Spectrum of resistance to antibacterial drugs of *Staphylococcus spp.* isolates depending on the ability to form biofilms

T. E. Denisenko[1*], *A. A.* Metla[1], *O. A.* Minkova[1], and *E. I.* Yarygina[1]

[1]Federal State Budgetary Educational Institution of Higher Education "Moscow State Academy of Veterinary Medicine and Biotechnology – MBA named after K.I. Scriabin", Moscow, Russia

Abstract. The paper presents the results of studying the spectrum of resistance to antibacterial drugs of *Staphylococcus spp.* isolates depending on their ability to form biofilms. Two methodological approaches were used to determine the causes of the low efficiency of antibiotic therapy used in livestock farms. The sensitivity of the isolated staphylococci to antibiotics was determined and the ability of these cultures to form biofilms was established. It was found that staphylococci isolated from goats were resistant mainly (more than 50% of isolates) to tylosin, penicillin, clindamycin, amikacin, lincomycin, gentamicin, meropenem and levofloxacin, from cattle - to azithromycin, clindamycin, meropenem, rifampicin, amikacin, and from poultry - to tylosin, penicillin, tetracycline, doxycycline, amikacin, azithromycin, clindamycin, ciprofloxacin, cefotaxime, lincomycin and gentamicin. It was found that all strains had multiple drug resistance to two or more groups of antibiotics. It was found that 60-67% of microorganism strains were potentially capable of forming biofilms. It has been proven that positive results of antibiotic susceptibility tests in vitro cannot be extrapolated to the predicted effectiveness of these drugs in vivo. The need to study isolated staphylococcal strains for antibiotic sensitivity and their ability to form a biofilm when selecting an optimal treatment regimen for farm animals and poultry is substantiated.

1 Introduction

In livestock and poultry farms free from dangerous diseases, infections are factorial or opportunistic diseases, the main role in the etiology of which is played by bacteria of the genus *Staphylococcus*. According to the authors, mastitis as the most common factorial diseases in cows and goats is caused by staphylococci. The most clinically significant coagulase-positive species in all types of farm animals and birds is S. aureus. On the other hand, according to Björk, S et al. (2019), and Waller KP, et al. (2011), over the past 20 years, the number of subclinical mastitis in cattle and small ruminants caused by coagulase-negative staphylococci has increased. Thus, according to Nelli, A. et al. (2022), subclinical mastitis occurs in dairy goats more often (5–30% or higher) than the clinical form (less than

* Corresponding author: denisenkote@yandex.ru

5%), with staphylococcus being the predominant pathogen in both cases. In particular, coagulase-negative staphylococci (CoNS), namely Staphylococcus caprae, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus chromogenes* and *Staphylococcus simulans*, most often cause subclinical mastitis and less often - clinical, while for *Staphylococcus aureus* the opposite is observed. Regarding the role of staphylococci in the disease in birds, some scientific works indicate that the problem of lameness in poultry has a complex etiology, but among infectious factors, staphylococci are considered one of the most important bacterial pathogens. Factorial diseases of dairy cattle and poultry, the etiologic agent of which is bacteria of the genus *Staphylococcus*, lead to significant economic losses due to increased feed conversion ratio (FCR), low productivity, culling and expensive and ineffective treatment.

According to Björk, S et al. (2019), and Waller KP, et al. (2011), factorial and opportunistic diseases of productive animals and birds are not amenable to treatment using the standard scheme [5-6]. According to the authors, this problem is associated with the diverse biochemical activity of bacteria, a wide range of pathogenicity factors that ensure their attachment, penetration and spread in the body, the ability to form biofilms and widespread resistance of bacteria to antimicrobial drugs.

