ETIOPATHOGENETIC ASPECTS OF SHEEP MASTITIS

ANNOTATION

Mastitis in sheep is a serious problem that leads to poor milk quality and is the cause of intestinal disorders in lambs. The etiology of mastitis in sheep is still poorly understood and there is no general opinion on the causative agent of mastitis. There is a little of information in the literature about biofilm-forming mastitis pathogens. The biofilm of microorganisms is the most important virulence factor and contributes to the protection of the pathogen from antimicrobial drugs, thereby reducing the effectiveness of therapeutic drugs.

This study aimed to determine the main causative agents of mastitis in sheep in two forms: subclinical and clinical, from farms of Almaty region, to identify biofilm-forming strains among them, and to study the pathological process in the udder by conducting histological studies.

132 samples of milk from various farms in the Almaty region were examined. To determine subclinical mastitis, the express test "Milkotest" was used.

The subclinical form of mastitis occurs in farms more often than the clinical form, 20.8% and 13.4%, respectively. In subclinical mastitis, coagulase-negative staphylococci dominated, and in the clinical form, cultures of S. aureus were more often isolated, cultures of E. coli were isolated to a lesser extent. The biofilm formation was determined by a statistical method. Among the isolated cultures, 149 isolates were able to generate biofilm. It has been shown that coagulase-negative staphylococci and S. aureus had a greater potential to produce a biofilm compared to the isolated E. coli cultures. It was also found that the nature of the inflammatory process and pathological and anatomical changes in the udder of animals in mastitis depend on the type of the pathogen.

Key words: mastitis in sheep, subclinical, clinical, S. aureus, coagulase-negative staphylococci (CoNS), biofilm formation.

Introduction. Mastitis in sheep has a strong economic impact on the country's agriculture and on animal welfare in sheep farming.

Sheep mastitis manifests itself in two forms: clinical and subclinical. With clinical mastitis, the color of milk changes; clots are present in the structure of milk, and the number of leukocytes in the substance also increases. In clinical cases, visual inspection of the udder may reveal swelling, heat, pain, and induration. Clinical mastitis can occur during any lactation and dry period, but most often, severe forms of clinical mastitis appear approximately 2-4 weeks after lambing or weaning [1]. In subclinical mastitis, there are no clinical signs of the disease, except for an increased number of somatic cells and the presence of pathogenic organisms in the milk, as well as an inflammatory reaction, which is diagnosed using screening or laboratory tests. Pathogens, causative agents of subclinical and clinical mastitis, are diverse, but Staphylococcus spp. are the most commonly diagnosed pathogens of mastitis in sheep. Studies conducted in dairy herds have shown that in more than 70% of cases of mastitis in sheep, staphylococci
were isolated as the main cause of infection [2;3;4]. Streptococcus spp., Enterobacteriaceae, Pseudomonas aeruginosa, Mannheimia haemolytica, Corynebacteria and fungi also can be causal agent of mastitis in sheep, but in studies was detected considerably less [5;6].

In research by Queiroga, M.C. there was a high occurrence of subclinical mastitis (32.2%) and a relatively low prevalence of clinical mastitis (1.7%). More interestingly, mechanically milked sheep were found to have a higher prevalence rate than hand milked animals [7].

According to Aliyev, Yu.A.: the percentage of patients with mastitis varies from 3.2 to 15.8, an average of 6.5%. From the above, it can be said that subclinical mastitis is more common than the clinical manifestation [8].

Biofilm formation is an important in the pathogenesis of mastitis [9]. Biofilm-forming bacteria are more tolerant to opsonophagocytosis and conventional antibiotics, compared to their planktonic counterparts biofilm-forming bacteria are 100-1000 times less susceptible to antibiotics [10;11]. This can lead to chronic or recurrent infections in the herd. As a result of biofilm formation, it becomes more and more difficult to treat and eradicate the disease, which makes this problem more urgent [11;12;13].

The economic damage caused by mastitis is formed not only due to a decrease in milk, meat and wool productivity of diseased ewes, culling of animals, the death of newborn lambs, but also often the death of sheep.

Materials and Methods. Research was carried out in sheep farms of Almaty region and in the laboratory "Microbiology Virology and Immunology", KazNARU. The disease was diagnosed by clinical examination of sheep's mammary gland and laboratory examination of milk. Subclinical mastitis of sheep was determined by "Milkotest" test.

The diagnostic tool "Milkotest" is based on the effect of anionic and nonionic surfactants (surfactants), that are part of the test on the cell membrane of somatic cells, leading to a violation of its integrity and the release of cell contents into the external environment. This changes the viscosity (consistency) of raw milk, possibly a visible change in the color of the mixture. The test is carried out using a special plate, into which milk and 2 ml of the product are poured to the level. The formation of a gel and a change in the color of the milk indicates inflammation and is considered a positive reaction.

