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BIODIVERSITY OF SPECIES AND ANTIMICROBIAL RESISTANCE OF BOVINE MILK WITH CLINICAL AND SUBCLINICAL MASTITIS

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ABSTRACT

This article was discussed the problems biodiversity of cow mastitis. The purpose of the work was to conduct a statistical analysis of microbiological data milk samples from cows with mastitis in order to ensure the targeted use of antibiotics on Ukrainian farms. Also, the article reveals the problems of the aseptic sampling technique for analysis, which complicates their identification. The obtained milk samples from 20 farm of Ukraine were examined using microbiology methods. As a result, 41% of all isolated bacteria were contagious mastitis agents: 15% of *Staphylococcus aureus* and 26% of *Streptococcus agalactiae*, and 59% were environmental mastitis agents. The most bacteria refer to Gram (+) microflora, namely streptococci (22.5% - *Streptococcus spp.* (*S.agalactiae* not include) and coagulase-negative stain (CNS) of staphylococci (14% - *Staphylococcus spp.* CNS). 18.5% of all isolated isolates are identified as Gram (-) microflora: for by Gram (-) microflora: 11% - *E.coli*, 6% - *Pasteurella spp*., 1.5% - *Proteus spp*. Mastitis caused by fungi (yeast), accounted for 1.5% of all diagnosed mastitis. Most of the isolates were sensitive to amoxicillin + clavulanic acid and gentamicin -93, 5%. The smallest number of isolates were sensitive to tylosin- 20,9% and streptomycin- 48,3%. A significant percentage (83.8% - 54.8%) of obtained isolates were sensitive to rifampicin, amoxicillin, bacitracin, cloxacillin, trimethoprim, florfenicol, ampicillin, lincomycin, cephalexin, enrofloxacin, neomycin, penicillin.

Keywords: biological diversity; *Streptococcus agalactiae*; intramammary infection; profile of antibiotics; bacteriological cultivation

INTRODUCTION

The biological diversity of different groups of microorganisms varies greatly on Earth. The literature data indicate a wide variety within the population strain of bacteria, which is an important factor in adapting bacteria to unstable environmental conditions (**Swift et al., 2004**). There is an opinion that the widespread use of antibiotics and disinfectants in agriculture owning to lead to the uncontrolled spread of these resistant microorganisms in ecosystems (**Theuretzbacher et al., 2013**).
Mastitis is a common multifactorial disease of dairy cattle, but its main cause is still the penetration of bacteria into the parenchyma (**Sudhan et al., 2010**).
Contagious agents were shown in previous studies have the ability to latent infection and are the root cause of subclinical inflammation of the mammary gland (**Riekerink et al., 2006**). There are many contagious microorganisms that cause inflammation of the mammary gland, such as *Prototheca spp*., *Corynebacterium bovis*, but the main contagious agents are *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma bovis*., with the exception of some mycoplasma infections that may come from other parts of the body and spread on the system level. These three organisms enter the mammary gland through the nipple channel and rapidly spread from cow to cow during milking (**Keefe et al., 1997**).
Environmental mastitis– caused by opportunistic bacteria *Escherichia coli*, *Klebsiella spp*., *Pseudomonas spp*., *Proteus spp*., *Serratia spp*., gram-positive bacilli, yeasts, *Streptococcus spp.* (except *S. agalactiae*), *Staphylococcus spp.* (except *Staph. aureus*), which spread mainly outside the milking parlor (**Hogan at al., 1997**); (**Blum et al., 2017**).
Cultivation of bacteria in milk samples determines the status of infection. Depending on the pathogen, its sensitivity and resistance to antibiotics and the clinical condition of the udder, veterinarians choose the appropriate method of treatment in each particular case (**Dohoo et al., 2011a**). The goal of microbiological study is to identify microorganisms investigated present in milk. The results of bacteriological study make sense in many respects: they provide targeted use of antibiotics, inform about the resistance of pathogens, etc. (**Dohoo et al., 2011b**). The basic requirements for microbiological study are taking milk samples in aseptic conditions and before starting antibiotic therapy. In addition, the point of sampling has a great impact on the results of bacteriological study (**Andersen et al., 2010**).
It is considered that approximately 20-25% of bacteriological studies of acute forms of mastitis have negative results. It is shown, during the analys is of milk from cows with severe cases of clinical mastitis, 70% of cases received negative results of bacteriological examination (**Ganguly et al., 2017**). As a result, microscopic examination of milk precipitate, phagocyte grams of negative bacilli in leukocytes have often been detected. After freezing, thawing and cultivation, in some cases, it was possible to isolate the pathogen (**Czyżak-Runowska et al., 2018**).
Furthermore, it is noted that SCC can be used as a biomarker for prompt diagnosis of both clinical and subclinical mastitis as well as to ascertain the effectiveness of the therapeutic regimen. This will ensure early detection of subclinical mastitis and will enable successful implementation of mastitis control programmes to ensure quality milk production (**Das et al., 2018**).
The purpose of the work was to study the variety of pathogenic and conditionally pathogenic flora found in milk of cows, as well as its antibiotic sensitivity.
To achieve the goal, the following tasks were set:

