the main objectives is to reduce the use of antibiotics in animal husbandry by improving the quality of life and conditions of animals. In this regard, it is recommended to apply good animal husbandry and animal handling practices in livestock enterprises and during animal transportation; improving animal welfare (for example, ensuring an optimal microclimate, high-quality water, appropriate ventilation and distribution of areas) at all stages, including production, transportation and slaughter; the use of locally adapted breeds that are more resistant to diseases and stress, or animals selected for disease resistance (resistant animals will require fewer antimicrobial treatments); compliance with veterinary and sanitary, sanitary and hygienic rules, biosafety measures at enterprises to prevent the use of medicines; compliance with strict measures to combat diseases (for example, vaccinations); the use of feed ingredients/additives that increase the efficiency of feed conversion to exclude the use of antibiotics as growth stimulants; the rejection of feed ingredients with anti-nutritional properties; the use of modern waste disposal methods. In addition, it is necessary to organize and conduct monitoring and supervision of the spread of antibiotic-resistant bacteria, including the assessment and identification of trends and sources of antimicrobial resistance in bacteria; the discovery of new mechanisms of antimicrobial resistance; providing data necessary for the analysis of risks to animal and human health; providing a basis for practical recommendations for the protection of animal and human health; providing information for monitoring antimicrobial prescriptions in agricultural organizations and the reasonable use of recommendations; evaluating and determining the effectiveness of measures to combat antibiotic resistance.

The development of modern microbiology is closely related to the improvement of methods for identifying pathogenic microorganisms. Significant progress has been made in this field, primarily through coordinated research in chemistry, immunology, and genetics. Special methods have been developed that allow detailed study of the genetic apparatus of microorganisms, as well as the identification of specific antibodies and antigens using serological techniques.

As for the novelty of the work done, in order to solve such an urgent problem of modern microbiology, *S. agalactiae* strains were isolated from cow's milk in the Kostanay region. We studied and evaluated the level of resistance of isolates to the main groups of antibiotics. The obtained research results are recommended for use in dairy farms of Kostanay region.

Materials and methods

The study of the phenotypic characteristics of *S. agalactiae* and the research on antibiotic resistance were carried out at the base of the Microbiology Laboratory of the Biotechnology Research Institute of

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The biological characteristics of streptococci in the milk samples were identified using classical microbiological methods. The antibiotic susceptibility testing was performed using the disk-diffusion method in Mueller-Hinton agar.

The susceptibility of the bacteria to antimicrobial agents was tested using the disk-diffusion method. Interpretation was carried out according to the EUCAST guidelines, version 11.0 [8], MIC 4.2.1890–04 MIC. Determination of the susceptibility of microorganisms to antimicrobial agents [9].

The tests were conducted and prepared in accordance with the requirements of the State Standard of the AIS 26809-86 "Milk and Milk Products. General Requirements for Preparation, Sampling, and Trials". Adherence criteria, research methods, and preparation for testing" [10].

Microbiological study of *S. agalactiae.*

Morphology and bacterial characteristics of colonies of the isolated S. agalactiae strains were identified microscopically and through biochemical properties, after proper cultivation.

Selective incubation of milk samples for preliminary streptococcal isolation was performed at a temperature of 35-37 °C for 18-24 hours, promoting the growth of streptococci. Further, the selective streptococcal agar was used, followed by CHROMagar Mastitis GP, CHROMagar Step B S1, and S2 agars for the isolation of different types of streptococci to maximize their growth. In blood agar, the majority of *S. agalactiae* strains form β-hemolytic colonies which are smooth, shiny, and yellow, while α-hemolytic or non-hemolytic colonies manifest differently. On CHROMagar Step B, colonies appeared as greenish.

The differences in the morphology of the colonies of *S. agalactiae* were identified through biochemical methods, comparing them with colonies of other types of streptococci and bacteria.

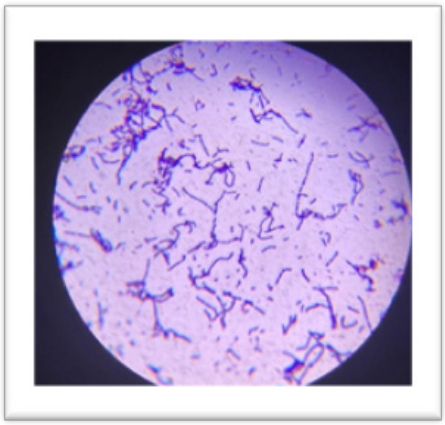
A CAMP test was performed, in which a streak of beta-toxin-producing *S. aureus* was placed at the center of blood agar. Then, perpendicular streaks of the tested beta-hemolytic streptococcal strains were made in such a way that they did not intersect the streaks of the hemolytic staphylococcal strain. The cultures were incubated at 35 ºC in a 5% CO_2 atmosphere. After incubation, the hemolytic area, indicative of the projection of the lysis zone formed by both streptococci and staphylococci, was observed where the erythrocytes had lysed.

Results

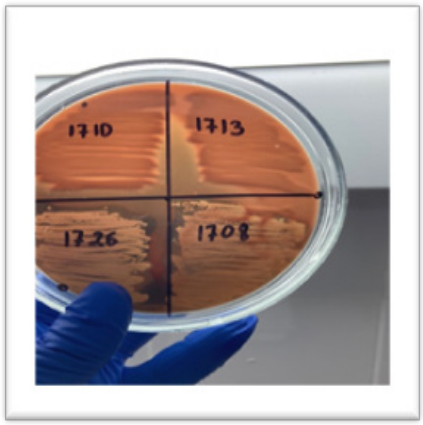
The results of the study indicate that out of 631 milk samples from animals in the region's dairy farms, 35 of them were found to be *S. agalactiae* isolates.

The characteristics of the *S. agalactiae* isolates were examined, showing that they possess the necessary properties for the growth and development of streptococci, including the presence of blood, lactose, and glucose in suitable environments.

Furthermore, the morphology and microscopic examination of the colonies of the *S. agalactiae* isolates led to the identification of their biochemical traits (figure 1).



Colored by Gram method streptococci



Hemolysis of red blood cells in blood agar

Growth of mastitis pathogens CHROMagar Mastitis GP

CHROMagar Step B *S.agalactiae* purple

Figure 1 – Morphology and biochemical properties of growths of *S. agalactiae* strains in nutrient media

S. agalactiae ferments glucose, lactose, sucrose, and maltose, but does not ferment mannitol, inulin, sorbitol, or gelatin. It also shows hyaluronidase activity.

In addition, for the differentiation of *S. agalactiae,* the CAMP test was used, with some strains of *S. aureus* producing beta-toxin, which acts synergistically with a substance (CAMP factor) produced by *S. agalactiae*. According to the literature, not all S. agalactiae strains show a positive result in the CAMP test. In our studies, 29 out of 35 strains showed a positive result in the CAMP test (82.8%).

The EUCAST recommends the use of benzylpenicillin, norfloxacin, vancomycin, tetracycline, erythromycin, and levomycetin to treat *S. agalactiae*. However, due to the wide range of antibiotics used for treating mastitis in goats, we have tested 16 different antibiotics.

The bacterial susceptibility of the 35 divided *S. agalactiae* strains, as well as at least a few to one bacterial agent, has been tested. To assess the susceptibility of bacteria to antibacterial agents, a disc-diffusion method was employed using 5% defibrinated sheep blood Mueller-Hinton agar (figure 2).

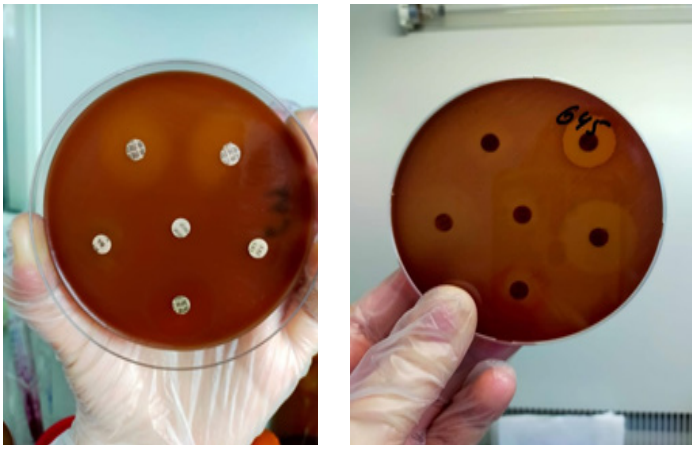


Figure – 2 Disco-diffuse method

The greatest susceptibility was demonstrated towards β-lactam agents (benzylpenicillin, amoxicillins, ampicillins), aminoglycosides (kanamycin, neomycin, gentamicin), tetracyclines (doxycycline), and the macrolide erythromycin within the tetracycline group (table 1).

Table 1– Antibioticogram of *S. agalactiae* strain

Isolates	Name of antibacterial drugs	Number of resistant strains	%
S. agalactiae	ampicillin	13	61,9
S. agalactiae	amoxicillin	17	80,9
S. agalactiae	benzylpenicillin	19	90,4
S. agalactiae	norfloxacin	6	28,5
S. agalactiae	vancomycin	5	23,8
S. agalactiae	streptomycin	9	42,8
S. agalactiae	kanamycin	16	76,1
S. agalactiae	neomycin	13	61,9
S. agalactiae	gentamicin	7	33,3
S. agalactiae	tetracycline	5	23,8
S. agalactiae	doxycycline	10	47,6
S. agalactiae	erythromycin	7	33,3
S. agalactiae	tylosin	9	42,8
S. agalactiae	sulfamethoxazole / trimethoprim	6	28,5
S. agalactiae	ciprofloxacin	5	23,8
S. agalactiae	chloramphenicol	4	19

As you can see from the table, the majority of *S. agalactiae* isolates were susceptible to benzylpenicillin (90.4%), amoxicillin (80.9%), kanamycin (76.1%), ampicillin and neomycin (61.9%), doxycycline (47.6%), tylosin (42.8%), gentamicin, and erythromycin (33.3%). The susceptibility profile of clinical and resistant strains of streptococci is shown in the figure 3.

