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Prevalence and sensitivity of contagious and environmental cow mastitis-causing pathogens to antibiotics in Ukrainian farms

Ruslan Zaritskyi, Yurii Zhuk, Denys Dreval, Vitalii Kovpak, Yurii Masalovych, Ivan Cheverda, Iryna Derkach, Taras Savchuk

ABSTRACT
Mastitis is considered the most common and problematic disease, resulting in significant economic losses due to reduced milk yields, reduced quantity and quality of milk, treatment costs, and premature culling of animals. One of the traditional methods of treating mastitis in cows is using antibiotics, which leads to the emergence of polyresistant strains of microorganisms, the so-called Superbugs. The emergence of Superbugs, which are not sensitive to most existing antibiotics, is a major concern in veterinary and humane medicine. This study aimed to identify pathogens isolated from the secretion of the mammary gland of cows with mastitis to determine their spread and sensitivity to antibiotics. The samples of secretion from the udder were examined by bacteriological method. The isolates were identified by conventional methods and by the modern method – mass spectrometry (MALDI-TOF MS). The sensitivity of the bacterial isolates to antibiotics was determined by the disc diffusion method (Kirby-Bauer). The results of studies of cow mammary gland secretion samples indicate that 49.2% of the isolates are contagious, and 50.8% are environmental ones. The most common among the isolates of mastitis-causing pathogens were *Streptoccocus agalactiae* – 16.9%, *Streptococcus uberis* – 10.9%, *Staphylococcus aureus* – 10.7%, E. Coli – 9.6%, *Corynebacterium bovis* – 7.3%, *Staphylococcus haemolyticus* – 4.8%, *Staphylococcus chromogenes* – 3.6%, *Streptoccocus dysgalactiae* – 3.4%. Mastitis is caused by algae and yeast – 1% of all detected pathogens, respectively. According to the results of the determination of the sensitivity of isolates of mastitis-causing pathogens to antibiotics, it was found that most isolates were sensitive to Amoxicillin, Ceftiofur, and Rifampicin, and least of them – to Neomycin, Tylosin, Tilmicosin, Bacitracin.

Keywords: mastitis, milk, contagious, environmental, pathogen, cows, antibiotic resistance

INTRODUCTION
Dairy products, especially milk, are among the most important food sources for most of the world's population. The growing global demand for dairy products necessitates an increase in the average milk yield per cow. The increase in milk yields resulted from genetic selection and an improvement in the technology of feeding and keeping cows **[1]**. Bovine mastitis is an inflammatory reaction of udder tissue caused by physical injury or infection **[2]**. Mastitis is considered the most common disease that leads to significant economic losses in the dairy industry due to decreased milk yields, shortage of milk, treatment costs, and premature culling of sick animals. It is one of the most important diseases on cattle dairy farms, which affects udders, as well as the quantity and quality of milk, the increase in the number of culling, and the death of affected animals **[3]**, **[4]**, **[5]**. Mastitis is a major concern for global milk production; in the works **[6]**, **[7]**, it was reported that the economic losses attributable to mastitis were approximately $1 billion to $2 billion per year in the U.S. dairy industry. Researchers **[8]** noted that in Canada, this figure was 400 million Canadian dollars. According to the authors **[9]**, annual economic losses due to mastitis in India amounted to 60,532.1 million Indian rupees. According to current

estimates, the costs associated with cow mastitis in Europe amount to 1.55 billion euros annually **[10]**. It should be noted that worldwide financial losses associated with mastitis are estimated at 53 billion US dollars **[11]**.

Mastitis is caused mainly by bacterial infections and is classified based on epidemiology into contagious and environmental mastitis. The first one is caused by bacteria such as *Staphylococcus aureus* or *Streptococcus agalactiae*, which are transmitted from an infected cow to a healthy one, usually during milking through the operator's hands, reusable towels, and/or a milking machine, which is a reservoir of bacteria **[12]**, **[13]**. Environmental mastitis is caused by pathogenic environmental microorganisms found in litter, soil, manure, and feed. These include *Escherichia coli*, *Klebsiella* spp. **[14]**.

One of the traditional methods of therapy for cow mastitis is using antibiotics **[15]**, **[16]**. The authors **[17]** found that due to the bacterial diversity associated with bovine mastitis and the infrequent pathogen identification, broad-spectrum antimicrobials against gram-negative and gram-positive microorganisms are regularly used in the dairy industry. After decades of antimicrobial use, bacterial resistance is a growing concern in veterinary and humane medicine. Monitoring patterns of sensitivity of clinical isolates is an important aspect of the One Health approach. In the European Commission Guidelines on the prudent use of antimicrobials in veterinary medicine, it is recommended to test isolated pathogens of mastitis for sensitivity to antibiotics before treatment of animals with antimicrobials to prevent the multiplication of resistant bacteria by rationally selecting appropriate antimicrobials.

This study aimed to identify the pathogens isolated from the samples of mammary gland secretion of cows with mastitis and to determine the sensitivity of the main mastitis pathogens to antibiotics in Ukrainian farms.

Scientific Hypothesis

We expect that isolates of mastitis-causing pathogens from mammary gland secretion of cows with mastitis will show various sensitivity and, in some cases, resistance to certain antibiotics. This will allow us to determine which of the listed antibiotics can be recommended for effective therapy for animals sick with mastitis and which ones cannot. Studying the mammary gland secretion of cows with mastitis, identifying pathogens, and determining their sensitivity to antibiotics is an effective means of increasing the production of high-quality and safe dairy products.

MATERIAL AND METHODOLOGY

Samples

The samples of mammary gland secretion of cows received during 2019-2023 for study in the Laboratory of Bacteriology and Pathology of the Center for Veterinary Diagnostics LLC were in sterile test tubes. The samples were collected from farms specialising in raising cattle and having a dairy production direction. The research was conducted on 168 dairy farms. Immediately after collection, the samples were cooled to a temperature of +2 to +4 °C and immediately transported to the laboratory within 12 hours. A total of 1506 samples were analyzed. A bacteriological examination of 1,506 samples of udder secretions taken from cows suffering from clinical and subclinical forms of mastitis revealed 1,257 samples as positive. 115 samples had a negative result - no growth of microorganisms. Contamination was detected in 134 samples of udder secretion (Figure 1).

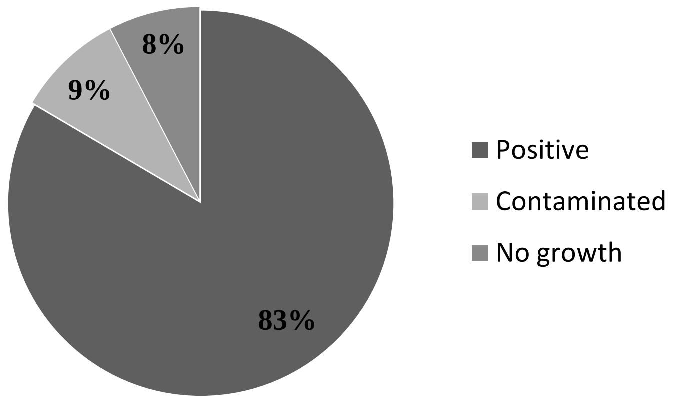

Figure 1 The results of the study of individual samples of the secretions taken from the udders.

