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O.V. HADZEVYCH[1*], A.P. PALIY[1], B.T. STEHNII[1], A.B. STEHNII[1], O.N. CHECHET[1], D.V. HADZEVYCH[1], A.P. PALII[2], O.V. PAVLICHENKO[2], R.V. SEVERYN[2], R.V. PETROV[3], L.P. LIVOSHCHENKO[3]

[1] National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine», 83 Pushkinska Str., Kharkiv, 61023, Ukraine

[2] State Biotechnological University, 44 Alchevskykh Str., Kharkiv, 61002, Ukraine

[3] Sumy National Agrarian University, 160 Herasyma Kondratieva Str., Sumy, 40021, Ukraine

* Author for correspondence; e-mail: olgagadzevych@gmail.com

ANTIBIOTIC RESISTANCE OF MICROBIOTAS OF FISHERY ENTERPRISES HYDRO ECOSYSTEMS

The aquatic environment is an integral part of biocenosis that directly affects its condition and safety in terms of epidemiology and epizootology. The study of the aquatic environment for the presence of pathogens and the quantitative characteristics of sanitary-indicative microorganisms is extremely important. The obtained data allow us to assess and predict the risks of infections, and to develop a plan of measures to prevent the spread of certain pathogens. ***The aim of the work.*** *To analyze the microbial state of the aquatic environment in different hydro ecosystems of fish farms in the Kharkiv region and to assess the presence of microbiological risks to public health.* ***Methods.*** *The research objects were 150 samples of water taken from different hydro ecosystems in the Kharkiv region. Water was taken from closed water supply systems (n = 30) and from ponds (n = 120), where commercial fish is bred for sale. The presence and number of sanitary-indicative microorganisms and pathogenic bacteria were determined by the bacteriological (cultural) method. Resistances to antibacterial drugs in selected sanitary-indicative microorganisms were determined using the Agar disk-diffusion method. Estimation of the reliability of the difference between the compared indicators was determined using Student's t-test.* ***Results.*** *The dominant sanitary-indicative microorganisms in the aquatic environment of fish farming were bacteria of the genus Citrobacter spp., Aeromonas spp., and Pseudomonas spp. The total bacterial contamination of water bodies ranged from* $1.9 \pm 0.50 \cdot 10^4$ *to* $2.1 \pm 1.20 \cdot 10^5$ *CFU in 1* cm^3 *of water. No pathogenic to humans bacteria have been detected. Isolated sanitary-indicative microorganisms had significant resistance to antibacterial drugs. Resistance to penicillins, sulfonamides, and nitrofurans was the highest (p = 0.0001). The percentage of penicillin resistance strains ranged from 81.5% to*

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87.0%, sulfonamide — from 74.1% to 94.4%, and nitrofuran — from 55.5% to 66.7%. Fluoroquinolone and cephalosporin resistance varied depending on the type of antibacterial substance, but it did not exceed 29.6%. ***Conclusions.*** *According to the research results for the aquatic environment of fish farms in the Kharkiv region, no pathogenic microorganisms were detected. However, it has been established that sanitary-indicating microorganisms (Citrobacter spp., Aeromonas spp., Pseudomonas spp.), which were dominant and had polyresistance to antibacterial drugs, may be risk factors for human health. Thus, the hydro ecosystems of fish farms have favorable conditions for the accumulation of bacterial strains resistant to antibiotics. Therefore, the use of antibacterial drugs should be scientifically justified and strictly controlled.*

Keywords: *hydro ecosystem, microbiological risks of the aquatic environment, sanitary-indicative microorganisms, antibiotic resistance.*

The state of health and physiological activity of any living organism directly depends on the conditions of keeping, feeding, and the epizootiological safety of the territory [1, 2]. The aquatic environment is of particular importance because human and animal organisms are in direct contact with it [3]. Clearly, water cannot be sterile, but it is important to determine which microbiota predominates in the aquatic environment and whether it can be a risk factor for the health of aquatic life, animals, and humans [4, 5].