Despite the emergence of new methods for diagnosing and treating animals, the number of diseases caused by staphylococci is only increasing. Due to the diversity of clinical manifestations of the infection, veterinary specialists have difficulties with diagnostics. In case of successful isolation and identification of the pathogen, according to therapeutic standards, it is necessary to conduct a study of the isolated strains to determine their sensitivity to antimicrobial drugs. But in the vast majority of cases, even specially selected antimicrobial therapy is ineffective. We assume that staphylococcal isolates have different sensitivity to antibiotics in vitro and in vivo, which is associated with their ability to form biofilms. This assumption posed several research questions for us: what is the number of staphylococci resistant to popular antibiotics common in livestock and poultry farms in Russia; how often are staphylococci found that cause factorial or opportunistic diseases in animals that are capable of forming a biofilm.

The aim of our study includes the investigation of the antimicrobial activity of nineteen most widely used antibiotics against isolates of staphylococci in a free-floating (planktonic) state, determination of the ability of the studied isolates to form biofilms and correlation of the obtained data to predict the effectiveness of antibiotic therapy.

2 Materials and methods

Materials. The objects of the study were cows, goats, broiler chickens. The choice of the object of the study is associated with the active consumption of products from these animals by humans as food or food raw materials. The subject of the study were isolates of bacteria of the genus *Staphylococcus*, isolated from the milk of cows and goats, as well as from the droppings of agricultural poultry for the period 2024-2025.

Methods. To isolate bacteria of the genus *Staphylococcus*, standard cultivation on nutrient dense media was used, identification was carried out by determining the cultural-morphological and biochemical properties. The study of sensitivity to antibacterial drugs was carried out by the disc diffusion method. The ability to form a biofilm was detected by sowing isolates on a nutrient medium with Congo red.

Procedure. Milk from cows and goats was collected in sterile plastic containers, after washing the udder and milking the first portions of milk into a separate container, thereby collecting milk for testing from the milk cistern of the udder. Fresh samples of bird droppings were collected in sterile plastic containers. The obtained samples of biomaterials

were transported to the laboratory on the day of collection, observing a gentle temperature regime, using a cooling element.

Milk samples were centrifuged, the supernatant was poured off, and the sediment was suspended with a pipette. The resulting sediment in a volume of 20 μl was distributed with a spatula on blood meat-peptone agar. The crops were cultivated at a temperature of +37 °C under microaerophilic conditions for 48 hours. Morphology and tinctorial properties were determined by staining a smear from each culture according to Gram. A catalase test was performed for each colony of gram-positive cocci by lowering a complete bacteriological loop of the culture into a drop of hydrogen peroxide with the subsequent appearance of air bubbles in the resulting suspension. The selected cultures of gram-positive and catalase-positive cocci were sown on yolk-salt agar (an elective nutrient medium) using the simple streak method. Cultivation was carried out under the conditions described above. Isolates corresponding to *Staphylococcus* bacteria by their cultural and morphological properties were identified as *Staphylococcus spp.* For species identification of staphylococci, biochemical properties were determined using the StaphyTest 24 test system (manufacturer Erba Lachema, Czech Republic). The obtained data on the cultural, morphological and biochemical properties of each isolate were interpreted using the AbisOnline electronic bacteria identifier.

Determination of susceptibility to antibacterial drugs by the disk diffusion method was performed as follows: 200 μl of the suspension of staphylococcal isolates with a turbidity of 0.5 according to McFarland were placed on Mueller-Hinton nutrient agar, distributed with a spatula, left for 5 minutes for complete absorption, then disks with antibiotics were placed along the perimeter of the Petri dish, after which they were cultivated for 24 hours at a temperature of +37 °C under aerobic conditions. The result of susceptibility to antibacterial drugs was taken into account by measuring the diameter of the zone of inhibition of microorganism growth. Sensitivity assessment and interpretation were carried out based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Biofilm formation in staphylococcal isolates was determined based on the detection of polysaccharide intercellular adhesin (PIA): a young culture of microorganisms was added to a dense nutrient medium containing Congo red and distributed over the surface using a streaking loop. The cultures were cultivated at a temperature of +37 °C under aerobic conditions for 48 hours. Black colonies that colored the surrounding medium black were defined as having high biofilm activity, black or brown colonies without coloring the medium were defined as having an average ability to form a biofilm. Staphylococci that had light brown colonies without coloring the medium were assessed as incapable of biofilm formation.

