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Comparison Of The Minimum Bactericidal Concentration Of Antibiotics On Planktonic And Biofilm Forms Of Staphylococcus Aureus: Mastitis Causative Agents.

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ABSTRACT

Staphylococcus aureus is the main causative agent of subclinical, clinical, relapsing and chronic mastitis in cows. The spread of staphylococcal mastitis in cows is associated with the ability of microorganisms to form biofilms. The treatment of this disease is usually reduced to the use of antibiotics based on laboratory tests to determine the sensitivity of planktonic forms of bacteria in vitro. However, traditional etiotropic antibacterial therapy is not always effective, despite the sensitivity of planktonic forms of bacteria – causative agents of mastitis to antibiotics. The purpose of the work was to compare the minimal bactericidal concentration of antibiotics concerning influence on planktonic and biofilm forms of S. aureus, isolated from cows with mastitis in Ukraine. It has been established that among the four tested antibiotics, tetracycline, enrofloxacin, amoxicillin with clavulanic acid and gentamicin, the lowest MBC on planktonic cells of S. aureus was found in amoxicillin with clavulanic acid and enrofloxacin, which ranged from 0,05 to 0,8 mg/mL. In antibiotics of tetracycline and gentamicin, MBC on planktonic cultures ranged from 0,4 to 12,5 mg/mL. It has been found that **MBECs** of antibiotics on S. aureus cells, which are formed in biofilm, are several times higher than those of planktonic forms of culture data. The most effective exposure to S. aureus cells in the biofilm was amoxicillin with clavulanic acid. In more than half of cultures of S. aureus, **MBECs** on biofilm forms was 7,5 times higher, compared with MBC on planktonic forms.

Keywords: Staphylococcus aureus, minimum bactericidal concentration, minimum biofilm eradication concentrations.

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INTRODUCTION

Mastitis is the main disease of cows [1, 2, 6]. Mastitis affects the quality and safety of milk, reduces profitability due to expenses to treatment and destruction of milk because of the risk of appearance of antibiotic residues [12]. Many gram-positive and gram-negative pathogens can cause mastitis [9, 14, 16]. Most often, Staphylococcus aureus is the causative agent, causing intramammary infections, which often remain undetected and poorly responsive to antibiotic treatment [1, 7, 13]. Despite the apparent positive in vitro antimicrobial susceptibility, treatment of animals with mastitis often leads to repeated cases of clinical and chronic manifestations of the disease [6, 20].

A distinctive feature of the staphylococcal property is that the infections, caused by them, are complicated by the formation of biofilms [1, 2]. As part of the biofilm, bacteria become more pathogenic, including resistance to antibiotics [10, 11]. In addition, biofilms reduce the effectiveness of antimicrobial peptides, which are important components of the host's internal immune system [22]. Subminimum inhibitory concentrations of antibiotics have also been shown to induce the formation of bacterial biofilm [5].

Staphylococcus aureus is the main causative agent of subclinical, clinical, relapsing and chronic mastitis in cows [1, 2]. The treatment of this disease is usually reduced to the use of antibiotics based on laboratory tests to determine the sensitivity of planktonic forms of bacteria in vitro. The antimicrobial therapy of this disease is currently based on analysis of antimicrobial susceptibility in accordance with CLSI standards. Traditionally, microbiologists estimate the effectiveness of antibiotic by measuring the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) [3, 4]. Virtually in all diagnostic laboratories, these measurements are carried out on freely floating, planktonic, laboratory phenotypes. These analyses measure only the concentration of chemotherapeutic agent needed to suppress the growth or elimination of planktonic bacteria. For some antibiotics, the concentration necessary to destroy biofilm forms of microorganisms may exceed the thousand times required to destroy planktonic bacteria of the exact same strain [8, 15]. Therefore, the use of typical laboratory studies to select chemotherapy for staphylococcal mastitis may be inadequate.

The purpose of the work – to compare the minimum bactericidal concentration of antibiotics concerning influence on planktonic and biofilm forms of S. aureus isolated from cows with mastitis in Ukraine.

MATERIALS AND METHODS

There were investigated 117 isolates S. aureus, isolated over the past three years in the study of 120 samples of the breast secretion of cows suffering from mastitis. Samples were sampled at dairy farms in Ukraine.

