

Drift of antimicrobial resistance of bacterial pathogens of the *Moraxella* group

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Abstract. The multiple resistance of bacteria to antibiotics leads to a decrease in the effectiveness of treatment methods for bacterial diseases, an increase in morbidity and mortality and, in some cases, to prolonged bacterial transmission. In this regard, the search for new antimicrobial agents and the need to improve veterinary approaches in therapeutic and preventive work with bacterioses is currently a priority task. The study is devoted to the assessment of antibiotic resistance in zoopathogenic moraxella pathogens of infectious bovine keratoconjunctivitis. The article analyzes the drug resistance of topical epizootic strains of *Moraxella bovis* and *M. bovoculi*. It was found that since the 1982 study and ending with the own results obtained at the present time, there has been a noticeable increase in the resistance of *M. bovis* and *M. bovoculi* to antibiotics. However, a still high level of sensitivity in moraxella is detected for antibiotics of the fluoroquinolone and cephalosporin groups, as well as for ampicillin and benzylpenicillin, the studied bacteria were the most sensitive despite the fact that resistance was recorded for oxacillin and methicillin from the same group.

1 Introduction

One of the most difficult and urgent problems in modern veterinary medicine is infectious bovine keratoconjunctivitis (IBK). Bovine keratoconjunctivitis is a disease, the main causative agent of which is *Moraxella bovis* [1,2,3], characterized by a high degree of spread, causing serious and purulent discharge from the eyes, photophobia, conjunctival vascular hyperemia, blepharospasm and iridospasm. In addition to *M. bovis*, *M. bovoculi* is in second place in terms of isolation from clinical samples, which has been described quite recently – since 2007 [4,5], and is considered one of the causative agents of IBK. Therefore, it is also very important to know the role of *M. bovoculi* in the etiology of infection in order to successfully and effectively combat the disease. To do this, you need to know the sensitivity of these pathogenic microorganisms to antibacterial drugs that are used as a means of choice in the treatment and therapy of IBK. Antimicrobials, which are registered as the main means of combating infectious keratoconjunctivitis, are few. For example, in Australia, cloxacillin, cephalonium, florfenicol and tetracycline have been registered for the treatment of IBK [6],

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in the USA only oxytetracylin, an antibiotic of the broad-spectrum tetracycline group, and tulathromycin, a macrolide antibiotic [7]. In general, studies on sensitivity to antimicrobial drugs *M. bovis* and *M. bovoculi* are few in the literature.

The diagnosis of IBK is established based on comprehensive epizootological, clinical, virological and serological studies with bacteriological examination to detect the presence of *M. bovis* and/or *M. bovoculi* in the selected samples. In addition, during laboratory diagnosis, it is necessary to exclude invasive rickettsiosis keratoconjunctivitis and eye disease mediated by avitaminosis [8].

Moraxella bovis is a gram-negative, acid-resistant and polymorphic (cells are more like beans) microorganism of the genus *Moraxella* of the family *Moraxellaceae* [9]. *M. bovis* has a tropism for mucous membranes and produces a toxin capable of destroying the skin of the animal's eye [1]. *Moraxella bovoculi* are gram-negative diplococci, occasionally cocci, in terms of enzymatic activity differ from other species of the genus *Moraxella* due to the presence of phenylalanine deaminase. This species was differentiated using genomic analysis, which showed that, judging by the sequence of the 16S-23S rRNA gene, the type strain of *M. bovoculi* is most closely related to *M. bovis* and *M. ovis* in the taxonomic rank of classical moraxella, and that, apart from the presence of phenylalanine deaminase activity, *M. bovoculi* does not differ from *M. bovis* in other parameters [5].

The high incidence of cattle leads to significant economic damage due to the culling of animals, a decrease in dairy productivity in cows and weight gain in calves, deterioration of animal feeding, which negatively affects the quality reproduction of the herd and production.

