UDC 619:616-07,619:579.62

MORAXELLA SPECIES DIVERSITY IN INFECTIOUS BOVINE KERATOCONJUNCTIVITIS IN NOTHERN KAZAKHSTAN

Kuibagarov M.A.[1], Kamalova D.K.[1], Shustov A.V.[1], Suminov A.A.[2], Karibaev T.B.[3], Ryskeldina A.Zh.[1], Shevtsov A.B.[1]

[1]National Center for Biotechnology
13/5, Korgalzhyn road, Astana, 010000, Kazakhstan
[2] "Genesis.KZ" LLP
13/5, Korgalzhyn road, Astana, 010000, Kazakhstan
[3]National Reference Center for Veterinary Medicine
22/3, 150 let Abaya str., Astana, 010000, Kazakhstan
marat.kuibagarov@gmail.com

ABSTRACT

The purpose of this work was to study bacterial species diversity in *Moraxella* involved in infectious bovine keratoconjunctivitis (IBK) in Northern Kazakhstan. DNA were isolated and *Moraxella* spp. cytotoxin A fragments were amplified in 29 samples taken from cattle with clinical signs of IBK. Nucleotide sequence analyses and phylogenetic studies showed that *Moraxella bovis* DNA was present in ten samples, *Moraxella bovoculi*/*Moraxella ovis* DNA was present in eight samples, and that mixed profiles (mixed-infection or contamination) were found in eleven samples. *Moraxella bovoculi*/*Moraxella ovis* were isolated from bovine biological material with clinical signs of IBK for the first time in Kazakhstan.

Keywords: *Moraxella* in infectious bovine keratoconjunctivitis, bacterial cytotoxin A analysis, *Moraxella bovis* in Northern Kazakhstan

---

INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK) or «pink eye» is an acute contagious disease characterized by lacrimation, hyperemia of conjunctival vessels, photophobia, serous-purulent discharge, opacity and ulceration of a cornea, deformation of an eyeball in a form of keratoconus, partial or total loss of vision in an affected animal's eye [1]. IBK is the most common eye disease of cattle and can affect all breeds of cattle, however, the highest morbidity is recorded in breeds with no pigmentation in the eye area (mostly Hereford lines) [2,3,4,5]. On the territory of Kazakhstan, this infection, in addition to Hereford, is most often registered with the Auliekol and Kazakh white-headed local breeds [6].

A cause of IBK in cattle is thought to be a combination of physical factors and a biological pathogen. The main causative bacterial agent of IBK which is most often isolated from sick animals is a bacterium *Moraxella bovis* [7,8,9]. However, there are also data which show a possibility of colonization of conjunctiva by *Moraxella bovis* opportunistically, without an appearance of typical clinical signs [10]. Among different microorganisms which can influence the IBK pathogenesis, there should be noted *Moraxella bovoculi*, *Mycoplasma spp*., some viruses which can either aggravate severity of the disease or serve as predisposing factors for infection [11, 12, 13, 14, 15, 16].

In recent years, IBK in Kazakhstan is often recorded in cattle on farms of various forms of ownership. However, information about the etiologic agent in the IBK cases varies and is

presented in limited sources. A purpose of this study was to describe specific diversity of bacterial pathogens which cause IBK.

The research was carried out at the Laboratory of Applied Genetics of the RSE "National Center for Biotechnology" of the Ministry of Education and Science of the Republic of Kazakhstan.

MATERIALS AND METHODS

For this study samples of total DNA were isolated from swabs taken from eyes of cattle with clinical signs of IBK. Collection of samples from the affected eyes of animals was performed by introducing dry sterile cotton swabs into the conjunctival sac. Swabs were placed separately in sterile test tubes and delivered to the laboratory in a cooling container with ice.

To the tubes, 1 ml of 0.5X TE buffer was added, vortexed vigorously for about 1 minute. The liquid was taken into an Eppendorf tube (1.5 ml), centrifuged at 10,000 rpm for 3 min. Portions (100 μl) of 0.5X TE buffer was added to the pellet, resuspended and used for DNA isolation using the “DNA-sorb-B” kit (InterLabService, Russia). A concentration of DNA was determined spectrophotometrically using a NanoDrop1000 spectrophotometer.

Analysis of PCR-amplified DNA fragments was carried by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Electrophoresis was performed in a horizontal electrophoresis chamber PowerPac, using BioRad Electrophoretic bath current source. A buffer 1X TAE was used as the electrophoresis buffer. Documenting of the results was done using a Gel Doc gel documentation system (Bio-Rad) and Quantity One software (Bio-Rad). Sizes (bp) of the PCR amplificates were determined by comparing their electrophoretic mobility in the gel with a mobility of marker’s ladder (DNALadder 1kb, Fermentas).