Chemicals

COLUMBIA LAB-AGAR + 5% KB blood agar (BioMaxima S.A., Poland).
API 20 E blood agar (BioMérieux, France).
Mueller–Hinton agar (Oxoid, Great Britain).
MacConkey agar (Oxoid, Great Britain).
Antibiotic discs (Oxoid, Great Britain; Condalab, Spain).
Erba indole test (Lachema, Czech Republic).
Oxidase test (HiMedia Laboratories, India).
Catalase test (Technopharm LLC, Ukraine).
Gram stain *Química Clínica Aplicada* (S.A., Spain).

Animals, Plants and Biological Materials

Cows were of various breeds (Holstein, Ukrainian black-and-white, jersey), of different ages, and differed in the number of days of lactation and productivity. No information regarding the size of the livestock, diet structure, maintenance technologies, watering, milking system, and milk production. The secretions were taken from the udders of cows with mastitis.

Instruments

Microbiological analyzer MALDI-TOF VITEK®MS (BioMérieux, France).
Mass spectrometry system VITEK MS KB V3.2.0 US Version (BioMérieux, France).
Petri dishes (TOV Micromed).
Microbiological loops (TOV Micromed).

Laboratory Methods

The bacteriological study was carried out by inoculating 0.1 ml of the test milk sample, which was applied with a microbiological loop to the blood agar, followed by cultivation under aerobic conditions at a temperature of +37 °C for 24-48 hours. If more than three species of microorganisms were sown, such a sample was considered to be contaminated and not further studied.

Conventional bacteriological methods carried out the identification of bacterial cultures obtained on blood agar and the MALDI-TOF method using VITEK ®MS device **[18]**. The VITEK MS KB V3.2.0 US Version database was used to analyze the mass spectra.

The sensitivity of isolates to antibiotics was determined using the Kirby-Bauer disc diffusion method **[19]** in vitro on Mueller-Hinton agar, using Oxoid commercial discs, with the following disc action: Amoxicillin (25 mg/disc), Enrofloxacin (10 mg/disc), Streptomycin (10 mg/disc), Trimethoprim/Sulfamethoxazole (25 mg/disc), Oxytetracycline (30 mg/disc), Ceftiofur (30 mg/disc), Ampicillin (10 mg/disc), Gentamicin (10 mg/disc), Neomycin (30 mg/disc), Lincomycin (15 mg/disc), Cloxacillin (5 mg/disc), Tylosin (30 mg/disc), Bacitracin (0.04 mg/disc), Cephalexin (30 mg/disc), Danofloxacin (5 mg/disc), Spiramycin (100 mg/disc), Marbofloxacin (5 mg/disc), Tilmicosin (15 mg/disc), Rifampicin (5 mg/disc), Cefquin (30 mg/disc). The results of the determination of the diameter of the growth retardation zone for each disc were recorded as sensitive, intermediate, or resistant.

Description of the Experiment

Sample preparation: The bacteriological study of 1,506 secretions taken from the cows' udders with clinical and subclinical forms of mastitis found that 1,257 samples were positive, 115 samples were negative – no growth of microorganisms, and 134 secretions were contaminated

Number of samples analyzed: 1,506 samples were analyzed.

Number of repeated analyses: All measurements were performed 5 times.

Number of experiment replication: The number of replicates of each experiment to determine one value was 5 times.

Design of the experiment: First, we chose farms that specialize in raising cattle and have a dairy-oriented productivity direction to take samples from each farm individually. The study was conducted on 168 dairy farms in Ukraine. 5 persons conducted all on-farm studies between January 2022 and October 2023. The maintenance conditions and milking procedures were evaluated and documented in a standardized data collection form. The milking pattern was recorded by observation during one milking period. Following the clinical examination of cow udders and the study of secretions using the California Mastitis Test, the samples of secretion of animals sick with mastitis were taken and placed in sterile test tubes. The samples were then cooled to +2 to +4 °C and immediately transported to the laboratory within 12 hours after sampling. The selected samples of secretions from udders were subjected to bacteriological study, identified with the subsequent study of the selected isolates for antimicrobial substances according to the methods **[18]**, **[19]**.

Statistical analysis

The results were evaluated using statistical software Statgraphics Centurion XVII (StatPoint, USA) – multi-factor analysis of variance (MANOVA), LSD test. Statistical processing was performed in Microsoft Excel 2016 in combination with XLSTAT. Values were estimated using mean and standard deviations. The reliability of the research results was assessed according to the Student's test.

RESULTS AND DISCUSSION

The results of the bacteriological study of individual samples of secretion from the udder (from the affected particles of the udder) showed that *Streptococcus agalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, *E. coli*, and *Corynebacterium bovis* were mostly isolated from the samples tested. During the study period, with associations taken into account, 1,351 isolates were isolated. The main isolates are presented in Table 1 and Figure 2.

Table 1 The mastitis-causing pathogens isolated from the udder secretion of cows with mastitis for 2019-2023.

Microorganisms	**Number of isolates**
Streptococcus agalactiae	211
Streptococcus uberis	136
Staphylococcus aureus	134
Escherichia coli	120
Corynebacterium bovis	91
Staphylococcus haemolyticus	60
Staphylococcus chromogenes	45
Streptococcus dysgalactiae	43
Corynebacterium spp.	40
Aerococcus viridans	39
Staphylococcus spp.	36
Trueperella pyogenes	31
Streptococcus parauberis	25
Staphylococcus equorum	23
Staphylococcus epidermidis	23
Staphylococcus xylosus	15
Staphylococcus sciuri	15
Enterococcus faecalis	15
Streptoccocus spp.	13
Pasteurella multocida	12
Prototheca spp.	12
Lactococcus lactis	11
Streptoccocus mitis	11
Staphylococcus arlettae	10
Micrococcus spp.	8
Enterobacter cloacae	8
Klebsiella pneumoniae	8
Enterococcus faecium	7
Corynebacterium xerosis	7
Staphylococcus simulans	6
Lactococcus garvieae	6
Klebsiella terrigena	6
Candida kefyr	6
Bacillus licheniformis	6
Enterobacter amnigenus	5
Pseudomonas aeruginosa	5
Total	**1249**

Figure 2 The microbial landscape of the mastitis-causing pathogens (%) isolated from udder secretion of cows during 2019-2023.

During bacteriological studies, a group of microorganisms was isolated quite rarely. The total number of isolates of such rare mastitis-causing pathogens was 102 isolates (Table 2).

