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JUSTIFICATION FOR THE EFFICACY OF THE BIOCIDE 'KREZONID' IN CONTROLLING BACTERIAL INFECTIONS

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Summary. The study aimed to experimentally substantiate the bactericidal activity of the biocidal agent 'Krezonid' (based on meta-cresol, lactic acid, and a quaternary ammonium compound) and to determine the minimum effective regimens for its use to completely inactivate Gram-positive and Gram-negative pathogenic bacteria, which are important in veterinary medicine and poultry farming, while simultaneously verifying the absence of a bacteriostatic effect. The study was conducted using the in vitro suspension method in accordance with the requirements of European standards (EN 1040:2005, EN 1656:2019, EN 12353:2021) and methodological recommendations for veterinary disinfectants. Standard strains of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 were used as test microorganisms. Working concentrations of the drug of 0.1%, 0.3%, 0.5%, and 1.0% were tested at exposure times of 10, 20, and 30 minutes. After contact, the samples were rinsed three times with saline, and cultures were inoculated onto tryptone-soy agar (to assess bactericidal activity) and into tryptone-soy broth with repeated re-inoculations over 72 hours (to rule out bacteriostasis). The results showed a clear dependence of efficacy on concentration and exposure time. A concentration of 0.1% did not ensure complete inactivation even after 30 minutes. At 0.3%, complete inactivation was achieved inconsistently, with occasional residual growth. A stable and reproducible bactericidal effect against both test cultures was observed at a concentration of 0.5% after just 20 minutes of exposure, and at 30 minutes in 100% of replicates. The maximum rate of action was noted at 1.0% — destruction of microorganisms after 10 minutes of contact. No bacteriostatic effect was observed under effective conditions: growth did not resume after repeated inoculations. The data obtained confirm the pronounced bactericidal (rather than bacteriostatic) activity of 'Krezonid' against Gram-positive and Gram-negative bacteria. The recommended minimum concentration is 0.5% for a 30-minute exposure (or 20 minutes under stable conditions); a 1.0% concentration for 10 minutes is optimal for rapid disinfection. The results allow us to recommend the product for use in veterinary and sanitary measures at industrial livestock and poultry facilities, provided that appropriate protocols are followed

Keywords: bactericidal activity, bacteriostatic effect, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, disinfection

Introduction. The rapid expansion of industrial livestock and poultry farming is accompanied by an increase in the biological load on the production environment, which creates favorable conditions for the circulation and accumulation of bacterial pathogens. Under such conditions, the effectiveness of veterinary and sanitary measures directly determines the level of epizootic stability of farms and the safety of animal-derived products (Melo et al., 2020; Wales et al., 2021).

One of the key elements of the biosecurity system is the use of disinfectants capable of ensuring rapid and complete inactivation of pathogenic and opportunistic microorganisms. At the same time, practical experience shows that a decrease in their effectiveness may accompany prolonged use of products with the same mechanism of action. Therefore, there is a need for a scientifically sound selection and periodic replacement of disinfectants within a given area (Ponomarenko et al., 2021; Paliy et al., 2020). At the same time, the costs of disinfection measures directly affect the production cost of livestock products.

In recent years, a decline in the sensitivity of microorganisms to certain disinfectants has been increasingly observed in production conditions due to their prolonged and repetitive use. The development of pathogenic bacteria's resistance to disinfectants negatively impacts the epizootic stability of farms and necessitates the regular rotation of products with different biocidal mechanisms of action (Rutala and Weber, 2019; Scicchitano et al., 2024).

The market for veterinary disinfectants features several dozen products from various chemical groups, among which those based on quaternary ammonium compounds predominate (Kovalenko and Nedosiekov, 2011; Ponomarenko et al., 2020). However, objective data on their actual effectiveness under livestock production conditions are often limited or promotional in nature, which complicates making an informed choice (Tarka and Nitsch-Osuch, 2021; Koti et al., 2024).

The development and implementation of effective, domestically produced biocidal agents with high antimicrobial activity, an acceptable toxicological profile, and affordability is particularly relevant (Kovalenko

et al., 2013; Paliy et al., 2018). In this context, the biocide 'Krezonid' shows promise; however, its efficacy requires scientific validation using standardized laboratory methods (Kovalenko et al., 2018).

The objective of this study is to experimentally investigate the bactericidal activity of 'Krezonid' at various concentrations and exposure times. Additionally, we aim to determine the minimum effective application conditions for completely inactivating Gram-positive and Gram-negative pathogenic and opportunistic bacteria that are significant in animal husbandry.