A selection of PCR-primers was carried out with a use of programs PrimerSelect (DNASTAR), BioEdit and a web-resource NCBI PrimerBlast. During the primer selection process, following key parameters were considered: similar annealing temperatures for forward and reverse primers, length of primers between 18-25 bp, low probability of formation of secondary structures.

Genotyping was performed by PCR-amplification of a fragment of a cytotoxin A gene followed by a sequence determination. Purification of the PCR products from primers was performed by enzymatic method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas). The products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The sequences were produced on an automatic genetic analyzer 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences were analyzed and assembled into contigs and alignments using SeqMan software (DNAStar). After that, uninformative sequences were removed (primers, and fragments having a low-quality index). The sequences obtained were identified using GenBank BLAST algorithm. Phylogenetic trees were constructed from the sequences deposited in the GenBank database. For phylogenetic studies a software Mega 5 was used. ClustalW algorithm was used to align the nucleotide sequences, and phylogenetic trees were constructed using the Neighbor-joining (NJ) method. Verification of the topology was performed using the "Bootstrap method", number of replications - 1000.

RESULTS

According to published literature, one of the main factors of moraxella pathogenicity is a cytotoxin A [17, 18, 19, 20, 21, 22]. The cytotoxin A gene is found in all three species of moraxella associated with a development of keratoconjunctivitis: *Moraxella bovis, Moraxella bovoculi, Moraxella ovis* (the genes named as *mbxA, mbvA u movA*, respectively). *Nucleotide similarity between the mbvA and movA sequences varies from 2 to 5%, and between mbxA*

from 77 to 82%. This level of nucleotide variability allows using the cytotoxin A nucleotide sequences for a selection of groups of primers for use in a species-specific detection of all three species. To calculate the primers, from the international database GenBank (April 2018), 52 deposited sequences of the *Moraxella spp* cytotoxin A gene were downloaded. As a result, a couple of primers (*Mor_cyto_F*3200 and *Mor_cyto_R*3675) were designed, which are specific (PrimerBlast, https://www.ncbi.nlm.nih.gov/tools/primer-blast) to the target gene of the three species of *Moraxella spp*.

For this study, from five farms of Akmola region (Central Kazakhstan), 29 ocular swab samples were taken from diseased cattle (belonging to breeds Kazakh white-headed, Holstein, Hereford). The cattle presented with varying degrees of clinical signs of IBK: *conjunctival hyperemia*, corneal erosion, corneal ulceration, internal membranes prolapsed (keratoconus) with loss of ocular substance (Fig. 1). Sampling was conducted during May-August 2018.

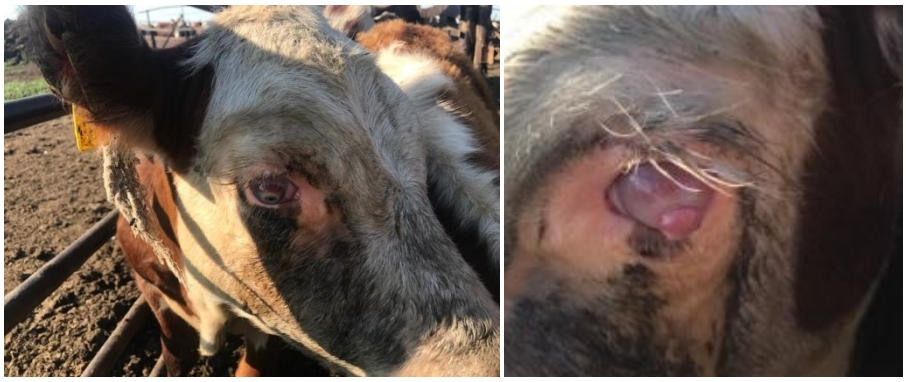
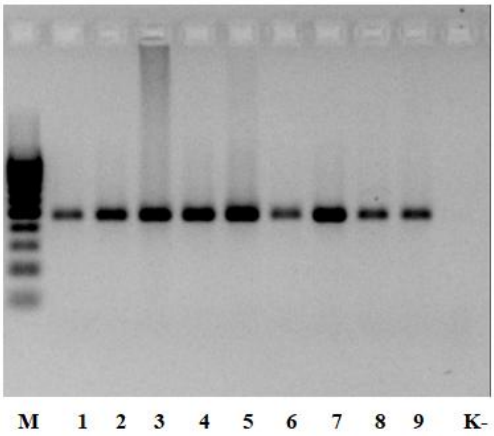


Fig. 1. Clinical signs of IBK in cattle

PCR on total DNA as the template was performed using the selected primers Mor_cyto_F3200 and Mor_cyto_R3675, under optimized conditions, which allow a fragment of a specific length (480 bp) of the cytotoxin A gene to be amplified. As an example, fig. 2 shows the results of amplification of 9 samples.