Table 2 Mastitis-causing pathogens that were rarely isolated during bacteriological study of udder secretion.

Microorganisms	Number of isolates
1	**2**
Staphylococcus cohnii, Staphylococcus auricularis, Staphylococcus vitulinus, Staphylococcus hyicus, Streptococcus suis.	4*
Staphylococcus capitis, Bacillus altitudinis, Moraxella osloensis,Citrobacter freundi, Corynebacterium amycolatum, Corynebacterium pilosum, Corynebacterium glutamicum, Corynebacterium pseudodiphtheriticum, Acinetobacter iwoffii,Candida crusei, Pantoea spp., Enterococcus durans.	3*
Serratias pp., Pseudomonas fluorescens,Corynebacterium aurimucosum.	2*
Staphylococcus warneri, Streptoccocus alactolyticus, Streptococcus salivarius, Streptococcus pluranimalium, Streptoccocus bovis, Streptoccocus pseudoporcinus, Streptoccocus equisimilis, Streptoccocus canis, Streptoccocus pyogenes, Streptoccocus gallolyticus, Corynebacterium confusum, Corynebacterium tuberculostearicum, Corynebacterium ammoniagenes, Corynebacterium freneyi, Corynebacterium casei, Corynebacterium jeikeium, Enterobacter ludwigii, Enterobacter aerogenes, Empedobacter brevis, Enterobacter spp., Bacillus pumilus, Bacillus altitudinis, Bacillus cereus, Lactococcus raffinolactis, Lactobacillus delbrueckii, Acinetobacter johnsonii, Acinetobacter ursingii, Candida tropicalis, Candida rugosa, Enterococcus italicus, Serratia grimesii, Citrobacter werkmanii, Pseudomonas spp., Macrococcus caseolyticus, Brevibacillus spp., Kocuria carniphila, Neisseria flava, Aeromonas sobria, Klebsiella oxytoca, Proteus mirabilis, Psychrobacter phenylpyruvicus, Raoutella terrigena, Carnobacterium maltaromaticum, Aerococcus vaginalis.	1*

Note: * – the number of isolates of each of the microorganisms specified.

The distribution of isolated contagious and environmental mastitis-causing pathogens is shown in Figure 3.

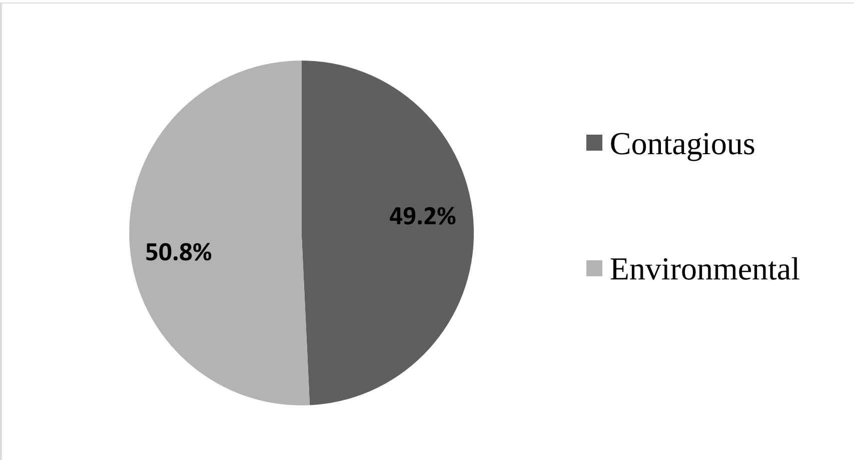

Figure 3 The distribution of isolated mastitis-causing pathogens according to their contagiousness.

The results of the determination of the sensitivity of isolated mastitis-causing pathogens to antibiotics are presented in Tables 3-11.

Table 3 Sensitivity of isolates to antibiotics.

Antibiotic	*Streptococcus agalactiae*	%	*Streptococcus uberis*	%	*Staphylococcus aureus*	%	*E. coli*	%
Amoxicillin (25 µg/disc)	198	93.8	128	94.1	82	61.2	74	61.7
Enrofloxacine (10 µg/disc)	102	48.3	95	69.9	119	88.8	101	84.2
Streptomycin (10 µg/disc)	91	43.1	22	16.2	95	70.9	68	56.7
Trimethoprim/ Sulfamethoxazole (25µg/disc)	148	70.1	100	73.5	116	86.6	98	81.7
Oxytetracyclin (30 µg/disc)	120	56.9	51	37.5	96	71.6	92	76.7
Ceftiofur (30µg/disc)	171	81.0	104	76.5	107	79.9	89	74.2
Ampicillin (10 µg/disc)	133	63.0	78	57.4	58	43.3	6	5.0
Gentamicin (10 µg/disc)	68	32.2	43	31.6	120	89.6	108	90.0
Neomycin (30 µg/disc)	29	13.7	21	15.4	98	73.1	59	49.2
Lincomycin (15 µg/disc)	180	85.3	62	45.6	103	76.9	0	0
Cloxacillin (5 µg/disc)	171	81.0	98	72.1	128	95.5	0	0
Tylosin (30µg/disc)	118	55.9	55	40.4	62	46.3	0	0
Bacitracin (0.04 µg/disc)	86	40.8	59	43.4	53	39.6	0	0
Cephalexine (30 µg/disc)	95	45.0	115	84.6	93	69.4	2	1.7
Danofloxacin (5 µg/disc)	49	23.2	49	36.0	94	70.1	83	69.2
Spiramycin (100 µg/disc)	140	66.4	74	54.4	60	44.8	0	0
Marbofloxacin (5 µg/disc)	94	44.5	87	64.0	121	90.3	113	94.2
Tilmicosin (15 µg/disc)	67	31.8	17	12.5	73	54.5	1	0.8
Rifampicin (5 µg/disc)	186	88.2	116	85.3	131	97.8	0	0
Cefquinome (30 µg/disc)	120	56.9	74	54.4	50	37.3	75	62.5

Table 4 Sensitivity of isolates to antibiotics.

