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Examination of urine microflora and resistance of isolated pathogens during inflammatory processes of the urinary tract in dogs

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Antibiotic-resistant bacteria are currently frequently isolated from pets and farm animals. The long-term irrational use of antibiotics for the treatment of animals and humans is underestimated and requires further attention and research, including in Ukraine. The aim of our study was to identify bacterial isolates and study their sensitivity to antibiotics in urinary tract inflammation in dogs. According to the statistical data of the VetForce system of the BTNAU clinic, out of 202 dogs examined in the clinic, 15 (7.43 %) were diagnosed with diseases with signs of urinary tract inflammation. It has been established that a large number of microorganisms, mainly *E. coli*, *Streptococcus urinae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, causes inflammatory processes of the urinary tract in dogs. A less common microorganism, *Klebsiella pneumonia*, proved to be resistant.

It was found that bacterial cystitis (the first group of animals) prevailed 1.5 times more often than in animals with urolithiasis. In dogs of the first group of 6–12 years old and over 12 years old, the percentage of the disease was higher compared to the group from 0.6 to 1.6 years old by 10.2 and 22.2 %, respectively.

Dogs of the second group, aged 1.6–12 years, suffering from urolithiasis (66.6 %), suffer from bacterial diseases 4 times more often compared to animals aged 0.6–1.6 years. *Streptococcus urinae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were isolated and identified from the urine of dogs in this group. In the associated form, *E. coli* and *Streptococcus pyogene* predominated in the urine of dogs. A resistant strain of *Klebsiella pneumonia* to amoxicillin, streptomycin, kanamycin, gentamicin and tetracycline was identified.

Antibiotics should be used rationally, taking into account that for the treatment of animals it is necessary to determine the sensitivity of the pathogen with which the animal was infected. Antibiotic therapy should be carried out until complete elimination, which is sometimes achieved through prolonged treatment.

Key words: microorganisms, distribution, pathogens, urine, dogs, resistance, antibiotics, urinary tract, bacteriological testing.

Problem statement and analysis of recent research. Despite the martial law in Ukraine, people continue to care about animals, including pets. They continue to care for them and treat them in case of illness. There is also a continued demand for animals as security. However, the presence of pathogens in the environment, the presence of transmission factors for these pathogens, and the presence of a favourable host lead to an infectious process, an infectious disease in an animal. Prevention and treatment of infections are of key importance for the health of animals, people and the environment.

Depending on the number of animals in an individual sector and the preventive protection measures taken, infectious diseases can spread rapidly, infecting dogs and their owners. Today, there is already evidence of keeping and rearing farm animals without antibiotics. The increase in the cost of such products does not meet the needs of consumers. Therefore, antibiotics continue to be used on livestock farms to treat animals, despite restrictions and recommendations to reduce their use [1–4].

Unfortunately, the widespread and long-term use of antibiotics has increased the pressure on natural selection among bacterial strains that are resistant to various antimicrobial drugs [5–6]. Antibiotic-resistant bacteria are now frequently isolated from humans, pets and farm animals [6]. In addition, antibacterial compounds and resistant bacteria are spreading beyond agricultural regions and accelerating the phenomenon of resistance in other bacteria, including those pathogenic to humans. Several other factors, such as climate change and biodiversity, and the development of tourism, force pathogens to evolve and adapt quickly to the environment [7]. Evolution in microorganisms is primarily a slow process that takes many generations. Bacteria can modulate their gene repertoire in just one generation and thus radically change their behaviour towards a particular antibacterial drug. Mobile genetic elements, such as plasmids, transposons and integrons, facilitate horizontal gene transfer between species and help bacteria evolve faster, including the acquisition of virulence factors or antibiotic resistance [6].

The emergence of resistance in microorganisms common to dogs is already threatening the effectiveness of antibiotics, including those historically used in medicine to alleviate problems caused by infections [5–8]. When discovering penicillin, Alexander Fleming observed organisms capable of resistance and demanded caution in its use [7–15]. The existence of antibiotic resistance among bacteria and the ineffectiveness of some drugs have already been proven [8–23]. The World Health Organisation (WHO) has announced that about 700,000 deaths each year are attributed to resistant and persistent pathogens that cause infectious diseases with complications and long-term treatment. If the problem of increasing bacterial resistance to antibiotics continues to grow at the same rate, the number could reach 10 000 000 deaths among the world's population by 2050 [24–26].