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A number of vaccines have been developed for the prevention of IBK, but the problem of effective and economically feasible treatment of animals with IBK remains unresolved. So, for example, by Russian scientists, Gaffarov H.Z. Dupleva L.Sh., Spiridonov G.N. et al. (2004), from the Federal Center for Toxicological, Radiation and Biological Safety, an inactivated associated hydroxylaluminium vaccine against bovine IBK based on *M. bovis* antigens and herpesvirus type I was developed, which was used in the prevention of IBK.

Vaccines have also been developed by foreign scientists for the prevention of IBK, so, according to Puch G.W.Jr., Hughes D.E., Schulz V.D. et al. (2021), the benefits of vaccines derived from *M. bovis* whole cells included a decrease in the colonization rate of *M. bovis* and protection from homologous strains of *M. bovis* after an experimental trial in which IBK was artificially induced [10].

However, despite years of research on vaccines against IBK, no vaccine has been distributed. Researchers have gained an understanding of the immunology of IBK and the antigenic nature of *M. bovis* and other pathogens, but despite great efforts, the factors contributing to the epizootic effectiveness of the vaccine remain unattainable [11].

Therefore, today the use of antibiotics is the priority method of treating IBK, and therefore there is a threat of antibiotic resistance. The scientific world community has recently been paying more and more attention to the problem of antibiotic resistance. Uncontrolled use of antibiotics, the occurrence of mutations in bacterial cells that cause vertical and horizontal transfer of altered genetic material, including resistance factors, between different types of bacteria are key reasons for the development of multiple resistance of zoopathogens to drugs used in treatment and prevention [12]. In other words, improper use of traditional treatment methods, such as the use of antibacterial chemicals, in case of IBK can increase antibiotic resistance, which, subsequently, can cause low treatment effectiveness, loss and decrease in product quality or the immunity of these bacteria to treatment with antibiotics due to the appearance of resistant strains. The importance of resistant pathogens of productive animals poses a threat, including to human health, since humans are the main consumers of livestock

products. Therefore, monitoring of antimicrobial sensitivity against both pathogenic and commensal bovine bacteria in case of IBK is relevant and recommended by the World Organisation for Animal Health (WOAH-OIE). To do this, it is necessary to follow the basic requirements for the management of organic livestock and the production of organic products, which include, in particular, limiting the use of antibiotics to prevent morbidity and stimulate productivity [13].

2 Purpose and objectives of the study

To study the sensitivity to antibiotics of *M. bovis* and *M. bovoculi*, new pathogenic isolates isolated by us, in comparison with foreign data. To achieve this goal, the following tasks were defined:

1. To analyze the results of sensitivity of zoopathogenic moraxella to antibiotics based on primary scientific data;
2. To conduct a study of the drug resistance of the isolates of infectious keratoconjunctivitis pathogens actually obtained;
3. To assess the spread of antibiotic resistance of moraxell pathogens and justify promising solutions.

3 Materials and methods

The paper uses theoretical research methods such as: topical problematic and retrospective review, systematic analysis of the results of primary scientific research based on articles by foreign authors from various databases of electronic libraries, analysis and synthesis, inductive conclusion, as well as routine methods of microbiological research.

Microorganisms were isolated from pathological material from cattle with signs of keratoconjunctivitis in different regions of the Russian Federation in the period 2022-2023, three of which were identified as *M. bovoculi* – 2 isolates and *M. bovis*.

A disco-diffuse method was used to test for antibiotic resistance. A suspension of bacterial cells corresponding to $1.5 \cdot 10^8$ CFU/ml was prepared from the daily culture. Meat peptone agar (MPA) was used for sowing by the continuous lawn method. 100 μ l of suspension was applied per MPA. Discs with the following antibiotics were used: ampicillin (10 mcg), gentamicin (120 mcg), tetracycline (30 mcg), enrofloxacin (30 mcg), benzopenicillin (10 units), neomycin (30 mcg), lefloxacin (5 mcg), cefuroxime (30 mcg), lincomycin (15 mcg), rifampicin (5 mcg), cefepim (30 mcg), trimethoprim/sulfamethaxazole (1.25/23.75 mcg), oxacillin (10 mcg), carbenicillin (100 mcg), kanamycin (30 mcg), levomycetin (30 mcg), ristomycin (30 mcg), methicillin (10 mcg). The results were evaluated according to the following criteria, where the diameter of the sterility zone is indicated in mm: more than 25 mm – high sensitivity, 15-25 mm – medium sensitivity, 10-15 mm – low sensitivity, <10 mm – resistance.