Antibiotic	*Corynebacterium bovis*	%	*Staphylococcus haemolyticus*	%	*Staphylococcus chromogenes*	%	*Streptococcus dysgalactiae*	%
Amoxicillin (25 µg/disc)	89	97.8	53	88.3	39	86.7	42	97.7
Enrofloxacine (10 µg/disc)	77	84.6	57	95.0	44	97.8	32	74.4
Streptomycin (10 µg/disc)	80	87.9	52	86.7	37	82.2	28	65.1
Trimethoprim/ Sulfamethoxazole (25µg/disc)	7	7.7	52	86.7	44	97.8	35	81.4
Oxytetracyclin (30 µg/disc)	83	91.2	55	91.7	41	91.1	29	67.4
Ceftiofur (30µg/disc)	87	95.6	59	98.3	45	100	37	86.0
Ampicillin (10 µg/disc)	66	72.5	32	53.3	30	66.7	33	76.7
Gentamicin (10 µg/disc)	83	91.2	56	93.3	45	100	31	72.1
Neomycin (30 µg/disc)	65	71.4	58	96.7	44	97.8	18	41.9
Lincomycin (15 µg/disc)	62	68.1	38	63.3	36	80.0	28	65.1
Cloxacillin (5 µg/disc)	46	50.5	58	96.7	45	100	42	97.7
Tylosin (30µg/disc)	65	71.4	45	75.0	23	51.1	26	60.5
Bacitracin (0.04 µg/disc)	57	62.6	31	51.7	19	42.2	23	53.5
Cephalexine (30 µg/disc)	50	54.9	58	96.7	45	100	33	76.7
Danofloxacin (5 µg/disc)	57	62.6	50	83.3	40	88.9	20	46.5
Spiramycin (100 µg/disc)	68	74.7	49	81.7	26	57.8	30	69.8
Marbofloxacin (5 µg/disc)	65	71.4	54	90.0	44	97.8	31	72.1
Tilmicosin (15 µg/disc)	63	69.2	43	71.7	22	48.9	29	67.4
Rifampicin (5 µg/disc)	86	94.5	58	96.7	45	100	41	95.3
Cefquinome (30 µg/disc)	61	67.0	37	61.7	35	77.8	32	74.4

Table 5 Sensitivity of isolates to antibiotics.

Antibiotic	*Corynebacterium spp.*	%	*Aerococcus viridians*	%	*Staphylococcus* spp.	%	*Trueperella pyogenes*	%
Amoxicillin (25 µg/disc)	28	70	36	92.3	28	77.8	29	93.5
Enrofloxacine (10 µg/disc)	28	70	14	35.9	30	83.3	22	71.0
Streptomycin (10 µg/disc)	28	70	9	23.1	31	86.1	20	64.5
Trimethoprim/ Sulfamethoxazole (25µg/disc)	5	12.5	25	64.1	25	69.4	8	25.8
Oxytetracyclin (30 µg/disc)	26	65	24	61.5	24	66.7	10	32.3
Ceftiofur (30µg/disc)	29	72.5	27	69.2	23	63.9	26	83.9
Ampicillin (10 µg/disc)	34	85	21	53.8	15	41.7	17	54.8
Gentamicin (10 µg/disc)	35	87.5	21	53.8	28	77.8	26	83.9
Neomycin (30 µg/disc)	24	60	10	25.6	29	80.6	1	3.2
Lincomycin (15 µg/disc)	24	60	10	25.6	19	52.8	27	87.1
Cloxacillin (5 µg/disc)	16	40	19	48.7	24	66.7	27	87.1
Tylosin (30µg/disc)	20	50	14	35.9	21	58.3	25	80.6
Bacitracin (0.04 µg/disc)	31	77.5	18	46.2	15	41.7	7	22.6
Cephalexine (30 µg/disc)	25	62.5	26	66.7	27	75.0	19	61.3
Danofloxacin (5 µg/disc)	22	55	5	12.8	22	61.1	11	35.5
Spiramycin (100 µg/disc)	25	62.5	21	53.8	24	66.7	26	83.9
Marbofloxacin (5 µg/disc)	28	70	11	28.2	30	83.3	23	74.2
Tilmicosin (15 µg/disc)	20	50	6	15.4	24	66.7	28	90.3
Rifampicin (5 µg/disc)	36	90	26	66.7	31	86.1	28	90.3
Cefquinome (30 µg/disc)	11	27.5	13	33.3	15	41.7	15	48.4

Table 6 Sensitivity of isolates to antibiotics.

Antibiotic	*Streptococcus parauberis*	*%*	*Staphylococcus equorum*	*%*	*Staphylococcus epidermidis*	*%*	*Staphylococcus xylosus*	*%*
Amoxicillin (25 µg/disc)	13	52	23	100	10	43.5	15	100
Enrofloxacine (10 µg/disc)	10	40	23	100	14	60.9	13	86.7
Streptomycin (10 µg/disc)	9	36	23	100	14	60.9	15	100
Trimethoprim/ Sulfamethoxazole (25µg/disc)	10	40	20	87	8	34.8	15	100
Oxytetracyclin (30 µg/disc)	4	16	22	95.7	12	52.2	15	100
Ceftiofur (30µg/disc)	12	48	20	87	14	60.9	13	86.7
Ampicillin (10 µg/disc)	15	60	19	82.6	5	21.7	4	26.7
Gentamicin (10 µg/disc)	5	20	23	100	15	65.2	15	100
Neomycin (30 µg/disc)	1	4	23	100	15	65.2	15	100
Lincomycin (15 µg/disc)	12	48	13	56.5	13	56.5	8	53.3
Cloxacillin (5 µg/disc)	16	64	22	95.7	13	56.5	13	86.7
Tylosin (30µg/disc)	8	32	20	87.0	16	69.6	7	46.7
Bacitracin (0.04 µg/disc)	11	44	7	30.4	8	34.8	3	20.0
Cephalexine (30 µg/disc)	16	64	23	100	15	65.2	13	86.7
Danofloxacin (5 µg/disc)	5	20	23	100	13	56.5	10	66.7
Spiramycin (100 µg/disc)	13	52	20	87	19	82.6	10	66.7
Marbofloxacin (5 µg/disc)	10	40	23	100	14	60.9	15	100
Tilmicosin (15 µg/disc)	2	8	18	78.3	10	43.5	9	60.0
Rifampicin (5 µg/disc)	14	56	23	100	22	95.7	13	86.7
Cefquinome (30 µg/disc)	2	8	16	69.6	12	52.2	11	73.3

Table 7 Sensitivity of isolates to antibiotics.

Antibiotic	*Staphylococcus sciuri*	%	*Enterococcus faecalis*	%	*Streptoccocus* spp.	%	*Pasteurella multocida*	%
Amoxicillin (25 µg/disc)	14	93.3	13	86.7	11	84.6	12	100
Enrofloxacine (10 µg/disc)	14	93.3	6	40.0	7	53.8	12	100
Streptomycin (10 µg/disc)	11	73.3	0	0.0	6	46.2	5	41.7
Trimethoprim/ Sulfamethoxazole (25µg/disc)	14	93.3	8	53.3	5	38.5	10	83.3
Oxytetracyclin (30 µg/disc)	10	66.7	4	26.7	5	38.5	11	91.7
Ceftiofur (30µg/disc)	10	66.7	2	13.3	8	61.5	10	83.3
Ampicillin (10 µg/disc)	3	20.0	6	40.0	8	61.5	8	66.7
Gentamicin (10 µg/disc)	14	93.3	4	26.7	7	53.8	6	50.0
Neomycin (30 µg/disc)	13	86.7	0	0.0	3	23.1	2	16.7
Lincomycin (15 µg/disc)	0	0	2	13.3	7	53.8	1	8.3
Cloxacillin (5 µg/disc)	13	86.7	0	0	5	38.5	6	50.0
Tylosin (30µg/disc)	9	60.0	1	6.7	3	23.1	4	33.3
Bacitracin (0.04 µg/disc)	5	33.3	5	33.3	4	30.8	1	8.3
Cephalexine (30 µg/disc)	14	93.3	2	13.3	3	23.1	11	91.7
Danofloxacin (5 µg/disc)	10	66.7	3	20.0	3	23.1	9	75.0
Spiramycin (100 µg/disc)	6	40.0	3	20.0	6	46.2	7	58.3
Marbofloxacin (5 µg/disc)	14	93.3	6	40.0	4	30.8	12	100
Tilmicosin (15 µg/disc)	13	86.7	0	0.0	1	7.7	9	75.0
Rifampicin (5 µg/disc)	13	86.7	5	33.3	4	30.8	12	100
Cefquinome (30 µg/disc)	10	66.7	1	6.7	2	15.4	11	91.7