The spread of resistant pathogens occurs from one animal species to another, from animals to humans. Therefore, the infection vector of dogs, which can spread these bacteria to farm animals and humans, is dangerous. Therefore, the One Health approach to combating the biotic and abiotic components involved in the phenomenon of antibiotic resistance is becoming even more relevant in veterinary medicine. Understanding how antibiotic resistance develops in microorganisms throughout the agri-food chain is essential for One Health. Scientists in the context of international projects, including the European Union project «Improving skills in laboratory practice for agri-food specialists in eastern Europe» (Ag-Lab), are considering the problem of antibiotic resistance development in the world. Unfortunately, while 125 countries have announced some kind of antimicrobial resistance awareness activities, only 36 of them are taking steps to address this problem in the agricultural sector [27–30]. This suggests that the impact of agriculture on antimicrobial resistance may be underestimated and requires further attention and research, including in Ukraine.

The aim of the study was to isolate, identify bacterial isolates and study their sensitivity to antibiotics in dogs with urinary tract inflammation.

The material for the study was urine from dogs with signs of urinary tract inflammation. We selected animals whose owners sought help at the BTNAU FVM clinic. The material for the study was dogs with signs of urinary tract inflammation (n=15), which were divided by age and breed into 2 groups (the first - with bacterial cystitis (n=6), the second – with urolithiasis (n=9). The study was conducted during 2021-2022, using the VetForce system of the BTNAU clinic. In order to establish the cause of the disease, biomaterial for bacteriological studies was taken from animals with a clear clinical picture of the disease. The material was delivered to the educational and research laboratory for molecular diagnosis of FVM in sterile dishes, not preserved. Since the laboratory is located next to the clinic, we did not use transport systems (ready-to-use media with swabs for transporting samples in MS651 poly-styrene tubes). The material collected from each animal was in separate tubes well sealed with sterile stoppers.

Materials were numbered, indicating the species, sex, name and location from which the material was taken. All materials were placed in boxes with refrigerants and plastic containers and delivered to the laboratory. For each material, an accompanying document was filled out according to the form provided by the laboratory. The accompanying documents indicated the type of biomaterial, data on the number of patients and animals, their species, age and nature of the disease, and the preliminary diagnosis made by a veterinary specialist. The biological material was examined in three sequences to ensure the reliability of the diagnosis.

Sampling of pathological material and preparation of accompanying documents was carried out in accordance with the "Rules for sampling pathological material, blood, feed, water and sending them for research" № 15-14/111 of 15 April 1997 [31]. Urine samples were collected in a volume of 15+5 ml, and sowing on nutrient media was performed upon admission to the laboratory. Bacteriological studies were performed in accordance with SOPs and general biosafety rules [32–35]. Urine samples were inoculated on nutrient media and microscopic examination of the urine sediment was performed. After the urine had been at rest for 1.5 hours (enough time for the sediment to fall to the bottom), it was collected and put into a centrifuge tube with the sample number written on it. Sterile tubes were used for each sample. 10 ml of dog urine collected from the bottom of the vessel was centrifuged for 5 min at 2000 rpm. The precipitate of dog urine was used for the preparation of preparations and sowing on nutrient media. The prepared preparations were stained by the Gram's method. The Gram staining protocol for microorganisms consisted of 4 main stages: 1) application of carbolic genicyanine violet solution for 1 min on the heat-treated smear preparation (dried and fixed) and rinsing with a soft, indirect stream of tap water; 2) applying a mordant – Lugol's solution for 1 minute and rinsing with a soft, indirect stream of tap water; 3) applying a decoloriser – 96° ethyl alcohol for 15 seconds and rinsing for 2 seconds with a soft, indirect stream of tap water; 4) by applying safranin for 30 seconds to 1 minute and rinsing with a soft, indirect stream of tap water. After that, they were dried and microscopy was performed [36]. To determine the presence of microorganisms in the urine and study their tinctorial properties, a study was performed using an immersion system with a magnification of X1000 [37].