3 Results

As a result of the study of biological material from cattle, goats and chickens, bacteria of the genus *Staphylococcus* were found. In cattle, staphylococci were isolated in 29% of cases (Figure 1) and were represented by two species – *Staphylococcus aureus* and *Staphylococcus epidermidis*. In samples of cow udder secretion, these types of bacteria were found both in monocultures and in associations with other opportunistic microorganisms. A total of 130 staphylococci isolates were obtained.

The results of determining the sensitivity to antibacterial drugs of staphylococcal isolates obtained from cows are presented in Figures 2 and 3, from goats - in 4 and 5, from poultry - in Diagrams 6 and 7.

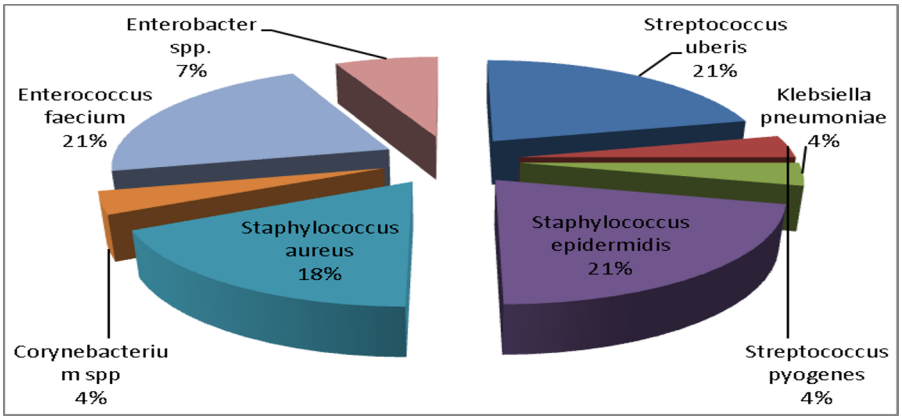

Fig. 1. The proportion of staphylococcal isolates obtained during microbiological examination of biological material from cattle.

Four types of staphylococci were isolated from goat milk: *Staph. aureus* - 6 isolates, *Staph. epidermidis* - 4, *Staph. xylosis* - 4, *Staph. hyicus* - 3. From samples of poultry droppings we obtained *Staph. aureus* - 7 isolates, *Staph. haemolyticus* - 3, *Staph. saprophyticus* - 2.

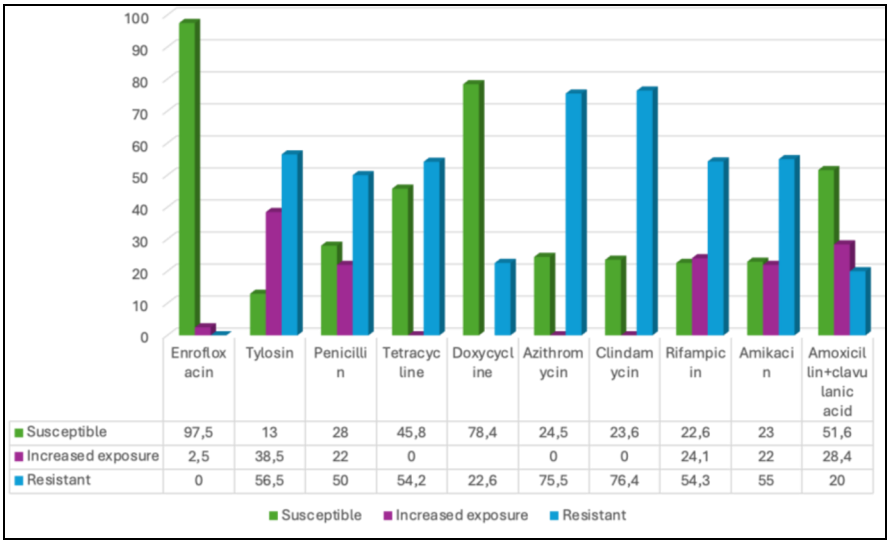

Fig. 2. Results of determination of sensitivity to antibacterial drugs of staphylococci isolated from cattle.