1. Determine the pathogens that cause mastitis within Ukraine.
2. Analyze antibiotic sensitivity and resistance of isolated mastitis agents.

MATERIALS AND METHODS

The obtained milk samples from 20 farm of Ukraine were examined using the bacteriological method. The isolation of aerobic bacteria from the milk samples under study was used blood agar (for isolation, identification and determine the type of hemolysis) produced by BioMerieuxtm (France). For pre-identification and selective isolation, McConkey Agar (for isolation Coliforms, E.coli), Mannitol Salt Agar (MSA) (for use as a selective and differential medium for the isolation of pathogenic staphylococci.), Edwards Agar (for the rapid isolation of Streptococcus agalactiae and other streptococci), Saburo Agar (for the cultivation of yeasts, moulds) manufactured by Himedia^{tm} (India) and Biolife^{tm} (Italy) were used. Bacterial staining was done by Gram's Method. Identification of isolated bacterial isolates was carried out using commercial test systems API 20E BioMerieux^{tm} (France) and STREPTOtest ^{16} ErbaLachematm (Czech Republic). Culture media and commercial tests systems were cultivated 37±1ºC in incubator, 18±2h in an aerobic environment.
Antibiotic sensitivity of the isolated isolates was identified using the disc in vitro diffusion method on the Mühler-Hinton agar with the use of standard commercial disks Amoxicillin-25mcg/disc, Amoxicillin+ClavulanicAcid-20mcg/disc+10mcg/disc, Gentamicin-10mcg/disc, Enrofloxacin-10 mcg/disc, Florfenicol-30 mcg/disc, Streptomycin-10 mcg/disc, Trimethoprim-5 mcg/disc, Ampicillin-10 mcg/disc, Penicillin G-10 units, Tylosin-15 mcg/disc, Neomycin-30 mcg/disc, Lincomycin-15 mcg/disc, Cloxacillin-30 mcg/disc, Rifampicin-5 mcg/disc, Bacitracin-10 mcg/disc, Cephalexin-30 mcg/disc manufactured by Himedia^{tm} (India) and Oxoid™ (Florfenicol). Select four to five similar colonies and transfer into suitable broth or saline to obtain turbidity equivalent to 0.5 McFarland barium sulphate standard. The standard ready-made inoculum requires agitation on a vortex mixer before each use. For proper turbidity adjustment, it is helpful to use a white background with contrasting black lines. Inoculate the agar plates directly from the suspension, spreading the inoculums as evenly as possible with sterile swab. Antimicrobial susceptibility test discs are then placed with the aid of flamed tweezers to the inoculated medium. Incubate

the test materials at 37˚ C for 18 hours and then measure the diameters of the inhibition zones surrounding the discs in millimeters.
For statistical processing methods used programs: Microsoft Excel, apiweb[tm] bioMerieux. The interpretation of antibiotic gram results we used some breakpoint tables for interpretation of zone diameters (version 7.1, 2017), developed by the European Committee on Antimicrobial Susceptibility Testing. The European Committee on Antimicrobial Susceptibility Testing. Available at: **http: //www.eucast.org.**