In the presence of pathogenic microbiota in water, it becomes a dangerous source of infection of macroorganisms. Infection can occur directly, in particular through direct contact with water, or indirectly — through food (fish, plants), which can be a factor in the pathogen transmission [6—11].

The official website of the State Food and Consumer Service of Ukraine, in the annual report for 2020, states that water was the cause of disease in the population in 3.8% of cases. Therefore, the constant study of the aquatic environment for the presence of pathogens and quantitative characteristics of sanitary-indicative microorganisms is an urgent task for relevant specialists. The obtained data allow us to assess and predict the risks of infections and to develop a plan of measures to prevent the spread of some pathogens [12]. An important task is to determine the distribution in the aquatic environment of microorganisms resistant to antibacterial drugs [13—19].

The research aimed to determine and analyze the microbial state of the aquatic environment in different hydro ecosystems of fish farms in Kharkiv region, to establish and assess the presence of microbiological risks to public health.

Materials and methods. In 2020—2021, 150 water samples were subjected to bacteriological analysis. Water was taken from closed water supply systems (CWSS) (n = 30) and in ponds (n = 120) in the Kharkiv region, where commercial fish is bred for sale. Bacteriological studies were conducted in the laboratory for the study of animal bacterial diseases of the National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine» (Kharkiv). Water samples were taken following the sanitary rules and regulations and examined no later than 2 hours after sampling. The presence and number of sanitary-indicative microorganisms and pathogenic microorganisms of bacterial etiology were determined in the samples. For the study, we used simple, selective and differential-diagnostic nutrient media, disks, and strips, manufactured by LLC «Pharmactive» (Ukraine) and «Himedia Laboratories Prv. Limited» (India). Isolated cultures were obtained from selective media. To study the morphological properties of microorganisms, smears were stained by a Gram stain. Erba Laachema commercial test kits (Czech Republic) were used to identify microorganisms such as ENTERO 24-, STAPHY 24-, STREPTO 16-, NEFERM 24-, ANAERO 23-, En-COCCUS 16-, CANDIDA 21- tests. The species affiliation of isolated cultures of microorganisms was determined by the cultural, morphological, and biochemical properties recommended by the «Bergey's Manual of Systematics Bacteriology»,

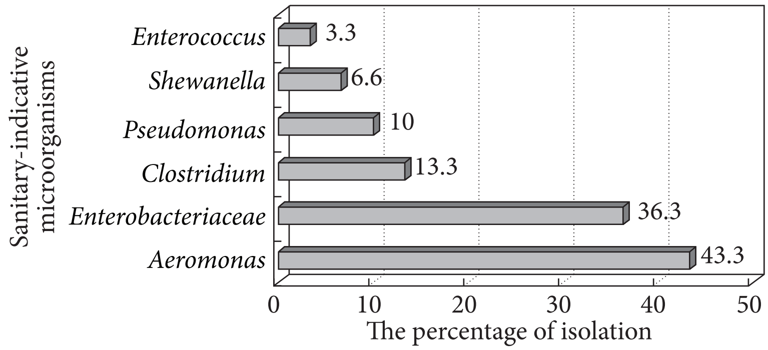

Fig. 1. The ratio of genera of sanitary-indicative microorganisms isolated in water from closed water supply systems, n = 30

2012 [20]. Ten-fold dilutions of water samples in buffer-peptone water (from 10^{-1} to 10^{-6}) were performed to determine quantitative indicators. The total microbial count (TMC) was determined by plating 1 cm^3 of the prepared water sample under agar. The presence of pathogenic factors and resistance to antibacterial drugs in selected sanitary-indicative microorganisms were determined according to [21]. The data obtained were necessary to establish the potential risks of infection in animals and humans.

