The effect of antimicrobials agents on the antilysocyme activity of enterobacteria in vitro

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Abstract. The fight against infectious animal diseases has resulted in a change in the etiological structure of infectious agents. This is associated with the variability of bacteria and the involvement of opportunistic microorganisms in the infectious process. The emergence and development of the pathological process are largely determined by the reduction in the colonization resistance of the gastrointestinal tract shell and the persistent properties of microorganisms. This paper aims to study the antilysozyme activity of enterobacteria isolated from sick calves with signs of acute intestinal disorders and evaluate the effect of antimicrobial drugs on the antilysozyme activity of enterobacteria in vitro. For this purpose, the suspension of an enterobacteria culture was incubated together with the studied antibiotic, followed by the determination of the anti-lysozyme activity and statistical data processing. Data analysis showed that the anti-lysozyme activity of enterobacteria, when exposed to antimicrobial agents, depended on the agent under study and was determined by the specific characteristics of the microorganism. Most of the studied antibiotics had a predominantly inhibitory effect on the antilysozyme activity of *Escherichia coli*, and the persistent potential of bacteria of the Proteus genus was enhanced by the same antimicrobial agents.

1. Introduction

Pathologies of the digestive system continue to occupy one of the leading places in the structure of morbidity and mortality of young farm animals [1]. Digestive disorders in young cattle are traditionally classified as pathologies of non-infectious etiology. However, this classification does not correspond to reality. The existing concept of the etiology and pathogenesis of calves’ digestive system pathology considers it as a multi-factorial process, with increasing importance being attached to the infectious component. The increase in the share of diseases caused by opportunistic microorganisms is due to a violation of the balance between the body, its microflora, and the environment. Gastrointestinal diseases are often caused by mixed microflora. This complicates the course of diseases, as well as the choice of medicines. The fight against infectious animal diseases has resulted in a change in the etiological structure of infectious agents. This is due to the constant variability of bacteria and the involvement of opportunistic microorganisms in the infectious process. These microorganisms are present as commensals in the normal microflora of animals. Undoubtedly, the reduction in the colonization resistance of the gastrointestinal tract is of particular importance in the etiology and pathogenesis of acute intestinal disorders. When a microecological imbalance occurs, strains of persistent potential pathogenic bacteria accumulate in the body, which can weaken the body’s defenses and initiate the development of an infectious process.
Besides, in the occurrence and development of the pathological process, the resistant properties of microorganisms are of great importance [2, 3].

The pathogenic potential of conditionally pathogenic microorganisms is determined to a certain extent by their biological properties. The widespread use of treatment methods, vaccines, and immunostimulators has resulted in changes in the biological properties of microorganisms.

Antibiotics have profound and sometimes persisting effects on the intestinal microbiota, characterized by the diminished abundance of beneficial commensals and increased abundance of potentially detrimental microorganisms [4].

From a general biological point of view, a comprehensive study of persistent characteristics as pathogenicity factors is important. It is known that one of the leading factors of colonization of the host organism by microorganisms and subsequent persistence is the antilysozyme activity of microorganisms. Besides, the optimization of treatment regimens and the prevention of diseases caused by opportunist microorganisms is impossible without a comprehensive study of the persistent characteristics of antimicrobial resistance [5].

It is important to understand the mechanisms of the influence of various factors on the persistence potential of microorganisms. Assessment of persistence potential can be used for practical purposes – diagnostics and treatment of diseases. Understanding these effects will help adapt antibiotic treatment to minimize "collateral damage" [6].

It is known that the use of antimicrobial drugs in the treatment of infectious diseases results in changes in the sensitivity of microorganisms to them or the formation of resistance [7, 8].

It is a well-known fact that the persistence of microorganisms might influence their viability within the macro-organism, whereas the suppression of microbial persistence via drug preparations might greatly reduce therapeutic duration [9].

Thus, this study aims to analyze the antilysozyme activity of enterobacteria isolated from sick calves with signs of acute intestinal disorders and to evaluate the effect of antimicrobial drugs on the antilysozyme activity of enterobacteria in vitro.

2. Research methods

Experimental studies were conducted at the Department of Microbiology, Virology, and Immunology of the Federal State Budgetary Scientific Institution Far East Zone Research Veterinary Institute, Russia, Blagoveschensk.

Material for the study was selected in the base farm. The object of research is a red-mottled newborn young cattle of Holstein breed with signs of gastrointestinal diseases. In the selected farm, the incidence of newborn calves with gastrointestinal diseases is high.

To determine the etiology of the disease, a bacteriological study of the biomaterial from the rectum of the calves with signs of acute intestinal disorders was carried out, biomaterial was investigated using commonly-accepted bacteriological methods [10, 11]. The biomaterial was selected with a sterile swab, placed in a sterile saline solution, and immediately delivered for examination. Pure bacterial cultures were obtained by preparing ten-fold dilutions in saline solution of the initial suspension, followed by seeding on dense nutrient media. The following media were used in the study: meat-peptone agar, Ploskirev medium, Endo medium, bismuth-sulfite agar, salt agar, Chapek medium). The crops were incubated in a thermostat for 24-48 h at a temperature of 37 °C. Morphological properties of isolated cultures of microorganisms were studied by light microscopy. Identification of isolated pure cultures was performed by morphological, tinctorial, biochemical and serological properties. Nine samples of biological material from newborn calves with clinical signs of acute intestinal disorders were subjected to bacteriological research.