Primary cultures of samples, with the selection of pathogens, were performed on blood MPA, with subsequent re-cultures and the use of the API-test system. The study of QMAFAnM indicators (the quantity of mesophilic and facultative anaerobic microorganisms) was carried out using the meat-peptone agar (MPA) medium, the sensitivity to antibiotics of the selected isolates – by Muller-Hinton, disk diffusion method. This universal method is for a wide range of antimicrobial drugs and does not require the use of special equipment. The EUCAST method is a standardized method based on the principles defined in the report of the International Collaborative Antibiotic Susceptibility Study and the experience of expert groups around the world. The selection of antibiotics (amoxicillin, cephalexin, kanamycin, lincomycin, streptomycin, spiramycin, enrofloxacin, rifampicin, erythromycin, tetracycline, gentamicin, colic, etc.) and the determination of their limit values for the diameters of growth retardation zones were calibrated in accordance with the European limit values published by EUCAST and freely available on the EUCAST website http://www.eucast.org [38].

Müller-Hinton agar was prepared according to the manufacturer's instructions [38]. The medium was poured to a thickness of 4.0 ± 0.5 mm (approximately 25 ml for a 90 mm diameter round dish). The surface of the agar was dry before use, with no condensation on the surface. If necessary, the plates were dried at 20 – 25 °C for 8 – 10 h or at – 35 °C with the lid removed for 15 min. They were stored in a refrigerator at 4 – 8 °C.

Results of the study. According to the statistics of the VetForce system of the BTNAU clinic, out of 202 dogs examined in the clinic, 15 (7.43 %) were found to have diseases with signs of urinary tract inflammation, indicating the prevalence of this disease among this species of animals. We examined urine samples from animals with urinary tract inflammation caused by bacterial pathogens, depending on the age of the animals (Fig. 1).

The results of the study indicate that inflammatory processes of the urinary tract caused by bacterial pathogens were recorded in animals of different ages. However, the percentage of dogs with bacterial cystitis was 1.5 times higher than that of animals with urolithiasis (n=6 out of 15). It should be noted that these were mainly dogs older than 1.6 years. In the animals of the first, older age group (6 – 12 and over 12 years), the percentage of the disease was higher compared to dogs from 0.6 to 1.6 years old by 10.2 and 22.2 %, respectively.

In the animals of the second group with urolithiasis, the quantitative index was lower compared to the first group and no disease was observed in dogs aged 0.6 to 1.6 years. In dogs aged 1.6–12 years, the figures were identical and amounted to 16.7 %, while in animals older than 12 years, the disease was recorded 4 times more often compared to animals aged 1.6–12 years and amounted to 66.6 %.

It was found that the number of dogs with urinary tract inflammation caused by bacterial pathogens increases with age.

During the study, the clinic was visited by owners of dogs with signs of urinary tract disease of the following breeds: German Shepherd, French Buddhist, Shar Pei, Poodle, Cocker Spaniel, Golden Retriever, and Bull Terrier. Among the animals of the first group (Fig. 2), the disease was recorded in German shepherds, cocker spaniels and Sharps. In animals of the second group – in Cocker Spaniels and German Shepherds.

Thus, we found that in dogs, inflammatory processes of the urinary tract caused by bacterial pathogens were most often recorded in Cocker Spaniels and German Shepherds.