RESULT AND DISCUSSION

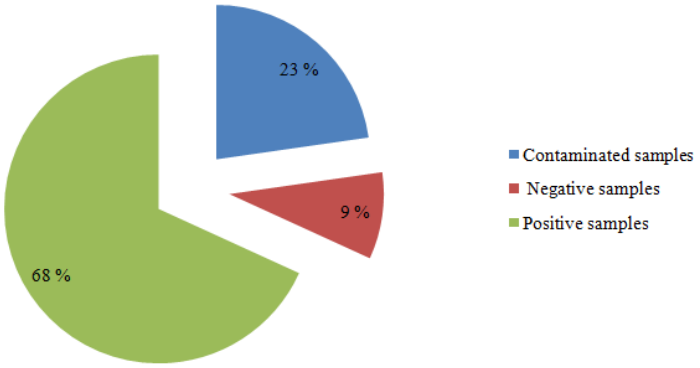

Figure 1 Percentage of milk samples taken for analysis

As seen from the data given in the Fig. 1, the number of milk samples amounted to 92, of which 20 samples contaminated (infected by exogenous microflora), which is 23% of the total number of samples. This is a fairly large percentage, which indicates the contravention of aseptic technology and the neglect of the rules for sampling milk for examination. In most cases, if more than 2 or 3 different types of microorganisms are plated from a sample of milk with a subclinical form of mastitis, such a sample is considered to be contaminated and is excluded from further examination (**Dohoo et al., 2011a**). This is due to the fact that theoretically any microorganism can cause the infection of the udder (**Dohoo et al. 2011b**) but it is unlikely that mastitis can be caused by more than 2-3 different microorganisms at the same time (**Reyher et al., 2011**).
There was a small percentage (9%) of negative samples, that is, those from which no microflora was allocated. This often happens, especially with the samples taken from cows with a clinical form of mastitis. This may be due to many reasons, the main of which include: significant fluctuations of pH in milk; the presence of a significant number of inflammatory cells and various chemicals that appear in milk during inflammation (enzymes, hormones, cellular debris, etc.) and inhibit bacterial microflora; periodic minor isolation of the agent with milk, or the absence of isolated agent (with mastitis caused by *S. aureus*); aseptic (non-infectious) nature of inflammation; violation of the conditions of storage and transportation of selected samples of milk (violation of the temperature control), etc. (**Degen et al., 2015**).

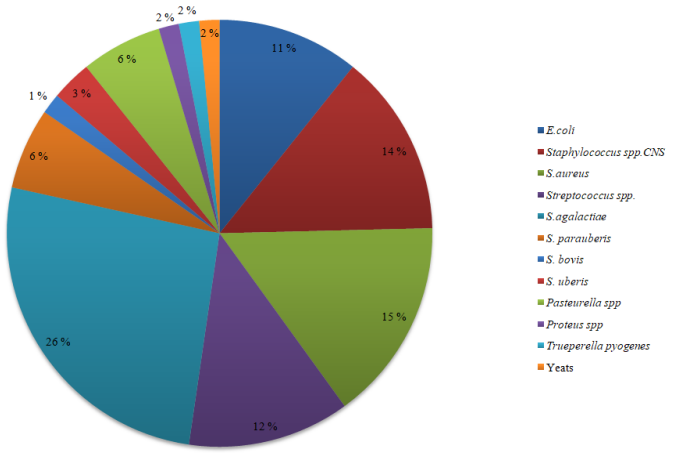

Figure 2 Results of biodiversity of individual milk samples (samples from the affected udder quarter) from cows with clinical and subclinical form of mastitis

