Nonspecific resistance to keratoconjunctivitis of moraxellosis etiology in young cattle

L F Sotnikova¹, N V Pimenov¹*, A V Goncharova¹, S F Nazimkina¹, M I Slozhenkina² and A A Mosolov²

¹Moscow State Academy of Veterinary Medicine and Biotechnology – K.I. Skryabin MVA, Moscow, Russia
²Volga Region Research Institute of Manufacture and Processing of Meat-and-Milk Production, Volgograd, Russia

*E-mail: pimenov-nikolai@yandex.ru

Abstract. The key problems in the study of the pathogenesis, therapeutic, and prophylactic tactics in keratoconjunctivitis caused by the Moraxella bacteria are the issues of immunoresistance of a susceptible organism and immunotropic reactions. The paper presents research data revealing the mechanisms of young cattle’s immunoreactivity to experimental M. bovis infection into the conjunctival sac. The immunoreactivity mechanisms in terms of the indicators, such as the level of A, M, and G immunoglobulins in the lacrimal fluid, lysozyme activity, the total protein concentration, and bactericidal activity in the lacrimal fluid and in the blood serum, have been found. There was shown systemic immobilization against the background of local manifestation.

1. Relevance
Obtaining high quality meat and dairy products is the cornerstone of efficient livestock breeding. Cattle diseases are wreaking havoc on the industry and cause a decline in productivity and product quality. A matter of great concern is mass keratoconjunctivitis that currently tends to spread. The etiology, pathogenesis, and clinical manifestation of keratoconjunctivitis in cattle have changed considerably and require a new approach to their study. Moreover, there is no consensus about the main etiological agent that causes mass eye damage in animals. At present, researchers have found a number of keratoconjunctivitis causative agents, i.e. rickettsia, viruses, telasia, chlamydia, and mycoplasma. Most authors in Russia and abroad adhere to the concept of bacterial etiology that considers Moraxella bovis – primary pathogenic agent—and reducing the natural resistance of cattle as important factors of the disease [1, 2, 3, 4].

Infectious keratoconjunctivitis (“pink eye”) is moraxellosis, an infectious disease caused by Moraxella bovis that initially was regarded as a highly contagious disease due to the low mortality and prevalence of chronic forms [5]. Nevertheless, the high economic damage caused by this pathology requires a comprehensive study of the problem [6, 7]. In the study of the pathogenesis and development of therapeutic and prophylactic tactics in moraxellosis keratoconjunctivitis, the issue of the immunoresistance of a susceptible organism is fundamental [8, 9], with a number of questions on etiology, pathogenesis, and young cattle’s immunological reactivity to keratoconjunctivitis caused by Moraxella bovis being insufficiently studied and requiring detailed and comprehensive research.
This circumstance provides scientific interest in revealing the regularities of the general and body’s local protection against ocular pathology using the example of young cattle as a model with a low immune status and predetermines the prognosis of the disease and effective and economical methods of its treatment and prevention.

2. Materials and research methods
In order to achieve unbiased evaluation of cattle’s general and local resistance to mass keratoconjunctivitis, indicators of natural resistance of animals in Test and Control groups were compared. We studied 20 black-and-white steers at the age of 5-6 months, i.e. 15 of them were in Test group and 5 heads were in Control group.

In animals with clinical signs of keratoconjunctivitis, a sterile swab stick was placed in the posterior fornix of the conjunctiva; the material was scraped, plated on 5% blood agar by the lawn method, and incubated at 37 °C for 36 hours. When examining the inoculum, attention was paid to typical gray-white transparent, round, with smooth edges and beta-hemolysis, 1-2 mm in diameter colonies that were subcultured on 5% blood agar and again incubated in the same way. Sterile saline solution was added to a Petri dish with cultured growth of Moraxella bovis, shook, and brought to the concentration of 2x10⁶ microbial bodies in 1 ml, using the turbidity standard.

Before infecting Test and Control steers, the conjunctival contents were studied with respect to Moraxella bovis. The steers were infected by injecting 0.1 ml of the prepared suspension into the epithelial layer of the cornea. Control animals were injected with 0.1 ml of sterile saline solution.

After infection, tear fluid was obtained from the diseased eye on days 1, 2, 3, 4, 7, 10, 15, 20, 25, and 30, and blood from jugular vein on days 1, 2, 5, 10, 15, 20, and 30.

