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Bacteriological Monitoring Of The Pathogens Of Mastitis In Dairy Complex Of The North-West Region Of The Russian Federation.

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ABSTRACT

The bacteriological study of samples, including milk cows with hidden and clinically manifested mastitis, vaginal swabs from cows, calves nasal swabs was conducted in the industrial dairy farming. Cultures were K. pneumoniae subsp. pneumoniae, K. pneumoniae subsp. ozaenae, cultures and K. oxytoca. In most cases isolated in association with Proteus mirabilis, Pseudomonas aeruginosa and Escherichia coli. Mastitis caused K.pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Morganella, Providensia of the varieties coliform mastitis. Klebsiella widely distributed in the environment and treat opportunistic microorganisms. Although Mastitis caused K.pneumoniae referred to environmentally the mastitis and the source of infection consider the environment, these bacteria are increasingly manifests itself as contagious pathogens which has marked the pathogenicity factors: the polysaccharide capsule, fimbriae, the ability to totsinoumstum, and hemolytic, the antilysocyme, anticomplementary, antiinterference, DNA asnou and LT-enterotoxigenic activity. The pathogenicity of Klebsiella species is not a sign, and labile characteristic of the particular strain. Klebsiella with different frequency and intensity capable of production of a number of pathogenicity factors, each of which performs a specific function and is in interaction with each other There is an urgent need for the rapid diagnosis of mastitis caused K.pneumoniae, for the purpose of effective treatment and preventive measures.

Keywords: Bacteriological monitoring, opportunistic microorganisms, Klebsiella pneumoniae subsp. pneumoniae, mastitis of cows, cultural and biochemical properties, virulence, DNA-knowing activity, adhesive activity.

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INTRODUCTION

In dairy farming continues to be a serious problem of the widespread inflammation of the mammary gland in cows. [1, 2, 3, 4]. Biological factors play a significant role in the etiology of mastitis. Virtually all microorganisms that infect the udder can, in the presence of predisposing conditions, cause pathological processes in the tissues of the breast. Therefore, when determining the causes of mastitis on the recommendation of the Commission of the International Federation of Dairy Cattle Breeding, the term "udder infections" should be used instead of the term "mastitis pathogens". [2, 14]. Many authors combine these microorganisms into two groups [14, 13, 12]. The first group is obligate parasites, unstable in the external environment, for example, Streptococcus agalactiae, Mycoplasma sp. They cause contagious mastitis and spread in the population of cows during milking with objects contaminated with microorganisms: napkins for washing the udder, hands of milkers, milking machines [30].

The second group is conditionally pathogenic microorganisms that retain their viability for a long time in the environment ("natural pathogens", "ground" pathogens"), yeast and others. [3, 12]. They cause opportunistic or enveronmental mastitis (eng. Environment - environment) [13, 14]. The primary habitat of bacteria causing opportunistic mastitis is the environment (feces, soil, hygiene items, sawdust, shavings, wood pellets, water, etc.). Often they are representatives of the resident intestinal microflora of animals and humans. Infection can occur both during the contact of the nipples with microorganisms during milking and during the periods between milkings (in contact with dirty litter or dirty floor). One type of coliform mastitis is Klebsiella mastitis, caused by Klebsiella and related microorganisms. Such species as K.pneumoniae, K.oxitoca, K.terrigena, K.planticola were previously attributed to the genus Klebsiella of the family Enterobacteriaceae. At present, some species: K.terrigena, K.planticola, and K.ornithinolytica are recommended to belong to the genus Raoultella. The species of K.pneumoniae is divided into 3 subspecies: K.pneumoniae pneumoniae, K.pneumoniae ozaenae, and K.pneumoniae rhinoscleromatis [4]. Klebsiellosti mastitis is increasingly common among dairy cattle in various countries. They can also play the role of an etiological factor in pneumonia, metritis, gastroenteritis, septic processes in humans and animals [2, 5]. Factors suggesting the occurrence of Klebsiella mastitis are adverse climatic conditions, as well as increased exploitation of highly productive animals in unsanitary conditions unfavorable to them, with year-round stall maintenance and no walking.

The pathogenicity of Klebsiella is not a species trait. Klebsiella with different frequency and intensity are capable of producing a number of pathogenicity factors, each of which performs certain functions and interacts with each other [3, 4]. The main pathogenicity factors of Klebsiella are a polysaccharide capsule, fimbria, which provide adhesion to epithelial cells and toxin formation [4, 7]. Adhesion to the epithelium is also mediated by plasmid factors encoding the formation of specific surface proteins [11, 13]. Of great importance in the pathogenesis of Klebsiella lesions are also the siderophore system of bacteria that binds Fe^{2+} ions and reduces their content in the tissues. In Klebsiella, iron chelators enterobactin (enterohelin) and aerobactin have been detected [7, 14]. Virulent K.pneumoniae often has hemolytic, antilysocyme, anti-complementary, anti-interferon, DNA-ase and LT enterotoxigenic activities [5, 6, 10].