Lanes: 1-9, DNA samples; M, molecular weight marker (Fermentas, 100-1000 bp, step 100 bp); K-, negative control of an amplification reaction

Fig. 2. PCR results of 9 DNA samples isolated from biological material

Specific PCR products were obtained in 29 samples. Sequencing was carried out for further species identification. In the sequencing electrophoregrams, for 9 samples superposition of signals was found at about 15% of the nucleotides, which indicated contamination of samples with DNA from different but closely related species. Comparison of these sequences with the reference sequences *M. bovis* Epp63 strain and *M. bovoculi* 237 strain made it possible to establish that the mixed signals are because of differing nucleotides between the species (fig. 3).

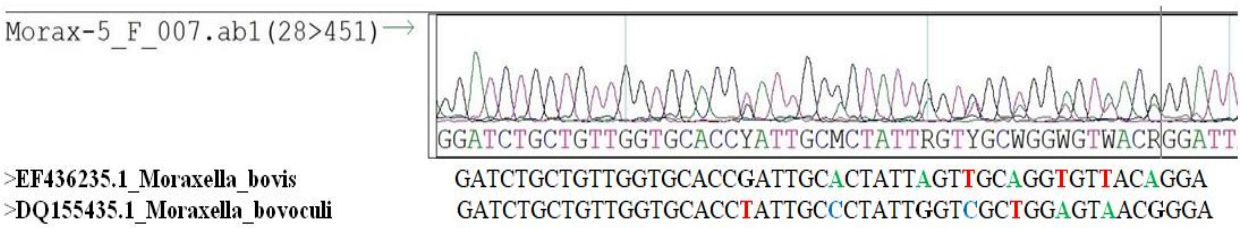


Fig. 3. Comparison of sequences from the field samples with the sequences from reference strains

The results allow concluding that in at least 11 samples two moraxella species are present concominantly: *M.bovis* and *M.bovoculi/M.ovis*. Also the analysis of the fragment of the cytotoxin A gene does not allow for reliable differentiation of *M. bovoculi* from *M.ovis*, since dissimilarity in the nucleotide sequences of the whole gene does not exceed 1.3% (fig. 4).

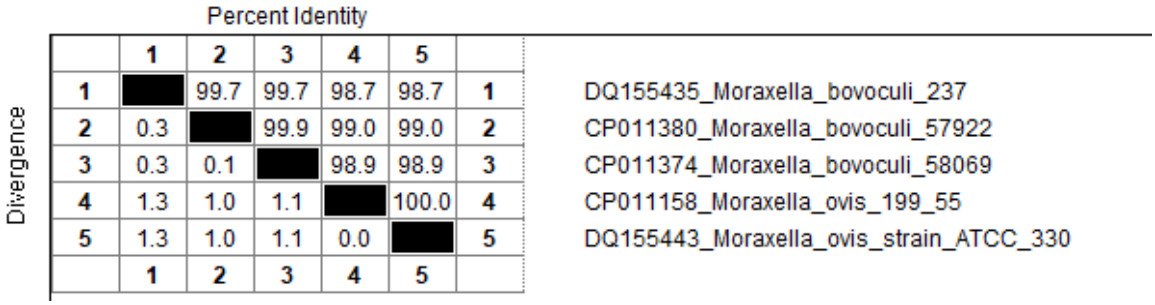


Fig. 4. Nucleotide similarity of *mbvA and movA* genes

The nucleotide sequences of the remaining 18 samples showed no signs of contamination with two or more species and were used to construct a phylogenetic tree (figure 5). The investigated sequences show grouping into two clades. The first clade includes the reference sequence of *M. bovis*, four samples from a farm No.1, two samples from a farm No.4 and four samples from a farm No.5. The second clade includes the reference sequences from *M. bovoculi* and *M. ovis*, as well as two samples from a farm No.1, four sample from a farm No.2 and by one samples from the farms Nos.5 and 3.