Tests for the susceptibility of microorganisms to antibacterial drugs were performed with a pure bacteria culture following current recommendations [22]. Mueller Hinton Agar nutrient medium manufactured by HiMedia (India) and standardized commercial disks with antibiotics manufactured by HiMedia (India), Farmaktiv LLC (Ukraine) were used to determine the antibiotic sensitivity of the isolated microorganisms.

The result of the antibioticogram was determined according to the criteria set out in EUCAST 2021 [23]. Statistical processing of the obtained data was performed using the computer program Microsoft Excel. Estimation of the reliability of the difference between the compared indicators was determined using Student's t-test.

Results. According to the study results, it has been found that the amount of microflora in CWSS and ponds varies depending on the season and the temperature of the aquatic environment (Table 1). The indicator of the total bacteriological contamination of water in CWSS and ponds in summer at a water temperature above 14 °C was higher than in spring and autumn, when the water temperature was below 10 °C.

In water from closed water supply systems (Fig. 1), sulfite-reducing clostridia (*Clostridium* spp.) were isolated in 4 samples (13.3% of cases), bacteria of the family *Enterobacteriaceae* — in 11 samples (36.3%), bacteria of the genus *Pseudomonas* — in 3 samples (10.0% of cases), bacteria of the genus *Shewanella* — in 2 samples (6.6%), bacteria of the genus *Aeromonas* — in 13 samples (43.3% of cases), and the presence of enterococci (*Enterococcus*) was found in 1 sample (3.3% of cases).

Bacteria of the genus *Citrobacter* (4 samples, 35%), *Enterobacter* (2 samples, 18%), *Serratia* (3 samples, 27%), *Proteus* (1 sample, 10%), and *Escherichia* (1 sample, 10%) dominated among

Table 1. **The results of determining the microbial count in water**

Indicator	Material type	Number of samples	Results	
			Summer period	Spring and autumn period
Total microbial count, CFU/cm^3	ater from CWSS ater from ponds	30 120	$4.5 \pm 0.96 \cdot 10^4$ $2.1 \pm 1.20 \cdot 10^5$	$1.9 \pm 0.50 \cdot 10^4$ $3.0 \pm 0.65 \cdot 10^4$

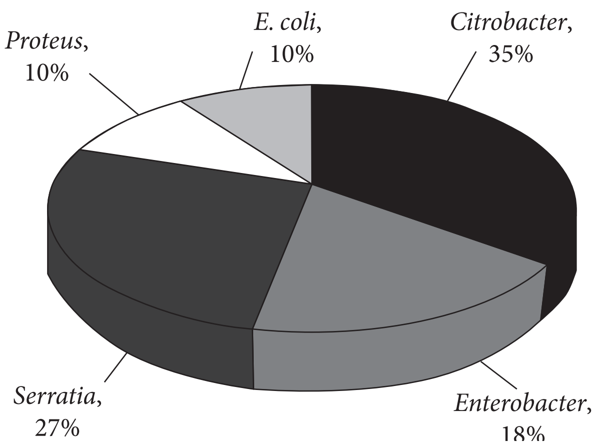

Fig. 2. The ratio of bacteria of the family *Enterobacteriaceae* isolated in the water from closed water supply systems (%)

the bacteria of the family *Enterobacteriaceae* in water from CWSS (Fig. 2).

The microflora of ponds was more diverse (Fig. 3). Bacteria of the family *Enterobacteriaceae* were isolated in 110 samples (92%), sulfite-reducing clostridia — in 24 samples (20% of cases), bacteria of the genus *Pseudomonas* — in 33 samples (27% of cases), bacteria of the genus *Aeromonas* — in 43 samples (35% of cases), the presence of enterococci was found in 32 samples (26% of cases) and coagulase-positive staphylococci — in 18 samples (15% of cases).