A study of the sensitivity of staphylococcal isolates obtained from cattle to antibiotics showed resistance of 75% of *S.aureus* isolates to drugs such as azithromycin, clindamycin, meropenem, rifampicin, and amikacin. *S.epidermidis* cultures were sensitive to most of the studied drugs, including those to which *S.aureus* was resistant.

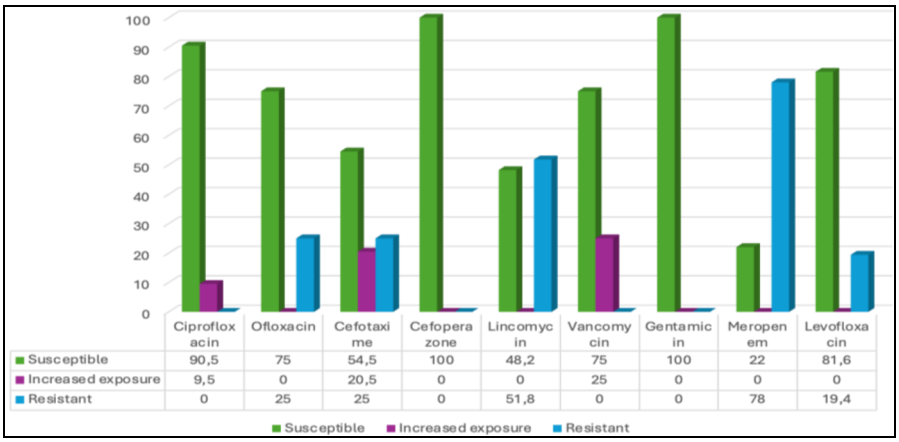

Fig. 3. Results of determination of sensitivity to antibacterial drugs of staphylococci isolated from cattle.

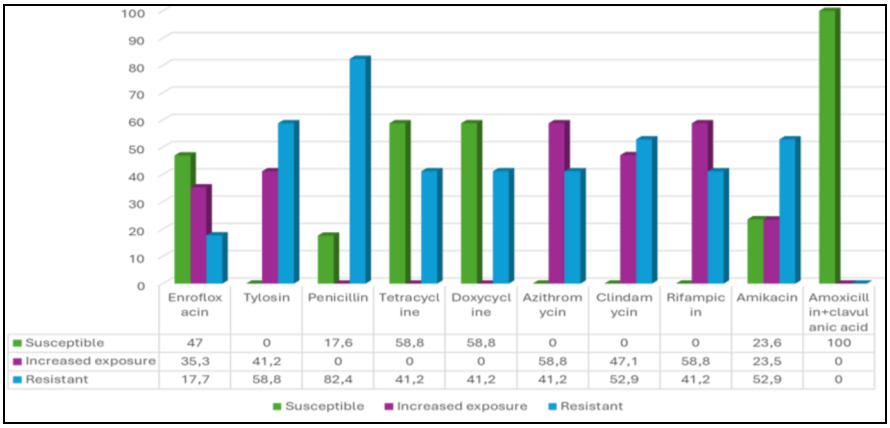

Fig. 4. Manifestation of varying degrees of sensitivity to antibacterial drugs in staphylococci isolated from goats.

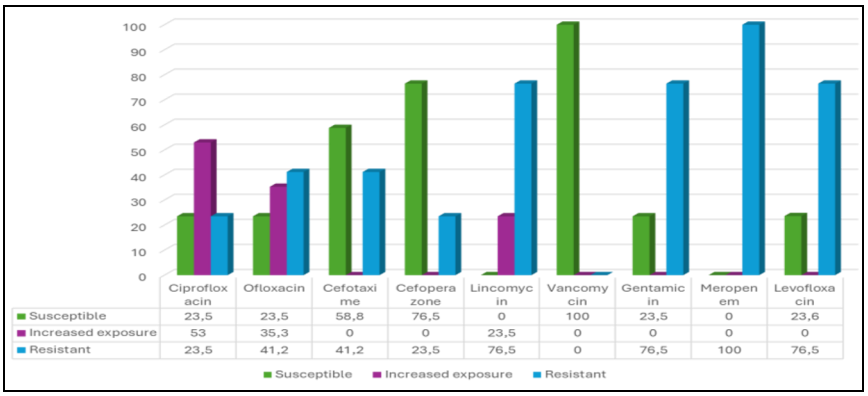

Fig. 5. Manifestation of varying degrees of sensitivity to antibacterial drugs in staphylococci isolated from goats.