The results were analyzed after 20 hours of thermostating at 37 °C. The experiment was performed in accordance with the Guidelines 4.2. 1890-04 [14].

4 Results of the study

One of the early studies of the sensitivity of *M. bovis*, the causative agent of IBK, to antibiotics was the work carried out by Webber J.J., Fales W.H., Selby L.A. (1982). Ими было протестировано 84 изолята *M. bovis*, 71 из которых были выделены от крупного рогатого скота в 54 эпизоотиях ИКК в штате Миссури в период с 1978 по 1981 год, а 13 оставшихся были получены из библиотеки штаммов They tested 84 isolates of *M.*

bovis, 71 of which were isolated from cattle in 54 epizootics of infectious keratoconjunctivitis in Missouri between 1978 and 1981, and the remaining 13 were obtained from the strain library of the National Center for Animal Disease Control, Ames, Iowa. Sensitivity was determined by both the disco-diffuse method and the broth microdilution method. Sensitivity was determined *in vitro* to 13 antimicrobial drugs. The results of the study were as follows: all tested *M. bovis* strains were resistant to cloxacillin, 68% of hemolytic isolates showed resistance to streptomycin, whereas all non-hemolytic isolates were sensitive to streptomycin. One hemolytic isolate was resistant to triple sulfonamides. The values of the minimum inhibitory concentration (MIC) for cloxacillin, gentamicin, penicillin and streptomycin were significantly ($P < 0.01$) higher for hemolytic isolates than for non-hemolytic isolates. The obvious uniform resistance to cloxacillin, determined by the disco-diffuse method, was confirmed in the determination of MIC: 97% of the tested hemolytic isolates had MIC > 2 mcg/ml, and in 3% the MIC was 1 mcg/ml. Of the streptomycin-resistant isolates determined by the disco-diffuse method, 87% of the MIC values of streptomycin were ≥ 128 mcg/ml. Isolates that were sensitive to streptomycin, according to the results of the disco-diffuse method, had corresponding values MIC ≤ 0.5 mcg/ml. Contrary to the conclusions of Le Goffic and Martel [15], none of the *Moraxella* isolate was resistant to kanamycin. The results of this study show that all isolates are resistant to cloxacillin and that 68% of hemolytic isolates are resistant to streptomycin, while *M. bovis*, on average, was sensitive to the rest of the antibiotics according to the results of both the disco-diffuse method and the broth microdilution method [16].

An *in vitro* study conducted by scientists from the USA Thomas R. S., Donald W. W., Carolyn S. W. (1998) on the sensitivity of *M. bovis* to seven antimicrobial drugs, as a result of which 55 strains of *M. bovis* were tested, showed that the isolates had the following average values of MIC antibiotics: ampicillin – 0.25 mg/l; ceftiofur – 0.125 mg/l; tilmicosin – 2 mg/l; tylosin – 8 mg/l; erythromycin – 1 mg/l; oxytetracycline – 0.5 mg/l and gentamicin – 0.5 mg/l [17]. We see that most antibiotics have low MIC levels, which indicates the clinical efficacy of the selected antibiotics.

Another study was conducted by Australian scientists from the University of Sydney, McConnell C. S., Shum L., House J. K. (2007), after a natural outbreak of IBK. Antimicrobial sensitivity was determined *in vitro* in accordance with the National Committee on Clinical Laboratory Standards (NCCLS) by the disco-diffuse method [18]. Sensitivity was tested to cloxacillin (5 mcg), cephalonium (30 mcg), florfenicol (30 mcg) and tetracycline (30 mcg). All isolates of *M. bovis* obtained for this study from cattle in New South Wales, Victoria, Tasmania and South Australia demonstrated full sensitivity to all antimicrobial drugs tested [6]. The complete sensitivity of *M. bovis* isolates to the tested antibiotics also indicates the high clinical efficacy of these drugs against IBK.