Materials and methods. Experimental studies investigating the bactericidal activity of the new biocide 'Krezonid', which is based on meta-cresol, lactic acid, and a quaternary ammonium compound, were conducted in the laboratory at the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine. These studies were performed according to the most recent methodological recommendations and regulatory documents for determining bactericidal activity and verifying the absence of bacteriostatic effects in veterinary disinfectants (Harkavenko et al., 2020; SE 'UkrNDNC', 2022a, 2022b, 2022c).

In the first stage of the study, the drug's primary bactericidal activity was evaluated *in vitro* using the suspension method. Standard strains from the institute's collection were used as test microorganisms: the Gram-positive culture *Staphylococcus aureus* ATCC 6538 and the Gram-negative culture *Pseudomonas aeruginosa* ATCC 15442 (Kovalenko and Nedosiekov, 2011; SE 'UkrNDNC', 2022a, 2022b, 2022c).

To prepare the inoculum, colonies from daily cultures were washed off the surface of tryptone soy agar (TSA) with sterile saline under aseptic conditions. The turbidity of the resulting bacterial suspensions was adjusted to a standard of 0.5–1.0 on the McFarland scale, corresponding to a microorganism concentration of $1.35–3.0\times10^8$ CFU/cm³.

Working solutions of the biocidal product 'Krezonid' were prepared immediately before the experiments according to the manufacturer's recommendations. The working concentrations used in the experiments were 0.1%, 0.3%, 0.5%, and 1.0%. The amount of disinfectant and water used was calculated based on preparing 100 ml of each working solution.

Suspension tests were conducted in triplicate for each concentration and each test culture. Sterile test tubes were filled with 4.5 cm³ of the appropriate working solution of the biocide, and 0.5 cm³ of bacterial suspension was added. The mixtures were thoroughly mixed and incubated for 10, 20, and 30 minutes.

After the microorganisms had been in contact with the biocide, the test cultures were washed three times with sterile saline solution to completely remove any remaining product. The washed cultures were brought to the original volume (4.5 cm³) by adding tryptone-soy broth, after which they were inoculated.

To determine bactericidal activity, 0.1 cm³ of the suspension was inoculated onto the surface of tryptone-soy agar, using three Petri dishes for each concentration and exposure. The plates were incubated in a thermostat at 37 ± 1 °C for 48 h, with preliminary results recorded after 24 h. To identify or rule out a bacteriostatic effect of the drug, parallel cultures were prepared by inoculating 0.1 cm³ of the suspension into test tubes containing tryptone-soy broth (TSB). Incubation was carried out for 72 h at a temperature of 37 ± 1 °C with daily reinoculations on fresh liquid nutrient medium every 24 h.

The results of the bactericidal activity of the test biocide 'Krezonid' were recorded after 24–48 h on TSA plates; to detect the bacteriostatic effect of the preparation, results were recorded after 24–72 h in TSB tubes. Growth of the test cultures was monitored by performing similar inoculations of bacterial suspensions that were not exposed to the biocide.

Results. Experimental studies have shown that the biocide 'Krezonid' exhibits pronounced bactericidal activity against the test strains *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442; however, the level of efficacy depended significantly on the concentration of the working solution and the duration of exposure.

According to the test results, when the preparation was applied at a concentration of 0.1%, a significant reduction in the viability of both test cultures was observed after just 10 minutes of exposure, as confirmed by a decrease in the number of colonies when plated on solid nutrient medium (Tables 1 and 2). However, complete inactivation of microorganisms under these conditions was not achieved, as isolated growth of colonies of both staphylococci and pseudomonads was observed after incubation.

Extending the exposure time to 20 and 30 min at a concentration of 0.1% led to a further reduction in the infectious load; however, even at the maximum exposure time, residual growth of the test cultures was observed, indicating that this treatment regimen was not sufficiently bactericidal.

When using a 0.3% working solution of the drug 'Krezonid', a significant increase in the antimicrobial activity of the drug was observed. Thus, after 20 min of exposure in cultures on solid medium, only isolated colonies of *Staphylococcus aureus* were detected, while the growth of *Pseudomonas aeruginosa* was significantly inhibited. As the contact time was increased to 30 min, the number of viable cells in both cultures decreased to minimal levels.