As can be seen from the presented diagrams, more than 50% of staphylococcus cultures isolated from goats were resistant to tylosin, penicillin, clindamycin, amikacin, lincomycin, gentamicin, meropenem and levofloxacin. The presented drugs belong to different generations of antibiotics, which shows a high prevalence of microorganisms with a wide range of resistance to both veterinary and medical drugs in goat farms.

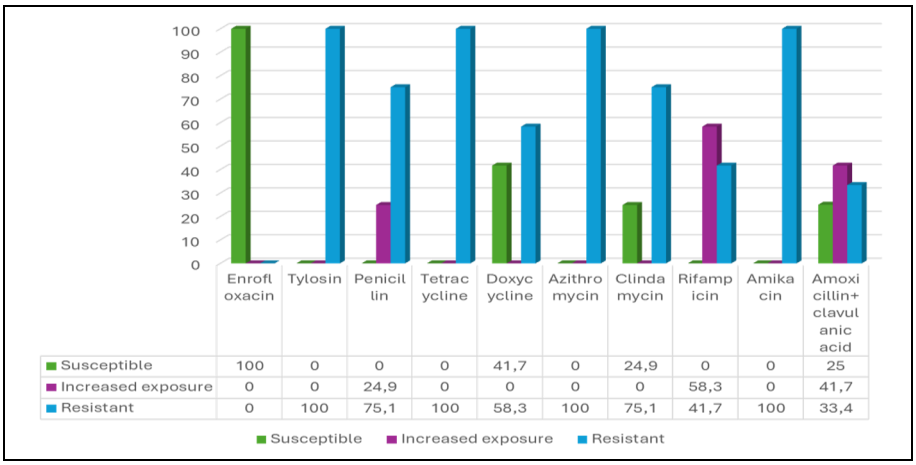

Fig. 6. Manifestation of different degrees of sensitivity to antibacterial drugs in staphylococci isolated from farm poultry.

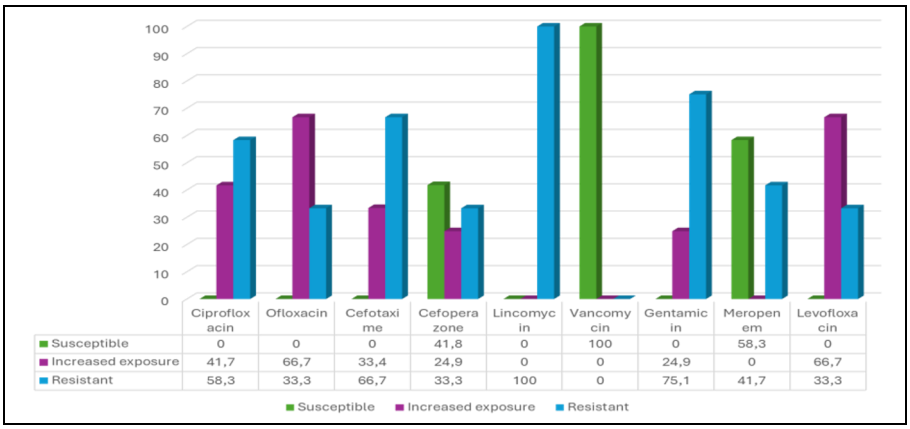

Fig. 7. Manifestation of different degrees of sensitivity to antibacterial drugs in staphylococci isolated from farm poultry.