When studying the pathogenesis of Klebsiella infection, virulence factors were found in Klebsiella pneumoniae strains associated with liver abscesses, including capsular polysaccharides K1 or K2, lipolysaccharides, hypermucoidity, adhesins and iron binding system. However, the presence and role of these factors in the pathogenesis of mastitis caused by Klebsiella pneumoniae is unknown [1, 2, 3, 4, 10, 11].

The mucoid strains of serotypes K1 or K2 were more virulent for mice than the nemcoid strains of the same serotypes. Klebsiella has 7 serologically different types of capsules and their degree of virulence can be related to the content of mannose in the polysaccharide capsule [12, 13].

The causative agents of opportunistic mastitis can form stable associations, mutually enhancing the negative impact on the organism of animals. Successfully combating such diseases can only have an idea about the dynamics of the formation and spread of such associations and their properties.

The purpose of this work is to conduct microbiological monitoring of priority pathogens in microbial associations during inflammatory processes of the udder of cows in the conditions of the animal-breeding complex for milk production in the North-West region of the Russian Federation.

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Research objectives: 1. Determine the architectonics of the microbial ecology of opportunistic mastitis in the cattle-breeding complex for milk production and determine the species spectrum and structure of priority pathogens. 2. To study the dynamics of changes in the species composition and pathogenic potential of microorganisms of the genus Klebsiella and their associated bacteria isolated on the farm for 6 years. 3. To monitor the dynamics of development of pathogens resistant to modern antibacterial drugs.

MATERIALS AND METHODS

Bacteriological monitoring was carried out in a cattle-breeding complex for the production of milk in the North-Western region of the Russian Federation in the period from 2012 to 2018. The objects of study were milk samples from sick and healthy cows, smears from the upper vaginal vault of cows, patients with vulvovaginitis and mastitis, smears from the nose of calves with rhinitis, bronchitis and bronchopneumonia, rectal smears from calves with enteritis, washes from milking glasses and equipment in the milking hall, bedding material, the air of livestock buildings. Smears and washings were taken with sterile, moistened cellulose probes and delivered for testing in the Amis transport medium (without activated carbon). Samples of litter material were delivered in sterile plastic containers. Air samples were taken by the sedimentation method (for MUK 4.2.734-99 "Microbiological monitoring of the working environment"), in the maternity ward and the milking parlor with an exposure of 1, 3 and 5 minutes. Primary crops were made on the medium Kessler, Endo, glucose-blood agar, yolk-salt agar Chistovich. The obtained pure bacterial cultures were tested on a complex of cultural and biochemical properties. The ability of Klebsiella to capsulation was studied on MPA medium with 1% glycerol and 1% glucose; hemolytic activity - on blood agar with human erythrocytes and sheep erythrocytes. The final identification and determination of sensitivity to antibiotics were performed using the automatic microbiological system "VITEC COMPACT 2". The presence of beta-lactamase was determined by the method of "double disks". The beta-lactamase class was established by PCR. DNA-aznuyu activity was determined using the ready-made environment "DNA agar" company "Pronadisa" production "Conda", Spain. Adhesion activity was tested on a model of human erythrocytes, while high-adhesion cultures of bacteria were considered, having an average adhesion index (SPA) with erythrocytes - over 4, medium adhesion - from 2.01 to 4.0, low-adhesive - from 1.01 to 2, 0 and non-adhesive - at SPA from 0 to 1.0. [1, 3]. The virulence of the isolated cultures was determined by staging a bioassay on white mice with the subcutaneous washout of a daily agar culture (500 thousand mt / cm^3) in a dose of 0.2 cm^3. The sensitivity to bacteriophages was tested using the “falling drop” method using “Pyobacteriophage polyvalent purified” (NPO Microgen, Ufa), “Bacteriophage cletoxiellus polyvalent purified liquid” (NPO Microgen, Ufa), “Klebsifag”, “Bacteriophage lab, bac”. (NPO Biomed, Perm), “Sekstafag (polyobacteria pyobacteriophage)” (NPO Biomed, Perm). The optimization of microbiological diagnostics of opportunistic mastitis by the proteometric method was carried out using MALDI-TOF-SM on the basis of the Pasteur Research Institute (St. Petersburg).