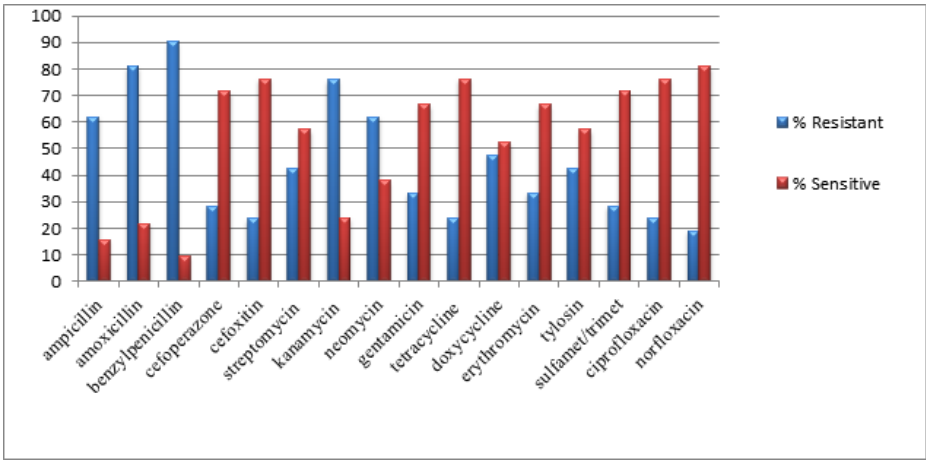


Figure 3 – Susceptibility (%) of sensitive and resistant strains of *S. agalactiae*

Discussion

According to our observations, the drugs of the natural penicillin group had the greatest activity, while resistance to tetracycline and erythromycin was quite high among all the studied groups. Fluorinated quinolones remain an effective treatment for streptococcal infections, however, an increase in the number of insensitive isolates to drugs of this group among *S. agalactiae* has been found [11].

The text you provided seems to report the results of a study that investigated 631 samples of raw milk for mastitis, identifying 35 *S. agalactiae* isolates. It details the antibiotic susceptibility of these isolates, indicating a high susceptibility to benzylpenicillin (90.4%), amoxicillin (80.9%), kanamycin (76.1%), and moderate susceptibility to ampicillin and neomycin. However, the isolates demonstrated lower susceptibility to doxycycline (47.6%), tylosin (42.8%), gentamicin, and erythromycin (33.3%).

The majority of the isolates showed high susceptibility to β-lactam antibiotics (penicillins and cephalosporins), while demonstrating lower susceptibility to aminoglycosides, tetracyclines, and macrolides. The study highlights the varying degrees of susceptibility, ranging from mono-resistance to multidrug resistance, among the isolates.

Conclusion

Thus, various mechanisms of antibiotic resistance and ways of their acquisition by bacteria significantly complicate the process of selecting effective antibiotic therapy both in agro-industrial organizations and in medical institutions. The mechanisms of acquired and natural antibiotic resistance are inherently complex and vary from species to species, from strain to strain of microorganisms. Basically, intraspecific and interspecific acquisition of antibiotic resistance genes is carried out through horizontal transfer - conjugation, transformation, and transduction. The main measures to combat antibiotic resistance include reducing the use of antibiotics by improving the quality of life and conditions of animals; organizing and conducting monitoring and surveillance of the spread of antibiotic-resistant bacteria; developing new antibiotics and test systems for the diagnosis of antibiotic resistance of bacteria.

Authors' Contributions

GKA and MZhA: Concept development, design and planning of the study, data collection and analysis, critical review of the article and final approval, research, statistical analysis. MZhA and SKD: Sampling and delivery of samples and conducting research. All the authors have read, reviewed and approved the final version of the manuscript.

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Using of immunochromatographic analysis to determine antibiotics in milk

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Abstract

Background and Aim. Failure to comply with the rules for the use of antibiotics and/or the tim-ing of milk production from cows subjected to antibiotic therapy leads to the entry of residual amounts of drugs into the human body with dairy products, causing various pathologies. For practice, simple but sufficiently sensitive and specific rapid tests are needed to determine the safety of milk. Such tests can be developed based on immunochromatographic analysis (ICA).

Materials and Methods. Colloidal gold (CG) particles were prepared according to the method of Wang et.al. (2014) and examined using transmission electron microscopy and UV-visible spectroscopy. CG particles were used to label antibodies against streptomycin (STR), tetracycline (TC) and/or chloramphenicol (CAP) according to the method of Yakovleva et al. (2012). The ICA test was developed based on the principle of competition of antibiotics in milk with analogues immobilized on a nitrocellulose membrane.

Results. The multiplex ICA test showed sensitivity at the level of the best world analogues, detecting STR, TC and CAP in milk in concentrations equal to or exceeding the maximum residue limits (MRL) for these antibiotics: 200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively. The advantage of the ICA test is its affordability, since the proposed technologies for the production of antibiotic-specific anti-bodies and immunoassay test line reagents significantly reduce the cost of domestic products, making them more accessible to the country's consumers.

Conclusion. A domestic ICA test system has been designed for simultaneous rapid analysis of milk for the content of streptomycin, tetracycline and chloramphenicol.

Keywords: antibiotic; immunochromatographic test; milk; streptomycin; tetracycline; chloramphenicol.

Introduction

Milk is rich in nutrients such as high-quality proteins, fats, lactose, phospholipids, vitamins, enzymes, minerals, which especially plays an important role in the nutritious nutrition of human [1]. All over the world, including in the Republic of Kazakhstan (RK), the demand for milk is growing every year. According to the National Bureau of Statistics of the Republic of Kazakhstan, the gross production of commercial raw milk in the country in 2022 amounted to 3 million 975 thousand tons [2]. Various veterinary drugs, including antibiotics, are used to prevent diseases and treat dairy cows [3]. However, if the rules for the use of antibiotics and the holding period for obtaining milk from cows subjected to injection of antibiotics are not followed, the remains of medicinal substances can enter the human body through milk and dairy products [4-6]. Residual amounts of antibiotics can cause allergic reactions,

dysbacteriosis, nephropathy, carcinogenic and mutagenic effects, and also cause the development of antibiotic resistance - one of the modern global medical problems [7]. In May 2015, the 68th World Health Assembly adopted the Global Action Plan on Antimicrobial Resistance, which reflects the global consensus that antimicrobial resistance poses a serious threat to human health. The World Health Organization (WHO) has established MRL of antibiotics to control drug residues in products of animal origin [8]. The gold standard for monitoring antibiotic residues in meat and milk is the microbiological method. It is simple to perform, but the result of the study is achieved no earlier than 3-4 hours. Modern methods, such as real-time polymerase chain reaction, enzyme-linked immunosorbent assay, gas chromatography and/or high-performance liquid chromatography methods require expensive equipment and trained personnel and cannot be used for rapid analysis of milk for antibiotic content in food safety laboratories at dairy processing plants or food markets [9]. Thus, practice requires simple to perform, but sufficiently sensitive and specific tests that allow one to determine the presence or absence of antibiotics in animal products in a few minutes [10]. Such tests can be developed using the principles of ICA [11].

The goal of our work was to design a domestic test system based on ICA for simultaneous rapid analysis of milk for the content of three antibiotics: streptomycin, tetracycline and chloramphenicol.

Materials and methods

Reagents. The following antibiotics were used in the work: streptomycin sulfate (Sintez, Kurgan, Russia), oxytetracycline hydrochloride (BioPharmGarant, Vladimir, Russia), chloramphenicol (Panreac, Barcelona, Spain). Conjugates of antibiotics with protein carriers, such as ovalbumin and BSA (Jackson ImmunoResearch Inc, Pennsylvania, USA), as well as rabbit polyclonal antibodies (pAb) were obtained by us previously [12].

To construct an ICA test to determine the residual amount of the above antibiotics and test its sensitivity, goat anti-rabbit IgG (Jackson ImmunoResearch Inc., Pennsylvania, USA), tetrachloroauric acid (HAuCl4) (Fluka, Basel, Switzerland), nitrocellulose membrane CNPC 15 MDI Easypack kits (Advanced Microdevices; Ambala Cantonment, India), and a commercial ICA test kit (Beijing Meizheng Bio-Tech Co., Beijing, China).

Synthesis and characterization of colloidal gold. CG particles with a diameter of 20 nm were prepared according to the method described by Wang et.al. [13]. Briefly, an aqueous solution of chloroauric acid (100 mL of 0.01% (w/v) AuCl3•HCl•4H2O) was heated to boiling point, followed by the addition of 2 mL of 1.0% (w/v) sodium citrate solution. The reaction solution was simultaneously stirred and gently boiled for 5 minutes until the color of the solution changed from straw yellow to red. The resulting CG solution was stored at 4 °C for several months and used for conjugation with the puri-fied antibody. CG particles were examined using transmission electron microscope (TEM) (Jeol, Tokyo, Japan) and UV-visible spectroscopy (Biochrom, Cambridge, UK) in the wavelength range 400–800 nm.

Preparation of a conjugate of CG nanoparticles with antibodies. To 10 ml of solution CG with pH 7.0-7.5 was added dropwise with stirring to 1 ml of pAb solution with a concentration of 20 μg/ml, incubated with constant stirring for 30 minutes at room temperature. Then BSA was added to the re-sulting solution to a final concentration of 0.1%, sucrose to a final concentration of 10%, as well as 0.01% sodium azide. To remove unbound antibodies, the conjugate was centrifuged (30 min, 11000g, 4 °C). The supernatant was removed, and the sediment was redissolved in the required volume of phosphate-buffered saline (PBS) containing 0.1% BSA, 10% sucrose, and 0.01% sodium azide [14]. The mixture was stored at +4 °C until use. The resulting solution was applied to a pad of fiberglass membrane (manufacturer's name, city, country) and dried at room temperature for 8 hours.

Construction of an immunochromatographic composite. A solution of PBS with sucrose and BSA was used as a working buffer for the sorption of conjugates onto a nitrocellulose membrane. To form test and control zones on the membrane using an automatic dispenser Easy Printer Model LMP-0.2 (Advanced sensor systems private limited, Ambala cantt, India) a solution of antibiotic conjugates with OVA and a solution of anti-species antibodies were applied. The following concentrations of rea-gents were used: antibiotic conjugated with OVA – 0.5 mg/ml, anti-species antibodies labeled with CG – 0.250 mg/ml. The membrane with the applied reagents was dried at room temperature for 8 hours un-til completely dry or at 37 °C for 2 hours. The finished composites on the membrane were cut into strips using special equipment “SS - Programmable Strip Cutter” (Advanced sensor systems private limited,

Ambala cantt, India) for cutting. The strips were stored at room temperature in hermetically sealed containers.

Sample preparation of milk. Antibiotic-free milk samples were collected from a black-and-white cow that was healthy and not injected with any antibiotics. Milk samples were centrifuged for 10 minutes at 5000 rpm. The fat was then separated from the skim milk [15] and added a certain amount of antibiotics. Mix the sample thoroughly before testing, transfer 200 µl of the sample to the well of polystyrene plate for ELISA (Medpolimer, St. Petersburg, Russia) for further research.