Staphylococci isolated from poultry showed resistance to tylosin, penicillin, tetracycline, doxycycline, amikacin, azithromycin, clindamycin, ciprofloxacin, cefotaxime, lincomycin and gentamicin. The fact of detection of a wide range of resistance of *Staphylococcus* isolates indicates irrational use of antibiotics in poultry farms. And detection of pathogens resistant to medical drugs, including new generations, may be the result of contamination of poultry with human pathogens.

3.1 Results of determination of biofilm formation in staphylococci

In the study of 130 staphylococci isolated from samples from cattle, the following results were obtained: 35% of staphylococcal isolates were found to have no ability to form a biofilm, as they showed growth in the form of light brown colonies; 22% of staphylococci that showed growth in the form of brown or black colonies without coloring the medium, the ability to form biofilm was assessed as average; 43% of isolates had a high ability, showing growth in the form of brown or black colonies with blackening of the medium (Figure 8).

In the study of 17 staphylococci isolated from samples from goats, 35% of staphylococcal isolates were found to have no ability to form biofilm, as they showed growth in the form of light brown colonies; In 30% of staphylococci that showed growth in the form of brown or black colonies without coloring of the medium, the ability to form biofilms was assessed as average; 30% of isolates had a high ability, showing growth in the form of brown or black colonies with blackening of the medium (Figure 8).

Of the 12 staphylococci isolated from samples of agricultural poultry, 33% of the staphylococcal isolates were found to have no ability to form a biofilm, as they grew as light-brown colonies; 17% of staphylococci, which grew as brown or black colonies without coloring the medium, had an average ability to form biofilms; 50% of the isolates had a high ability, growing as brown or black colonies with blackening of the medium (Figure 8).

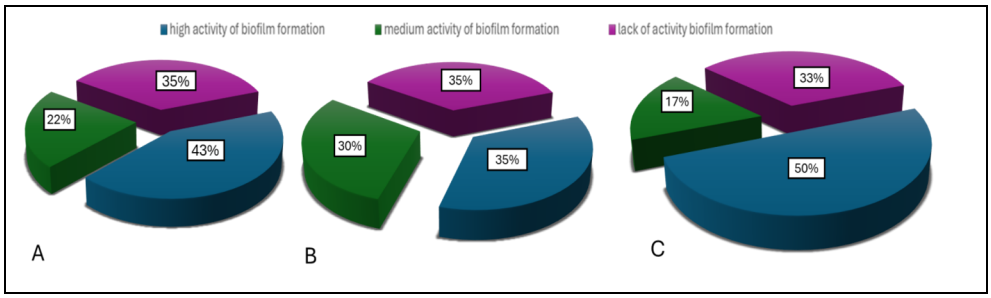

Fig. 8. Display of high and moderate ability or absence of biofilm formation: A – proportion of staphylococcal isolates isolated from cattle. B – proportion of staphylococcal isolates isolated from goats. C – proportion of staphylococcal isolates isolated from poultry.

3.2 Correlation of manifestation of resistance to antibacterial drugs and biofilm formation

Determination of biofilm formation in staphylococcal isolates was carried out based on detection of polysaccharide intercellular adhesin. The dependence of staphylococcal isolate resistance to antibacterial drugs on the presence of the ability to form biofilms during in vitro testing was established (Figure 9).