RESULTS AND DISCUSSION

The results of monitoring studies are presented in the table 1.

Table 1: Microorganisms isolated during the monitoring of the industrial animal breeding complex in 2012-2018

Type of material	2012-2014			2016-2018		
	Number of samples	Selected microorganisms	Number of cultures	Number of samples	Selected microorganisms	Number of cultures
Milk in acute and subacute form of mastitis before treatment	26	Klebsiella pneumoniae sp. pneumoniae Proteus mirabilis Pseudomonas aeruginosa Escherichia coli	15 5 4 10	40	Klebsiella pneumoniae sp. pneumoniae Routella Proteus mirabilis Pseudomonas aeruginosa Escherichia coli Providensia sp.	22 5 25 6 18 2 11

					Morganella morganii	
Milk with subacute mastitis after treatment	15	Klebsiella pneumoniae sp. pneumoniae Proteus mirabilis	8 4	15	Klebsiella pneumoniae sp. pneumoniae Proteus mirabilis	12 6
Milk with hidden mastitis	15	Klebsiella pneumoniae sp. pneumoniae Staphylococcus aureus	4 2	15	Klebsiella pneumoniae sp. pneumoniae Staphylococcus aureus	2 3
Milk Clinic. healthy cows	20	-	0	-	-	-
Vaginal smears of cows with mastitis and vulvovaginitis	15	Klebsiella pneumoniae sp. pneumoniae Staphylococcus aureus Streptococcus sp. Escherichia coli	4 10 5 9	5	Klebsiella pneumoniae sp. pneumoniae Staphylococcus aureus Staphylococcus epidermidis Escherichia coli	1 2 2 6
Vaginal swabs of clinically healthy cows	15	Escherichia coli Streptococcus sp.	4 4	5	Escherichia coli Streptococcus sp.	4 4
Smears from the nasal cavity of calves with diseases of the respiratory organs	10	Klebsiella pneumoniae sp. pneumoniae Klebsiella pneumoniae sp. ozenae Escherichia coli Proteus mirabilis	2 2 4 1	5	Klebsiella pneumoniae sp. pneumoniae Escherichia coli Proteus mirabilis Staphylococcus aureus	4 2 4 2
Rectal calf smears with gastroenteritis	4	Escherichia coli Proteus mirabilis Klebsiella oxytoca	4 3 4	5	Escherichia coli Proteus mirabilis Proteus vulgaris Morganella morganii Klebsiella oxytoca Pseudomonas aeruginosa	4 3 4 8 2 2
Nasal swabs of clinically healthy calves	10	Klebsiella oxytoca Escherichia coli Streptococcus sp. Staphylococcus aureus	4 10 5 6	5	Klebsiella oxytoca Escherichia coli Streptococcus sp. Staphylococcus aureus	2 5 3 1
Lavings from milking machines and equipment:						
before milking (clean)	5	-	0	5	-	0
after milking cows with mastitis	3	Klebsiella pneumoniae sp. pneumoniae	3	5	Klebsiella .pneumoniae sp. pneumoniae Morganella morganii	3 2
after milking and washing with detergent-disinfectant	5		0	5		0
Washes from the glass with a solution for treating nipples before milking (during use)	3	Klebsiella pneumoniae sp. pneumoniae	3	5	-	0
Washes from the glass with des. milking nipple solution	3	-	0	-	-	-

Wash off the carousel equipment in the milking parlor	5	Klebsiella pneumoniae sp. pneumoniae Klebsiella pneumoniae subsp. ozenae Klebsiella oxitoca Escherichia coli	5 3 4 1	-	-	-
Wash off the wall in the delivery room	1	-	0	-	-	-
Samples litter in the livestock room	5	Escherichia coli Klebsiella oxitoca Proteus vulgaris Proteus mirabilis	5 5 4 1	5	Escherichia coli Klebsiella oxitoca Proteus vulgaris Proteus mirabilis Pseudomonas aeruginosa	5 5 2 1 1
Air samples in the milking parlor	5	Klebsiella pneumoniae sp. pneumoniae Klebsiella .pneumoniae sp. ozaenae	3 1	5	Klebsiella pneumoniae sp. pneumoniae Escherichia coli	3 5
Air samples in the maternity ward	5	Escherichia coli	5	-	-	-
Total:	170		176	135		218

In 2012-2014, in the cattle-breeding farm, we carried out monitoring bacteriological studies of 170 samples of material taken from the above-listed loci. From the delivered material, microscopic, bacteriological and proteometric methods of research were used to isolate and identify 176 cultures of microorganisms of practical veterinary importance, including 47 cultures of K. pneumoniae subsp. pneumoniae, 6 cultures of K. pneumoniae subsp. ozaenae and 17 cultures of K. oxytoca. In most cases, they were isolated in association with Proteus mirabilis, Pseudomonas aeruginosa and Escherichia coli.