The activities of blood serum lysozyme and lacrimal fluid were determined by the E. Osserman’s method based on the lysozyme ability to lyse the cell walls of Mic. Lysodeikticus suspended in agar.

The bactericidal activity of blood serum in the lacrimal fluid was determined by the of O.A. Smirnova and T.A. Kuzmina’s method.

The contents of the G and M immunoglobulins in blood serum and A, M, and G immunoglobulins in the lacrimal fluid were found by the radial immunodiffusion technique.

The microbial count of the lacrimal fluid was determined by diluting the material with saline and seeding the resulting dilution on the beef-extract agar (BEA).

3. Results and analysis
In the acute period of the disease, keratoconjunctivitis of young cattle has been established to cause depression of the general condition, an increase in the general body temperature, cardiac acceleration, and rapid breathing. The WBC count rises by 5-7 thousand. In the subacute and chronic periods of the disease, these indicators decrease to the initial level.

Studies revealed the mechanisms of immunoreactivity to experimental moraxella infection into the conjunctival sac. Analysis of the humoral immunoglobulins of the lacrimal fluid of Test and Control steers found A, M, and G immunoglobulins; before infection, 55% of all immunoglobulins were A immunoglobulins, 36% were G immunoglobulins, and 14% were M immunoglobulins.

In the first half of the acute period of the disease (days 1-7, cellular infiltration and ulceration of the cornea), in the lacrimal fluid there was found a 2-fold decrease in immunoglobulin A (from 132±20 μg/ml to 70±3 μg/ml), 1.8 times decrease in immunoglobulins G (from 99 ±5 μg/mg to 59.5±5.8 μg/ml), and 1.5 times decrease of immunoglobulin M (from 39.5±0.5 μg/ml to 24±0.5 μg/ml), P<0.05 (table 1).

A steady increase in the studied parameters of the lacrimal fluid was established in the period from the symptom of red superficial corneal vessels (days 5-7-10) to the formation of a granulation barrier (days 10-14). The immunoglobulin M concentration reached its highest level on day 10 (62±0.2 μg/ml), immunoglobulin G on day 20 (240±7 μg/ml), immunoglobulin A on days 25-30 (155±74 μg/ml), P<0.05. Table 1 shows, that during the recovery period, all Test steers had higher content of immunoglobulins in the lacrimal fluid by 15% than Control steers.
A decreased content of immunoglobulin G on the first days of the disease was assumed to be caused by profuse lacrimation that resulted in dilution and washing out of the indicator under study. An increase in immunoglobulin in the second half of the acute process, apparently, was due to an increase in the porosity of the conjunctiva blood vessel wall, which lead to the diffusion of immunoglobulin into the lacrimal fluid. A stable increase in immunoglobulin in the subacute and chronic periods (inflammation signs in the anterior segment of the eye were not pronounced) may be caused by both its diffusion through the blood vessels walls and local synthesis of specific antibodies.

A stable increase in immunoglobulin A was most likely associated with the local synthesis of secretory immunoglobulins.

**Table 1.** Immunoglobulins A, M, and G in the lacrimal fluid in experimental keratoconjunctivitis caused by Moraxella bovis (μg/ml).

| Period       | Day | Immunoglobulins in lacrimal fluid | A   | P  | M   | G   | P  |
|--------------|-----|----------------------------------|-----|----|-----|-----|----|
| Before infection | 2   | 72.6 ± 6                         | <0.05 | 24 ± 0.5 | <0.05 | 63 ± 7 | >0.05 |
| Acute        | 3   | 77 ± 6                           | <0.05 | 45 ± 0.6 | <0.05 | 60 ± 5 | <0.05 |
|              | 4   | 60 ± 3                           | <0.05 | 38 ± 0.3 | <0.05 | 60 ± 3 | <0.05 |
|              | 7   | 85 ± 6                           | <0.05 | 52 ± 0.4 | <0.05 | 89 ± 0 | <0.05 |
|              | 10  | 72 ± 6                           | <0.05 | 62 ± 0.4 | <0.05 | 89 ± 0 | <0.05 |
| Subacute     | 15  | 93 ± 6                           | >0.05 | 58 ± 0.7 | 0.05  | 103 ± 10 | >0.05 |
|              | 20  | 109 ± 4                          | >0.05 | 44 ± 0.5 | >0.05 | 240 ± 7 | <0.05 |
| Chronic      | 25  | 119 ± 15                         | >0.05 | 40 ± 0.5 | >0.05 | 119 ± 15 | <0.05 |
|              | 30  | 155 ± 7                          | 0.05  | 40 ± 0.5 | >0.05 | 108 ± 6 | >0.05 |