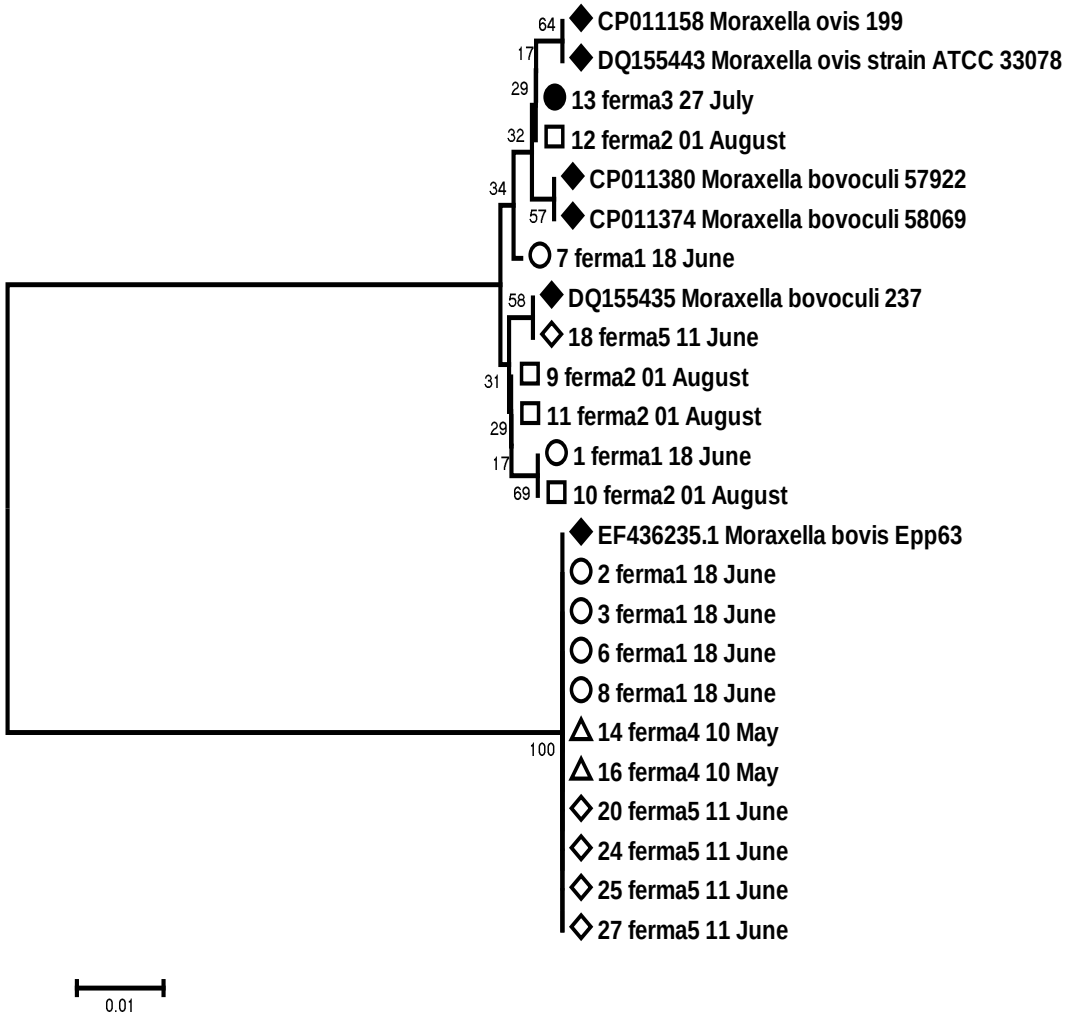


Fig. 5. Phylogenetic tree of the nucleotide sequences of *Moraxella spp* cytotoxin A gene

In the samples from four farms (out of five locations studied), at least two species of *Moraxella spp*. were identified. In the samples from one farm (No.2) only one species was identified: *M. bovoculi/M. ovis* (table 1).

Table 1. Species diversity of *Moraxella spp*.

Farm	Number of samples	Identified species		
		M. bovis	*M. bovoculi /M.ovis*	Mixed species
Farm 1	9	4	2	3
Farm2	4	0	4	0
Farm 3	2	0	1	1
Farm 4	4	2	0	2
Farm 5	10	4	1	5

CONCLUSION

There is still no accepted consensus among experts on an etiology of IBK. In connection with this and an absence of any general strategy for diagnosis and specific prevention of the disease, we can talk about formation of reservoirs of this infectious disease in Central Kazakhstan.