The study of antilysozyme activity (ALA) in enterobacteria was carried out according to the method of O V Bukharin [12], in modification of Y A Makarov et al. using the indicator culture of Micrococcus luteus and discs impregnated in lysozyme with a concentration of 1, 2, 3, 4 and 5 μg/ml. The method involves sowing the culture under study on the nutrient medium, while before incubation, disks impregnated with lysozyme are applied to the surface of the sown medium. Antilysozyme active cultures
are those whose growth is not inhibited around one or more disks. The maximum concentration of lysozyme in the disk, around which the growth of the culture is not inhibited, is taken as the level of antilysozyme activity [13].

To assess the influence of various factors on antilysozyme activity, the suspension of daily broth culture of enterobacteria was co-incubated with the studied antimicrobial agent under the conditions of a shaker incubator at 37°C for 60 min. Then the mixture was centrifuged at 3000 rpm, after which the culture was washed twice with normal saline and screened on the plain broth. Inoculations were incubated at 37°C for 16-18 h. After that, antilysozyme activity was determined in cultures. Instead of agents, an isotonic sodium chloride solution was used as a control [14].

Factors studied: ten antibacterial agents of various pharmacological groups, widely used in veterinary medicine to treat animals, in the standard concentration used to determine antibiotic sensitivity.

Statistical data processing was performed using the Microsoft Excel 7.0 application program.

3. Results

As a result of the bacteriological analysis of biomaterial samples obtained from the calves with signs of acute intestinal disorders, cultures of microorganisms belonging to the following genera were identified: *Escherichia*, *Proteus*, *Citrobacter*, *Enterobacter* and *Pseudomonas* – totally of 22 cultures (table 1). Therefrom, microorganisms classified as *Escherichia coli* - three cultures, *Proteus mirabilis* - six cultures and *Pseudomonas aeruginosa* - one culture had pathogenic properties. Other cultures did not exhibit pathogenicity.

Thus, the main infectious agents that cause gastrointestinal diseases in calves are representatives of the enterobacteriaceae family. Herewith, *Escherichia coli* and *Proteus mirabilis* have the greatest etiological significance - their specific gravity in the occurrence of digestive pathology was 33.3% and 40.0%, respectively.

| Type of microorganisms               | Overall |
|-------------------------------------|---------|
| *Escherichia coli*                  | 10      |
| *Proteus mirabilis*                 | 8       |
| *Enterobacter cloacae*              | 2       |
| *Citrobacter diversus*              | 1       |
| *Pseudomonas aeruginosa*            | 1       |
| Isolated microorganisms, overall    | 22      |

Analysis of antilysozyme activity showed that, the prevalence of the antilysozyme trait in the studied microorganisms was 85%, and differed in intensity levels (table 2). Overall, 58.8% of bacteria had a low level of antilysozyme activity, 11.7% had a medium level and 29.4% Overall, had a high level. The average statistical value of the antilysozyme trait in the isolated bacteria was $2.2 \pm 1.67 \mu g/ml$, which is more consistent with the average level.

In bacteria of the genus *Escherichia*, the level of antilysozyme activity was high in 30% of cases, in 20% - medium and in 50% - low. In bacteria of the genus *Proteus*, a high level of trait was detected in 16.7% of cases and low in - 83.3% of cases.

In bacteria of the genus *Citrobacter* isolated in a single case, a high level of ALA was noted. In bacteria *Enterobacter* and *Pseudomonas*, ALA was not detected.

The studied persistent characteristic is an indicator of the adaptive properties of microorganisms to the immune mechanisms of the host organism and the ability to inactivate lysozyme - a factor of natural defense of the body. The presence of this trait in bacteria ensures the long-term survival of the pathogen in the body and the chronization of the inflammatory process.

Changes of the biological properties of pathogens of intestinal infections, and a high incidence of newborn calves make it actual to monitor the persistent characteristics of microorganisms and to study
the factors that could suppress the persistent potential of a pathogen, thereby complicating its parasitization in the host organism.

**Table 2.** Distribution of microorganisms isolated from calves with acute intestinal disorders by levels of antilysozyme activity.

| Type of microorganisms        | The number of studied cultures | The number cultures with ALA | Number of crops with a level ALA | 1-2 μg/ml | 3-4 μg/ml | 5 μg/ml |
|------------------------------|--------------------------------|-----------------------------|---------------------------------|-----------|-----------|---------|
| *Escherichia coli*           | 10                             | 10                          | 5                               | 2         | 3         |
| *Proteus mirabilis*          | 8                              | 6                           | 5                               | -         | -         | 1       |
| *Enterobacter cloacae*       | 2                              | -                           | -                               | -         | -         | -       |
| *Citrobacter diversus*       | 1                              | 1                           | -                               | -         | -         | 1       |
| *Pseudomonas aeruginosa*     | 1                              | -                           | -                               | -         | -         | -       |
| **Overall**                  | **22**                         | **17**                      | **10**                          | **2**     | **5**     |         |

Therefore, we set up an experiment to study the influence of some antimicrobial agents on the antilysozyme activity of pathogenic enterobacteria in vitro. The data obtained in the experiment are presented in the table (table 3).