The literature data and our study results enabled assuming that in the acute period, the immunoglobulin M entered the lacrimal fluid out from the developed network of lymphatic vessels of the circular muscle of the eye (the large molecular weight of the immunoglobulin makes it possible to diffuse the indicator from the blood vessels). The inflammatory edema made the endothelium of the lymphatic vessels swell, the mesendothelial connections open, and the lumen of the lymphatic vessels expand, so proteins with a large molecular weight penetrated from the focus of inflammation, with inflammatory conjunctival edema and immunoglobulin M in the lacrimal fluid simultaneously decreasing. However, we revealed no dramatic response in the content of immunoglobulins M and G in the blood serum in both Test and Control steers, and the existing minor fluctuations were within the physiological range.

Thus, our data on changes in immunoglobulins in the lacrimal fluid and their constant content in the blood serum supported the hypothesis of an independent synthesis of secretory and specific immunoglobulins involved in the local immunity.

If we admit a local system of synthesis of secretory and specific immunoglobulins that exercise protective functions in the eye, a change in their level in the lacrimal fluid can to a certain extent serve as a prognostic test and contribute to the development of rational therapy.

Of course, immunoglobulins are not the only regulator of the pathological process in the organ of vision; however, along with other nonspecific protective factors (such as lysozyme, etc.), immunoglobulins are determined as a necessary diagnostic indicator that determines the use of immunomodulating medications.

For a deeper and more comprehensive analysis of the mechanisms of general and local protection of the body of young cattle from keratoconjunctivitis, the lysozyme activity of the lacrimal fluid and blood serum was studied as one of the most important factors of the body's nonspecific defense against infection.

The lysozyme activity of the lacrimal fluid in Test and Control steers before the disease was found to be 306±72 μg/ml. On the first 2-3 days of illness in Test steers, the lysozyme activity of the lacrimal
fluid increased to 1013±130 μg/ml, on days 5-6 (infiltration and ulceration of the cornea) the level of the studied indicator was at a very low level of 24±7 μg/ml, P<0.05 or without activity (table 2).

**Table 2.** Lysozyme activity of the lacrimal fluid in experimental M. Bovis keratoconjunctivitis.

| Disease period | Day | Lysozyme activity (μg/ml) | P   | Control | P   |
|----------------|-----|---------------------------|-----|---------|-----|
|                |     | Test                      |     |         |     |
| Before infection|     | 306 ± 72.8                | -   | 306± 72.8| -   |
| Acute          | 2   | 491 ± 31.3                | >0.05 | 450± 20.0| -   |
|                | 3   | 1013 ± 130                | <0.001 | 411± 34.0| -   |
|                | 4   | 24.4 ±7.7                 | <0.01 | 369 ± 20.0| >0.05|
|                | 7   | 480 ± 45                  | >0.05 | 320 ±21.0| -   |
|                | 10  | 524.3 ± 108               | >0.05 | 310 ± 22.0| -   |
| Subacute       | 15  | 584 ± 108                 | <0.05 | 308 ±10.6| -   |
|                | 20  | 624 ± 63                  | <0.05 | 309 ±12.3| -   |
| Chronic        | 24  | 416 ± 56.7                | >0.05 | 315 ±17.5| -   |
|                | 30  | 410 ±33                   | >0.05 | 320 ±15.6| -   |

Table 2 shows, that starting from day 10 after infection, the lysozyme activity had a stable tendency to increase and reached 624±63 μg/ml on day 20, then its content decreased to the Control steers level of 410±33 μg/ml on day 30.