In 2016-2018, we carried out bacteriological monitoring studies of 135 samples of biological material in the same farm. As a result, 218 cultures of bacteria assigned to priority pathogens were isolated and identified, including 47 cultures of K. pneumoniae subsp. pneumoniae, 9 cultures K. oxytoca, 5 cultures Raoutella.

We found that for six years in the inflammatory processes of the udder of cows in the conditions of the dairy cattle breeding complex, priority pathogens were microorganisms belonging to the group of coliform bacteria, in particular, to the genus Klebsiella.

The architectonics of the microbial ecology of opportunistic mastitis in this cattle-breeding farm was determined. Microbial associations of Klebsiella pneumoniae sp. Are the basis for the formation of a stable microecological system of cow udder for clinical, subclinical, and hidden mastitis. pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, partially with Staphylococcus aureus, Morganella, Providensia, Raoutella and other species.

Thus, it has been established that the overwhelming majority of the members of these microbial associations are “natural”, opportunistic pathogens capable of maintaining their long-term viability in the environment. This is confirmed by the results of numerous studies of environmental objects carried out by us in the household, from which representatives of the above-mentioned microbial associations were regularly identified. The table shows that their negative impact is not limited to pathological effects on the udder of lactating cows. These microbial associations are also distinguished from animals with vulvovaginitis and from calves with diseases of the respiratory organs.

The research results show that for 6 years of observations, the architectonics of microbial ecology on the farm has not undergone fundamental changes, despite the measures taken to treat animals, disinfect livestock facilities and equipment. This may be due to the peculiarities of the biological properties of the priority pathogen, which in the surveyed household are the highly virulent Klebsiella pneumoniae subs.pneumoniae strains.

Klebsiella species K. pneumoniae subsp. pneumoniae were small, immobile, gram-negative rods, located singly, in pairs or in a chain of three cells, had a capsule that was clearly visible under microscopy. With growth on the GRM-broth, they caused a uniform opacification, thin mucous film on the surface. After sowing on agar medium, visually the growth of colonies could be observed already in 2-3 hours. Microscopy (x400) of young 2-3-hour colonies on MPA medium contained bacteria in the colony loop-like (unlike the cultures of K.pneumoniae subsp. Ozaenae, which were located in the 2-3-hour colony in concentric rows). Gray and white, translucent, shiny, merging mucous colonies with a diameter of 3-4 mm grew on the MPA after 18-24 hours. On the Endo medium, the studied cultures formed very large colonies with a diameter of up to 12–15 mm, lactose-varied, lush, rising above the surface of the medium by 5-6 mm and higher, forming, after touching with a bacteriological loop, mucous cords of 10 cm or more. The cultures continued to grow actively at a temperature of 20-23 °C, after 48 hours the mucus layer became so powerful that a part of the colonies broke off from the surface of the medium in an inverted Petri dish and formed bunches and cords on the lid. 3-4 days after seeding, the mucous substance of the colonies was compacted in the form of a rubbery adhesive mass, forming a matrix. On the Endo medium with the addition of 5% milk, the colonies of this Klebsiella population reached a diameter of 20-22 mm in 48 h of incubation. On the blood agar, the cultures studied gave medium and large gray mucous colonies, the medium in the cup became dark brown, and a narrow (1-2 mm) β-hemolysis zone appeared directly around the colonies.

In the study of the biochemical properties of selected cultures of Klebsiella pneumoniae subsp. pneumoniae determined that they do not secrete indole and hydrogen sulfide, grow slowly on Simmons citrate medium, do not possess phenylalanine deaminase, give a negative reaction with methyl red and positive (or doubtful) Voges-Proskauer, slow down the milk. Using the analyzer "VINEK COMPACT 2", found that they belong to 2 different Klebsiell populations of the same species, differing in biochemical activity. At the same time, both variants showed an atypical result of a differentiation test for urease (-) and lysine decarboxylase (-). The cultures of the first variant slowly digested lactose.

Cultures of Klebsiella pneumoniae subsp. pneumonie, isolated from the secretion of the mammary gland during mastitis, were virulent for white mice and caused their death within 4-6 days after subcutaneous infection at a dose of 0.2 ml.