In 2011, a study was conducted by a group of scientists from the USA Angelos J. A., Ball L. M., Byrne B. A. in order to find out the minimum inhibitory concentrations of antimicrobials for *M. bovoculi*. 57 field isolates cultured *in vitro* from the eyes of sick animals in California from 2002 to 2007 were used for the study. As a result of the study, the minimum inhibitory concentrations necessary to suppress the growth of 90% of microorganisms (MIC₉₀) were determined for the following 18 antibiotics: danofloxacin and enrofloxacin: ≤ 0.12 mcg/ml; ampicillin and ceftiofur: ≤ 0.25 mcg/ml; penicillin: 0.25 mcg/ml; gentamicin: ≤ 1 mcg/ml; chlortetracycline, oxytetracycline and thiamulin: 1 mcg/ml; florfenicol: 0.5 mcg/ml; trimethoprim-sulfamethoxazole: $\leq 2/38$ mcg/ml; clindamycin: 2 mcg/ml; neomycin and tilmicosin: ≤ 4 mcg/ml; tulatromycin: 4 mcg/ml; spectinomycin and tylosin: 16 mcg/ml; and sulfadimethoxine: > 256 mcg/ml [19]. A small MIC₉₀ indicates the clinical efficacy of the selected antibiotics as a means of combating IBK.

In 2014, Russian scientists Subbotin V.V. and Karaichentsev D.V. conducted a sensitivity study of 27 *M. bovis* isolates isolated from sick animals from farms located in the Belgorod

and Kursk regions. As a result of the conducted studies, the following conclusions were made: *M. bovis* cultures showed sensitivity to moxifloxacin, MPC = 0.01–0.04 mcg/ml, gentamicin 0.25–1.0 mcg/ml, levofloxacin 0.1–0.8 mcg/ml, lincomycin 0.1–0.4 mcg/ml, benzylpenicillin 0.25–0.5 mcg/ml, streptomycin 0.5–1.1 mcg/ml, oxytetracycline 3.2–6.4 mcg/ml, oxacillin 4.5–9.0 mcg/ml, roxithromycin 5.8–11.6 mcg/ml. However, at the same time, the isolates of *M. bovis* turned out to be resistant to sulfadimesine, phthalazole, and nystatin, as the MIC turned out to be more than 100 mcg/ml [20]. We observe, on average, a low MIC, which indicates the effectiveness of the tested drugs. However, the MIC of oxytetracycline increased by about 4.5 times, which may indicate the emergence of resistance to this antibiotic.

In 2015, scientists from Brazil Maboni G, Gressler L. T., Espindola J. P. et al. a study was conducted in which the sensitivity profiles of 32 strains of *Moraxella spp.*, namely 10 strains of *M. bovis*, 11 strains of *M. bovoculi* and 11 strains of *M. ovis* to a number of antimicrobial drugs were compared. Because of the study, the minimum inhibitory concentrations necessary to suppress the growth of 90% of organisms (MIC₉₀) were determined for the following 10 antibiotics: enrofloxacin: 0.06 mcg/ml; florfenicol, gentamicin, neomycin, ceftiofur: 1 mcg/ml; ampicillin and penicillin: 2 mcg/ml; oxytetracycline: 8 mcg/ml; cefoperazone and cloxacillin: ≥ 32 mcg/ml is for *M. bovis*. In addition, for *M. bovoculi*: ampicillin and penicillin: ≤ 0.015 mcg/ml; ceftiofur: 0.03 mcg/ml; enrofloxacin, florfenicol, gentamicin: 1 mcg/ml; neomycin: 2 mcg/ml; cefoperazone: 4 mcg/ml; oxytetracycline: 12 mcg/ml; cloxacillin: > 32 mcg/ml [21]. We can observe that most strains have proven to be highly sensitive to a number of antimicrobials, but some strains have shown relative resistance. For example, *M. bovis* is relatively resistant to cefoperazone and cloxacillin, and *M. bovoculi* to cloxacillin and to a lesser extent to oxytetracycline. However, on average, both species showed high sensitivity to the tested antibiotics, from which it can be concluded that these drugs are still highly clinically effective.