Table 8 Sensitivity of isolates to antibiotics.

Antibiotic	*Lactococcus lactis*	%	*Streptoccocus mitis*	%	*Staphylococcus arlettae*	%	*Micrococcus* spp.	%
Amoxicillin (25 µg/disc)	10	90.9	7	63.6	4	40	8	100
Enrofloxacine (10 µg/disc)	7	63.6	6	54.5	10	100	8	100
Streptomycin (10 µg/disc)	4	36.4	8	72.7	9	90	7	87.5
Trimethoprim/ Sulfamethoxazole (25µg/disc)	5	45.5	10	90.9	10	100	5	62.5
Oxytetracyclin (30 µg/disc)	5	45.5	7	63.6	5	50	6	75
Ceftiofur (30µg/disc)	8	72.7	11	100	9	90	8	100
Ampicillin (10 µg/disc)	6	54.5	8	72.7	0	0	7	87.5
Gentamicin (10 µg/disc)	8	72.7	5	45.5	10	100	8	100
Neomycin (30 µg/disc)	5	45.5	1	9.1	10	100	6	75
Lincomycin (15 µg/disc)	5	45.5	11	100	2	20	7	87.5
Cloxacillin (5 µg/disc)	2	18.2	10	90.9	8	80	7	87.5
Tylosin (30µg/disc)	5	45.5	11	100	8	80	6	75
Bacitracin (0.04 µg/disc)	1	9.1	8	72.7	0	0	7	87.5
Cephalexine (30 µg/disc)	2	18.2	9	81.8	8	80	7	87.5
Danofloxacin (5 µg/disc)	5	45.5	5	45.5	10	100	8	100
Spiramycin (100 µg/disc)	5	45.5	11	100	6	60	6	75
Marbofloxacin (5 µg/disc)	7	63.6	9	81.8	10	100	8	100
Tilmicosin (15 µg/disc)	3	27.3	9	81.8	3	30	5	62.5
Rifampicin (5 µg/disc)	2	18.2	11	100	10	100	8	100
Cefquinome (30 µg/disc)	5	45.5	7	63.6	8	80	0	0

Table 9 Sensitivity of isolates to antibiotics.

Antibiotic	*Enterobacter cloacae*	%	*Klebsiella pneumoniae*	%	*Enterococcus faecium*	%	*Corynebacterium xerosis*	%
Amoxicillin (25 µg/disc)	4	50	0	0	7	100	7	100
Enrofloxacine (10 µg/disc)	8	100	8	100	0	0	7	100
Streptomycin (10 µg/disc)	7	87.5	5	62.5	2	28.6	4	57.1
Trimethoprim/ Sulfamethoxazole (25µg/disc)	8	100	8	100	3	42.9	6	85.7
Oxytetracyclin (30 µg/disc)	7	87.5	7	87.5	6	85.7	7	100
Ceftiofur (30µg/disc)	5	62.5	7	87.5	0	0	4	57.1
Ampicillin (10 µg/disc)	0	0	0	0	1	14.3	7	100
Gentamicin (10 µg/disc)	8	100	8	100	3	42.9	7	100
Neomycin (30 µg/disc)	7	87.5	6	75	1	14.3	7	100
Lincomycin (15 µg/disc)	0	0	0	0	3	42.9	4	57.1
Cloxacillin (5 µg/disc)	0	0	0	0	0	0	7	100
Tylosin (30µg/disc)	0	0	0	0	2	28.6	4	57.1
Bacitracin (0.04 µg/disc)	0	0	0	0	3	42.9	5	71.4
Cephalexine (30 µg/disc)	0	0	0	0	0	0	7	100
Danofloxacin (5 µg/disc)	8	100	8	100	0	0	4	57.1
Spiramycin (100 µg/disc)	0	0	0	0	4	57.1	4	57.1
Marbofloxacin (5 µg/disc)	8	100	8	100	0	0	6	85.7
Tilmicosin (15 µg/disc)	0	0	0	0	0	0	4	57.1
Rifampicin (5 µg/disc)	0	0	0	0	3	42.9	7	100
Cefquinome (30 µg/disc)	8	100	8	100	0	0	6	85.7

Table 10 Sensitivity of isolates to antibiotics.

Antibiotic	*Staphylococcus simulans*	%	*Lactococcus garvieae*	%	*Klebsiella terrigena*	%	*Bacillus licheniformis*	%
Amoxicillin (25 µg/disc)	6	100	6	100	0	0	2	33.3
Enrofloxacine (10 µg/disc)	5	83.3	2	33.3	5	83.3	6	100
Streptomycin (10 µg/disc)	5	83.3	0	0	1	16.7	4	66.7
Trimethoprim/ Sulfamethoxazole (25µg/disc)	5	83.3	0	0	3	50	6	100
Oxytetracyclin (30 µg/disc)	5	83.3	3	50	3	50	6	100
Ceftiofur (30µg/disc)	5	83.3	2	33.3	5	83.3	0	0
Ampicillin (10 µg/disc)	5	83.3	2	33.3	0	0	0	0
Gentamicin (10 µg/disc)	6	100	1	16.7	6	100	6	100
Neomycin (30 µg/disc)	6	100	1	16.7	2	33.3	6	100
Lincomycin (15 µg/disc)	6	100	0	0	0	0	0	0
Cloxacillin (5 µg/disc)	6	100	0	0	0	0	1	16.7
Tylosin (30µg/disc)	6	100	0	0	0	0	2	33.3
Bacitracin (0.04 µg/disc)	1	16.7	0	0	0	0	0	0
Cephalexine (30 µg/disc)	6	100	0	0	1	16.7	3	50
Danofloxacin (5 µg/disc)	6	100	0	0	3	50	5	83.3
Spiramycin (100 µg/disc)	2	33.3	0	0	0	0	5	83.3
Marbofloxacin (5 µg/disc)	6	100	1	16.7	6	100	6	100
Tilmicosin (15 µg/disc)	3	50	0	0	0	0	2	33.3
Rifampicin (5 µg/disc)	6	100	0	0	0	0	4	66.7
Cefquinome (30 µg/disc)	4	66.7	1	16.7	3	50	0	0

Table 11 Sensitivity of isolates to antibiotics.