Testing milk for antibiotic content. The ICA test strip sample pad was immersed in a well containing 200 µl of milk sample for 3 minutes. After 10 minutes, the test strip was removed from the well, placed on a dry surface, and the results of milk analysis for the presence of STR, TC and CAP were visually recorded. The results were recorded after 10 min. For statistical processing, all measurements were performed three times and in triplicate.

Statistical processing of results. Statistical analysis of test specificity were carried out according to the method described by Akinshina Yu.A. [16].

Results

CG particles were obtained by reducing chlorauric acid with sodium citrate. In order to obtain CG particles with a diameter of about 20 nm, a certain proportion of reagents were observed. The re-sulting CG had a wine-red color. After cooling the solution, it was poured into vials, the pH was meas-ured, and the CG particles were examined with a spectrophotometer and a transmission electron micro-scope. The concentration of hydrogen ions in the solution was 6.5. Spectrophotometric analysis of CG is a very important characteristic of its properties, which determine its suitability for use in immunochromatographic tests. The results of the study are shown in Figure 1.

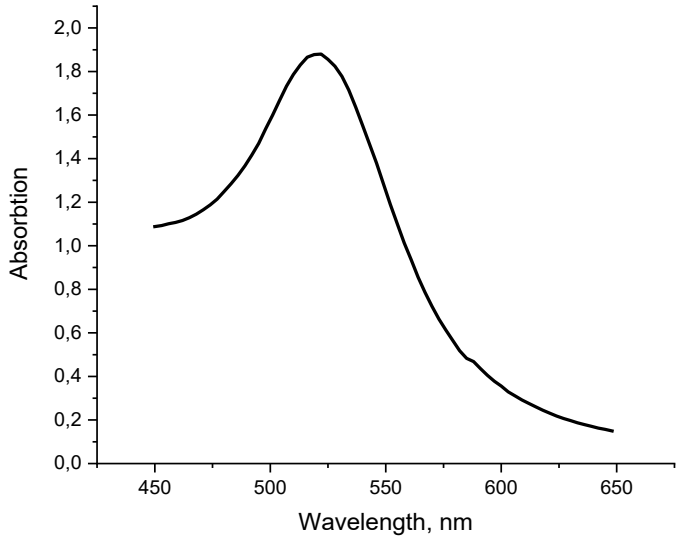

Figure 1 – Spectrophotometry of CG particles with a diameter of 20 nm

As can be seen from Figure 1, spectrophotometric analysis of CG particles showed the presence of one peak in the ultraviolet spectrum at OD520 with optical density = 1.8 (Figure 1).

The TEM image indicates that the CG particle is well dispersed (Figure 2)

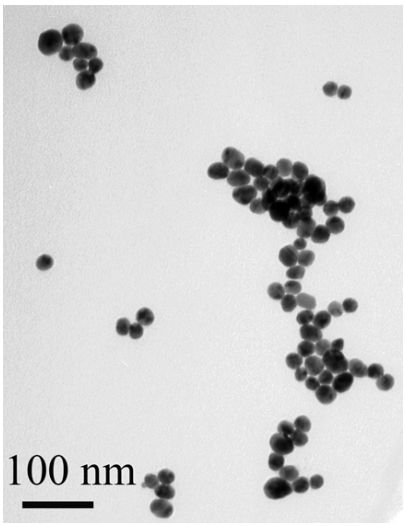

Figure 2 – Fragments of micrographs of CG particles

From Figure 2 it follows that the CG particles were almost the same diameter in the range 14 - 26 nm, and the average short circuit diameter was 19.47±2.5nm, which provided a good basis for using it as a label for pAb specific to the antibiotics used.

To simultaneously test milk samples for the presence of TC, STR and CAP, a multiplex competitive ICA test was prepared, the principle of which is shown in Figure 3.

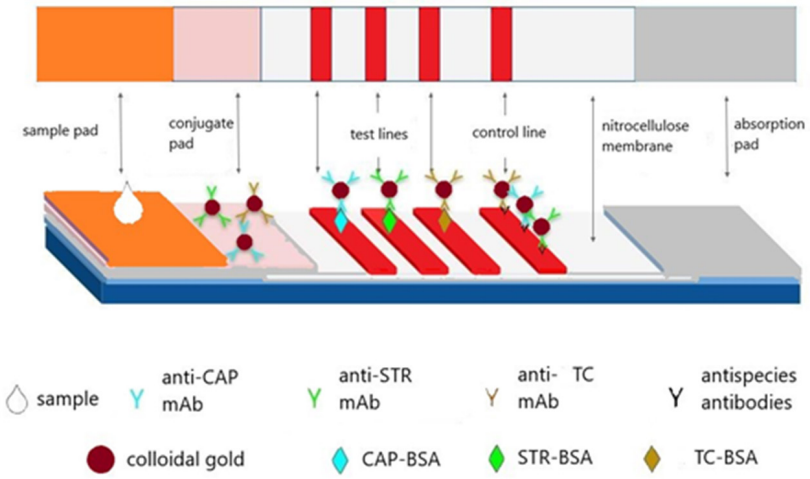

Figure 3 – Principle of competitive immunochromatographic test for determining TC, STR and CAP

The principle of the test is based on the competition of the analyte of the test sample and the antibiotic immobilized on the membrane with a carrier protein for binding to the antigen-binding site of pAb labeled with CG. Staining the control line confirms that there is a sufficient volume of the introduced sample and the correctness of the research methodology. If the ICA result is negative, i.e. if there is no antibiotic in the milk and/or its concentration does not exceed the MRL for STR, TC and CAP (200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively) [17,18], the control line and the test line corresponding to the antibiotic (TC, STR and/or CAP) are colored red. A positive reaction of the test system is characterized by the absence of staining of the test line of the corresponding antibiotic and the absence of staining of the control line. The manifestation of only the control line indicates that the content of all three antibiotics in the tested milk exceeds the MRL.

The specificity of the developed ICA test was determined on milk samples containing various concentrations of antibiotics STR, TC, and CAP (Figure 4).

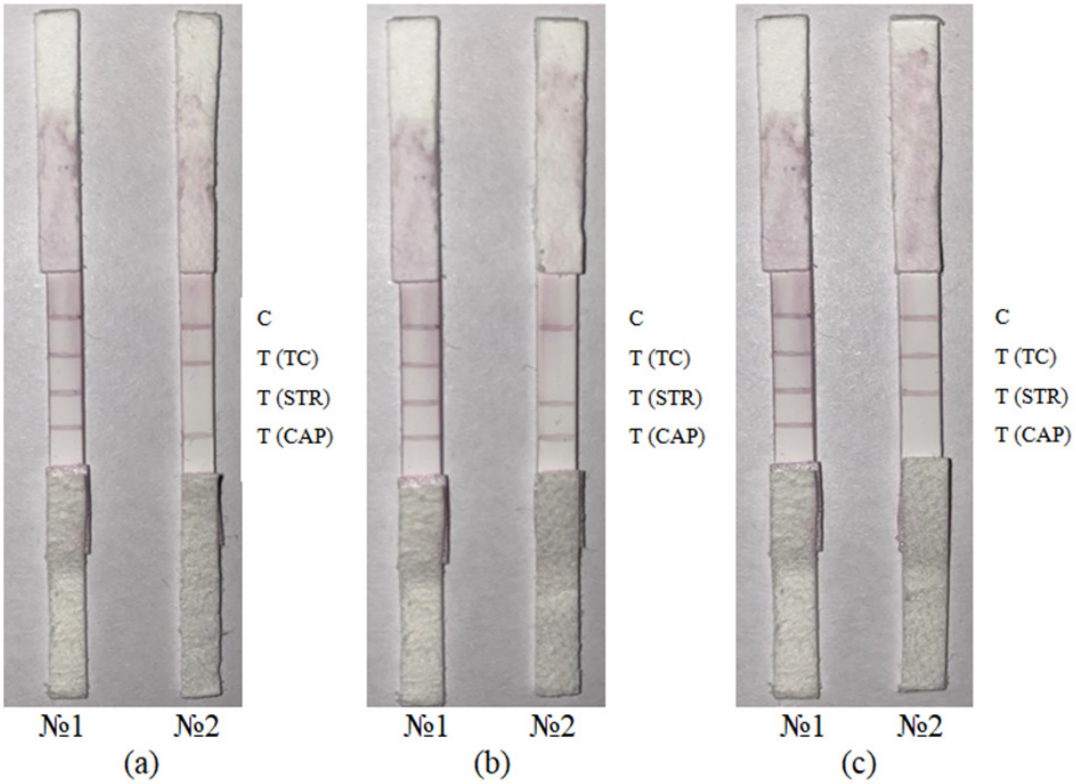

Figure 4 – Sensitivity of the developed ICA test
C- control line, T – test line; a, b, c, - №1: milk samples without antibiotics; a - №2: TC - 0 ng/ml, STR - 200 ng/ml, CAP -0 ng/ml; b - №2: TC - 10 ng/ml, STR - 0 ng/ml, CAP -0 ng/ml; c - №2: TC - 0 ng/ml, STR - 0 ng/ml, CAP - 0.3 ng/ml

From Figure 4 it can be seen that milk samples containing STR, TC and CAP within the MRL (200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively) give a positive reaction to the presence of the corresponding antibiotics. It should be noted that each of the prepared ICA test strips detected the presence of one antibiotic at a concentration equal to the MRL, but did not give positive results when examining milk samples containing other antibiotics.

The diagnostic value of the developed competitive ICA test was determined in comparison with a commercial analogue - the Pioneer Meizheng Bio-tech (China) express test kit, designed to determine residual amounts of four antibiotics, including STR, TC and CAP, in milk. For each analysis, milk samples containing one specific antibiotic at a specific concentration were used. The first sample was supplemented with the antibiotic TC at a concentration of 10 ng/ml, the second sample was supplemented with STR at a concentration of 200 ng/ml, and the third sample was supplemented with CAP at a concentration of 0.3 ng/ml. A commercial test kit was used according to the manufacturer's instructions provided.

The results of comparative studies of milk samples for antibiotics using a home ICA test and its foreign analogue are shown in Figure 5.