A 3-fold increase in the lysozyme activity in tears on days 2-3 may be caused by the hyperfunction of the lacrimal glands that are one of the main sources of lysozyme synthesis, with the acini of individual lacrimal glands lobules that remained vegetative before the introduction of the antigen being involved in the active secretory process. However, being constantly excessively stimulated, functional capabilities of any biological system are limited and followed by a drop in activity and exhaustion. Excessive stimulation of lacrimation leads to a decrease in synthetic activity and an increase in hydrolytic processes, which explains the low lysozyme level on days 5-6. A steady increase in lymphocytic activity in the period of from 7-10 to 20 days was probably caused by accumulation of a large number of macrophages—a source of lysozyme—in the focus of inflammation. Moreover, we did not exclude lysozyme produced by the lacrimal gland. It should be noted that we did not established a significant change in the lysozyme activity of blood serum in both Test and Control steers (table 3).

**Table 3.** Lysozyme activity of blood serum in experimental M. bovis keratoconjunctivitis.

| Day      | Lysozyme activity (μg/ml) |
|----------|---------------------------|
| Before infection | 720.5 ± 8.4             |
| 4        | 810.5 ±10.8              |
| 7        | 815.5 ± 12.8             |
| 10       | 820.73 ± 10.2            |
| 15       | 828.6± 15.6              |
| 20       | 825.2 ± 20.3             |
| 25       | 830.3 ±7.5              |

Note: p >0.05

Thus, the change in the lysozyme activity of the lacrimal fluid with a constant content of lysozyme in the blood serum indicated a great importance of lysozyme as a factor of local nonspecific eye protection.

Inflammation is known to be an initial response that develops when tissues are damaged and pathogenic agents act on the body. Local and general manifestations of the inflammatory process can affect the immune response. The inflammatory response is accompanied by an increase in acute protein deficiency in the blood plasma.
The results obtained indicate that the level of total protein in the blood serum of Test steers was notably affected and manifested in an increase (P<0.05) in the total protein concentration in the acute period of the disease (9.92±0.45 mg/ml on day 7 and 10.09±0.87 mg/ml on day 15) compared with the recovery period (8.92±0.24 mg/ml on the day 20, P<0.05) and Control group (8.61±0.37 mg/ml, P<0.05) regardless of the clinical course of the disease (table 4).

Our study results suggest that a significant 15% increase in total serum was due to proteins of the acute phase of inflammation or adaptive proteins synthesized in the liver. A decrease in total blood serum protein in transition from the acute period to the subacute period of the disease was caused, in our opinion, by a decrease in the albumin fraction resulting from the inhibition of its synthesis that indicated the hepatotropic role of the pathogen.

Thus, an increase in the total protein of blood plasma is inextricably linked with the inflammatory phenomena in the conjunctiva and cornea, which indicated certain mechanisms of the general protection involved into the inflammatory process.

The data analysis of the total protein content showed an inverse relationship between the content of the studied indicator in the lacrimal fluid and blood serum.

A decrease in the protein of the lacrimal fluid (from 7.14±0.13 mg/ml to 4.67±0.8 mg/ml) on day 2 was probably caused by profuse lacrimation, dilution, and leaching of protein fractions with conjunctival surface (table 4).

Table 4. Concentration of total protein in the lacrimal fluid in experimental keratoconjunctivitis.

| Disease period | Day | Control, mg/ml | P | Test, mg/ml | P |
|----------------|-----|----------------|---|-------------|---|
| Before infection | 2   | 7.14±0.13      | <0.05 | 7.0±0.8     | >0.05 |
| 1              | 3   | 4.67±0.8       | <0.05 | 7.2±0.14    | >0.05 |
| 2              | 4   | 3.71±0.67      | <0.05 | 7.1±0.13    | >0.05 |
| 3              | 7   | 3.48±0.68      | <0.05 | 7.2±0.05    | >0.05 |
| 4              | 10  | 3.95±0.86      | <0.05 | 7.2±0.33    | >0.05 |
| 5              | 15  | 4.20±0.02      | <0.05 | 7.3±0.1     | >0.05 |
| 6              | 20  | 4.75±0.11      | <0.05 | 7.1±0.1     | >0.05 |
| 7              | 25  | 4.26±0.68      | <0.05 |            |     |
| 8              | 30  | 5.30±0.05      | <0.05 |            |     |
| 9              |     | 6.11±0.13      | >0.05 |            |     |

The increase in the total protein of the lacrimal fluid in the subacute and chronic periods was caused, in our opinion, by a decrease in lacrimation and inflammatory signs of the anterior segment of the eye. We found no significant changes in the total protein of blood serum and lacrimal fluid in Control steers. Considering the above, we can claim about the perfect plasticity of the body’s defense mechanism, which confirmed the hypothesis that young cattle’s keratoconjunctivitis caused by Moraxella bovis is a polysystemic disease.