In pond water (Fig. 4), *Citrobacter* (42 samples, 35%), *Enterobacter* (32 samples, 26%) and *Proteus* (24 samples, 20%) were also persistent microorganisms from the family *Enterobacteria-*

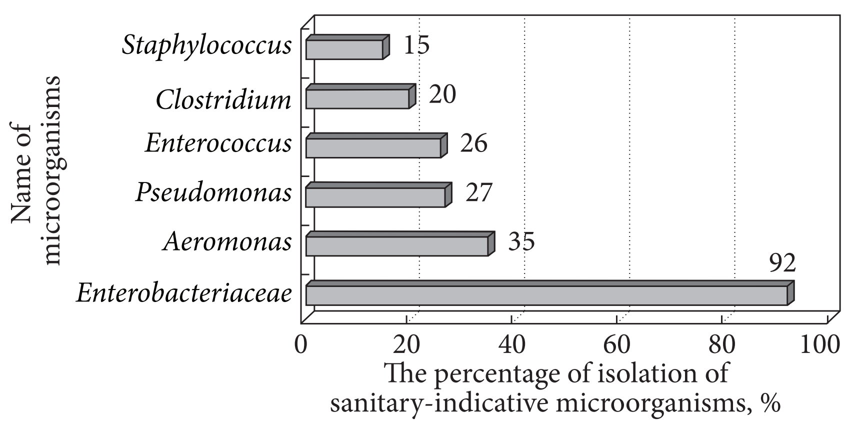

Fig. 3. The ratio of genera of sanitary-indicative microorganisms isolated from ponds, n = 120

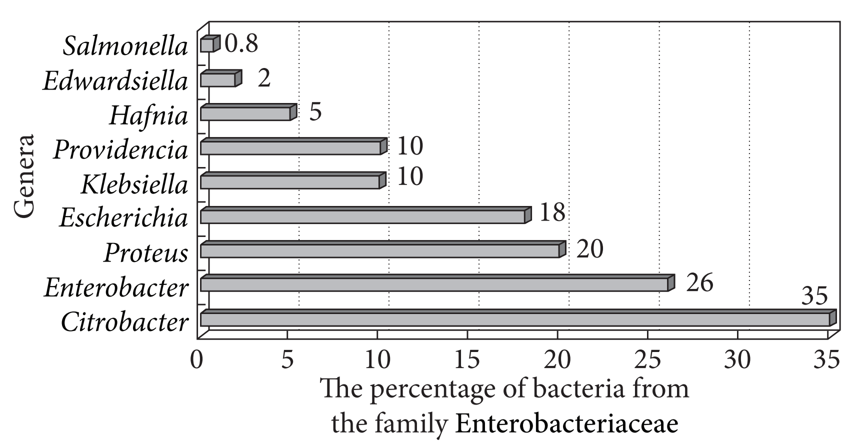

Fig. 4. Percentage of bacteria of the family *Enterobacteriaceae* that were isolated from pond water

ceae. In addition, *Escherichia* (22 samples, 18%), *Edwardsiella* (2 samples, 2%), *Klebsiella* (12 samples, 10%), *Providencia* (12 samples, 10%), *Salmonella pullorum* avian species (1 sample, 0.8%), and *Hafnia*, which was characterized by hemolytic properties (6 samples, 5%) were isolated. In 21% of cases, several species of dominant enterobacteria were isolated simultaneously.

Thus, the water from CWSS was more favorable (p = 0.5210) in terms of the total bacterial contamination and the spread of sanitary-indicative microorganisms than the water from ponds. No virulent microorganisms were isolated from CWSS water, and no pathogenic factors, including adhesive, hemolytic, and toxigenic properties, were detected in isolated sanitary-indicative bacteria.

A strain of avian salmonella, *Hafnia*, which had hemolysins and coagulase-positive staphylococci in 15% of the cases, was isolated from pond water. Bacteria of the *Escherichia coli* group were isolated by 55.7% more than from CWSS water, and enterococci and sulfite-reducing clostridia were isolated by 22.7% and 6.7% more than from CWSS water, respectively (Table 2). In contrast to water ponds, *Shewanella* spp. was isolated in water from SWSS, and the number of isolated aeromonads was 8.3% higher than from water ponds (Table 2).