The maximum bactericidal effect at a concentration of 0.3% was achieved after 30 minutes of exposure, during which no growth of the test cultures on solid medium was observed in most replicates. At the same time, in isolated cases, when samples were inoculated after contact with the drug, weak growth was observed, which did not allow us to conclude unequivocally that this regimen was fully bactericidal for all microorganisms studied.

Table 1 — Study of the bactericidal activity and bacteriostatic effect of the biocide 'Krezonid' on test cultures of *Staphylococcus aureus* ATCC 6538

<table>
<tr><td rowspan="5">Working concentrations of 'Krezonid', %</td><td colspan="6">Assessment of the growth of test cultures on nutrient media following treatment with various concentrations of the biocide 'Krezonid' at different exposure times</td></tr>
<tr><td colspan="6">Cultivation of test microbial cultures on solid and liquid media in a thermostat at a temperature of 37 ± 1°C for a period of:</td></tr>
<tr><td colspan="4">48 hours</td><td colspan="2">72 hours</td></tr>
<tr><td colspan="3">Bactericidal activity of 'Krezonid'</td><td colspan="2">Bacteriostatic effect following treatment with 'Krezonid'</td></tr>
<tr><td colspan="2">Tryptone-soy agar (solid culture medium)</td><td>Growth control</td><td>Tryptone-soy broth (liquid culture medium)</td><td>Growth control</td></tr>
<tr><td colspan="7">Multiplicity of studies, number of Staphylococcus aureus ATCC 6538 culture tubes</td></tr>
<tr><td colspan="7">Exposure 10 minutes</td></tr>
<tr><td></td><td colspan="3">TSA plates' numbers</td><td rowspan="5">Continuous growth</td><td colspan="2">TSB plates' numbers</td><td rowspan="5">Severe turbidity, sediment</td></tr>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>1</td><td>2</td><td>3</td></tr>
<tr><td>0.1</td><td>Continuous growth on the surface of the TSA</td><td>Growth of colonies across the entire surface of the TSA plate</td><td>Continuous growth on the surface of the TSA</td><td colspan="3" rowspan="2">Medium turbidity, growth (+)</td></tr>
<tr><td>0.3</td><td colspan="3">Growth of colonies across the entire surface of the TSA plate</td></tr>
<tr><td>0.5</td><td>11 isolated colonies on the TSA surface</td><td>9 isolated colonies on the TSA surface</td><td>5 isolated colonies on the TSA surface</td><td colspan="3">No growth</td></tr>
<tr><td>1.0</td><td colspan="3">No colony growth</td><td colspan="3">No growth</td></tr>
<tr><td colspan="7">Exposure 20 minutes</td></tr>
<tr><td>0.1</td><td colspan="3">Growth of colonies across the entire surface of the TSA plate</td><td rowspan="3">Continuous growth</td><td colspan="2">Medium turbidity, growth (+)</td><td rowspan="3">Severe turbidity, sediment</td></tr>
<tr><td>0.3</td><td>3 isolated colonies on the TSA surface</td><td>5 isolated colonies on the TSA surface</td><td>7 isolated colonies on the TSA surface</td><td colspan="2">No growth</td></tr>
<tr><td>0.5
1.0</td><td colspan="3">No colony growth</td><td colspan="2">No growth</td></tr>
<tr><td colspan="7">Exposure 30 minutes</td></tr>
<tr><td>0.1</td><td colspan="3">Growth of individual colonies on the surface of TSA</td><td rowspan="3">Continuous growth</td><td colspan="2">Medium turbidity, growth (+)</td><td rowspan="3">Severe turbidity, sediment</td></tr>
<tr><td>0.3
0.5
1.0</td><td colspan="3">No colony growth</td><td colspan="2">No growth</td></tr>
</table>

The results of the evaluation of the bactericidal activity of the drug 'Krezonid' against a culture of *Staphylococcus aureus* ATCC 6538 indicate that complete inactivation of the staphylococcus is achieved when the drug is used at concentrations of 0.5% or higher for an exposure time of at least 20 min. At lower concentrations, partial growth inhibition was observed, which did not guarantee complete elimination of the pathogen.

The use of the biocide at a concentration of 0.5% provided a stable and reproducible bactericidal effect. After 20 min of exposure across all replicates, there was no growth of *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Further increasing the contact time to 30 minutes did not result in the appearance of viable cells, confirming the complete inactivation of the test cultures.