Concerning the spectrum of isolated pathogens, 41% of all isolated isolates were contagious mastitis agents: 15% of *S. aureus* and 26% of *S. agalactiae* (pathogenic), and 59% were environmental (conditionally pathogenic) mastitis agents. The most of bacteria refer to Gram (+) microflora that causes environmental mastitis, namely streptococci (22.5% - Streptococcus spp.) and staphylococci (14% - *Staphylococcu spp.*). 18.5% of all isolated isolates are identified as Gram (-) microflora: for by Gram (-) microflora: 11% - *E.coli*, 6% - *Pasteurella spp*., 1.5% - *Proteus spp*. Mastitis caused by fungi (yeast), accounted for 1.5% of all diagnosed mastitis. It was shown in the Fig. 3

Table 1 Distribution of the different number of isolated mastitis agents by their sensitivity to various antibiotics

Active substance of antibiotic	*Staphylococcus aureus*		*Streptococcus agalactiae*		*Escherichia coli*		*Staphylococcus spp (CNS)*		*Streptococcus spp.*		*Streptococcus parauberis*		*Streptococcus bovis*		*Streptococcus uberis*		*Pasteurellla spp.*	
	№	**%***	**№**	**%**	**№**	**%**	**№**	**%**	**№**	**%**	**№**	**%**	**№**	**%**	**№**	**%**	**№**	**%**
Amoxicillin	10	**100**	17	**100**	0	**0**	7	**78**	6	**75**	4	**100**	1	**100**	2	**100**	3	**75**
Amoxicillin+ClavulanicAcid	10	**100**	17	**100**	6	**86**	8	**89**	7	**87.5**	3	**75**	1	**100**	2	**100**	4	**100**
Gentamicin	10	**100**	16	**94**	7	**100**	9	**100**	7	**87.5**	3	**75**	1	**100**	1	**50**	4	**100**
Enrofloxacine	8	**80**	7	**41**	6	**86**	9	**100**	4	**50**	0	**0**	0	**0**	0	**0**	4	**100**
Florfenicol	9	**90**	11	**65**	4	**57**	8	**89**	4	50	3	**75**	0	**0**	0	**0**	3	**75**
Streptomycin	5	**50**	9	**53**	2	**29**	8	**89**	3	**37.5**	0	**0**	0	**0**	0	**0**	3	**75**

Trimethoprim	10	**100**	13	**76**	7	**100**	8	**89**	3	**37.5**	2	**50**	0	**0**	0	**0**	3	**75**
Ampicillin	9	**90**	15	**88**	1	**14**	5	**55.5**	5	**62.5**	3	**75**	1	**100**	0	**0**	3	**75**
Penicilillin	8	**80**	12	**70.5**	0	**0**	4	**44**	4	**50**	3	**75**	0	**0**	0	**0**	3	**75**
Tylosin	3	**30**	5	**29**	0	**0**	1	**11**	1	**12**	0	**0**	0	**0**	0	**0**	3	**75**
Neomycin	9	**90**	10	**59**	0	**0**	9	**100**	3	**37.5**	0	**0**	0	**0**	0	**0**	4	**100**
Lincomycin	9	**90**	16	**94**	0	**0**	8	**89**	2	**25**	2	**50**	0	**0**	0	**0**	4	**100**
Cloxacillin	9	**90**	17	**100**	0	**0**	9	**100**	4	**50**	3	**75**	1	**100**	2	**100**	3	**75**
Rifampicin	10	**100**	17	**100**	0	**0**	9	**100**	6	**75**	4	**100**	0	**0**	2	**100**	4	**100**
Bacitracin	9	**90**	17	**100**	0	**0**	8	**89**	6	**75**	3	**75**	1	**100**	2	**100**	3	**75**
Cephalexin	10	100	14	82	0	0	7	78	3	37.5	2	50	1	100	1	50	3	75
Total of isolate	**10**		**17**		**7**		**9**		**8**		**4**		**1**		**2**		**4**	

Note: *% – percentage of susceptible isolates in relation to the total number of isolates

Table 2 Distribution of the total number of isolated mastitis agents by their sensitivity to various antibiotics