Biological properties and the presence of pathogenic factors were determined in the selected sanitary-indicative microorganisms in order to determine possible risk factors for the health of aquatic organisms and humans. According to the results of these studies, it was found that the sanitary-indicative microorganisms isolated from both CWSS and ponds had significant resistance to antibacterial drugs (Table 3). The highest resistance (p = 0.0001) was observed to penicillins, sulfonamides, and nitrofurans.

The level of resistance to penicillins in isolated from CWSS microorganisms was 81.5% and in microorganisms isolated from pond water — 87.0%. The percentage of resistance to

Table 2. **The results of determining the presence of sanitary-pathogenic microorganisms in different hydro ecosystems of fish farms in Kharkiv region**

Indicators	Water	Number of positive samples, %	The difference between the indicators (%)
Bacteria of the Escherichia coli group	From CWSS (n=30) From ponds (n=120)	11 / 36.3 110 / 92.0	55.7 higher in ponds
Sulfite-reducing *Clostridium* spp.	From CWSS (n=30) From ponds (n=120)	4 / 13.3 24 / 20	6.7 higher in ponds
Coagulase-positive *Staphylococcus* spp.	From CWSS (n=30) From ponds (n=120)	0 18 / 15.0	15.0 higher in ponds
Pseudomonas spp.	From CWSS (n=30) From ponds (n=120)	3 / 10.0 33 / 27.0	17.0 higher in ponds
Aeromonas spp.	From CWSS (n=30) From ponds (n=120)	13 / 43.3 43 / 35.0	8.3 higher in CWSS
Enterococcus spp.	from CWSS (n=30) From ponds (n=120)	1 / 3.3 32 / 26.0	22.7 higher in ponds
Salmonella spp.	From CWSS (n=30) From ponds (n=120)	0 1 / 0.8	0.8 higher in ponds
Shewanella spp.	From CWSS (n=30) From ponds (n=120)	2 / 6.6 0	6.6 higher in CWSS

Table 3. **Resistance to antibacterial drugs of sanitary-indicative microorganisms (bacteria of the *Escherichia coli* group, aeromonads, and pseudomonads) isolated from different hydro ecosystems in the Kharkiv region**

Antibiotics	Resistance of sanitary-indicative microorganisms isolated from			
	closed water supply systems (n = 27)		ponds (n = 54)	
	Number of resistance	%	Number of resistance	%
Penicillins				
Amoxicillin (10 mg)	22	81.5	47	87.0
Cephalosporins				
Cefotaxime (30 mg)	3	11.1	11	23.4
Ceftazidime (30 mg)	4	14.8	16	29.6
Tetracyclines				
Tetracycline (30 mg)	13	48.1	42	77.8
Doxycycline (30 mg)	13	48.1	42	77.8
Oxytetracycline (30 mg)	13	48.1	40	74.1
Fluoroquinolones				
Enrofloxacin (10 mg)	2	7.4	4	7.4
Ofloxacin (5 mg)	6	22.2	2	3.7
Ciprofloxacin (5 mg)	5	18.5	3	5.5
Gatifloxacin (5 mg)	5	18.5	6	11.1
Norfloxacin (10 mg)	8	29.6	11	20.3
Levofloxacin (5 mg)	6	22.2	5	9.2
Aminoglycosides				
Gentamicin (10 mg)	12	44.4	5	9.2
Kanamycin (30 mg)	9	33.3	11	20.3
Amikacin (30 mg)	14	51.8	13	24.1
Neomycin (30 mg)	8	29.6	9	16.7
Amphenicols				
Levomycetin (30 mg)	13	48.1	36	66.7
Nitrofurans				
Furazolidone (50 mg)	15	55.5	36	66.7
Sulfonamides				
Sulfadiazine (25 mg)	20	74.1	51	94.4
Sulfamethoxazole (25 mg)	20	74.1	51	94.4
Other				
Fosfomycin (200 mg)	16	59.2	47	87.0
Trimethoprim-sulfamethoxine (25 mg)	17	62.9	50	92.6