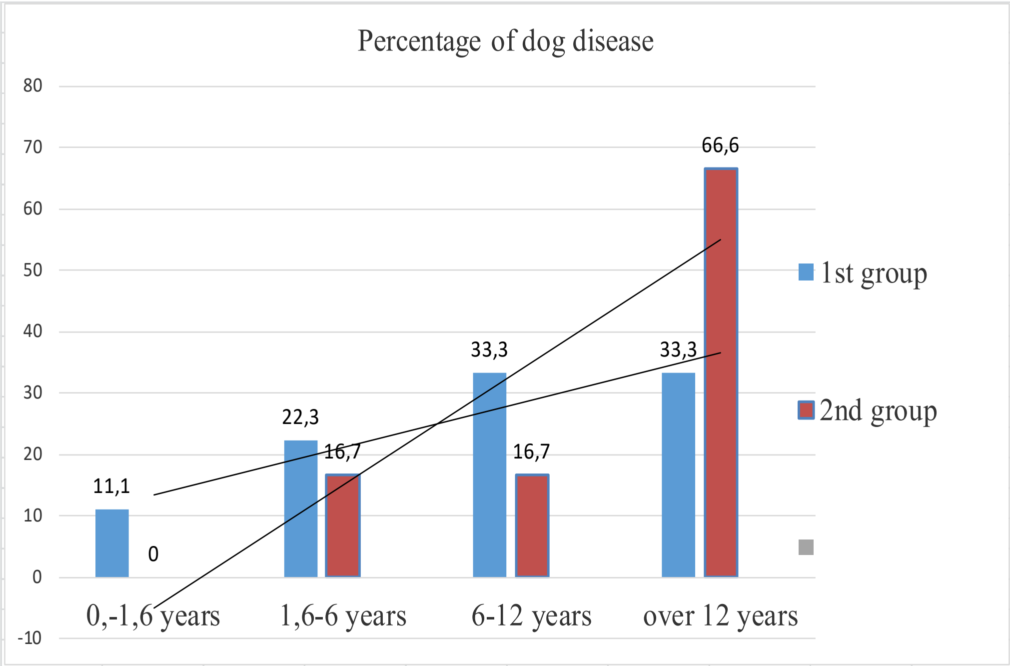

Fig. 1. **Inflammatory processes of the urinary tract caused by bacterial pathogens depending on the age of dogs,** n= 15.

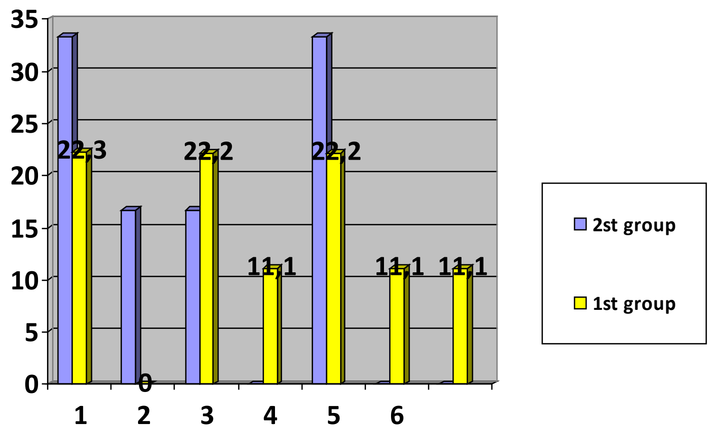

Fig. 2. **Distribution of morbidity in dogs with signs of inflammation of the urinary system, depending on the breed of dogs** (1 – German Shepherd, 2 – French Bulldog, 3 – Shar Pei, 4 – poodle, 5 – Cocker Spaniel, 6 – Golden Retriever, 7 – Bull Terrier).

We established that the quantity of mesophilic aerobic and facultative anaerobic microorganisms (QMAFANM) in animal urine in animals of the 1st and 2nd groups was probably higher than 10^5, which is a normative indicator (1-st group Lim = 7 x 10^4 / 7.3 x 10^6 CFU, 2-nd Lim = 4 x 10^4 / 3 x 10^6 CFU), which indicates an increase in the number of microorganisms (Fig. 3).

The results of the table show that during the study, a gram-negative, facultative anaerobic bacterium *Escherichia coli* was detected. It should be noted that this microorganism was detected more often than others.

Streptococci (*Streptococcus spp.*) were detected in dog urine samples - they were arranged in a chain and stained purple by Gram's stain.