The active substance of antibiotic	**Number of sensitive isolates**	**%***
Amoxicillin+clavulanic acid	58	93,5
Gentamicin	58	93,5
Rifampicin	52	83,8
Amoxicillin	50	80,6
Bacitracin	49	79
Cloxacillin	48	77,4
Trimethoprim	46	74,1
Florfenicol	42	67,7
Ampicillin	42	67,7
Lincomycin	41	66,1
Cephalexin	41	66,1
Enrofloxacine	38	61,2
Neomycin	35	56,4
Penicillin	34	54,8
Streptomycin	30	48,3
Tylosin	13	20,9

Note: *% – percentage of susceptible isolates in relation to the total number of isolates

As seen from the data given in the Tab. 2 and Tab. 3, most of the isolates were sensitive to Amoxicillin + Cl. Acid and Gentamicin -93, 5%. The smallest number of isolates were sensitive to tylosin- 20,9% and streptomycin- 48,3%. A significant percentage (83.8% -54.8%) of obtained isolates were sensitive to: rifampicin, amoxicillin, bacitracin, cloxacillin, trimethoprim, florfenicol, ampicillin, lincomycin, cephalexin, enrofloxacine, neomycin, penicilli.
The sensitivity of *E. coli* isolates is somewhat different. Thus, all 7 isolates of this agent were resistant to amoxicillin, penicillin, tylosin, neomycin, lincomycin, cloxacillin, rifampicin, bacitracin, and cefalexin. 6 out of 7 isolates were resistant to ampicillin and 5 out of 7 to streptomycin. All isolated *E.col* isolates were sensitive to gentamicin and trimetoprim (100%) and a significant percentage (86%) - to amoxicillin + clavulanic acid and enrofloxacin.

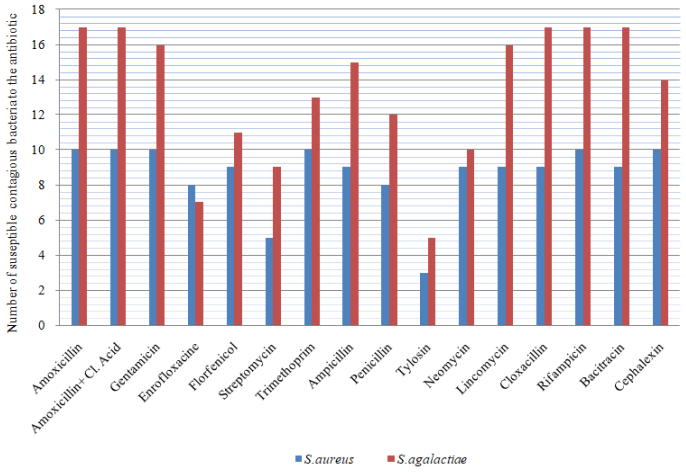

Figure 3 Antibiotic sensitivity of contagious mastitis pathogens (*Streptococcus agalactiae, Staphylococcus aureus*). It is shown in the Fig. 4 antibiotic sensitivity of contagious agents such as *Staph. aureus* and *S. agalactiae* was approximately identical. Most isolates of contagious agents were susceptible to amoxicillin, amoxicillin + clavulanic acid acid, gentamicin, ampicillin, lincomycin, cloxacillin, rifampicin, bacitracin, cephalexin and trimetoprim; and resistant to tylosin and streptomycin.

CONCLUSIONS

A significant percentage of samples (22%) that were submitted for examination were contaminated, suggesting an inappropriate sampling technique and contravention of the conditions of storage and transportation of samples for laboratory testing. In order to prevent contamination of samples, the existing recommendations should be clearly observed. We studied the diversity of biological isolates in milk samples conducted by us; indicate that strains streptococci which account for 48.5% of all diagnosed case is able to primer case the cause of mastitis. Among Gram-negative pathogens causing mastitis *E. coli* constitute a majority, but in relation to the total number of diagnosed mastitis the percentage of mastitis caused by *E. coli* amounted to only 11%. With regard to the findings, the antibiotic of choice is amoxicillin + cl. acid and gentamicin. The antibiotic of the last choice was tylosin.

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