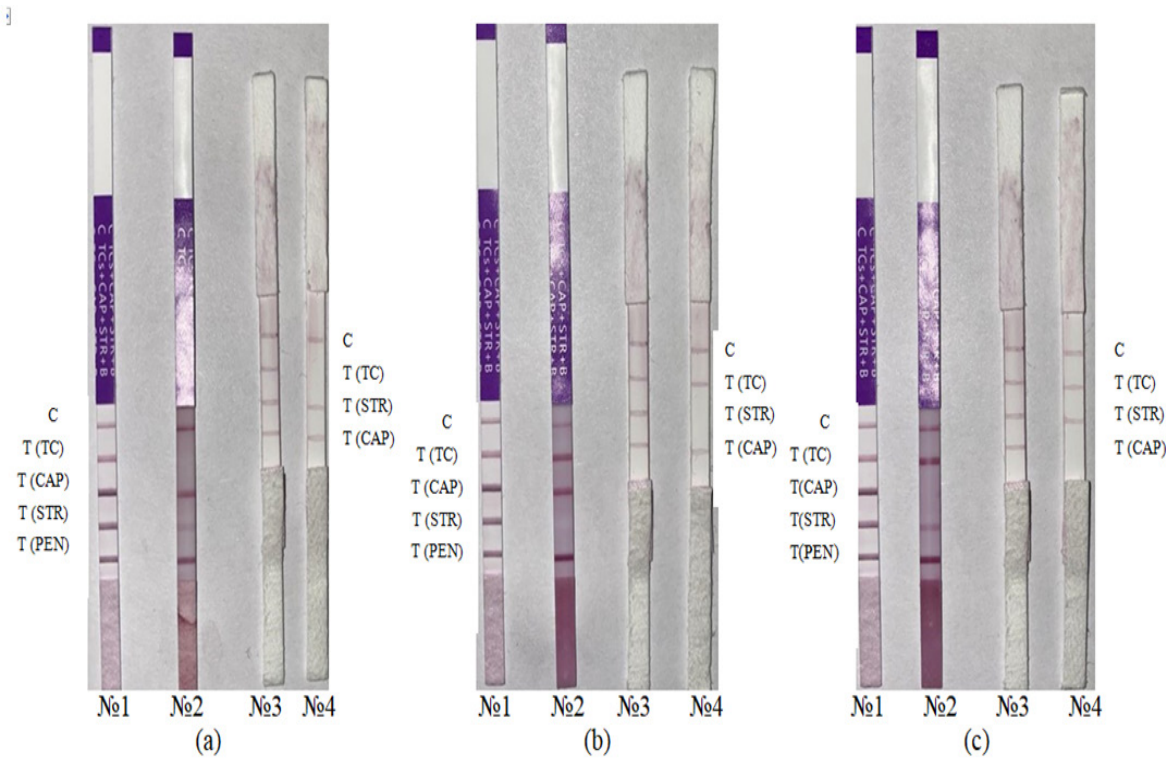

Figure 5 – Images of home ICA test strips and Pioneer Meizheng Bio-tech (China)
C- control line, T – test line; a, b, c, - №1 and № 3: milk samples without antibiotics; a - №2 and № 4: TC - 10 ng/ml, STR - 0 ng/ml, CAP -0 ng/ml; b - №2 and №4: TC - 0 ng/ml, STR - 200 ng/ml, CAP-0 ng/ml; c - № 2 and № 4: TC - 0 ng/ml, STR - 0 ng/ml, CAP -0.3 ng/ml

As the results of the comparative test showed, the absence of antibiotics in milk samples № 1 and № 3 was confirmed in both tests, while in samples № 2 and № 4 with antibiotic concentrations at the MRL, both home and commercial ICA tests gave positive results.

Milk samples from healthy cows (n=46), which were not administered antibiotics and/or other drugs, were examined for the presence of antibiotics STP, CAP, TC using domestic and commercial ICA tests. All samples were negative. The measured specificity was 100%.

Thus, the ICA test we developed in terms of its characteristics in detecting STR, TC and CAP in milk is not inferior to an imported commercial analogue and can be used at milk collection points, at milk processing plants and food markets for express determination of the safety of milk in terms of its content it contains the most widely used antibiotics.

Discussion

To reliably guarantee the quality of milk, it is necessary to control the content of residual amounts of medicinal drugs in it, primarily antibiotics, which are widely used in dairy farming for the prevention and treatment of diseases. Residual amounts of antibiotics in milk that exceed the MRL are not only harmful to human health, but also create a problem in the production of dairy products, inhibiting the growth and development of lactic acid bacteria. For rapid screening of food products for contamination with antibacterial drugs, enzyme-linked immunosorbent assay (ELISA) is becoming increasingly common. This test is recommended by European Union Directive (EC) 2002/657 for the determination of residues of veterinary drugs in animal products in the European Union [19]. However, this test is not used in practice, since the cost of milk analysis is very high. In addition, the equipment of domestic veterinary and sanitary laboratories in food markets leaves much to be desired. In this work, we have developed a more practical and cheaper test that can be used not only in poorly equipped laboratories, but also by the consumer himself. It is based on the use of a competitive ICA variant to detect the three most commonly

used antibiotics in milk - streptomycin, tetracycline and chloramphenicol in concentrations exceeding the MRL. In the post-Soviet space, GOST 32254-2013: Interstate standard "Milk" was developed to determine residual amounts of antibiotics in milk. The standard establishes the requirements for ICA tests for the rapid determination of penicillin, TC, CAP, STR in milk. In addition, there is GOST 32219-2013: Interstate standard "Milk and dairy products", developed taking into account the main regulations of the international standard ISO 18330:2003 "Milk and milk products – Guidelines for the standardized description of immunoassays or receptorassays for the detection of antimicrobial residues", which establishes high-quality immunological methods for the determination of antibiotics using ICA kits from manufacturers from foreign countries. However, imported diagnostics are still not used in food safety laboratories due to their high cost. For example, the average cost of one analysis using the ICA test PROQUITEST (Spain) is 1600 tenge [20]. Therefore, we need domestic express diagnostic kits that will be competitive in the market of veterinary drugs not only in sensitivity and specificity, but also in price offers. The developed domestic ICA test showed the same sensitivity as the imported analog Pioneer Meizheng Bio-tech (China), detecting STR, TC and CAP within the maximum MRL for these antibiotics: 200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively. The advantage of our diagnostic test is its affordability, since the manufacturing technologies we offer for ICA test components, namely pAb against STR, TC and CAP, as well as test line reagents, significantly reduce the cost of the domestic test system, making it more attractive to consumers in the country.

Conclusion

An ICA test has been developed for the simultaneous detection of STR, TC and CAP residues in milk, the content of which is equal to and/or higher than the MRL. The test is easy to use and is characterized by the rapidity of obtaining milk analysis results. The sensitivity of the test at the level of foreign analogues and proven technologies for obtaining pAb and manufacturing the antibiotic + carrier ICA test conjugate serve as the basis for its commercialization with the aim of introducing it into diagnostic practice in food safety laboratories of dairy processing enterprises and food markets. Our further research will be aimed at replacing pAb with monoclonal antibodies, which will standardize the ICA test and overcome the disadvantages inherent in polyclonal antibodies.

Authors' Contributions

ZhA and AJ: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. AB: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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Ixodes ticks of Kostanay region: biodiversity and distribution

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Abstract

Background and Aim. All over the world ixodes ticks are known as carriers and keepers of causative agents of some dangerous diseases in animals and humans. A large role in the research is given to the circulation of ixodes ticks. The fauna of ixodes ticks is a group of parasitic arthropods which has not been researched well enough in the northern region of Kazakhstan. The goal of the research was to identify ixodes ticks within the area of Kostanai region. The objectives were to determine species composition and their geographical distribution in Kostanai region.

Materials and Methods. The research work was carried out on the territory of Kostanay region. Ixodic ticks were collected using a flag. The determination of the type of ticks was carried out using light microscopy based on morpho-anatomical features

Results. In the course of research ticks of various species were found, such as *Dermacentor, Hyalomma* and *Rhipicephalus.* Two species of ticks are widespread in Kostanay region: *D. reticulatus Fabricius* -51.3% and *D. marginatus Sulzer* - 46.5%, single specimens were found as well, *D. niveus Neumann* – 0.9%, *H. scupence Sulzer*– 0.9% and *Rh. schulzei* Olenev - 0.4%. They were found in the southern area of the region.

Key words: Ixodes ticks; Kostanai region.

Introduction

Ixodes ticks are reservoirs and carriers of viral, bacterial, parasitical and fungous diseases. Their diverse epidemiological roles are influenced by various factors like environment and species composition and how well every species has been studied. Some of the diseases that can be transmitted by these ticks include tick-borne encephalitis, tick-borne borreliosis, hemorrhagic fever, ehrlichiosis, anaplasmosis, rickettsiosis, tularemia, and babesiosis. They are of significant concern to both medical and veterinary fields [1]. It is possible for a single tick to carry multiple pathogens from different groups [2, 3, 4].

The expansion of the ranges of different types of ticks, capable of spreading pathogens of infectious diseases in humans, farm animals and domestic animals, is registered today in many regions of the world, and it is a threatening phenomenon. The research of newly emerging natural foci of these diseases requires a comprehensive scientific approach. It is equally important for healthcare systems, sanitary and epidemiological control services in different countries to pay close attention to this issue.

Out of big diversity there are six genera of ixodes ticks that can be found in Kazakhstan, they are *Dermacentor spp, Ixodes spp, Rhipicephalus spp, Haemaphisalis spp, Hyalomma spp,* and *Boophilus spp* [5, 6]. Although ixodes ticks have been well-studied in the central, southern, southeastern, and western regions of Kazakhstan, the fauna of ixodes ticks in northern Kostanay region has yet to be fully explored.

In this connection, the aim of the work is to determine the species composition of ixodes ticks and their geographical distribution within Kostanay region.

Materials and methods

Ixodes ticks collected from various landscapes and climatic zones in three cities and eight areas of Kostanay region were used as a research material. The process of picking ixodes ticks took place between 2017 and 2019. It was done during the period of their activity, which typically spans from April to November, starting with the period of snow melting. Depending on vegetation biomass on the areas being surveyed, different methods were used to pick ticks; tick flags were used on meadows and in forest areas with high grass, while tick drags were used in steppes and low-grassy meadows (Figure 1). Ticks picked from different areas were placed separately in test tubes to ensure accurate identification and analysis.

Figure 1 – Picking ticks

Binocular microscope MBS-10 (Lytkarinsky Optical Glass Factory, c. Lytkarino, Russia) and determinants of Kerbebaeva E.I. (1998), Kapustina V.F. and Yakimenko V.V. (2013) were used to identify the species of ticks. Species validation of ixodes ticks was done by experts at National University of Life and Environmental of Ukraine, Kiev. The ticks (n=1756) were identified to the species level using morphological keys and the life-cycle stage and sex were determined. Where tick identification based on morphology was uncertain, sequencing was used; if the species could still not be reliably identified, the tick was excluded from the study. This approach allowed for accurate identification of the species of ticks and mites, which is essential for further analysis of their potential impact on human and animal health in the region [7]. Molecular genetic studies were carried out in the laboratory of the Scientific Research Center of Applied Biotechnology, Kostanay city and «National Center of Biotechnology» SC of the Ministry of Education and Science of the Republic of Kazakhstan.