It was found that the studied cultures of *Escherichia coli* were initially characterized by the following average values of antilysozyme activity - 2.3 μg/ml, and *Proteus mirabilis* – 1.0 μg/ml.

Antimicrobial agents influenced the antilysozyme activity of enterobacteria of the genera *Escherichia* and *Proteus* in different directions.

**Table 3.** The investigation of the effect of antimicrobial factors in antilysozyme activity of enterobacteria in vitro, n=3.

| Antimicrobial agents       | Antilysozyme activity, μg/ml | *Escherichia coli* | *Proteus mirabilis* |
|---------------------------|------------------------------|--------------------|----------------------|
| Control                   | 2.3                          | 1.0                |
| Chloramphenicol, 30 μg    | 1.7                          | 2.0^               |
| Ceftriaxone, 30 μg        | 1.7                          | 2.0^               |
| Enrofloxacin, 5 μg        | 0                            | 2.0^               |
| Kanamycin, 30 μg          | 2.3                          | 3.6^               |
| Ciprofloxacin, 5 μg       | 0.3                          | 2.0^               |
| Amoxicillin/clavunate, 20/10 μg | 0    | 2.0^               |
| Tetracycline, 30 μg       | 2.7^                         | 1.7^               |
| Cefotaxime, 30 μg         | 0.7                          | 2.7^               |
| Ofloxacin, 5 μg           | 1.7                          | 1.3^               |
| Benzylpenicillin, 6 μg    | 0.3                          | 0.7                |

Note: ^ – increase of the attribute value in comparison with the control.

The main part of the studied antibiotics produced a greater or lesser inhibitory effect on the antilysozyme activity in *Escherichia coli*. Only tetracycline slightly increased the manifestation of the trait by 1.1 times. The effect of kanamycin remained indifferent.

The persistent potential of bacteria of the genus *Proteus* was enhanced under the most antimicrobial agents. So ceftriaxone, enrofloxacin, kanamycin, ciprofloxacin, amoxicillin / clavunate and ofloxacin
increased antilysozyme activity: min 0.7 times and max 3.6 times. The inhibitory effect is slightly expressed in relation to benzylpenicillin, which on the background of the general stimulating effect of the studied factors on the persistent properties is of no great consequence.

Gram-negative bacteria have multiple persistence factors, so they can establish temporary or long-term colonization of the host organism [9, 15].

The infectious agents identified in the experiment cause massive gastrointestinal diseases in calves and have a high proportion in the occurrence of digestive pathology. Lysozyme plays a key role in the innate immune system of animals and humans. It has a powerful antimicrobial effect against a wide range of microorganisms, hydrolyzing peptidoglycan, a vital component of the bacterial cell wall. Gram-negative bacteria produce various types of lysozyme inhibitors that allow them to survive the bactericidal effects of lysozyme when their outer membrane is permeable. The antilysozyme activity of enterobacteria is part of the general mechanism of lysozyme resistance of microorganisms [16, 17].

Lysozyme is very important for resolving inflammation in the mucous membranes. Therefore, the authors believe that understanding the mechanism of influence of antimicrobial agents on the antilysozyme activity of enterobacteria will help act on microorganisms reducing their pathogenic potential.

Further clinical implementation of the results of such studies will reduce the bacterial load on the body, increase the effectiveness of therapy, and will have economic benefits.

4. Conclusion

As a result of the studies, it was found that enterobacteria: *Escherichia coli* and *Proteus mirabilis* have the greatest etiological significance in the occurrence of massive gastrointestinal diseases in the base farm - their specific weight in the occurrence of digestive pathology was 33.3% and 40.0%, respectively.

The antilysozyme activity of enterobacteria isolated from the calves with acute intestinal disorders manifested itself heterogeneously and had different levels of intensity.

After exposure to enterobacteria of various antimicrobial agents, antilysozyme activity was determined by the species characteristics of the microorganism and to a lesser extent depended on the studied agent.

An increase in the indicator of anti-lysozyme activity in *Proteus mirabilis* after interaction with antibacterial drugs clearly indicates an increase in the ability of bacteria to destroy an important protective barrier – the lysozyme. This increases the pathogenic aggressiveness of the microorganism. Given the high ability of microorganisms to adapt to endogenous factors of host protection, this can significantly affect the course and development of the infectious process.

Our research, using the example of the effect of antimicrobial drugs on the anti-lysozyme activity of some enterobacteria, clearly shows that drugs can modify the biological properties of microorganisms by increasing or suppressing the degree of their manifestation.

The obtained experimental data are the basis for understanding the mechanisms of the relationship of microorganisms with antimicrobial agents and the theoretical background for the development of approaches to the treatment and prevention of infections caused by opportunistic enterobacteria.

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