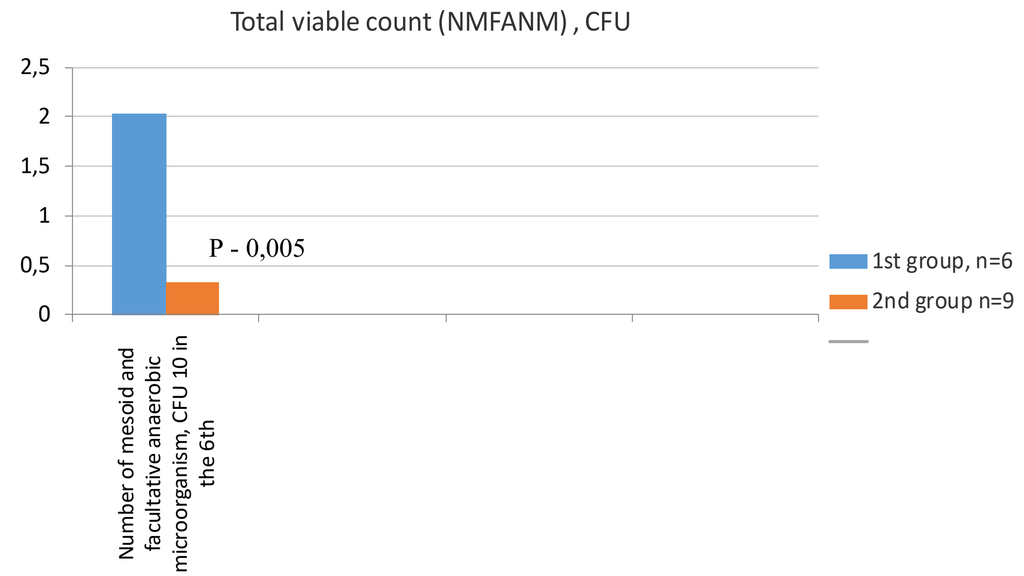

Fig. 3. **Indicators of CFU in the urine of pre-lactating animals, the first group relative to the second.**

During the bacteriological examination of the urine of dogs of the first and second groups, we isolated and identified the bacteria *Sarcina urinae*, which were found mainly in alkaline samples. We also isolated sapophytic urinary streptococci - *Streptococcus urina*: large diplococci arranged in a chain were isolated from alkaline urine. In addition to them, *E. coli, Ps. aeruginosa* and pus-forming bacteria were detected: *Streptococcus pyogenes, Staphylococcus aureus, etc*. (Table 1).

Table 1 – **Urine microflora**

Isolated microflora from animal urine, n = 15	
Monoinfection, n = 9	Association of microflora, n = 6
Streptococcus urinae	*E. coli, Sarcina urinae, Staphylococcus aureus*
E. coli (3)	*E. coli, Streptococcus pyogenes*
Pseudomonas aeruginosa (2)	*Proteus spp., Saccharomyces spp.*
Staphylococcus aureus s	*Pseudomonas aeruginosa, E. coli,*
C. *and lbicans*	*E. coli, Streptococcus pyogene, Proteus spp.*
Streptococcus pyogenes	*Streptococcus urinae, E. coli, Klebsiella pneumonia*

Examining the urine sediment under the microscope, we found yeast fungi *(Saccharomyces)* in the form of oval shiny nucleated formations arranged individually, in groups or in chains. New cells were formed on individual cells of yeast-like fungi by budding. Unlike erythrocytes, which have similarities to fungi, yeast fungi were not destroyed by the addition of a drop of acetic acid to the preparation. In addition, when comparing the results of the two groups - 55.6 and 66.7 per cent versus 44.4 and 33.3 per cent, respectively - the infection in the dogs' organisms caused by bacteria of the same species prevailed (Fig. 4).

We found that *Escherichia coli, Streptococcus urinae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were most often isolated from dog urine (Fig. 5).

Thus, we found that in both groups, pathogens were predominantly isolated as monoinfections: *E. coli* and *Pseudomonas aeruginosa*. However, it should be noted that in group 2, monoinfection in dogs (66.7 %) was 10.1 % higher compared to the first group (55.6 %). In addition, in animals with bacteria isolated in associations, they prevailed in the first group and amounted to 44.4 %, compared to 33.3 % in the second group. In the associated form, *Escherichia coli* and *Streptococcus pyogenes* prevailed. In order to prescribe the treatment of animals, the next stage of research was to determine the sensitivity of the isolated microorganisms to antibiotics (Fig. 6).

The isolates were sensitive (the zone of growth retardation was 1 – 3 mm higher) to Cephalexin, and one was weakly sensitive.

Thus, we have established that a large number of microorganisms cause urinary tract inflammation in dogs, but mainly *Escherichia coli, Streptococcus urinae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, *Klebsiella pneumonia*, a less common microorganism in animal urine, was resistant.

Discussion. The results of bacteriological diagnostics are necessary for the diagnosis, as well as for the correct choice of drugs for use. If the degree of bacteriuria does not exceed 10^3 microbial cells in 1 ml of urine, this indicates the absence of inflammation, urine contamination; if the bacteriuria is 10^4 microbial cells in 1 ml of urine, the result is doubtful and it is recommended to repeat the study; the degree of bacteriuria was 10^5 or more microbial cells. During the initial examination of urine samples from sick dogs, we found more than 10^5 (9×10^5, 4×10^6 CFU) in groups 1 and 2, indicating bacteriuria [39].

Some scientists claim that enterococci are found in the urine. Infection with enterococci mainly occurs with water. The maximum permissible level for water contamination with enterococci is 7 CFU per 100 ml of water. It is believed that this provides a high correlation of enterococci in urban sewage. However, we did not detect enterococci in samples from sick animals. The main feature of enterococci is that they are classified as opportunistic pathogens. About 20 species are known (*E. pallens, E. gilvus, E. faecalis, E. faecium, E. avium, etc.*) [40–41].