In determining the adhesive properties of isolated Klebsiella cultures, they found that they exhibit pronounced adhesive activity on the model of native human erythrocytes. The average adhesion index is more than 4. The participation rate of erythrocytes in the adhesive process was high, it was taken as 100. [1, 3].

Selected cultures of Klebsiella possessed pronounced DNA heat activity. 10 minutes after application of 8-10 ml of IN solution of HC1 to the growth zone of Klebsiella on DNA agar, the width of the depolymerization zone on DNA agar was 5-7 mm.

Tested cultures of Klebsiella pneumoniae subsp. pneumoniae (option 1) was resistant to antimicrobial drugs of several classes: sulfonamides, co-trimoxazole, tetracycline, aminoglycosides (tobramycin, gentamicin, amikacin), β-lactams (ampicillin, amoxiclav, cefepimu, ceftectime, cefacimine, cefacimine, β-lactam (ampicillin, amoxiclav, cefepimu, cefaximone, cefepamine, cyfax), β-lactams (ampicillin, amoxiclav, cefepimu, cefaximone, cefacimine, β-lactam (ampicillin, amoxiclav, cefepime, cefaximone, cefepamine), β-lactams (ampicillin, amoxiclav, cefepimu, cefacin, cefacimine, β-lactam (ampicillin, amoxiclav, cefepimu, cefacin, cefepamine, β-lactam) a) class CTXM-1. Tested cultures of Klebsiella pneumoniae subsp. pneumoniae (option 2), isolated from these milk samples, showed resistance only to ampicillin.

Tested cultures of Klebsiella pneumoniae subsp. pneumoniae of both variants showed sensitivity to the drug “bacteriophage klebsiellous polyvalent purified liquid” (NPO Microgen, Ufa); other tested commercial preparations containing bacteriophages were insensitive.

Culture of the 1st variant K. pneumoniae subsp. pneumoniae deposited by us in the collection of the fgbi "VGNKI", the registration number of the strain in the collection VSSHM-B-288-M.

The tested culture of K. oxytoca did not possess virulence for mice, was sensitive to the vast majority of tested ABP with the exception of ampicillin.

Culture Ps. aeruginosa and P. mirabilis, isolated in association with Klebsiella, were also virulent (caused the death of white mice within 2-4 days after infection) and are resistant to the most commonly used classes of ABP: fluoroquinolones (nalidixic acid, ciprofloxacin), sulfanilamides, co-trimoxazole, tetracycline, polymyxin, aminoglycosides (streptomycin, gentamicin), nitrofurantoin, β-lactams (amoxiclav, ampicillin, cefotaxime). Cultures of E.coli did not cause the death of mice.

As a result of prolonged persistence and passage to sensitive animals for 6 years, the pathogenic potential of pathogens has increased. The period of time from subcutaneous infection of white mice with the Klebsiella pneumoniae subsp.pneumoniae culture to death was reduced to 24-48 hours. The ability of Klebsiella pneumoniae subsp.pneumoniae strains to form a powerful polysaccharide biofilm has significantly increased, from 4 to 2 days the time from the onset of formation of mucous Klebsiell colonies to the appearance of a dense rubber-like matrix protecting them from external influences has accelerated. K. pneumoniae subsp. pneumoniae has retained resistance to disinfectants and detergents; it is not destroyed by the nipple treatment used in the household for milking before milking; it is not sensitive enough to the preparations that process the equipment and the milking room.

CONCLUSION

The studied strains of priority pathogens - pathogens of inflammatory processes in cows and calves in livestock farms - have a pronounced ability to form capsular mucous substances, show hemolytic activity, high adhesive activity, produce deoxyribonuclease, are resistant to the most frequently used classes of ALD, have advanced β-lactamase spectrum. Klebsiella-studied strain (Klebsiella pneumoniae subsp. Pneumoniae) produces beta-lactamase class CTXM-1. This suggests them to be one of the etiological factors of mastitis and vulvovaginitis of cows, as well as respiratory diseases of calves in this economy.

As a result of the research, the architectonics of the microbial ecology of opportunistic mastitis in the animal breeding complex was determined, the species spectrum and structure of priority pathogens were established as the basis for the formation of the microecological system.

The dynamics of changes in the species composition and pathogenic potential of microorganisms isolated in the economy indicates the existence of a stable pathological microbiocenosis, including natural coliform and associated bacteria. 3. The ability to biofilm formation and the associated virulence of pathogen associations increase, and their resistance to modern antibacterial drugs develops.

The results of local microbiological monitoring are a model of trends in global dynamics in the spectrum of the microbial landscape and the level of antibiotic resistance of the main pathogens. This is relevant and important for both veterinary science and practical public health.

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