Results

The study was in 2017-2021. Of these, 1756 ticks were identified to species including 5 species of the Ixodidae, although 32 submitted ticks could not be analysed because of damage during collection or transport. Submitted ticks most were adults, with 67,1% adult females and 32.5% adult males. Juvenile ticks were 0.4% nymphs.

We were doing the research on the species identification of ixodes ticks, which were collected in various landscapes and climatic zones of Kostanay region.

Kostanay region covers the area of 196,000 km2 or approximately 8% of the total area of Kazakhstan. The region is quite vast, stretching 700 km from the north to the south and 300-400 km from the west to the east. The topography of the region is predominantly flat, the area borders with the West Siberian Lowland in the north, the Torgai Plateau in the south, the Trans-Ural Plateau in the west and the small hills of Saryarka in the south-west. Kostanay region borders with several other regions of Kazakhstan, including North Kazakhstan, Akmola, Aktobe, Karaganda and Ulytau, as well as with three regions of Russia - Kurgan, Chelyabinsk and Orenburg.

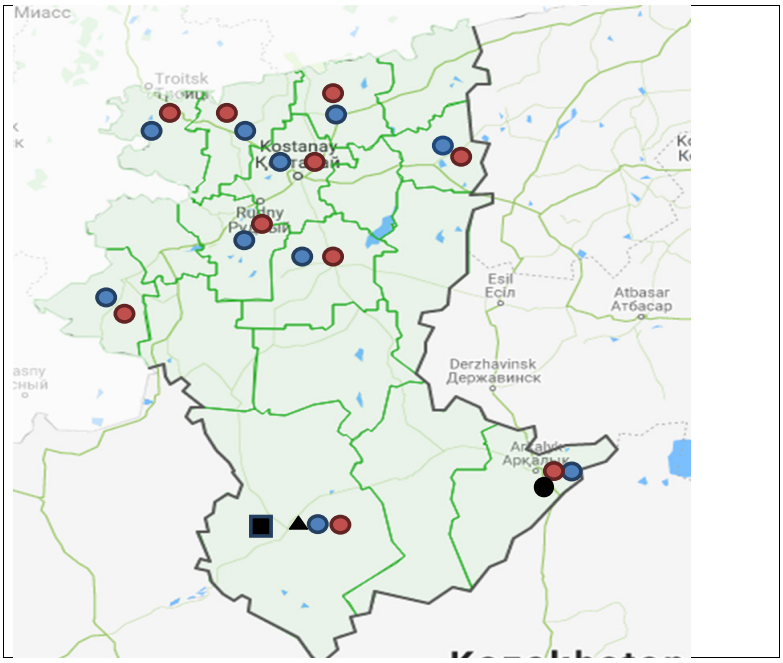
The areas where ticks were collected were: Kostanay, Fedorovsky, Karabalyksky, Auliekolsky, Sarykolsky, Mendykarinsky, Zhitikarinsky, Dzhangeldinsky areas and the cities of Kostanay, Rudnyi and Arkalyk.

The distribution of these ixodes ticks across the region varied and depended on the landscape and geographical characteristics of each area (Figure 2).

While doing the research of the ixodes tick fauna in Kostanay region, we identified 3 genera and 5 species of these ticks.

The data of the species composition of ixodes ticks within the area of Kostanay region are presented in Figures 3-7.

It can be seen that D. *reticulatus Fabricius* were 45% in 2017, 56.6% were picked in 2018, and 56.1%. were picked in 2019. In 2017, 52.8% of ticks D. *marginatus Sulzer* were picked, 42% and 41.3% were picked in 2018 and 2019 respectively. Also, in the southern areas some single specimens of D. *niveus Neumann* were found, 0.7% were picked in 2017, 0.5% were picked in 2018 and 1.4% were picked up in 2019. As for H. *scupence Shulzei* picks, in 2017 0.9% were picked, 0.5% - in 2018 and 1,2% - in 2019. As for *Rh. Schulzei Olenev*, 0.6% were found in 2017. 0.4% - in 2018 - (Figure 3).



- Kostanay region is marked with a black line - other areas are marked with green lines						
	●	*D.reticulatus*	▲	*H. scupence*	■	*Rh. schulzei*
	●	*D.marginatus*	●	*D. niveus*		

Figure 2 – The map of geographical distribution of ixodes ticks in Kostanay region.

Thus, in Kostanay region there are three genera: Dermacentor, Hyalomma and, Rhipicephalus. At the same time, the genus Dermacentor is represented by three species: *D. reticulatus, D. marginatus,* and *D. niveus*. Of these, the most common species in the region are ticks of *D. reticulatus* (51.3%) and *D. marginatus* (46.5%). The ticks of D. niveus were 0.9%, the ticks of *H. scupence* were 0.9% and *Rh. Schulzei* were 0.4%.

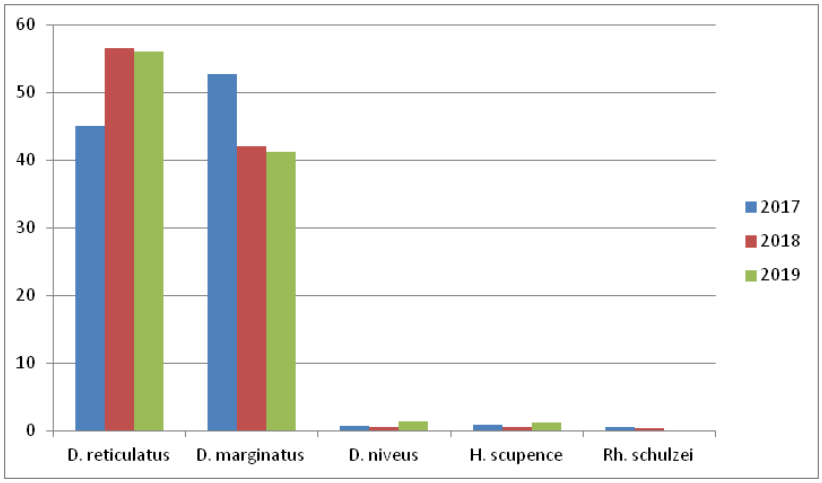


Figure 3 – Species composition of ixodes ticks in Kostanay region from 2017 to 2019

In ticks of the genus *Dermacentor*, there is a scutum on the dorsal side of the body with a characteristic enamel-like pigment (marble pattern). The color of the cuticle is dark brown. The posterior end of the body has 9-11 festoons (Figure 4). The peritremes are oval, with a dorsal process.

Dermacentor reticulatus has a hexagonal gnathosome base (with lateral projections) There is a thorn pointing backwards on the dorsal side of the 2nd segment of the palpi. The palps are angular, the outlines of the proboscis together with the palps are hexagonal (Figure 4, the spike is marked with a red arrow). In males, the scutum covers the entire dorsal part of the body (Figure 5).

Dermacentor marginatus there is no thorn on the dorsal side of the 2nd segment of the palpi. The palps are smooth, the outlines of the proboscis together with the palps are quadrangular (rectangle). The color of the cuticle of a hungry individual is dark brown. The dorsal process of the peritreme is well developed, has a chitinous thickening of the lateral margin (Figures 6 and 7).

Dermacentor niveus (Figures 8) the dorsal process of the peritreme is well developed (Figures 9), without thickening on the lateral margin.

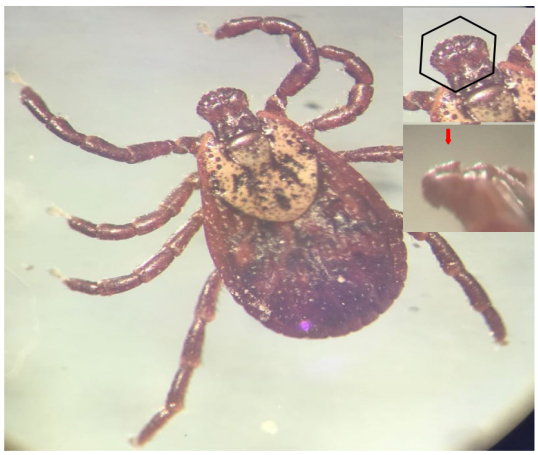


Figure 4 – *D.reticulatus* Female

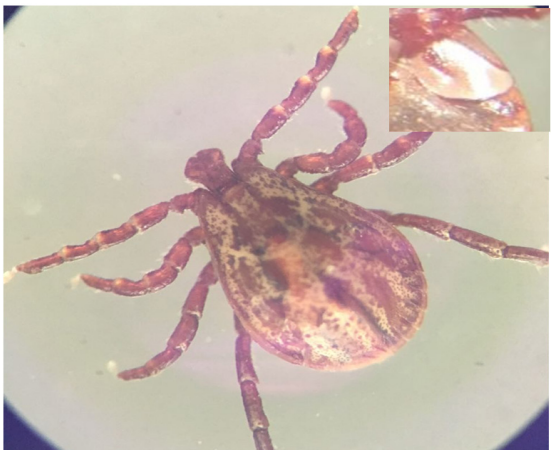


Figure 5 – *D.reticulatus* Male

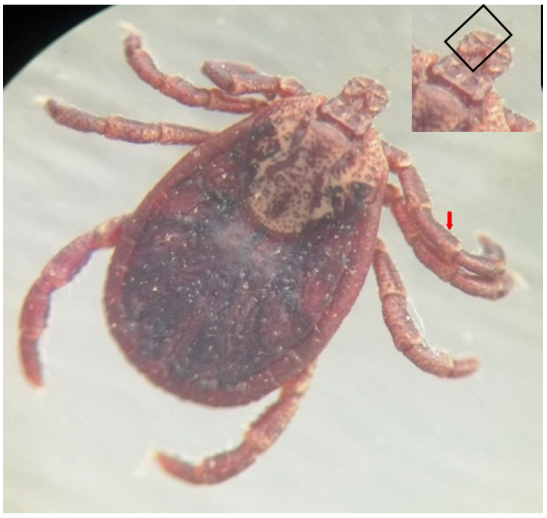


Figure 6 – *D. marginatus* Female

Figure 7 – *D. marginatus* Male

Figure 8 – *D. niveus* Female Dorsal side

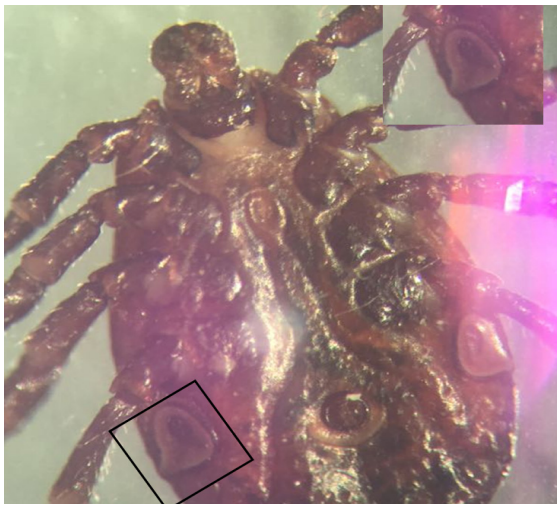


Figure 9 – *D. niveus* Female Ventral side

Since ticks of the species *D. reticulatus* (Figure 10) are widely spread in moderately humid and moderately dry climates, they are common in the areas of birch and pine forests, including steppe zones, i.e. Karabalyk area (12.8%), Fedorovsky area (11.1%), Mendykarinsky area (8.3%), as well as in the cities of Kostanay (31.6%) and Rudny (8.2%).