There is little information on a species diversity of bacteria isolated from animals with clinical signs of IBK in the territory of Kazakhstan. In rare publications, data appear on an

isolation of only one species (*Moraxella bovis*) which was confirmed by bacteriological and serological methods [6, 25]. For a long time it was believed that from the moraxella genus the only species taking part in a pathogenesis of IBK is *Moraxella bovis*. However, based on the fact that vaccines utilizing antigen from the sole species *Moraxella bovis* have not always been effective, search for the other involved species were initiated. Accordingly, *Moraxella bovoculi* was isolated and recognized relatively recently. In the summer of 2002, in Northern California, Angelos J.A. isolated hemolytic gram-negative cocci from calves with signs of conjunctivitis. After extensive bacteriological, biochemical and molecular-genetic analyzes the microorganism was classified as a new species *Moraxella bovoculi*, the nomenclature of which was published on April 5, 2005 [16, 26].

In recent years, an increasing number of cases of a so-called «Winter Pinkeye» had been reported, and epizootologic signs of the disease are distinctive in that the disease is registered off-season, when majority of pathogenic factors are excluded (extreme UV light, flying insects, wind, plants, dust, etc.) [27, 28]. What exactly causes the winter IBK and what are the factors that increase its spread, are currently not clear. The causing factor may appear to be a moraxella of a different species or maybe changes in a quantitative ratio of the known species play a role.

In a large portion of the examined samples, a presence of sole or mixed species was determined: *M.bovis* was detected in 10 samples (34%), *M. bovoculi/M.ovis* in 8 samples (27%), and 11 samples contained mixtures of *M. bovis* and *M. bovoculi/M.ovis* (37%).

In conclusion, attention should be paid to the occurrence of IBK in the non-typical seasons such as winter. The obtained data can be used in a development of methods for genetic diagnosis, prevention and treatment of IBK.

Acknowledgements

This study was funded by the Ministry of Education and Science of Kazakhstan by BR05236307 STP "Development of new tools and innovative technologies for agriculture and veterinary medicine" for 2018-2020 years.

REFERENCES

1. Spiridonov G.N. Infectious keratoconjunctivitis of cattle // Problems of prevention and control of especially dangerous, exotic and poorly studied infectious animal diseases. Proceedings of the international scientific production conference dedicated to the 50th anniversary of VNIIVViM. Pokrov, 2008, vol. 2, pp. 195-197.

2. Frisch J.E. The relative incidence and effect of bovine infectious keratoconjunctivitis in Bos indicus and Bos taurus cattle. *Anim Prod*, 1975, vol. 21, pp. 265-274.

3. Snowder G.D, Van Vleck L.D, Cundiff L.V, et al. Genetic and environmental factors associated with incidence of infectious bovine keratoconjunctivitis in preweaned beef calves. *J Anim Sci*, 2005, vol. 83, pp. 507-518.

4. Webber J.J, Selby L.A. Risk factors related to the prevalence of infectious bovine keratoconjunctivitis. *J Am Vet Med Assoc*, 1981, vol. 179, pp. 823-826.

5. Ward J.K, Neilson M.K. Pinkeye (bovine infectious keratoconjunctivitis) in beef cattle. *J Anim Sci, 1979*, vol. 49, pp. 361-366.

6. Ivanov N.P., Sultanov A.A., Bakiyeva F.A., Sattarova R.S., Egorova N.N. Moraxellosis of cattle in Kazakhstan // PSU named after S. Toraigyrov. News of NAS Republic of Kazakhstan. A series of agricultural sciences, 2016, no. 5.

7. Alexander D. Infectious bovine keratoconjunctivitis: A review of cases in clinical practice. Veterinary Clinics of North America: *Food Animal Practice*, 2010, vol. 26, pp. 487-503.

8. O'Connor A.M., Shen H.G., Wang C., Opriessnig T. Descriptive epidemiologyof *Moraxella bovis*, *Moraxella bovoculi* and *Moraxella ovis* in beef calves with naturally occurring infectious bovine keratoconjunctivitis (pinkeye). *VeterinaryMicrobiology*, 2012, vol. 155, pp. 374-380.

9. Lepper A.W., Barton I.J. Infectious bovine keratoconjunctivitis: Seasonal variation in cultural, biochemical and immunoreactive properties of *Moraxella bovis* isolated from the eyes of cattle. *Australian Veterinary Journal*, 1987, vol. 64, pp. 33-39.