Providing early protection of the body from the contagious matter, humoral factors thereby give the body time to form a more perfect specific immunity [9]. Bactericidal activity is a total action score of the humoral defense factors.

The study results obtained indicate that the bactericidal activity of the blood serum in Test steers was significantly affected and manifested in its increase in the acute period of the disease to 98.09±1.47% on day 10 (P<0.05) compared with the recovery period (72.8±7.3%) and Control group (70.54±5.7%) (table 5).

We also established a direct relationship between the severity of clinical signs in the anterior segment of the eye and the bactericidal activity of the lacrimal fluid.

A decrease in the bactericidal activity of the lacrimal fluid in the acute period of the disease (from 89.22±16.6% to 34.4±4.32% on day 7) reduced the resistance of the conjunctiva to pathogenic
microflora and promoted the corneal ulcers. A decrease in the bactericidal activity of the lacrimal fluid may be caused due to the inhibition of local humoral protection factors by microbial toxins.

Thus, the inflammation in the anterior segment of the eye directly depends on local ocular stability.

**Table 5.** Bactericidal activity of blood serum in experimental Moraxella bovis keratoconjunctivitis.

| Day   | Control, %       | Test, %       |
|-------|------------------|---------------|
| Before infection | 70.16 ± 4.95 | 70.16 ± 4.95 |
| 4     | 74.35 ± 5.65    | 77.53 ± 6.1  |
| 10    | 70.54 ± 5.7     | 98.09 ± 9.47x|
| 20    | 72.62 ± 6.73    | 72.62 ± 6.73 |
| 25    | 70.80 ± 5.6     | 72.80 ± 7.73 |
| 30    | 70.60 ± 3.95    | 72.65 ± 4.52 |

Note: x: p<0.05.

**4. Conclusions**

Thus, in the first half of the acute period of conjunctivitis—days 1-7 after infection, there was found a 2.5 times decrease in bactericidal activity in the lacrimal fluid (up to 34.4±4.3%), total protein 2 times (up to 3.48±0.8 mg/ml), IgA 2 times (up to 70±3 μg/ml), and IgG 1.8 times (up to 59±5.8 μg/ml). The lysozyme content increased 3-4 times on day 2 of illness and decreased to a low level (up to 24.4±7.4 μg/ml) on days 5-7. After a one-day 1.5 times decrease, the IgM level was gradually increasing. Against the background of a decrease in the indices of local protection, there was registered an increase (8 times) in bacterial contamination of the conjunctiva.

In the second half of the acute period of the disease (days 7-14), a gradual increase in the contents of IgG (from 59.5±5.8 to 108±19.8 μg/ml), IgA (from 7±3.9 to 97±6.3 μg/ml), lysozyme (from 24.4±7.4 to 524±39 μg/ml), bactericidal activity (from 34.4±4.3 to 50.8±13.1%), and total protein (from 3.48±0.8 to 4.75±1.1 mg/ml) was found. The bacterial sensitization of the conjunctiva decreased from 4036000±7700 to 232200±9600 microbial bodies/ml.

The bactericidal activity in Test group showed an increase in the blood serum of up to 100% and total protein 1.4 times in comparison with Control group; the lysozyme activity and the contents of IgG and M were at the initial level.

The subacute period was characterized by the activation of local defenses of the body, i.e. a further increase in the levels of Ig A and G to 109±4 and 240±7 μg/ml, respectively, and bactericidal and lysozyme activities by 61.6±12.4% and 624±63.6 μg/ml, respectively. In the blood serum, there was revealed a decrease in total protein and bactericidal activity to 8.92±0.24 mg/ml and 72.6±6.78, respectively. During this period, no significant changes in M and G immunoglobulins or lysozyme activity of Test steers were observed in comparison with Control steers. In the chronic period, a decrease in both local and general factors of natural resistance was established.

The data obtained make it possible to expand understanding of the pathogenetic and immunotrophic mechanisms of keratoconjunctivitis of moraxellosis etiology and improve the treatment and prophylactic tactics, taking into account the revealed needs to correct nonspecific resistance reactions to a susceptible model - young cattle at the age of puberty (unstable hormonal and adaptogenic mechanisms).

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