Antibiotic	*Enterobacter amnigenus*	%	*Pseudomonas aeruginosa*	%
Amoxicillin (25 µg/disc)	1	20	0	0
Enrofloxacine (10 µg/disc)	4	80	3	60
Streptomycin (10 µg/disc)	2	40	0	0
Trimethoprim/ Sulfamethoxazole (25µg/disc)	5	100	0	0
Oxytetracyclin (30 µg/disc)	2	40	0	0
Ceftiofur (30µg/disc)	2	40	0	0
Ampicillin (10 µg/disc)	0	0	0	0
Gentamicin (10 µg/disc)	5	100	5	100
Neomycin (30 µg/disc)	4	80	0	0
Lincomycin (15 µg/disc)	0	0	0	0
Cloxacillin (5 µg/disc)	0	0	0	0
Tylosin (30µg/disc)	0	0	0	0
Bacitracin (0.04 µg/disc)	0	0	0	0
Cephalexine (30 µg/disc)	0	0	0	0
Danofloxacin (5 µg/disc)	5	100	3	60
Spiramycin (100 µg/disc)	0	0	0	0
Marbofloxacin (5 µg/disc)	5	100	5	100
Tilmicosin (15 µg/disc)	0	0	0	0
Rifampicin (5 µg/disc)	0	0	0	0
Cefquinome (30 µg/disc)	2	40	0	0

The sensitivity of isolates to antibiotics is shown in Table 12.

Table 12 Sensitivity of isolates to antibiotics.

Antibiotic	Number of sensitive isolates		Number of resistant isolates	
	units	%*	units	%*
Amoxicillin (25 µg/disc)	976	**78.1**	273	21.9
Enrofloxacine (10 µg/disc)	879	**70.4**	370	29.6
Streptomycin (10 µg/disc)	702	56.2	547	43.8
Trimethoprim/ Sulfamethoxazole (25µg/disc)	827	66.2	422	33.8
Oxytetracyclin (30 µg/disc)	803	64.3	446	35.7
Ceftiofur (30µg/disc)	961	**76.9**	288	23.1
Ampicillin (10 µg/disc)	625	50.0	624	50.0
Gentamicin (10 µg/disc)	839	67.2	410	32.8
Neomycin (30 µg/disc)	590	47.2	659	**52.8**
Lincomycin (15 µg/disc)	683	54.7	566	45.3
Cloxacillin (5 µg/disc)	803	64.3	466	35.7
Tylosin (30µg/disc)	586	46.9	663	**53.1**
Bacitracin (0.04 µg/disc)	468	37.5	781	**62.5**
Cephalexine (30 µg/disc)	728	58.3	521	41.7
Danofloxacin (5 µg/disc)	648	51.9	601	48.1
Spiramycin (100 µg/disc)	670	53.6	579	46.4
Marbofloxacin (5 µg/disc)	884	**70.8**	365	29.2
Tilmicosin (15 µg/disc)	484	38.8	765	**61.2**
Rifampicin (5 µg/disc)	941	**75.3**	308	24.7
Cefquinome (30 µg/disc)	655	52.4	594	47.6

Note: * – the percentage relative to the total number of the isolates obtained – 1.249.

According to the data of Tables 1, 2 and Figures 2, 3, it was found that 615 (49.2%) isolates were accounted for contagious (infectious) cow mastitis-causing pathogens: *Streptoccocus agalactiae* – 211 (16.9%), *Streptococcus uberis* – 136 (10.9%), *Staphylococcus aureus* – 134 (10.7%), *Corynebacterium bovis* – 91 (7.3%), *Streptococcus dysgalactiae* – 43 (3.4%).

634 (50.8%) isolates were accounted for as environmental mastitis-causing pathogens, the main of which were *E. coli* – 120 (9.6%), *Staphylococcus haemolyticus* – 60 (4.8%), and *Staphylococcus chromogenes* – 45 (3.6%).

As can be seen from the antibioticograms obtained (Tables 3-11), the largest number of isolates, among the most common mastitis-causing pathogens, showed sensitivity to the following antibiotics:

– *Streptococcus agalactiae* – to Amoxicillin 198 (93.8%), Rifampicin 186 (88.2%), Lincomycin 180 (85.3%), Ceftiofur and Cloxacillin 171 (81%), respectively, Trimethoprim/sulfamethoxazole 148 (70.1%);

– *Streptococcus uberis* – to Amoxicillin 128 (94.1%), Cephalexin (84.6%), Rifampicin (85, 3%), Ceftiofur 104 (76.5%), Trimethoprim/sulfamethoxazole 100 (73.5%), Cloxacillin 98 (72.1%), Enrofloxacin 95 (69.9%);

– *Staphylococcus aureus* – to Rifampicin 131 (97.8%), Cloxacillin 128 (95.5%), Marbofloxacin 121 (90.3%), Gentamicin 120 (89.6%), Enrofloxacin 119 (88.8%), Trimethoprim/sulfamethoxazole 116 (86.6%), Ceftiofur 107 (79.9%), Lincomycin 103 (76.9%), Neomycin 98 (73.1%), Oxytetracycline 96 (71.6%), Streptomycin 95 (70.9%), Danofloxacin 94 (70.1%), Cefalexin 93 (69.4%);

– *E. coli* – to Marbofloxacin 113 (94.2%), Gentamicin 108 (90%), Enrofloxacin 101 (84.2%), Trimethoprim/sulfamethoxazole 98 (81.7%) isolates, to Oxytetracycline 92 (76.7%), Ceftiofur 89 (74.2%), Danofloxacin 83 (69.2%);

– *Corynebacterium bovis* – to Amoxicillin 89 (97.8%), Ceftiofur 87 (95.6%), Rifampicine 86 (94.5%), Gentamicin and Oxytetracycline 83 (91.2%), Streptomycin 80 (87.9%), Enrofloxacin 77 (84.6%), Spiramycin 68 (74.7%).

The largest number of resistant isolates showed sensitivity to the following antibiotics:

– *Streptococcus agalactiae* and *Streptococcus uberis* – to Neomycin;

– *Staphylococcus aureus* – to Cefquinome

– *E. coli* – to Lincomycin, Cloxacillin, Tylosin, Bacitracin, Spiramycin, Rifampicin;

– *Corynebacterium bovis* – to Trimethoprim/sulfamethoxazole.