sulfonamides was 74.1% and 94.4% and to nitrofurans — 55.5% and 66.7 %, respectively. The lowest resistance (p=0.0000) in the isolated sanitary-indicative microorganisms was observed for fluoroquinolones and cephalosporins. The level of resistance to fluoroquinolones in sanitary-indicative microorganisms isolated from CWSS water ranged from 7.4% to 29.6%, and in microorganisms isolated from pond water — from 3.7% to 20.3%. From 11.1% to 14.8% of microorganisms isolated from CWSS water and from 11.1% to 29.6% isolated from ponds were resistant to cephalosporins.

Discussion. The concentration of living organisms in a limited area poses a threat of accumulation of a large number of pathogens in the environment [24-26]. High microbial pollution of the aquatic environment increases its epidemiological and epizootological danger and, as a consequence, reduces the natural resistance of fish and promotes the emergence of bacterial diseases and microbial contamination of the internal organs of aquatic organisms [27].

There is little information in the available scientific literature on the etiology of sanitary-indicative microorganisms that dominate in the hydro ecosystems of Ukraine. However, there are reports that water can be dangerous, in particular under anthropogenic influence [13, 27]. It is noted that in the Egyptian ponds, 80% of all diseases are associated with water of low microbiological quality. Bacterial contamination of fish is a real threat to aquaculture systems, causing serious damage, mortality, and great economic losses among aquatic organisms. Contamination of fish during fishing with some entero-pathogenic bacteria then contaminates food, making it unfit for human consumption, or even harmful to consumers [27, 28]. Among the most common microorganisms in the aquatic environment of the ponds, there were bacteria of the genus *Pseudomonas* spp. (22.5 — 25,0%), *E. coli* (15,0 — 35,0%), *Proteus* spp. (up to 14.2% of cases), *Klebsiella* spp. (11,7 — 25,0%), *Citrobacter* spp. (10,0 — 15,0%) and *Salmonella* spp. (up to 1.6% of the cases) [27].

The most common microorganisms in the contamination of fish products were *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Provedencia* spp., *Serratia* spp., *Klebsiella* spp., which were isolated in an amount of $1.8 \cdot 10^2$ to $2.7 \cdot 10^4$ CFU in 1g of product [29]. There are studies that show that the infectious dose of bacteria pathogenic to living organisms is from 10^4 to 10^6 CFU [30]. Therefore, the risk of transmission of pathogens through aquaculture should be controlled, in particular through continuous monitoring and sanitation of water bodies.

The results of our research showed that *Citrobacter, Enterobacter, Proteus* and *E. coli* are the persistent bacteria in the ponds of the Kharkiv region. The total bacterial contamination of water bodies ranged from $1.9 \pm 0.50 \cdot 10^4$ to $2.1 \pm 1.20 \cdot 10^5$ CFU in 1 cm^3 of water. Human pathogens bacteria have not been isolated.

If we compare the percentage of resistance of microorganisms isolated from the water of fish farms with the resistance of microorganisms isolated from livestock farms in Ukraine, it is lower [31]. The resistance of microorganisms isolated from fish farm water to fluoroquinolones did not exceed 29.6%, penicillins — 87.0%, tetracyclines — 77.8%, and amphenicols — 66.7%.