Because of our own research, the following data were obtained:

Ampicillin and benzylpenicillin from the penicillin group showed high activity against all three cultures. Carbenicillin, belonging to the same group of antibiotics, showed an average effect on *M. bovis*, but it proved ineffective for *M. bovoculi* strains. Beta-lactam antibiotics from the penicillin group, oxacillin and methicillin, proved to be completely ineffective against the studied bacteria.

As for the aminoglycoside group, gentamicin and neomycin showed average activity against *M. bovis* and *M. bovoculi*. Kanamycin, in turn, showed lower activity and, unlike other tested aminoglycoside antibiotics, had a low effect on *M. bovis*.

Levofloxacin, belonging to the third generation of fluoroquinolones, had a very high effect on all tested isolates. However, enrofloxacin from the same group of antibiotics had lower activity in relation to *M. bovoculi*.

Antibiotics related to the cephalosporin series have also shown positive results. Despite the difference in generations, cefuroxime and cefepime had a higher effect on bacteria of the genus *Moraxella*.

A slight decrease in the sensitivity of *M. bovis* to tetracycline was revealed. The activity in relation to *M. bovoculi* was within acceptable limits.

As expected, rifampicin, used mainly against mycobacteria and non-spore-forming anaerobes, had practically no effect against *Moraxella*. This antibiotic has less activity against Gram-negative bacteria.

The sulfonamide antibacterial combination trimethoprim/sulfamethoxazole had average activity against all the studied bacteria. There is a decrease in sensitivity for *M. bovoculi*.

Despite its high activity against gram-positive bacteria, lincomycin from the lincosamide group had an average activity against *M. bovis*. However, at the same time, both strains of *M. bovoculi* were completely resistant to this type of antibiotics.

Levomyces had high activity, with the exception of one strain of *M. bovoculi* (medium activity).

Ristomycin had an average activity to epizootic isolates, despite the claimed properties of activity only to gram-positive bacteria.

5 Discussion

Studies conducted in the United States have demonstrated unambiguous sensitivity of *M. bovis* to ampicillin, cephalosporin, nitrofurans, penicillin, sulfonamides, tilmicosin and trimethoprim sulfonamide, but different sensitivity to cloxacillin, erythromycin, gentamicin, oxytetracycline streptomycin. Conversely, *M. bovis* has demonstrated resistance to tylosin and lincomycin [16,17]. All Australian isolates of *M. bovis*, without exception, showed complete sensitivity to cloxacillin, cephalonium, florfenicol and tetracycline [6].

Isolates of *M. bovis* isolated in our country showed sensitivity to moxifloxacin, gentamicin, levofloxacin, lincomycin, benzylpenicillin, streptomycin, oxytetracycline, oxacillin, roxithromycin, but at the same time proved resistant to sulfadimisine, phthalazole, nystatin [20]. Brazilian isolates of *M. bovis* were found to be sensitive to enrofloxacin, florfenicol, gentamicin, neomycin, ceftiofur, ampicillin, penicillin, oxytetracycline, but relatively resistant to cefoperazone and cloxacillin [21]. Strains of *M. bovoculi* from Brazil showed relative resistance to oxacillin and, to a lesser extent, to oxytetracycline, but were sensitive to ampicillin, penicillin, ceftiofur, enrofloxacin, florfenicol, gentamicin, neomycin and cefoperazone [21]. Isolates of *M. bovoculi* from the USA turned out to be sensitive to norfloxacin and ciprofloxacin, ampicillin, ceftiofur, penicillin, gentamicin, chlortetracycline, oxytetracycline, thiamulin, florfenicol, trimethoprim-sulfamethoxazole, clindamycin, neomycin, tilmicosin, tulathromycin, spectinomycin, tylosin, but resistant to sulfadimethoxine [19].