10. Pugh G.W., Jr., McDonald T.J. Identification of bovine carriers of Moraxellabovis by comparative cultural examinations of ocular and nasal secretions. *American Journal of Veterinary Research*, 1986, vol. 47, pp. 2343-2345.

11. Rosenbusch R.F. Influence of mycoplasma preinfection on the expressionofMoraxella bovis pathogenicity. *American Journal of Veterinary Research*, 1983, vol. 44, pp. 1621-1624.

12. Langford E.V., Leach R.H. Characterization of a mycoplasma isolated frominfectious bovine keratoconjunctivitis: *M. bovoculi* sp. nov. *Canadian Journal of Microbiology*, 1973, vol. 19, pp. 1435-1444.

13. Levisohn S., Garazi S., Gerchman I., Brenner J. Diagnosis of a mixed mycoplasma infection associated with a severe outbreak of bovine pinkeye in young calves. *Journal of Veterinary Diagnostic Investigation*, 2004, vol. 16, pp. 579-581.

14. Naglic T., Sankovic F., Madic J., Hajsig D., Seol B., Busch K. Mycoplasmasassociated with bovine conjunctivitis and keratoconjunctivitis. *Acta Veterinaria Hungarica*, 1996, vol. 44, pp. 21-24.

15. Angelos J.A. *Moraxella bovoculi* and infectious bovine keratoconjunctivitis:Cause or coincidence? Veterinary Clinics of North America: *Food Animal Practice*, 2010, vol. 26, pp. 73-77.

16. Angelos J.A., Spinks P.Q., Ball L.M., George L.W. *Moraxella bovoculi* sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. *International Journal of Systematics and Evolutionary Microbiology*, 1986, vol. 57, pp. 789-795.

17. Barber D.M., Jones G.E., Wood A. Microbial flora of the eyes of cattle. *Veterinary Record*, 2007, vol. 118, pp. 204-206.

18. Galva˜o K., Angelos J.A. Ulcerative blepharitis and conjunctivitis in adult dairy cows and association with *Moraxella bovoculi*. Can Vet J, in press.

19. Jayappa H.G., Lehr C. Pathogenicity and immunogenicity of piliated and nonpiliated phases of *Moraxella bovis* in calves. *Am J Vet Res*, 1986, vol. 47, pp. 2217-2221.

20. Annuar B.O, Wilcox G.E. Adherence of *Moraxella bovis* to cell cultures of bovine origin. *Res Vet Sci*, 1985, vol. 39, pp. 241-246.

21. Hoien-Dalen P.S., Rosenbusch R.F., Roth J.A. Comparative characterization of the leukocidic and hemolytic activity of *Moraxella bovis*. *Am J Vet Res*, 1990, vol. 51, pp. 191-196.

22. Kagonyera G.M., George L.W., Munn R. Cytopathic effects of *Moraxella bovis* on cultured bovine neutrophils and corneal epithelial cells. *Am J Vet Res*, 1989, vol. 50, 10-7.

23. Kagonyera G.M., George L.W., Munn R. Light and electron microscopic changes in corneas of healthy and immunomodulated calves infected with *Moraxella bovis*. Am J Vet Res, 1988, vol. 49, pp. 386-395.

24. Rogers D.G., Cheville N.F., Pugh G.W. Jr. Pathogenesis of corneal lesions caused by Moraxella bovis in gnotobiotic calves. *Vet Pathol*, 1987, vol. 24, pp. 287-295.

25. Uskenov R.B., Domanov D.I., Mukhanbetkaliev E.E., Eshzhanov T.E. Monitoring of infectious keratoconjunctivitis in the northern region of Kazakhstan. Proceedings of the International Scientific Conference «Modern Methodology of Science and Education». September 18, 2017, Warsaw, Poland, 2017, vol. 4, pp. 93-95.

26. Angelos, J.A., Ball L.M. Differentiation of *Moraxella bovoculi* sp. nov. from other coccoid *moraxellae* by the use of polymerase chain reaction and restriction endonuclease analysis of amplified DNA. *J. Vet. Diagn.Invest,* 2007a, vol. 19, pp.. 532-534.

27. Winter Pinkeye in Cattle. Livestock Update, January 2000. Available at: https://www.sites.ext.vt.edu/newsletter-archive/livestock/aps-00_01/aps-0161.html (accessed 26 September 2018).

28. Winter Pinkeye. Available at: https://addisonlabs.com/wp-content/uploads/2016/02/WinterPEVetAdvant.pdf (accessed 26 September 2018).