'Krezonid' demonstrated the highest antimicrobial activity at a concentration of 1.0%. For all exposure times studied (10, 20, and 30 min), no growth of the test microorganisms was detected in either solid or liquid culture media, indicating the rapid and intense bactericidal action of the product.

Analysis of the results presented in Table 2 showed that *Pseudomonas aeruginosa* exhibits slightly higher resistance to the tested biocide compared to *Staphylococcus aureus*. At the same time, the use of 'Krezonid' at a concentration of 0.5% ensured the complete absence of *Pseudomonas aeruginosa* growth after 20 and 30 min of exposure, whereas at a concentration of 1.0%, the bactericidal effect was achieved after just 10 min of contact.

An important aspect of the study was to determine the product's potential bacteriostatic effect. Based on the results of repeated inoculations into a liquid culture medium after prior contact with 'Krezonid' at effective concentrations, no resumption of growth was observed in the test cultures.

Table 2 — Study of the bactericidal activity and bacteriostatic effect of the biocide 'Krezonid' on test cultures of *Pseudomonas aeruginosa* ATCC 15442

<table>
<tr><td rowspan="6">Working concentrations of 'Krezonid', %</td><td colspan="6">Assessment of the growth of test cultures on nutrient media following treatment with various concentrations of the biocide 'Krezonid' at different exposure times</td></tr>
<tr><td colspan="6">Cultivation of test microbial cultures on solid and liquid media in a thermostat at a temperature of 37±1°C for a period of:</td></tr>
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<tr><td colspan="2">Tryptone-soy agar (solid culture medium)</td><td>Growth control</td><td>Tryptone-soy broth (liquid culture medium)</td><td>Growth control</td></tr>
<tr><td colspan="5">Multiplicity of studies, number of Pseudomonas aeruginosa ATCC 15442 culture tubes</td></tr>
<tr><td colspan="7">Exposure 10 minutes</td></tr>
<tr><td></td><td colspan="3">TSA plates' numbers</td><td rowspan="2">Continuous growth</td><td colspan="2">TSB plates' numbers</td><td rowspan="2">Severe turbidity, sediment</td></tr>
<tr><td>1</td><td>2</td><td>3</td><td>1</td><td>2</td><td>3</td></tr>
<tr><td>0.1</td><td colspan="3" rowspan="2">Continuous growth on the surface of the TSA</td><td rowspan="3">Continuous growth</td><td colspan="2" rowspan="2">Medium turbidity, growth (+)</td></tr>
<tr><td>0.3</td></tr>
<tr><td>0.5</td><td colspan="3">Growth of individual colonies on the surface of TSA</td><td colspan="2" rowspan="2">No growth</td></tr>
<tr><td>1.0</td><td colspan="3">No colony growth</td><td rowspan="3">Severe turbidity, sediment</td></tr>
<tr><td colspan="7">Exposure 20 minutes</td></tr>
<tr><td>0.1</td><td colspan="3">Continuous growth on the surface of the TSA</td><td rowspan="3">Continuous growth</td><td colspan="2">Medium turbidity, growth (+)</td></tr>
<tr><td>0.3</td><td>9 isolated colonies on the TSA surface</td><td>Growth of individual colonies on the surface of TSA</td><td>No colony growth</td><td>Medium turbidity, growth (+)</td><td>No growth</td></tr>
<tr><td>0.5</td><td colspan="3" rowspan="2">No colony growth</td><td colspan="2" rowspan="2">No growth</td></tr>
<tr><td>1.0</td></tr>
<tr><td colspan="7">Exposure 30 minutes</td></tr>
<tr><td>0.1</td><td colspan="3">Growth of individual colonies on the surface of TSA</td><td rowspan="3">Continuous growth</td><td colspan="2" rowspan="2">Medium turbidity, growth (+)</td><td rowspan="3">Severe turbidity, sediment</td></tr>
<tr><td>0.3</td><td>Growth of individual colonies on the surface of TSA</td><td>8 isolated colonies on the TSA surface</td><td>6 isolated colonies on the TSA surface</td></tr>
<tr><td>0.5</td><td colspan="3" rowspan="2">No colony growth</td><td colspan="2" rowspan="2">No growth</td></tr>
<tr><td>1.0</td></tr>
</table>

This indicates an absence of bacteriostatic activity and confirms the bactericidal mechanism of the drug's antimicrobial action.

These results are consistent with the current understanding of how combined-type biocidal compounds act, and confirm that the biocidal agent 'Krezonid' is appropriate for sanitising poultry facilities, provided the recommended concentrations and exposure times are adhered to.