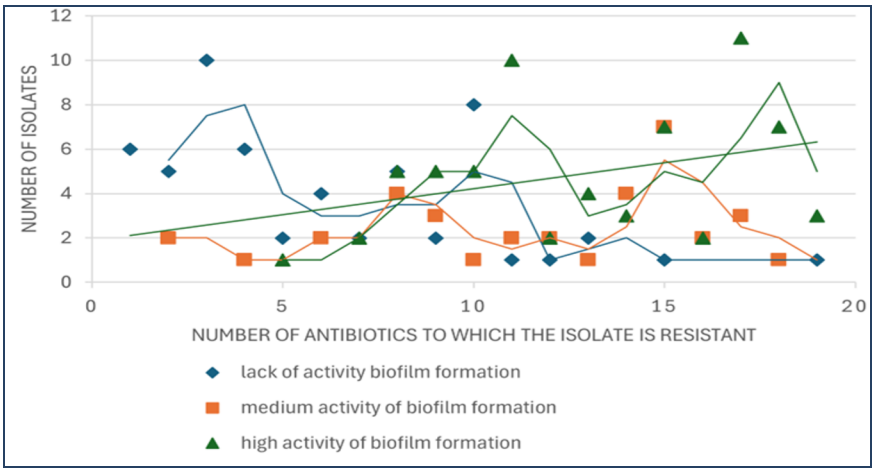

Fig. 9. Correlation of the degree of resistance to different antibiotics and the ability to form biofilm in staphylococcal isolates.

Y-axis - number of staphylococcal isolates. X-axis - number of antibiotics to which staphylococcal isolates showed resistance.

Values: ◆ - no ability; ■ - average ability; ▲ - high ability. Straight green line - exponent, demonstrating the general trend of dependence of resistance and high ability to form biofilms. Broken lines help to focus attention on the dependence of the indicators of the number of antibiotics, isolates and ability to form biofilms.

4 Discussion

An important step towards reducing the frequency of antibiotic use and increasing the success of etiotropic therapy is the introduction of a combination of antibiotic susceptibility tests and biofilm formation ability tests into the laboratory research protocol. As can be seen from the results of our study, staphylococcal cultures isolated from different types of farm animals show different degrees of resistance to antimicrobial drugs. Thus, bacteria isolated from cattle showed a smaller spectrum of antibiotic resistance than pathogens from goats and poultry. At the same time, it was noted that *S.aureus* isolates showed greater resistance.

The analysis of the spectrum of resistance to antimicrobial drugs showed that staphylococci isolated from goats and poultry were resistant to various pharmacological groups of antibiotics (*Lincosamide, Penicillins, Tetracyclines, Phenicols, Flouroquinolone, Cephalosporin, Glycopeptide, Macrolide*), both veterinary and medical. This may be due to both irrational antibiotic therapy for infectious pathologies of animals in goat and poultry farms and contamination with microorganisms from personnel and environmental objects. The fact of using medical drugs for the treatment of animals is also possible. Given the ability of staphylococci to horizontally transmit genetic information encoding resistance to certain drugs, a fairly rapid spread of polyresistant strains of microorganisms within a livestock enterprise is possible. Unsatisfactory sanitary conditions and the lack of comprehensive microbiological control aggravate the situation.

The fight against infectious pathologies of farm animals is further complicated by the ability of a number of microorganisms to form biofilm. Such formations prevent the drug from penetrating into the sites of colonization by microorganisms and do not provide the expected therapeutic effect. At the same time, microorganisms are able to form primary resistance to the drug used.

It is known that pathogenic staphylococci most often form biofilm in the sites of colonization of tissues and organs of the body of a sick animal. When culturing in laboratory conditions, including on nutrient media recommended for setting up a test for sensitivity to antibacterial drugs (Muller-Hinton medium), biofilm formation is not observed. Therefore, the results of tests to determine the sensitivity to antimicrobial drugs in biofilm-forming cultures of microorganisms will not be entirely correct. Our studies have shown that more than 50% of isolated staphylococcal cultures were able to form biofilm. Therefore, for these isolates, the results of antibiotic susceptibility testing may be ineffective when using highly sensitive drugs for therapeutic purposes. Therefore, we recommend introducing a biofilm formation test into routine microbiological diagnostic studies of staphylococcal infections in animals and correlating the results of this test with the results of antibiotic susceptibility testing.

5 Conclusion

As a result of the conducted studies of material from cattle, goats and poultry, we isolated staphylococci of the species *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Staphylococcus hyicus, Staphylococcus haemolyticus, Staphylococcus saprophyticus*.

The degree of resistance to various pharmacological groups of antimicrobial drugs depended on the type of staphylococcus and the type of animal from which the culture was isolated. Thus, the pathogens isolated from goats and poultry showed the widest spectrum of resistance.