According to the results of experimental studies (Table 12), most of the isolates were sensitive to Amoxicillin – 78.1%, Ceftiofur – 76.9%, Rifampicin – 75.3%, Marbofloxacin – 70.8%, Enrofloxacin – 70.4%. At the same time, most of the isolates were resistant to Bacitracin – 62.5%, Tilmicosin – 61.2%, Tylosin – 53.1%, Neomycin – 52.8%.

A significant percentage (70.8 – 50%) of the isolates obtained were sensitive (in descending order) to Marbofloxacin – 70.8%, Enrofloxacin – 70.4%, Gentamicin – 67.2%, Trimethoprim/sulfamethoxazole – 66.2%, Oxytetracycline and Cloxacillin 64.3%, Cephalexin – 58.3%, Streptomycin – 56.2%, Lincomycin – 54.7%, Spiramycin – 53.6%, Cefquin – 52.4%, Danofloxacin – 51.9%, Ampicillin – 50%.

During the bacteriological study of udder secretion from cows with mastitis, algae were isolated 12 times, 1% of the main isolates. Yeasts were sown 14 times, among which the isolates of *Candida kefyr* were the most common.

Mastitis - mammary gland inflammation, is one of the most common diseases of cattle worldwide **[45]**, **[46]**.

The most common reason for using antimicrobial drugs on dairy farms is treating cows for mastitis **[47]**, **[48]**. In addition, the use of broad-spectrum antimicrobials affects the development of resistance to a greater extent than narrow-spectrum antimicrobials **[49]**.

Antimicrobial drugs have been used for about sixty years to treat animals with mastitis. They are often prescribed without a preliminary test to identify the causative agent and determine its sensitivity, which is a rather important part of therapy **[50]**, **[51]**.

The irrational use of antimicrobials has initiated a rapid evolutionary process of bacterial resistance through natural selection and has led to an increase in the frequency and spread of bacterial antimicrobial resistance (AMR). The global emergency and the use of antimicrobials in cows have raised questions about alternative treatment approaches, but the main method of mastitis treatment remains the use of antimicrobials **[52]**, **[53]**.

The studies conducted by the authors **[20]**, **[21]**, **[22]** showed that the most common mastitis-causing pathogens were *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Escherichia coli;* the same data were provided by the researchers from Slovakia **[23]**, which completely coincide with the results of our research and is confirmed by the fact that these pathogens have the most widespread impact on dairy farms not only in Ukraine but also abroad. The studies conducted in Poland **[24]** demonstrated that contagious pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus* were most often isolated from mastitis milk, these microorganisms are the most common infectious agents that cause intramammary infection in dairy cows, our study results indicated that the most common pathogens isolated from milk of cows with mastitis were

Streptococcus agalactiae – 16.9%, *Streptococcus uberis* – 10.9% and *Staphylococcus aureus* accounted for 10.7% of the 1249 main isolates that we selected. Scientists **[25]** found that *Streptococcus agalactiae* was most often isolated in 1 out of 188 cases of mastitis. The high sensitivity of these bacteria to Cloxacillin was established experimental studies, which were conducted by us, indicating that *Streptococcus agalactiae* was isolated in 211 samples of mastitis milk, and the highest sensitivity of microorganisms was found to amoxicillin, rifampin, and lincomycin. The predominant isolated bacteria on dairy farms in Romania **[26]** were *Staphylococcus* spp. – 43.1%, *Streptococcus* spp. – 22.42%, *E. coli* – 13.79%, *Enterococcus* spp. – 8.62%, *Corynebacterium* spp. – 7.75%, and *Enterobacter* spp. – 4.31%. *S. hycus, S. chromogenes*, *S. xylosus*, and *S. capitis* were identified with a lower proportion and accounted for 36.0% of the isolated strains of staphylococci and 15.51% of the total isolates. *Corynebacterium bovis* and *Corynebacterium* spp. were isolated in a proportion of 7.75% of all isolates identified in this study, which partially coincided with the results of our studies. Gram-positive bacteria generally have low susceptibility to most antimicrobials tested **[27]**, **[28]**. The sensitivity of gram-negative bacteria to penicillins and quinolones was quite high. At the same time, resistance to macrolides, aminoglycosides, and tetracyclines was observed **[29]**, **[30]**, when analyzing the antibiotic susceptibility patterns, which we obtained, we recorded that such groups of antimicrobial agents as aminoglycosides and macrolides showed the lowest sensitivity to the isolated isolates, therefore we do not recommend using them to treat cows with mastitis. In Tanzania, the most common bacteria isolated from mastitis milk were *Staphylococcus aureus* (36.8%), *Pseudomonas aeruginosa* (17.8%), *Staphylococcus epidermidis* (16.1%), *Klebsiella* spp. (9.5%), *Micrococcus* spp. (6.3%) and *E. coli* (4.9%) **[31]**, our study results indicated that *Staphylococcus aureus* was detected in 10.7% of isolates, *Pseudomonas aeruginosa* – 0.4%, *Staphylococcus epidermidis* – 1.8%, *Micrococcus* spp. – 0.6% and *E. coli* 9.6%. Several researchers **[32]** informed that the prevalence of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* was 30%, 17%, and 3.5%, respectively. Most (90%) *S. aureus* resisted penicillin, while only 10% of strains resisted oxacillin. Almost half (40%) of *E. coli* strains showed resistance to streptomycin **[33]**, **[34]**, when analyzing the sensitivity of the isolated pathogens to antimicrobial agents, it was noted that 62.7% of *Staphylococcus aureus* isolates were resistant to cefquinome, and 93.8% of isolates were sensitive to amoxicillin, all 120 E. coli isolates were resistant to lincomycin, cloxacillin, tylosin, bacitracin, spiramycin, and rifampicin, which can be explained by the natural resistance of microorganisms to some antimicrobial substances, as well as may be due to the presence of pathogenicity factors such as biofilms, adhesins, as well as some enzymes that inhibit antibiotic.

Researchers **[35]**, **[36]** found that, among the contagious mastitis-causing pathogens, the most common were *Streptococcus agalactiae* – 24.1%, *Staphylococcus aureus* – 18.4%, *Corynebacterium* spp. – 7.2%, *Streptococcus dysgalactiae* – 5.6%, *Streptococcus uberis* – 2.2%. Environmental mastitis-causing pathogens accounted for 42.5% of the total number of isolates. The mastitis caused by yeasts accounted for 1.4%. The greatest sensitivity of the isolates was to Ceftiofur, Amoxicillin/Clavulanic acid, Rifampicin, Amoxicillin, Gentamicin, Ampicillin, Bacitracin, Cephalexin, Cloxacillin, Enrofloxacin, Trimethoprim/Sulfamethoxazole, Oxytetracycline, Lincomycin. The least sensitive – to Spiramycin, Tylosin, Streptomycin, Neomycin, Marbofloxacin, Tilmicosin, and Danofloxacin, which coincided with the results of our studies.