The etiology of mastitis in sheep was studied by means of bacteriological examination of milk from 67 sheep, 14 of which had subclinical mastitis and 9 clinically manifested mastitis in sheep, according to the "Methodological recommendations for examination of milk and udder fluid of cows" [15].

For histological studies, pathological material was obtained from organs and tissues. Organs 0.5-1 cm thick were fixed for histological examination. The fixed materials were soaked in 70%, 80%, 90%, 96% (1), 96% (2) alcohols, dehydrated and pressed into paraffin. From the prepared paraffin blocks, using a semi-automated microtome ERV 3100 (Australia), thin slices 5-7 μm thick were obtained.

Biofilm was determined by the statistical method [16]. 4 mL of 12-hour cultures at a dilution of 1:100 was sterilely added to plastic Petri dishes and placed in the thermostat for 48 hours. After that, the contents of the Petri dishes were carefully removed without damaging the formed biofilm by carefully adding 4 ml of distilled water and 400 μl of a 1% alcohol solution of the violet crystal and left at room temperature for 45 minutes. The stained Petri dishes were washed three times with distilled water and left to dry completely at room temperature (+20-+25°C). As a result, a visually detectable stained biofilm was obtained [16].

Research results. The study of the prevalence of subclinical (hidden) forms of mastitis in sheep in farms of Almaty region was performed by testing sheep milk using the diagnostic tool "Milkotest".

A total of 132 milk samples from 67 sheep were tested. First of all, animals were examined in keeping with the requirements of clinical practice, then milk was collected. Anamnestic information on ewes were collected from the record journal, as well as from interviews with operating personnel and livestock specialists.

Milk with mastitis contains an increased number of leukocytes and mostly has an alkaline reaction. Under the influence of "Milkotest" on leukocytes, the consistency of milk and color change.

Consideration of the results when working with "Milkotest": 1) blue-violet color, liquid consistency - negative reaction; 2) the formation of a light transparent gel that disappears after 10 seconds, the color of the mixture has orange-red threads - the number of somatic cells is from 170,000 to 500,000; 3) the formation of a disappearing light transparent gel, the color of the mixture has orange or burgundy inclusions - the number of somatic cells is from 500,000 to 1,000,000; 4) pronounced changes in the mixture, pronounced gelation, the gel adheres to the well of
the test plate, has a filamentous structure, the main color is yellow with reddish inclusions - the number of somatic cells is from 1,000,000 to 5,000,000.

Mastitis was diagnosed by using the Milkotest test and clinical examination of all animals taken under observation. The results are presented in table 1.

Table 1 shows that subclinical mastitis occurs in farms of the republic and accounts for 20.8% of the total number of examined animals. During a clinical examination, sheep mastitis was diagnosed in 9 animals, which amounted to 13.4%.

Table 1 – Results of diagnostic tests for mastitis in sheep

<table>
<thead>
<tr>
<th>Number of sheep examined</th>
<th>Methods of diagnostics</th>
<th>Number of animals positive for mastitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>«Milkotest»</td>
<td>Subclinical form (20,8%)</td>
</tr>
<tr>
<td></td>
<td>Clinical examination</td>
<td>Clinical form (13,4%)</td>
</tr>
<tr>
<td>67</td>
<td></td>
<td></td>
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</tbody>
</table>

In addition to these studies, 132 sheep milk samples were investigated to determine the extent of subclinical forms of mastitis. The results of milk samples from sheep with Milkotest are shown in Table 2.

Table 2 – Results of an investigation on the presence of subclinical mastitis in sheep

<table>
<thead>
<tr>
<th>Sample examination method</th>
<th>Number of examined samples</th>
<th>Identified positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>«Milkotest»</td>
<td>132</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20,8%</td>
</tr>
</tbody>
</table>

As can be seen from Table 2, subclinical mastitis in farms can reach significant rates (36.3%). It is known from the literature that subclinical mastitis is mostly chronic, is not noticed by farmers and can lead to loss of milk yield and deterioration of milk quality [17].

To determine and study the etiological nature of mastitis in sheep, a bacteriological study of all milk samples obtained from sheep with clinical and subclinical forms of mastitis, as well as healthy animals, was conducted.