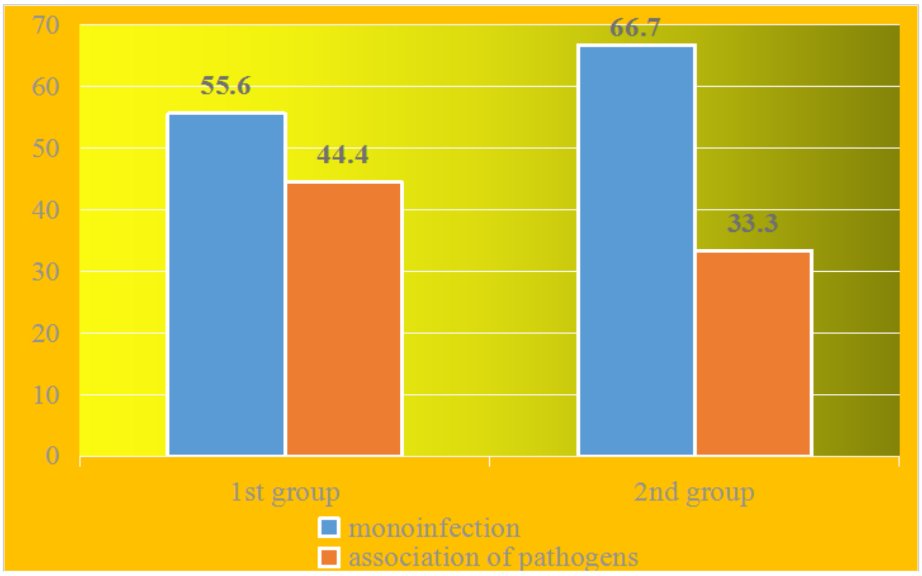

Fig. 4. **Percentage of isolated isolates, relative to groups, %.**

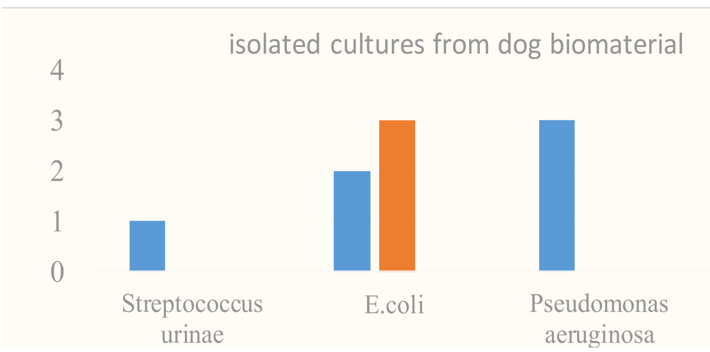

Fig. 5. **Isolated cultures from the urine of experimental dogs.**

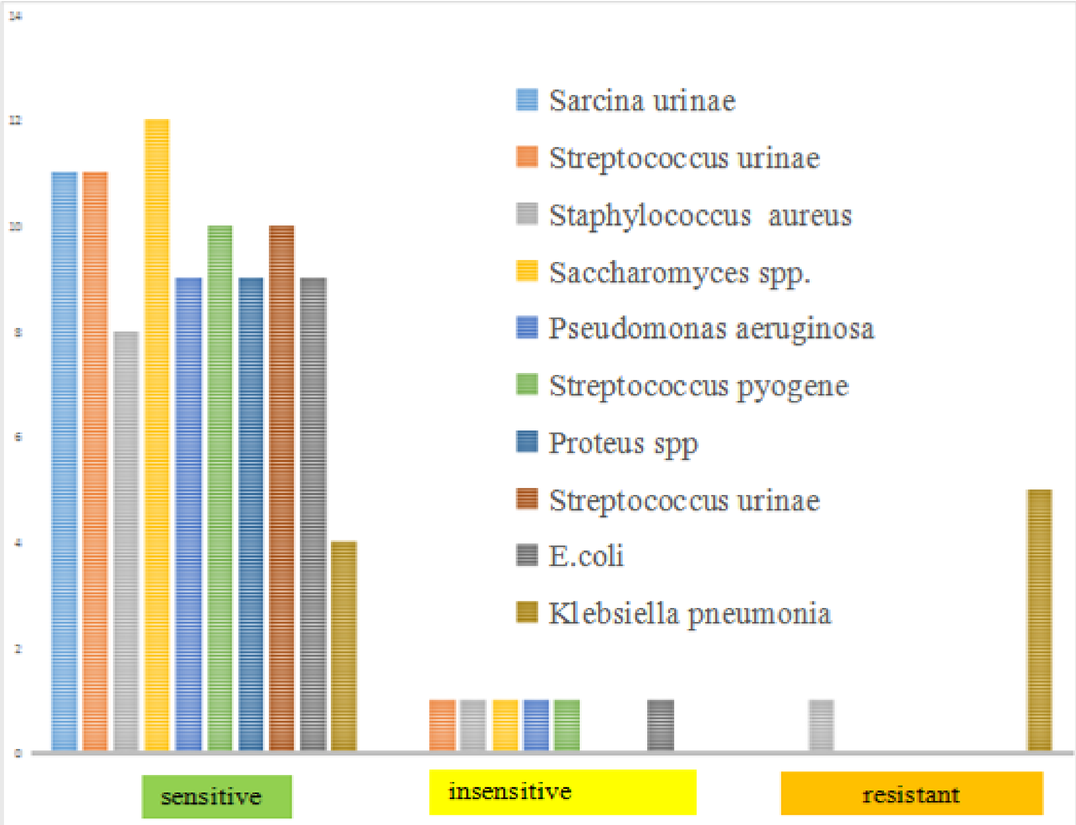

Fig. 6. **Determination of the sensitivity of microorganisms isolated from urine to antibiotics.**

The liver suffers from the effects of toxins synthesised by bacteria: it does not have time to neutralise harmful substances. The metabolites then enter the bloodstream and cause a picture of hepatic encephalopathy. An increase in the amount of QMAFAnM leads to diseases of the genitourinary system, causes cystitis, inflammation of the urinary bladder becomes the cause of the development of pyelonephritis [41].

A urinary tract infection is the presence of microorganisms in the urinary tract above the bladder sphincter, which is normally sterile. Significant bacteriuria, which we found in the urine of all groups, all animals, indicated a urinary tract infection - the number of live bacteria of one strain in 1 ml of urine. Under normal conditions, the urinary tract is sterile, with the exception of the urethra, which is home to mainly saprophytic coagulase-negative staphylococci (*Staphylococcus epidermidis*), vaginal bacilli (*Haemophilus vaginalis*), non-haemolytic streptococci, corynebacteria, and lactic acid bacteria (*Lactobacillus*) [42–43].

Pathogenic microorganisms colonise the urinary system mainly by ascending pathways. The first step in the development of an ascending urinary tract infection is the colonisation of the urethra by uropathogenic bacteria. This is easier in females, where the vagina is the reservoir of uropathogenic microorganisms; the distance between the urethra and the anus is also smaller. The next step is the penetration of bacteria, often during sexual intercourse [43].