Our studies have shown the presence of moderate water pollution in the CWSS and the presence of sanitary-indicative microorganisms. Bacteria of the genus *Citrobacter, Enterobacter,* and *Serratia* dominated. The isolated microorganisms did not have evident pathogenic factors, except for resistance to antibacterial drugs, in particular to penicillins (81.5%), sulfonamides (74.1%), nitrofurans (55.5%), tetracyclines (48.1%), and amphenicols (48.1%). According to our research, we can conclude that the CWS systems have favorable conditions for the accumulation of resistant strains. Therefore, the use of antibacterial drugs in such a system must be scientifically justified and strictly controlled. Sanitary-indicative microorganisms isolated from ponds were more resistant to tetracyclines (p=0.0000) and cephalosporins (p=0.2094) than to microorganisms isolated from CWSS.

Analyzing the data of the scientific literature on this issue, we can note that the spread of antibiotic resistance in bacteria around the world is large-scale and increasing [32—34]. The percentage of resistant bacteria varies depending on the country, region, and level of urbanization. For example, sulfonamides and fluoroquinolones have been reported to be present in China's surface water ecosystems. There are warnings about the isolation from the water of some fish farms in Europe of multidrug-resistant strains that are directly involved in food chains, threatening the population's health [35]. *Escherichia coli* strains isolated on the coast of Italy were resistant to tetracyclines (28%), penicillins (16.5%), ampicillin (16.5%), trimethoprim-sulfamethoxazole (13%), streptomycin (7%), and trimethoprim-sulfamethoxine (13%) [36].

Multidrug-resistant strains are also isolated from the marine ecosystems of Italian fish farms. Isolated bacteria in 17% of the cases were resistant to tetracycline, 7 % — to trimethoprim-sulfadiazine, 0.3 % — to flumequine (fluoroquinolone antibiotic) [37]. Other scientists also report high anthropogenic pressure on water habitats and the spread of multidrug-resistant strains in rivers, lakes, and ponds [35, 38]. In India, *E. coli* isolates resistant to eight antibiotics have been isolated from the river at points adjacent to the city [39]. Reliable ($p<0.05$) seasonal and spatial fluctuations of antibiotic-resistant isolates of *E. coli* in water and sediments have been found [40].

Thus, our research and analysis have confirmed the scientific literature data that any hydro ecosystem can be a source of danger to animal, aquatic and human health, and it is an indicator of sanitary-epidemiological and environmental problems due to the spread of microorganisms resistant to antibacterial drugs.

Hence, the prescription and use of antibacterial drugs, in particular in aquaculture, requires a comprehensive science-based approach. Recently, several highly effective and alternative means of treatment and control of diseases caused by microorganisms have been developed. They include the use of new gene editing technology (CRISPR/Cas9), genetically modified bacteriophages, engineered peptides, nanoantibiotics, vaccines, probiotics, and eubiotics [41—43].

To overcome the spread of antibiotic-resistant forms of microbiota, it is necessary to carry out joint work with scientists, specialists of diagnostic institutions, and owners and workers of livestock farms. Livestock workers must clearly understand that the use of antibacterial drugs for prophylactic purposes and without diagnosis with the determination of susceptibility of pathogens is unreasonable and environmentally hazardous. In fish farming, it is economically impractical to treat aquatic organisms due to the rapid spread of pathogens in the aquatic environment, especially in dense populations of living organisms. The main efforts and veterinary measures should be aimed at disease prevention, control of the quality of the aquatic environment, health of aquatic organisms, and compliance with the norms and parameters of their keeping.

Only a comprehensive vision for assessing and eradicating the risks of the spread of antibiotic-resistant microorganisms through water bodies can provide the solution to this urgent health problem.

Conclusions. According to the research results for the hydro ecosystems of fish farms in the Kharkiv region, no pathogenic microorganisms were detected. It has been established that sanitary-indicative microorganisms (*Citrobacter* spp., *Aeromonas* spp., and *Pseudomonas* spp.), which were dominant and had polyresistance to antibacterial drugs, have been identified as risk factors for human health. Favorable conditions for the accumulation of antibiotic-resistant microorganisms are created in hydro ecosystems. The use of antibacterial drugs should be scientifically justified and strictly controlled.