To explore the morphological and biochemical properties of cultures, milk samples were inoculated on MPA (meat-peptone agar), salt agar, blood agar, on a medium for determining DNase, and also on Endo medium. Samples were mixed well, two or three loops of milk were streaked onto meat-peptone agar and incubated at 37°C for 24-48 hours. All cultures were then subjected to bacteriological analysis. Depend on growth features on media and microscopy from individual colonies first selection of cultures was made. Staphylococci were identified founded on colony morphology, Gram stain, catalase test, and hemolysis on blood agar. Isolates of coagulase-negative staphylococci based on a negative coagulase reaction in rabbit plasma and by inoculation on saline agar with mannitol, and S. aureus isolates differed in the presence of coagulase and DNase.

67 milk samples from healthy sheep and animals with clinical and subclinical mastitis were examined; the results of the microbiological examination are shown in Table 3

The data of the table 3 demonstrate that during microbiological evaluation of milk samples from 67 sheep, 176 cultures were isolated, of which 56 (32%) were from ewes with clinical mastitis, 109 (62%) were subclinical, and 13 (6%) were healthy.

Table 3 – Cultures isolated from sheep milk

<table>
<thead>
<tr>
<th>Total samples examined of them from animals with:</th>
<th>Name of cultures and quantity</th>
<th>Total isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CoNS (Coagulase-negative staphylococci)</td>
<td>E. coli</td>
</tr>
<tr>
<td>Clinical mastitis</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Subclinical mastitis</td>
<td>85</td>
<td>15</td>
</tr>
</tbody>
</table>
Healthy

<table>
<thead>
<tr>
<th></th>
<th>6</th>
<th>5</th>
<th>0</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>The total amount</td>
<td>105</td>
<td>33</td>
<td>38</td>
<td>176</td>
</tr>
</tbody>
</table>

Coagulase-negative staphylococci (105 isolates) and *S. aureus* (38 isolates) play the leading role in the etiology of ewe mastitis. In foreign publications, it was also noted that with mastitis, staphylococci are predominantly detected in milk [18;19;20]. Especially in clinical forms of mastitis, *S. aureus* were most often isolated.

It is worth noting that coagulase-negative staphylococci (66 isolates) and *E. coli* (7 isolates) were isolated in the milk of sheep with subclinical mastitis in the highest number. Pathoanatomic changes in the udder of sheep depend on the form of mastitis and the type of pathogen. Thus, with serous mastitis, the macroscopic picture is characterized by a significant increase in the volume of the affected lobes, and, accordingly, the asymmetry of the right and left lobes of the udder. When cut, the surface is moist, shiny and relatively pale in color.

The segments of the udder appear in the form of gray-red fields separated from each other. The organ is strongly compacted, which is especially well felt when it is cut: the normal udder is of a soft elastic consistency and escapes from the knife, as a result of which it is difficult to dissect, but with mastitis it is easily cut. The cut surface gives a different picture of changes, depending on the nature of the inflammatory process. Interlobular and interalveolar connective tissue was edematous. Histological examination revealed that the alveolar lumens were filled with homogeneous exudate, which contained single polymorphonuclear leukocytes and cells of desquamated epithelium (Fig. 1).

Figure 1 – Leukocyte infiltration of the intralobular stroma with purulent fusion. G.E. 100x

The macroscopic picture in catarrhal mastitis was characterized by the following features: the cut surface is drier, granular due to the increase in lobules, has a yellowish color, when pressed, a cloudy, pus-like secret is separated. Histologically, desquamation of the epithelium and emigration of leukocytes are observed in the alveoli. There is edema and infiltration of leukocytes and histiocytes in the interlobular connective tissue (Fig. 2).

Figure 2 – Leukocyte infiltration of the intralobular stroma with purulent fusion. G.E. 100x

In purulent mastitis, the mammary cistern is filled with purulent exudate. The mucosa is covered with flaps of necrotic tissue. The lumen of alveoli also contains an exudate containing a large number of...
polymorphonuclear leukocytes and slightly desquamated epithelial cells. The interstitial tissue is infiltrated with leukocytes and histiocytes (Fig. 3).

Figure 3 – Purulent mastitis. H.E. 100x

Analysis of the results of pathoanatomic studies has shown that with mastitis caused by *Staphylococcus*, inflammation of the parenchyma is more purulent and gangrenous in nature. The secret of the udder is watery, flaky and has an admixture of pus.

In mastitis, the etiology of which was represented by *E. coli*, an acute inflammatory process of the parenchyma was observed and characterized by pronounced edema and necrotic phenomena.

According to R.R. Pederson, biofilm formation by microorganisms occurs not only on the surfaces of environmental objects, but also in animals [21].

In this regard, the next objective of our study was to investigate the ability of CoNS, *E. coli* and *S. aureus* isolates to form biofilms (Table 4).