Discussion. The experimental data obtained demonstrate the high bactericidal activity of the biocide 'Krezonid' against the standard test strains *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442, which are of key epizootic importance in livestock and poultry farming. The observed dependence of the product's efficacy on the concentration and duration of exposure is consistent with general patterns of action for chemical disinfectants and the requirements of current European standards for evaluating bactericidal activity.

The partial inhibition of growth in test cultures at a concentration of 0.1%, even with prolonged exposure, indicates that this treatment regimen is insufficient for the complete inactivation of microorganisms. Similar results are reported in the works of other authors, who note that reducing the concentration of biocidal agents below the minimum effective level may lead to the survival of part of the bacterial population and create conditions for the development of adaptive resistance (Koti et al., 2024).

A significant increase in bactericidal activity accompanied an increase in the concentration of 'Krezonid' to 0.3%. However, the residual growth of test cultures observed in isolated replicates following a 30-minute exposure indicates that this regimen cannot be considered fully reliable for practical use under conditions of high microbial load. This is particularly important given the known higher natural resistance of *Pseudomonas aeruginosa* to many disinfectants, as

confirmed by the results of numerous studies (Wlazlo et al., 2020).

It has been established that using the preparation at a concentration of 0.5%, with an exposure time of at least 20 minutes, provides a stable and reproducible bactericidal effect against both test microorganisms. It is this regimen that can be considered the minimum effective and technologically feasible for implementation in veterinary and sanitary practices. The results obtained are consistent with the literature, which emphasizes that to ensure the complete elimination of both Gram-positive and Gram-negative bacteria, it is necessary to use disinfectant concentrations capable of causing irreversible damage to cellular structures (Van Haute et al., 2015).

'Krezonid' exhibited its highest antimicrobial activity at a concentration of 1.0%, ensuring complete inactivation of the test cultures with minimal exposure. This rapid action is a significant advantage of the product, particularly in production settings where the length of sanitation breaks is limited. However, the feasibility of using the maximum concentration must be evaluated in terms of economic factors, the potential impact of organic contamination, and biosafety requirements.

The absence of a bacteriostatic effect following repeated inoculations confirms that 'Krezonid' acts through a bactericidal mechanism rather than by temporarily inhibiting microbial growth. This is of fundamental importance in preventing the formation of resistant bacterial populations, and is consistent with current recommendations for selecting disinfectants for livestock facilities (Curran et al., 2019).

Thus, the results of the studies confirm the potential of the biocide 'Krezonid' as an effective disinfectant for preventing bacterial infections in poultry and livestock farming. At the same time, the data obtained highlight the importance of adhering to scientifically sound concentration and exposure regimens and the need for further studies under production conditions.

Conclusions. Laboratory tests have shown that the biocide 'Krezonid' exhibits significant bactericidal activity against Gram-positive and Gram-negative microorganisms, in particular the standard test strains *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442.

The effectiveness of the product's bactericidal action directly depends on the concentration of the working solution and the duration of exposure. The minimum effective regimen, which ensures complete inactivation of both test cultures without residual growth, is the use of 'Krezonid' at a concentration of 0.5% for a 30-minute exposure.

Use of the biocide at a concentration of 1.0% provides a rapid and stable bactericidal effect against the microorganisms under study after just 10 min of exposure, indicating the high intensity of the product's biocidal action.

Based on the results of the bacteriostatic effect tests, it was concluded that, upon coming into contact with effective concentrations of 'Krezonid', the microorganisms did not resume growth in the liquid culture medium. This confirms the absence of a bacteriostatic effect and the presence of a bactericidal inactivation mechanism.

The experimental data obtained allow the biocide 'Krezonid' to be recommended for use in preventive veterinary and sanitary measures at poultry facilities, provided scientifically sound concentration and exposure regimens are followed.

Prospects for further research. Further scientific research should focus on studying the efficacy of the biocide 'Krezonid' in industrial settings, considering various types of organic contamination and technological surfaces. Particular attention should be paid to evaluating the product's virucidal and fungicidal activity, as well as determining its effectiveness against bacterial pathogens circulating on farms with varying levels of biosecurity.

Additionally, investigating the possibility of using 'Krezonid' for the aerosol disinfection of premises in the presence of animals and poultry is promising, as is evaluating the product's toxicological parameters and its impact on the microbiocenoses of the production environment during prolonged use.

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