On dairy farms in south-eastern Australia **[44]**, **[37]**, the studies of mastitis-causing pathogens showed that 472 samples (15.5%) out of 3.044 studied samples of cow mammary gland secretion were contaminated, and no growth was noted in 27.5% of the samples. The most common pathogens from clinical samples of mastitis were *Streptococcus uberis* (39.2%), *Staphylococcus aureus* (10.6%), *Escherichia coli* (8.4%), *Streptococcus dysgalactiae* (6.4%), our studies indicated that during the bacteriological examination of 1506 milk samples, 134 (9%) samples were found to be contaminated, the most frequently identified pathogens from the mammary gland secretion of cows with mastitis were *Streptococcus agalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, *Escherichia coli*, which amounted to 16.9%, 10.9%, 10.7%, and 9.6%, respectively, *Streptococcus dysgalactiae* was isolated in only 3.4% of isolates.

In Ethiopia **[38]**, **[39]**, the most predominant isolated bacterial mastitis-causing pathogens were *Staphylococcus aureus* (42.6%), *Streptococcus* spp. (26.2%) and *Escherichia coli* (11.5%), *Salmonella* spp. (3.3%) and *Klebsiella pneumoniae* (1.6%) were less isolated, as for the pathogen of salmonellosis, we have never detected *Salmonella* spp. during our studies, however, *Klebsiella pneumoniae* was detected in 8 (0.6%) milk samples. Other studies conducted in Ethiopia **[40]**, **[41]** demonstrated that the dominant mastitis-causing pathogens were *Staphylococcus aureus* (40.3%), *Streptococcus* spp. (24.3%), *Staphylococcus* spp. (12.5%), E. *coli* (8.3%), *Staphylococcus hyicus* (3.5%), and *Staphylococcus intermedius* (1.4%). The authors Awandkar et al. **[42]**, and Mahmoud and Yassein **[43]** reported that the prevalence of mastitis caused by yeasts was 1.09%, which also coincided with the results of our studies.

It should be noted, that when analyzing the microbial identification data by the MALDI-TOF MS method, 91.5% of isolates were identified to the species level, and 8.5%, which is 115 isolates, were identified only to the genus level. Most of the unidentified microorganisms are composed of gram-positive microflora – 85.2%.

Nonnemann et al. indicate that 500 isolates of microorganisms isolated from the milk of cows with mastitis, 93.5% of which were identified to the species level, and 6.5% were identified only to the genus level, for example, 4 out of 6 Acinetobacter, 2 out of 9 *Corynebacterium* and 2 in 11 *Bacillus* were identified only at the genus level **[54]**.

In Brazil, for the identification of 380 bacteria isolated from milk samples from bovine patients with mastitis, MALDI-TOF MS showed a typing rate of 95.5%, and the accuracy for identifying *Staphylococcus* isolates was 93.2% **[55]**.

Implementation of national mastitis control programs and evaluation of their effectiveness are mandatory. Some countries are ahead of others in improving approaches to mastitis treatment and controlling antimicrobial consumption on dairy farms; Their expertise can guide the development of further strategies. The health of cows and udders should be regularly monitored, farm management indicators should be improved, risk factors for mastitis should be identified and reduced and infectious agents should be minimized, the use of antimicrobials should be reduced, to develop and implement more effective control measures, alternative farming systems and/or to reduce the consumption of cattle products **[56]**, **[57]**.

The results of our research provide valuable information on the prevalence and sensitivity of cow mastitis pathogens to antibiotics on Ukrainian farms. A comprehensive research approach to pathogen identification, as well as a detailed analysis of antibiotic resistance, fills an important gap in veterinary medicine and farm management practices.

CONCLUSION

The spread of contagious cow mastitis-causing pathogens in the farms of Ukraine is at the level of 49.2%. The most common pathogens are *Streptoccocus agalactiae, Streptococcus uberis, Staphylococcus aureus, Corynebacterium bovis, Streptococcus dysgalactiae.* Environmental mastitis-causing pathogens account for 50.8% of all isolates, among which the most common are *E. coli*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes.* Only 1% of the diagnosed mastitis-causing pathogens are caused by algae (*Prototheca* spp.) and yeasts. The results of the determination of the sensitivity of isolates to 20 antibiotics showed the largest percentage of resistance to Bacitracin, Tilmicosin, Tylosin, Neomycin. The greatest percentage of sensitivity of isolated mastitis-causing pathogens was to Amoxicillin, Ceftiofur, Rifampicin. Thus, these antibiotics can be recommended for inclusion in therapy protocols for cows with mastitis in Ukrainian farms. However, this applies only to the mastitis caused by contagious pathogens, as there is a very large species diversity among environmental pathogens (Gram-positive and Gram-negative microflora), and, accordingly, a large diversity in antibiotic sensitivity; therefore, it is necessary to develop a treatment protocol only based on individual antibioticograms obtained in each case. Further studies are planned to expand the range of mastitis-causing pathogens and improve the analysis of their sensitivity to antimicrobial substances.

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Contact Address:

*__Ruslan Zaritskyi__, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Obstetrics, Gynecology, and biotechnology of animal reproduction, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: zaritskyi_rv@nubip.edu.ua
ORCID: https://orcid.org/0000-0003-1074-3118

Yurii Zhuk, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Obstetrics, Gynecology, and biotechnology of animal reproduction, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: zhuk_yv@nubip.edu.ua
ORCID: https://orcid.org/0000-0002-8827-6313

Denys Dreval, Center of Veterinary Diagnostics LLC, Laboratory of Bacteriology and Pathanatomy, Kaisarova Str., 15-A, 03041, Kyiv, Ukraine,
E-mail: bacteriology@cvd.com.ua
ORCID: https://orcid.org/0000-0003-4184-3189

Vitalii Kovpak, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Obstetrics, Gynecology, and biotechnology of animal reproduction, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: vitkovpak@ukr.net
ORCID: https://orcid.org/0000-0003-2419-1246

Yurii Masalovych, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Obstetrics, Gynecology, and biotechnology of animal reproduction, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: masalovich@nubip.edu.ua
ORCID: https://orcid.org/0000-0002-0986-0854

Ivan Cheverda, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Obstetrics, Gynecology, and biotechnology of animal reproduction, Vystavkova Str., 16, 03121, Ukraine,
E-mail: cheverda_im@nubip.edu.ua
ORCID: https://orcid.org/0009-0000-7545-307X

Iryna Derkach, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Pharmacology, Parasitology and Tropical Veterinary Medicine, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: derkach_im@nubip.edu.ua
ORCID: https://orcid.org/0000-0002-0149-7923

Taras Savchuk, National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Department of Surgery and Pathophysiology acad. I.O. Povazhenko, Vystavkova Str., 16, 03121, Kyiv, Ukraine,
E-mail: t_sav4uk@nubip.edu.ua
ORCID: https://orcid.org/0000-0002-7351-5684

Corresponding author: *