The infectious agent can enter the urinary system in three ways: a) haematogenous - from distant sources, b) lymphogenous – from the genital organs, intestines, c) ascending - from the urinary tract [44]. The effect of infection on the stone formation process is reduced to three main points. The infectious agent, being sufficiently virulent, causes inflammatory changes in the urinary system. This is evidenced by the physicochemical parameters of urine: a violation of the ratio of hydrophilic and hydrophobic colloids, a decrease in surface tension, a decrease in the solubility of minerals, the appearance of desquamated epithelium, etc. [45–46]. Formation of mucus around the microorganisms, which, together with rejected epithelial cells, forms the core of the future calculus, and infected urine acquires a stable pH [47–48]. For example, the enzymes *Proteus spp., Pseudomonas and Klebsiella spp*. break down urea into water and ammonia, which leads to a shift in urine pH to 7.5– 9.0. Despite the significant progress in the study of urolithiasis and the availability of a significant amount of literature on this issue, the question of the origin of urinary tract stones still remains one of the most complex and unexplored.

Antibiotic therapy should be carried out until complete elimination, which is achieved through long-term treatment. According to veterinarians, long-term treatment requires the alternation of two or three drugs at the same time. This ensures a better bacteriolytic effect and reduces the possibility of developing resistant strains of bacteria. Local application of antibiotics in the form of an instillation into the urinary tract is also not recommended, as its mucous membrane has no absorption capacity. Consequently, the administered antibiotics will dissolve in the urine and be excreted [49].