The high degree of sensitivity to ampicillin and cephalosporins is also compared with our own data. In the case of trimethoprim/sulfonamide and gentamicin, we can observe a decrease in activity, the MIC of gentamicin for *M. bovis* increased from 0.5 mcg/ml to 1 mcg/ml [17,21]. From this, presumably, it can be concluded that there is a possible correlation between the tendencies of resistance to the previously mentioned antibiotics. From the data of a 1982 study, it was found that none of the isolates was resistant to kanamycin [16], but in their own studies, low and medium sensitivity of *M. bovis* and *M. bovoculi* to this drug can be observed, which once again indicates the development of the process of bacterial resistance to antibiotics. Continuing the analysis of data from the USA, it can be noted that in comparison with other antibiotics, a large MIC was needed for oxytetracycline, it increased from 0.5 mcg/ml to 8 mcg/ml for *M. bovis* [17,20,21], but according to the results of our research, it is clear for our geographical area that the tetracycline antibiotic has an upper limit of medium activity against *M. bovoculi* and lower, but still effective, against *M. bovis*. The data of Brazilian researchers are similar to the results of our analysis – ampicillin, gentamicin and enrofloxacin also show high activity against *M. bovis* and *M. bovoculi* [21], however, there is an increase in MIC ampicillin and gentamicin against *M. bovis* by exactly two times [17,21]. High and medium sensitivity to tetracycline is confirmed by an Australian study [6].

Analyzing our data with studies from neighboring regions of Russia, we can observe a difference in sensitivity to lincomycin, expressed by a decrease in sensitivity. A decrease in the effectiveness of gentamicin is progressing, MIC is effective for treatment, but, at the same time, against the background of other studied antibiotics, it can be seen that its concentration for the suppressive effect of *Moraxella* increases. It was also found that the results of their own research show resistance to doxycycline, as in the case of studies in the Kursk and Belgorod regions conducted by V.V. Subbotin et al. (2014) [20].

6 Conclusions

Based on the results of the practical part of the study, the following can be noted:

1. *M. bovoculi* was not sensitive to oxacillin, methicillin, carbenicillin and lincomycin.
2. *M. bovis* was resistant only to oxacillin and methicillin, which indicates a lower degree of antibiotic resistance compared to *M. bovoculi*.
3. Still a high level of sensitivity in *Moraxella* is detected for antibiotics of the fluoroquinolone group and cephalosporins.
4. Ampicillin and benzylpenicillin have the greatest effect on the studied bacteria, despite the fact that resistance was recorded for oxacillin and methicillin from the same group.
5. In the general assessment, there is a tendency to decrease the sensitivity of *M. bovis* and *M. bovoculi* to antibiotics.

Thus, from the analysis of primary scientific data and our own research, we can conclude that benzylpenicillin, ampicillin, tetracycline and enrofloxacin, drugs of the fluoroquinolone group and cephalosporins are most effective in infectious bovine keratoconjunctivitis. In the case of a number of other drugs, progress has been made in the geographical drift of antibiotic resistance. Doxycycline has become a common ineffective antibiotic for a number of geographical areas.

Despite the effectiveness of antimicrobial therapy, the treatment of affected livestock has many disadvantages, and therefore the prevention of IBK is preferable [17]. However, the issue of long-term specific prevention has not yet shown obvious success. Alternative means of metaprophylaxis of the spread of IBK in disadvantaged areas should probably be considered. Taking into account our retrospective analysis, we observe differences in sensitivity to antimicrobials, in particular, a drift in antibiotic resistance, which, in the future, will lead to the emergence of strains with widespread drug resistance. In this regard, it is promising to consider bacteriophages [22] as a means of antibacterial therapy and short-term prevention of IBK in susceptible herds in disadvantaged areas and threatened areas.

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