Table 4 – Prevalence of biofilm-forming strains among pathogens of clinical and subclinical forms of sheep mastitis

<table>
<thead>
<tr>
<th>The total number of isolates</th>
<th>Biofilm forming isolates</th>
<th>Including</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>176</td>
<td>149</td>
<td>85</td>
</tr>
</tbody>
</table>

The study found that 149 (85%) of the 176 cultures studied formed biofilms, including 91.4% of coagulase-negative staphylococci, 89.4% of *S. aureus* and 57.8% of *E. coli* capable of forming biofilms. Among the cultures studied, *S. aureus* and coagulase-negative staphylococci were the most capable of biofilm formation. The intensity of biofilm formation was more pronounced in *S. aureus* (Fig. 1.b) followed by coagulase-negative staphylococci (Fig. 1.a) and then less pronounced biofilm formation in *E. coli*. Similar results are reflected in the results of studies conducted in Greece, where it was also found that *S. aureus* strains form a biofilm more frequently than coagulase-negative staphylococci [22].
Conclusions. Studies have found that mastitis is widespread among sheep in the farms of the Almaty region, with subclinical mastitis accounting for 20.8% out of number of animals studied, and clinical mastitis - 13.4%.

The study of etiology of mastitis in sheep showed that the dominant pathogens of mastitis are coagulase-negative staphylococci (59.7%) and S. aureus (21.6%), but in lower degree - E. coli (18.8%). In the etiology of clinical mastitis, S. aureus plays an important role, and in the subclinical form, coagulase-negative staphylococci and E. coli were more prevalent. Of the total number of isolates, coagulase-negative staphylococci (85%) and S. aureus (89.4%) showed the highest activity in biofilm formation. E.coli formed the least biofilm compared to staphylococci. With mastitis caused by S. aureus, inflammation of the parenchyma is observed to a greater extent of a purulent and gangrenous nature, while with mastitis, the etiology of which is represented by E. coli, an acute catarrhal inflammatory process of the parenchyma was observed. The results obtained allow us to conclude that it is necessary to revise the protocol for the treatment of mastitis with the development of means for irradiating biofilms and safe for the physiological state of the udder of animals.

REFERENCES

10 Gomes F. Saavedra M.J., Henriques M. Bovine Mastitis Disease/ Pathogenicity: Evidence of the Potential Role of Microbial Biofilms. 7.
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анықталды
процессінің экспресс
алынған биоқабықsha клиникалық
ықпал вирулентті және себебі
казылардың маңызды
жоқ дақылдарымен ауыратын
малдың ауырында, сондықтан
еди." Алматы облысында
анықтау және мастиитпен
малдың ауырында
кемділгін тімендетеді.

Бул зерттеуңдің мақсаты
- Алматы облысындағы шарашылыктарындағы койлардың клиникалық және субклиникалық мастииттің негізді қоздырғыштарын анықтау, сондықтан қозылардың молардасына
биоқабықша түзетін мастиит қоздырғыштарына арналық алғашқы, сонымен қатар, мастиитпен қысқартуға үшін қалқан қысқартьқтарға және молардың араласына байланысты.

Биоқабықша тұзу қабілеті статистикалық еңісіп анықтайды. Окшашланған осінділердің
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РЕЗЮМЕ
Мастиит овеч является серьезной проблемой, которая приводит к ухудшению качества молока и является причиной кишечных расстройств ягнят. Этиология мастиита овец еще плохо изучена и не единого мнения о возбудителе мастиита. В литературе есть небольшое
количество информации о возбудителях маститов, образующих биопленку. Биопленка микроорганизмов является важнейшим фактором вирулентности и способствует защите возбудителя от антимикробных препаратов, тем самым снижая эффективность лечебных препаратов.

Целью данного исследования было определение основных возбудителей клинического и субклинического мастита овец в хозяйствах Алматинской области, выявление среди них биопленкообразующих штаммов, а также изучение патологического процесса в вымени проведением гистологических исследований. Было исследовано 132 проб молока из различных хозяйств в Алматинской области. Для определения субклинического мастита был использован экспресс тест «Милкотест». Субклиническая форма мастита встречается в хозяйствах чаще по сравнению с клинической формой, 20,8% и 13,4% соответственно. При субклиническом мастите доминировали коагулаза-негативные стафилококки, а при клинической форме чаще были изолированы культуры S. aureus, в меньшей степени были выделены культуры E. coli.

Способность образовывать биопленку определяли статистическим методом. Из общего числа выделенных культур 149 изолятов имели способность к биопленкообразованию. Было установлено, что коагулаза-негативные стафилококки и S. aureus обладают большей способностью образовывать биопленку, по сравнению с выделенными культурами E. coli. Также установлено, что характер воспалительного процесса патологоанатомические изменения в вымени животных при маститах зависят от вида возбудителя.