Conclusions. 1. Bacterial pathogens cause inflammatory processes of the urinary tract, which are recorded mainly in German Shepherds and Rocker Spaniels of all ages, especially those older than 1.6 years. Bacterial cystitis prevails by 1.5 times compared to the figures for animals with urolithiasis. In dogs of the first group of 6–12 and older than 12 years, the percentage of the disease was higher compared to the group from 0.6 to 1.6 years by 10.2 and 22.2 %, respectively.

2. Dogs aged 1.6–12 years suffering from urolithiasis (66.6 %) suffer from bacterial diseases 4 times more often than animals aged 1.6–12 years. *Streptococcus urinae, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus* were isolated and identified from dog urine. In the associated form, they predominated in the urine of dogs: *E. coli and Streptococcus pyogene*.

3. *Klebsiella pneumonia* was found to be resistant to amoxicillin, streptomycin, kanamycin, gentamicin and tetracycline.

Information on conflict of interest. The authors (Iryna Rublenko, Iryna Chemerovska, Maria Bolibrukh, Svitlana Taranuha, Serhii Rublenko) of the article "Examination of urinary microflora and resistance of isolated pathogens during inflammatory processes of the urinary tract in dogs" claim no conflict of interests regarding their contribution and research results. The study was conducted in accordance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Official Journal of the European Union L276/33, 2010), as well as in accordance with the Law of Ukraine "On Protection of Animals from Cruelty" of 28.03.2006, № 27, Art. 230, Order of the Ministry of Education and Science № 416/20729 of March 16, 2012 "On Approval of the Procedure for Conducting Research and Experiments on Animals by Scientific Institutions" and approved by the Ethics Committee of Bila Tserkva National Agrarian University (conclusion № 2 of 31.05.18, protocol № 1). The materials of the article may be published.

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Вивчення мікрофлори сечі та резистентності виділених збудників за запальних процесів сечовивідних шляхів у собак

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Стійкі до антибіотиків бактерії, наразі, часто виділяють від домашніх улюбленців та сільськогосподарських тварин. Тривале нераціональне застосування антибіотиків, з метою лікування тварин та людей, недооцінене і потребує подальших досліджень, зокрема на території України. Мета дослідження – ідентифікація бактеріальних ізолятів та вивчення їх чутливості до антибіотиків за запальних процесів сечовивідних шляхів у собак. За статистичними даними системи *VetForce* клініки БНАУ, із 202 собак, які були обстежені у клініці, у 15 (7,43 %) виявлено захворювання з ознаками запальних процесів сечовивідних шляхів. Встановлено, що запальні процеси сечовивідних шляхів у собак спричинює ряд мікроорганізмів, це, переважно, *E. coli, Streptococcus urinae, Pseudomonas aeruginosa* та *Staphylococcus aureus.* Резистентним виявився менш поширений мікроорганізм – *Klebsiella pneumonia.*

Встановлено, що бактеріальний цистит (перша група тварин) переважає у 1,5 рази, порівняно з показниками хворих тварин на сечокам'яну хворобу. У собак першої групи 6–12 та старших 12 років відсоток захворювання був вищим, порівняно з групою від 0,6 до 1,6 років на 10,2 та 22,2 %, відповідно.

Собаки другої групи, віком 1,6–12 років, хворі на сечокам'яну хворобу (66,6 %), хворіють на бактеріальні захворювання у 4 рази частіше порівняно з тваринами 0,6–1,6 років. Із сечі собак цієї групи виділено та ідентифіковано *Streptococcus urinae*, *Escherichia coli*, *Pseudomonas aeruginosa* та *Staphylococcus aureus*. В асоційованій формі у сечі собак переважали *E. coli* та *Streptococcus pyogene*. Виявлено резистентний штам *Klebsiella pneumonia*

до амоксициліну, стрептоміцину, канаміцину, гентаміцину та тетрацикліну.

Слід раціонально застосовувати антибіотики, враховувати, що для лікування тварин необхідно визначати чутливість патогену, яким інфікувалася ця тварина. Антибактеріальну терапію доцільно проводити до повної ліквідації, яка досягається інколи за тривалого лікування.

Ключові слова: мікроорганізми, поширення, патогени, сеча, собаки, резистентність, антибіотики, сечовивідні шляхи, бактеріологічне дослідження.

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