The species of *D. marginatus* (Figure 11) ticks were found all over, they were noticed in Kostanay area - 10.9% and in Auliekol area - 10%, and in the city of Kostanay - 15.5%, but they predominated in more southern arid areas of the steppe and semi-steppe, i.e., in Zhitikarinsky area -24.5 %. As well as, 8.2% of ticks were picked in Dzhangeldinsky area, and 9.1%. were picked in the city of Arkalyk.

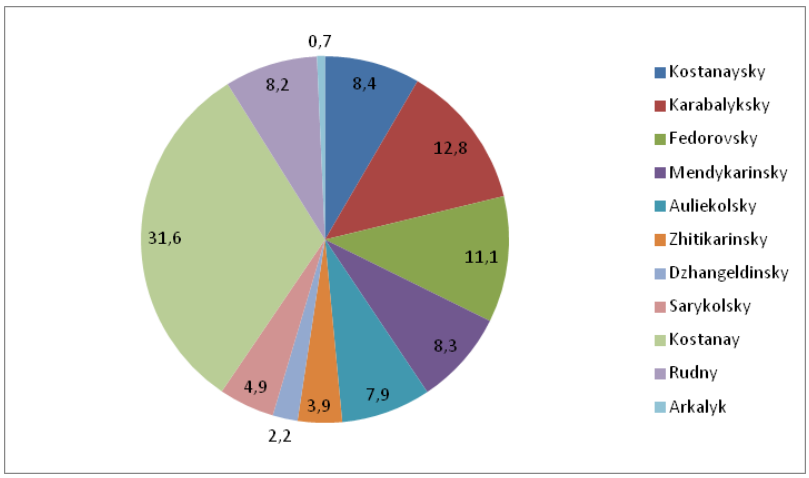


Figure 10 – Distribution of *D.reticulatus* ticks within Kostanay region

Rare species of ixodes ticks were identified in this natural area: the species of *D. niveus* were picked in the city of Arkalyk - 0.9%, and the species of *H. scupenceand* and *Rh. schulzei* were found in Dzhangeldinsky area - 0.9% and 0.4%, respectively.

Various species of ixodes ticks were found in Dzhangeldinsky area (Figure 12) and in the city of Arkalyk (Figure 13).

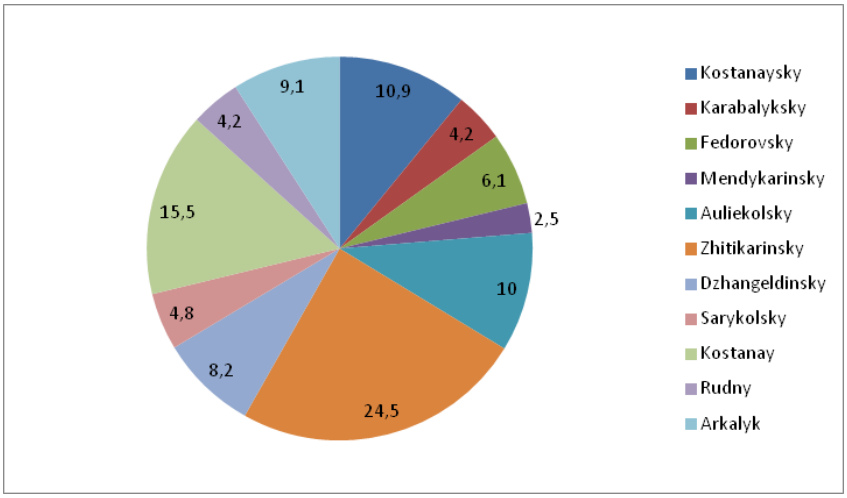


Figure 11 – Distribution of *D.marginatus* ticks within Kostanay region

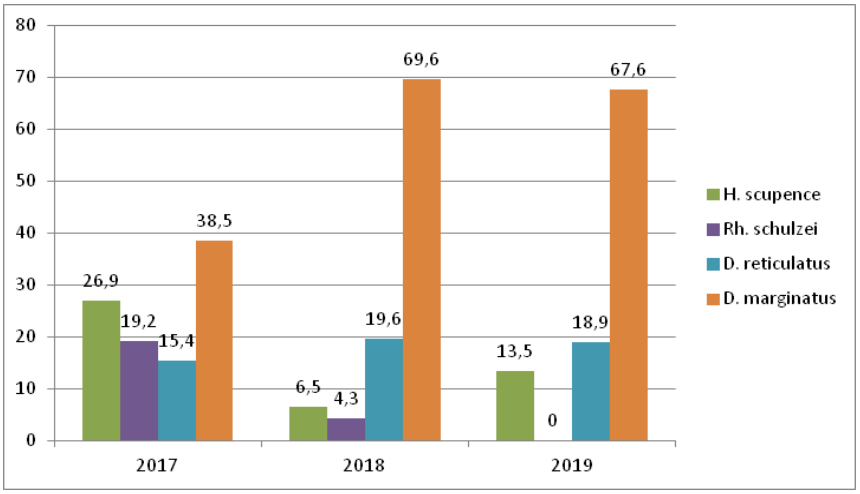


Figure 12 – Ixodes ticks of Dzhangeldinsky area

As can be seen from Figure 12, in the Dzhangeldinsky district, one species of ticks, *D. marginatus,* predominated within all years; twice as many of them were picked in 2018 and 2019 than in 2017.

In the city of Arkalyk the species of tick *D. niveus* were found, and they were not found anywhere else in the region.

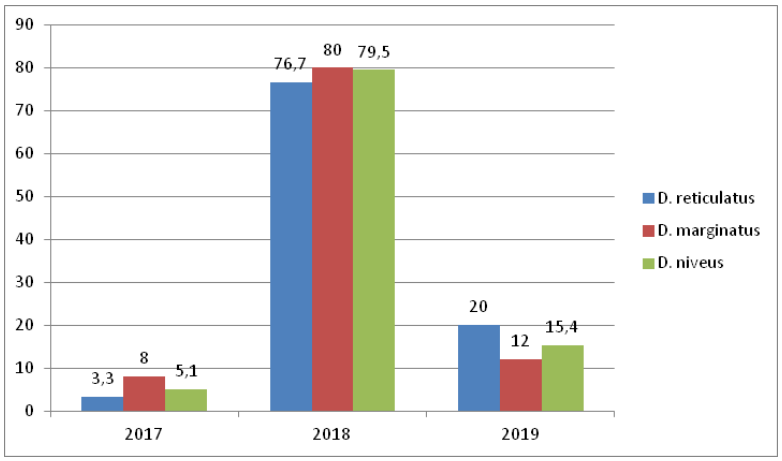


Figure 13 – Species diversity of ixodes ticks in Arkalyk

A lot of ticks were found in pasture areas where farm animals graze.

According to the results of our research, it was discovered that the density of ixodes ticks within the city was slightly lower than in the suburbs, the possible reasons can be the sparse vegetation in the city, mowing of lawns and a small number of hosts of pre-imaginal phases.

Discussion

Ixodes ticks exist all over the globe; one of the species, Ixodesuriae, lives even in the Arctic and the Antarctic [8]. Many studies have been conducted and it has been proved that Ixodes ticks make a major contribution to the spread of infectious, bacterial, parasitic and other diseases. To clarify the causes and conditions for the existence of a natural focus of any vector-borne disease it is essential to know the species composition and environment of the main sources and vectors of the pathogens according to the classification of E.N. Pavlovsky. (1964), [9]. Human influence on the environment has caused the transformation of nature and climate that accordingly has led to the changes in the habitats of ixodes ticks [10]. A comprehensive study of the family of Ixodidae ticks is of scientific and practical importance.

Of the 50 species of ixodofauna in Kazakhstan, there are 22 species in the west of the Republic of Kazakhstan. Five genera of ixodes ticks: *Ixodes, Haemophysalis, Dermacentor, Rhipicephalus, Hyalomma* were found in West-Kazakhstan part of the country. Four genera of ixodes ticks: I*xodes, Haemophysalis, Dermacentor, Rhipicephalus* and 12 species were identified in central Kazakhstan. A greater species diversity of ixodes ticks - *D. reticulatus* and *D. marginatus, D. niveus* (0.9%), *H. scupence* (0.9%) and *Rh. Schulzei* (0.4%) was discovered in Kostanay region in the south-western area, on the borderline of Aktobe and Karaganda areas. The quantity the ticks found was not large, it is the most likely ixodids inhabit and they are introduced from the neighboring southwestern areas.

In the border zones with the Russian Federation in the northern part of Kostanay region, species diversity is not significant and it is represented by only two species, which were found everywhere in the region, they were *D. reticulatus* and *D. marginatus.* It is worth noting that two species of ixodes ticks were identified in Chelyabinsk region, they were *D. reticulatus* and *I. persulcatus* [11].

In general, the climate in Kazakhstan is sharply continental, but when moving to the southern part of the country it becomes milder - subtropical. And unlike in the northern regions where the winter is long and harsh, in the southern areas the winter is shorter and the summer is hot and long. Due to the climatic conditions in the south of Kazakhstan, 35 species of blood-sucking ticks have been registered, 23 species are carriers of 18 species of pathogens of piroplasmosis. In general, large areas with dense herbaceous vegetation, an abundance of hosts for ixodes ticks in the Republic of Kazakhstan will contribute to the spread of vector-borne diseases of animals and humans.