Biofilm formation of staphylococcal cultures was noted in more than 50% of isolates, while this feature was found in all studied Staphylococcus species and did not depend on the type of host organism

A correlation was noted between the presence of a wide range of resistance of staphylococcal cultures and the ability to form biofilms. This indicates the possible influence of this feature on the results of *in vitro* antibiotic susceptibility tests.

Based on the studies conducted, we recommend the introduction of a biofilm formation test into the protocol of microbiological studies in the diagnosis of staphylococcal infections in animals.

References

1. M. Madigan, J. Martinko, Brock Biology of Microorganisms (11th ed.), Prentice Hall (2005)
2. P.J. Quinn, M.E. Carter, B.K. Markey, G.R. Carter, Staphylococcus species, In: Clinical veterinary microbiology, Edinburgh: Mosby, 118-26 (2000)
3. M. Setiabudy, D.A.P.S. Masyeni, A.A.G.I. Indraningrat, Biofilm Formation in Staphylococcus aureus and Coagulase-Negative Staphylococcus. Folia Medica Indonesiana, **59** (**3**), 222-228 (2023) https://doi.org/10.20473/fmi.v59i3.44598
4. S. Björk, R. Bage, B.M. Kanyima, Characterization of coagulase negative staphylococci from cases of subclinical mastitis in dairy cattle in Kampala, Uganda, Ir Vet J, **67**, 12 (2014) https://doi.org/10.1186/2046-0481-67-12
5. K.P. Waller, A. Aspan, A. Nyman, Y. Persson, U.G. Andersson, CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis, Vet Microbiol, **152** (**1–2**), 112-116 (2011)

6. L. Phophi, I.-M. Petzer, D.N. Qekwana, Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory, BMC Veterinary Research, **15**(**1**) (2019) doi:10.1186/s12917-019-2175-3
7. V. Silva, E. Correia, J.E. Pereira, C. González-Machado, R. Capita, C. Alonso-Calleja, G. Igrejas, P. Poeta, Biofilm Formation of Staphylococcus aureus from Pets, Livestock, and Wild Animals: Relationship with Clonal Lineages and Antimicrobial Resistance, Antibiotics, **11**, 772 (2022) https://doi.org/10.3390/antibiotics11060772
8. J. Azeredo, N.F. Azevedo, R. Briandet, N. Cerca, T. Coenye, A.R. Costa, C. Sternberg, Critical review on biofilm methods, Critical Reviews in Microbiology, **43**(**3**), 313–351 (2016)
9. S. Park, J. Ronholm, Staphylococcus aureus in Agriculture: Lessons in Evolution from a Multispecies Pathogen, Clinical Microbiology Reviews, **34**, **2** (2021)
10. A.M. Almansour, M.A. Alhadlaq, K.O. Alzahrani, L.E. Mukhtar, A.L. Alharbi, S.M. Alajel, The silent threat: Antimicrobial-resistant pathogens in food-producing animals and their impact on public health, Microorganisms, **11**(**9**), 2127 (2023)
11. A. Nelli, C. Voidarou, B. Venardou, K. Fotou, A. Tsinas, E. Bonos, G.C. Fthenakis, I. Skoufos, A. Tzora, Antimicrobial and Methicillin Resistance Pattern of Potential Mastitis-Inducing Staphylococcus aureus and Coagulase-Negative Staphylococci Isolates from the Mammary Secretion of Dairy Goats, Biology, **11**(**11**), 1591 (2022)
12. G. M. Szafraniec, P. Szeleszczuk, B. Dolka, Review on skeletal disorders caused by Staphylococcus spp. in poultry. Veterinary Quarterly, **42**(**1**), 21–40 (2022)
13. S. Woudstra, N. Wente, Y. Zhang, S. Leimbach, M.K. Gussmann, C. Kirkeby, V. Krömker, Strain diversity and infection durations of Staphylococcus spp. and Streptococcus spp. causing intramammary infections in dairy cows, Journal of Dairy Science, **106**(**6**), 4214-4231 (2023)