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О.В. Гадзевич[1], А.П. Палій[1], Б.Т. Стегній[1], А.Б. Стегній[1], О.М. Чечет[1],
Д.В. Гадзевич[1], А.П. Палій[2], О.В. Павліченко[2], Р.В. Северин[2], Р.В. Петров[3], Л.П. Лівощенко[3]

[1] Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини»,
вул. Пушкінська, 83, Харків, 61023, Україна
[2] Державний біотехнологічний університет,
вул. Алчевських, 44, Харків, 61002, Україна
[3] Сумський національний аграрний університет,
вул. Герасима Кондрат'єва, 160, Суми, 40021, Україна

АНТИБІОТИКОРЕЗИСТЕНТНІСТЬ МІКРОБІОТИ ГІДРОЕКОСИСТЕМ РИБНИЦЬКИХ ПІДПРИЄМСТВ

Водне середовище є невід'ємною частиною біоценозу і безпосередньо впливає на його стан та благополуччя з точки зору епідеміології та епізоотології. Дослідження водного середовища на наявність патогенів та кількості санітарно-показових мікроорганізмів є вкрай важливим. Отримані дані дозволяють оцінити та спрогнозувати ризики виникнення інфекцій та розробити план профілактичних заходів для недопущення поширення тих чи інших збудників захворювань. **Мета.** Проаналізувати мікробний стан водного середовища у різних гідроекосистемах рибницьких підприємств Харківської області та оцінити наявність мікробіологічних ризиків для здоров'я населення. **Методи.** Об'єктами досліджень були 150 проб води, відібраної з різних гідроекосистем Харківської області. Воду відбирали з установок замкнутого водопостачання (n = 30) та у ставках (n = 120), де розводять товарну рибу. Наявність та кількість санітарно-показових мікроорганізмів та патогенних бактерій визначали бактеріологічним (культуральним) методом. Резистентність до антибактеріальних препаратів у відібраних санітарно-показових мікроорганізмів проводили диск-дифузійним методом на агарі. Оцінку достовірності різниці порівнюваних показників визначали за допомогою t-критерію Стьюдента. **Результати.** Домінуючими санітарно-показовими мікроорганізмами у водному середовища рибництва були бактерії роду *Citrobacter* spp., *Aeromonas* spp. та *Pseudomonas* spp. Загальна бактеріальна забрудненість водойм знаходилась у діапазоні від $1{,}9 \pm 0{,}50 \cdot 10^4$ до $2{,}1 \pm 1{,}20 \cdot 10^5$ КУО в 1 см^3 води. Патогенних для людей бактерій не було виявлено. Ізольовані санітарно-показові мікроорганізми проявляли суттєву резистентність до антибактеріальних препаратів. Найбільшу резистентність (p=0,0001) відмічали до пеніцилінів, сульфаніламідів та нітрофуранів. Відсоток резистентних до пеніцилінів штамів становив від 81,5 % до 87,0 %, до сульфаніламідів — від 74,1 % до 94,4 %, до нітрофуранів — від 55,5 % до 66,7 %. До фторхінолонів та цефалоспоринів резистентність варіювала в залежності від виду антибактеріальної речовини, але вона не перевищувала 29,6 %. **Висновки**. За результатами досліджень з водного середовища рибницьких підприємств Харківської області патогенних мікроорганізмів не було виявлено. Проте встановлено, що факторами загрози для здоров'я людей можуть бути санітарно-показові мікроорганізми (*Citrobacter* spp., *Aeromonas* spp., *Pseudomonas* spp.), які домінували та мали полірезистентність до антибактеріальних препаратів. Таким чином, гідроекосистеми рибницьких підприємств мають сприятливі умови для накопичення антибіотикорезистентних штамів бактерій. Тому застосування антибактеріальних препаратів повинно бути науково виправданим та суворо контрольованим.

Ключові слова: *гідроекосистема, мікробіологічні ризики водного середовища, санітарно-показові мікроорганізми, резистентність до антибіотиків*