Conclusion

Having completed the research on ixodes ticks in Kostanay region, it has been identified that there are five species of ixodes mites from three genera of *Dermacentor, Hyaloma*, and *Rhipicephalus*. The most numerous genus was *Dermacentor,* with 901 specimens of *D. reticulatus*. The species of *D. marginatus* were high in numbers and were picked on dogs and found all over the area. In the spring and autumn periods their number can reach 100%. The next species *D. marginatus* were found all over the place and 818 specimens in number. It is essential to mention that the species of ixodes ticks *D. niveus, H. scupense*, and *Rh. schulze*i were found in the smallest numbers in the southern area of Kostanay region.

Authors' Contributions

A.Zh. and R.R. conceived and planned the research. A.Zh. and R.R. carried out the experiments. A.Zh., A.Sh., Z.A. and Zh.A contributed to sample preparation. A.Zh., R.R., K.S. and A.Sh., contributed to the interpretation of the results. A.Zh. took the lead in writing the manuscript. R.R. corrected the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Histological studies of muscle tissue in swine sarcocystosis in the northern Kazakhstan

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Abstract

Background and Aim. At present there is no information about the prevalence of sarcocysto-sis among pigs in Kazakhstan, so we aimed to study pork meat for the presence of sarcocysts in the muscles of pigs in the Kostanay region.

Materials and Methods. Pieces of heart muscle, neck, oesophagus, and diaphragm legs taken from pig carcasses served as material for the study. Sarcocysts presence in muscle slices was deter-mined by viewing the samples stained with methylene blue under a microscope. Morphological analysis of muscle tissues included histological and histochemical methods.

Results. Muscle samples from 71 pig carcasses were examined. The intensity of sarcocystosis in pigs was 42.2%, and the intensity of invasion was 8.43 cysts. The highest infection with sarco-cysts was found in sows 3-5 years old (29.6%). The predominant sarcocysts localisation was found: in the heart - 23.9%, oesophagus - 12.7%, and the diaphragm legs - 5.6%. Cervical muscles from the same animals were free of sarcocysts. *Sarcocystis suicanis* species was detected Pathological chang-es in muscle fibres were detected in the examined muscle slices. Swellings and inflammatory pro-cesses of focal or diffuse character, as well as serous and less often purulent reactive myositis with infiltration and admixture of eosinophils or lymphocytes were noted. Examination of slices revealed an immuno-allergic reaction leading to disruption of the heart histological structure, the oesophagus fibres and the diaphragm legs.

Conclusion. Such animal muscles studies for sarcocystosis have not been conducted in the Kostanay region. The prevalence of infection from the number of the studied livestock were deter-mined. According to studies in animals, the infection prevalence increases with age, which is associ-ated with increased contact of pigs with primary hosts. According to the histological studies results, we have established inflammatory processes and muscle lesions caused by exposure to the product of parasite vital activity.

Key words: histology; microscopy; northern region of Kazakhstan; pig; sarcocystosis.

Introduction

Currently, more than two hundred species of *Sarcosystis* known as the most common parasites of domestic animals. According to researchers from different countries of the world, the extensiveness of Sarcocystis infection of pigs ranges from 3 to 36% worldwide; in particular, S. suihominis causes the disease in Germany, Austria, Japan, Malaysia, Argentina [1-3].

In Kazakhstan, information about animal sarcocystosis dates back to the 70-80s of the last century. It was only in 2008 that new information on sheep sarcocystosis was registered in Western Kazakhstan. To date, studies on pigs in the Kostanay region have not been conducted, and there is no information on the prevalence of this invasion. The lifetime diagnosis of sarcocystosis is very difficult, and postmortem diagnosis does not always allow the establishment of the causative agent, only with additional studies

[4-6]. Therefore, the purpose of our study was to identify *Sarcosystis spp.* in the muscles of pigs kept in LLP "Barvinovskoye", Sarykol district, Kostanay region, by microscopy and histological studies.

Materials and methods

The material for the study were pieces of the heart muscle, neck, oesophagus, and legs of diaphragm weighting no more than 50 grams, taken from pig carcasses at the slaughterhouses of Kostanay in the period from August to October 2023. Visually examined 71 carcasses and selected 284 samples of muscle tissue. Further studies were conducted at the Research Institute of Applied Biotechnology, A. Baitursynuly Kostanay Regional University, and laboratory of patholoanatomical bureau.

From the selected pieces of muscle tissue, thin slices of no more than 5x5x2 mm were cut with curved eye scissors and prepared for examination by compressor microscopy. Next, the muscle pieces were spread on synthetic mesh and soaked in 0.2% aqueous solution of methylene blue for 20-30 minutes. After staining, the muscle pieces, along with the synthetic mesh, were placed on fil-ter paper for drainage and removal of excess dye. The samples were kept on the filter paper for only a few seconds. The stained muscle pieces were immersed in 1.5% acetic acid solution for 15-20 minutes to clarify the samples. Gentle stirring was carried out to avoid sticking the samples. The samples were again placed on filter paper for drainage and then transferred to a glass compressor consisting of two glass plates tightened with screws and viewed under a microscope [7].

During microscopic examination, the number of sarcocysts in each slice was counted, and the intensity of invasion was determined by counting sarcocysts in 28 slices of muscle tissue; further, the invasion was conditionally classified as weak (up to 50), medium (above 50-200) and strong (above 200 sarcocysts). A total of 284 slices of muscle tissue from pigs belonging to "Barvinovsko-ye" LLP, Sarykol district, Kostanay region, were subjected to the study.

Morphological analysis of biopsy material included histological and histochemical methods of research; for this purpose, muscle tissue samples were sent to the laboratory of the regional patho-logical anatomical bureau of Kostanay. The muscular tissue samples were taken by biopsy in 1×1 cm, after which the material was fixed in 10% formalin solution, and then poured into paraffin ac-cording to the standard technique. Serial microtome sections 4-6 μm thick were deparaffinized in warm water according to the standardized scheme and stained with hematoxylin and eosin. Histo-logic evaluation of the severity of morphologic changes was carried out by semi-quantitative method using scores, where 1 "+" - weakly expressed, 2 "+" - moderately expressed, 3 "+" - strongly ex-pressed, 4 "+" - very strongly expressed.

Results

Muscles from 71 pig carcasses (sows 3-5 years old, young pigs 6-12 months old) were examined for the presence of sarcocysts. During visual inspection of pig carcasses, no pathological changes or macrocysts were found. Samples of muscle tissue infected with sarcocystis were sent to Kostanay regional patholo-anatomical bureau for histological examination. The biopsy specimen was taken 1×1 cm, after which the material was fixed in 10% formalin solution and then embedded in paraffin according to the standard technique. Serial microtome sections 4-6 μm thick were deparaffinized in warm water according to the standardized scheme and stained with hematoxylin and eosin.

Histologic studies revealed sarcocysts in all muscle tissue samples (Table 1).

Table 1 – Infection of pig muscle with sarcocysts

Muscle tissue samples	Number of samples	
	examined	infected
Cardiac	71	17
Oesophagus	71	9
Cervical	71	0
Diaphragm	71	4

The intensity of the invasion amounted to 8.43 cysts. The highest number of sarcocysts was found in cardiac muscle, slightly less in the oesophagus and diaphragm legs. Of the muscle samples examined, 17 cardiac muscle samples (23.9%) were the most infected, 9 oesophagal samples (12.7%) and 4 diaphragm leg samples (5.6%) were less infected. Cervical muscles appeared free of sarcocyst infection. The age-specific sarcocyst infection of pigs is presented in Table 2.

Table 2 – Infection of pigs by age

Animal groups	Range of age	Number of samples		Percentage of infection, %
		examined	infected	
Sows	3-5 years	47	21	29.6
Piglets	6-12 months	24	9	12.6
Total		71	30	42.2

Percentage of infection amounted to 42.2% (Table 2). The highest infection was found in sows 3-5 years old, while the young animals were less infected.

When comparing the size of the detected cysts, it was found that the largest cysts were found in the diaphragm and the smaller ones in the heart (Table 3).

Sarcocysts in cardiac muscle were oval-shaped with rounded ends, in the oesophagus oblong, in diaphragm legs elongated with pointed ends, sometimes spindle-shaped. At morphometry, the size of cysts varied from small to large; cysts in cardiac muscle averaged 0.32±0.67×0.5±0.43, oesophagus 2.11±3.12×1.01±1.13, diaphragm legs 13.33±1.56×12.28±1.65 μm. According to morphometric features (wall thickness, presence of septa), the detected cysts corresponded to the description of *Sarcosystis suicanis* species [8, 9].

Table 3 – Dimensions (μm) of detected pig sarcocysts

Muscle tissue samples	length×width (max - min)	average M±m
cardiac	0.14–0.35×0.2–0.37	0.24±0.67×0.28±0.43
esophagus	3.24–3.35×0.7–1.17	3.29±3.12×0.93±1.13
cervical	-	-
diaphragm	8.58-10.55 x 13.30-16.72	9.56±1.56×9.01±1.65

Samples of muscles infected with sarcocysts were sent for histological studies, where patho-logical changes of muscle fibers were detected in the examined slices. In the sample (Fig. 1-3) from the heart, oesophagus and diaphragm stained with hematoxylin and eosin, longitudinal sections and partially transverse sections of muscle fibers were determined in most fields of view.

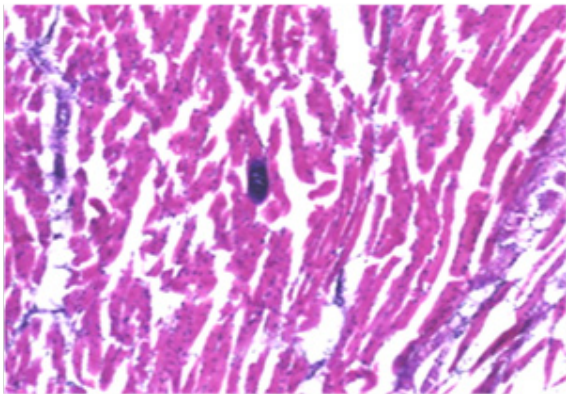

Figure – 1 *Sarcocystis* in cardiac muscle, second generation meronth filled with endozoites, hematoxylin and eosin staining ×200

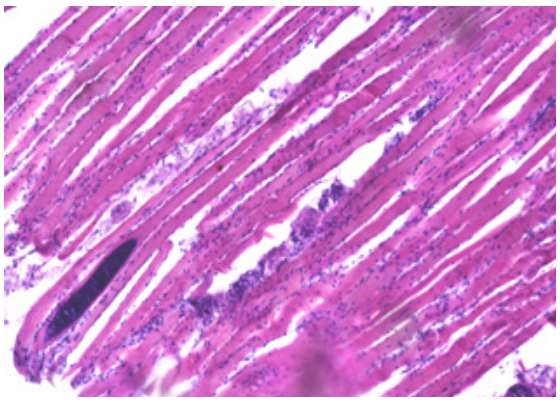

Figure – 2 *Sarcocystis* in the esophagus, hematoxylin and eosin staining ×200

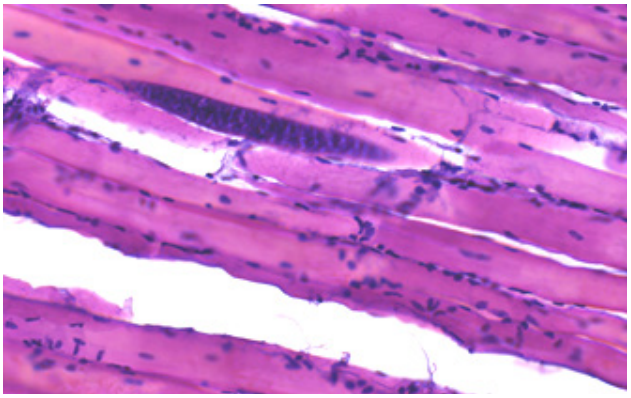

Figure 3 – *Sarcocystis* in the diaphragm hematoxylin and eosin staining ×200

The histologic section (Fig. 1) showed an area of intensive lesion with sarcocysts (5-6 larval capsules). Transverse striation was absent in the affected muscle fibers; sarcoplasm had a connective tissue capsule of oval and round shape around the juvenile stage of cyst development. On the periphery of the cyst capsule, there was nonspecific inflammation in the form of lymphohistiocytic infiltration with the admixture of lymphocytes. The degree of inflammation of muscle tissue at a distance from the lesion area is visualized as focal serous myositis.

In preparations from the oesophagus and diaphragm (Figs. 2, 3), sections of transverse striat-ed muscle tissue were determined. Longitudinal slices of muscle fibers were visible in most fields of view. The muscle to adipose tissue ratio was 18:1 and 23:1. Epimysium was not detected. The perimysium of samples taken from the esophagus was of normal thickness and structure; in the dia-phragm, it was thickened due to the proliferation of the connective-tissue component, which indicates fibrosis. The density of vessels was moderate, and single nerve trunks were determined. The density of endomysial slits was average, containing a moderate number of the capillary lumen and fibroblast nuclei. The sarcoplasm was slightly thickened, and muscle fiber nuclei were located parallel to the periphery of the longitudinal line of the muscle fiber. Transverse striation in unaffected muscle fibers was preserved, myoglobin saturation was sufficient, and muscle fibers were thinned. Five sarcocysts were found on the affected area of the histological section, where transverse striation in the affected muscle fibers was absent. The sarcoplasm had a connective tissue capsule that was oval and round in shape around the juvenile stage of cyst development. On examination of the periphery of the cyst capsule, nonspecific inflammation in the form of lymphohistiocytic infiltration with an admixture of lymphocytes was determined. The degree of inflammation of muscle tissue at a distance from the lesion area was visualized as focal serous myositis. A fragment of muscle fiber was red-brown in color, with fat and connective tissue layers, atrophic changes of parenchymatous cells, the proliferative reaction of lymphoid tissue were determined, which, apparently, were caused by intoxication of the organism with decay products of muscle fibers affected by sarcocysts and its viability. The inflammatory reaction was accompanied by microscopic lesions in the heart and diaphragm. The detection of cysts in the heart muscles indicates a defenseadaptive reaction of the organism of sick animals.

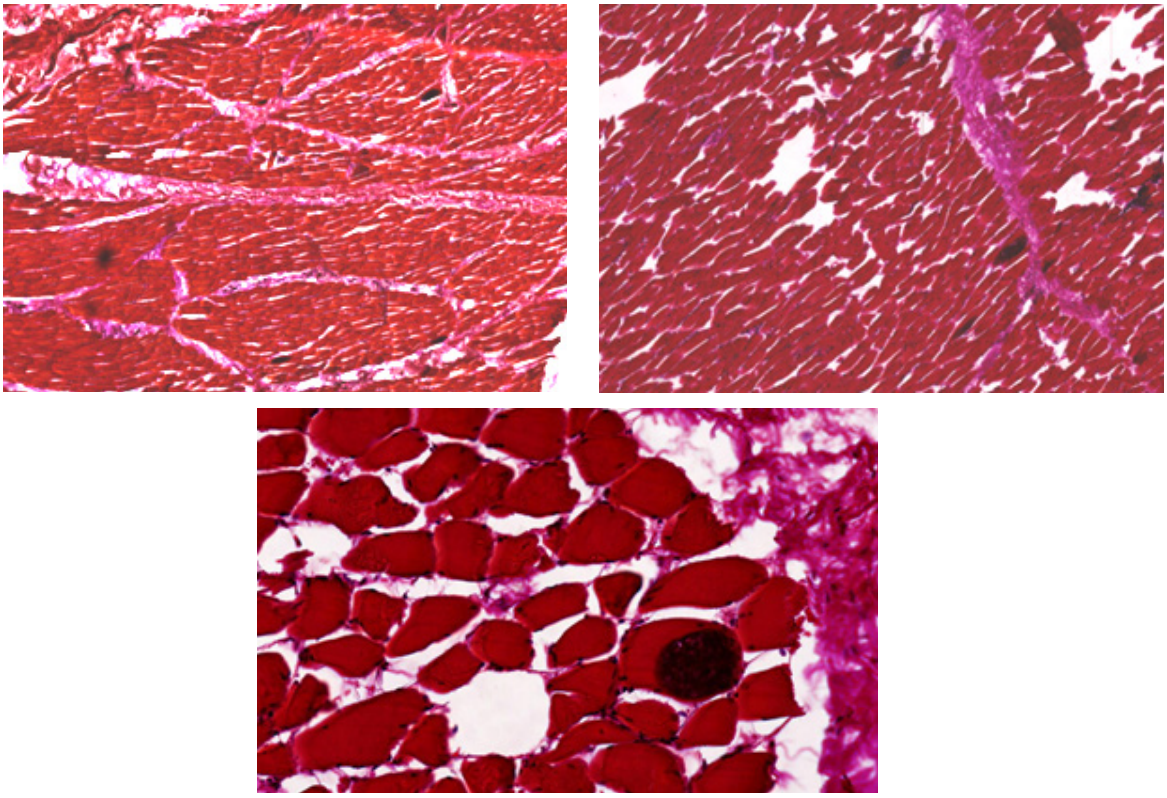

Figure 4-6 – *Sarcocysts* by Van Gieson staining, ×200

At additional histochemical staining by Van- Gieson (Fig.4 - 6), the fragments of muscle fibers were red-brown, with fat and connective tissue layers. Fibrous fibers were bright crimson, density and areas of occurrence were increased due to concentric structures on the periphery of neuro-vascular bundles and due to the thickening of perimysium fibers deeply embedded in endomysial spaces. The reaction of muscle fibers was greenish-brown, indicating an inflammatory process in the tissues.

Discussion

The obtained data testify to pigs' average extensiveness and weak intensity of invasion. Analysis of histological sections of muscle tissue of the studied animals showed that the presence of cyst capsules with endozoites was noted in the sections. A small number of connective tissue fibers around the cysts with hardly visible capsules was revealed, due to which we assume *Sarcocystis suicanis* invasion.

According to Maslennikova O.V., *Sarcocystis miescheriana (S. suicanis)* in wild boars was registered in Belarus in the muscles of the diaphragm and heart with a relatively low intensity of invasion - 9.2%, and in Ukraine - 46.5% [10]. In pigs in the Omsk region, the extensiveness of sarcocystosis invasion is high and ranges from 26.3 to 75.0% [11]. There are no data on the prevalence of sarcocystosis in pigs in Kazakhstan in general and in the Kostanay region in particular.

It is known that domestic pigs and wild boars are intermediate hosts of several species of sarcocysts pathogens: *S.suicanis, S.suifelis, S.suihominis*. Their definitive hosts are dogs, cats and humans [12-14].

In histological studies, sarcocysts were identified in all muscle tissue samples. The total infection of muscle tissue samples was 42.2%. The highest infection was found in sows 3-5 years old (29,6%), and young animals were less infected at 12,6%. The predominant localization of sarcocysts in the heart, esophagus, diaphragm of pigs was determined: in the heart - 23,9%, oesophagus - 12,7%, and legs of the diaphragm - 5,6%. Cervical muscles from the same animals were free of sarcocysts. The size of sarcocysts in pigs corresponds to *S. suicanis* [13, 15, 16].

Muscle sections showed edema and inflammatory processes of focal serous or diffuse character, serous and less often purulent reactive myositis with infiltration and admixture of eosinophils or lymphocytes. Examination of slices revealed immune-allergic reaction leading to disruption of the histological structure of the heart, oesophagal fibers and legs of the diaphragm. Sarcocysts can be

detected only by microscopic examination due to their small size. According to Salimov V.A. and others [17], when muscle tissue is affected by sarcocysts, carcasses become more contaminated with microflora, meat matures poorly, taste and nutritional properties deteriorate, and shelf-life decreases.

Conclusion

In this way, we have established for the first time the infection of pigs with sarcocystosis on the territory of the Kostanay region.The prevalence amounted to 42.2% of the number of the studied livestock. The intensity of invasion amounted to 8.43 cysts. During the examination of pig muscle samples, *S. suicanis* species was detected. In sows of 3-5 years of age, the infestation rate was 29.6%, while young animals were less infected and the infection rate was 12.6%. Localization of sarcocysts was observed mainly in the heart, oesophagus and diaphragm of pigs: 23.9% in the heart, 12.7% in the esophagus and 5.6% in the legs of the diaphragm. No sarcocysts were found in neck muscles. Histologic studies revealed inflammatory processes and muscle lesions in the form of focal serous myositis, most likely caused by exposure to the product of sarcocysts vital activity. To reduce the extensiveness and intensity of defining hosts defeat by sarcocystis invasion, we recommend not to allow feeding raw meat of pigs to dogs, cats and other carnivorous animals. Given the economic damage and epizootic and epidemic significance of the disease, it is necessary to ensure timely pre-ventive measures against the leading carriers of infection.

Authors' Contributions

ZhB and OT: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. KS and RR: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the fi-nal manuscript".

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Conflicts of Interest

Authors have no conflict of interest to declare.

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Using indirect hemagglutination assay for the diagnosis of cattle brucellosis

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