Selection and identification of S. aureus was performed using BD Baird Parker Agar according to standard methods. Tenfold dilutions of samples were sown on cups for primary isolation. They were incubated in aerobic conditions for 42-48 h at a temperature of 36±1°C and were performed readings after 18-24 and after 42-48 hours. To confirm the presence of S. aureus, tests were used for catalase, coagulase, oxidase, for the fermentation of D-mannitol, production of deoxyribonuclease, and acetoin [17].

To determine the ability to form a biofilm, a pure culture of isolated strain was seeded into holes of immunological plate in the amount of at least 10^5 CFU/ml. The plate was incubated at 37±1°C for 3 days. If during this period a biofilm was formed – surface or bottom-up growth in the hole, which gave a film, which at the removal of the medium settled on the walls, then the strain was considered film-forming.

Determination of the minimum bactericidal concentration of antibiotics on planktonic microorganisms was carried out according to standard methods [3, 4].

The study of sensitivity of microorganisms in the biofilm form to antibiotics was carried out on daily microbial biofilms, grown in plastic Petri dishes. After 24 h of incubation of cultures, the dishes were washed 3 times from the planktonic (not attached) microorganisms with sterile phosphate buffer and were added 5 cm^3 of freshly prepared antibiotics. After exposure, the antibiotics were poured out, the dishes were washed three times with sterile phosphate buffer, was introduced 5 cm^3 of sterile 0,9% sodium chloride solution and with sterile tampon were thoroughly washed off the walls and bottom of the microbial biofilm cup. From the dishes, 1,0 cm^3 of suspension was taken, a number of ten-fold dilutions were prepared, cultures of 1,0 cm^3 of each breeding in Petri dish were sown, poured MPA and incubated at 37^0C during 24-48 h for the determination of the number of bacteria [2].

Statistical processing of results was carried out using methods of variation statistics using the program Statistica 6.0 (StatSoft Inc., USA). Nonparametric methods of research were used (Wilcoxon criteria, Mann-Whitney criteria). The arithmetic mean (x), the standard error of the average (SE) were determined. The difference between the comparable values (SE) was considered to be significant for $P < 0{,}05$.

RESULTS

We analysed active antibacterial substances in the composition of anti-mastitis agents and found that most pharmacists use antibiotics of penicillin range (penicillin, amoxicillin with clavulanic acid), fluoroquinolone – enrofloxacin, tetracycline and aminoglycoside – gentamicin. Taking into account the analysis data, these antibiotics were used in further studies concerning influence on planktonic and film-forming forms of S. aureus. After all, a typical disease, caused by microorganisms (staphylococci, streptococci, intestinal and sinusoidal sticks) that can form a biofilm, include the subclinical form of mastitis [2, 20]. At the first stage of study, MBC of antibiotics were identified in relation to planktonic forms of S. aureus, isolated from cows with mastitis (Table 1). In the study were selected cultures that were resistant to antibiotics in the disco diffusion test.

Table 1: MBCs of antibiotics for plankton cultures of S. aureus.

Antibiotic Concentrations, mg/mL	Tetracycline		Enrofloxacin		Amoxicillin / Clavulonic Acid		Gentamicin	
	n =42	%	n =59	%	n=52	%	n=48	%
100	0	-	0	-	0	-	0	-
50	0	-	0	-	0	-	0	-
12,5	9	21,4	0	-	0	-	5	10,4
6,2	1	2,4	0	-	0	-	1	2,1
3,1	2	4,7	0	-	0	-	3	6,2
1,5	18	42,8	0	-	0	-	26	54,2
0,8	6	14,3	3	5,0	2	3,8	8	16,6
0,4	6	14,3	9	15,2	28	53,8	5	10,4
0,2	0	-	35	59,3	4	7,7	0	-
0,1	0	-	7	11,8	8	15,4	0	-
0,05	0	-	5	8,5	10	19,2	0	-

n – number of cultures studied.

From data of Table 1, it is evident that the minimum bactericidal concentration, acting on planktonic cells of S. aureus, was in amoxicillin with clavulanic acid. At the same time, MBC fluctuated in the range of concentrations from 0,05 to 0,8 mg/mL. In 19,2% of cultures, MBC amounted to 0,05 mg/mL, in more than half of cultures (53,8%) amounted to 0,4 mg/mL and only in 3,8% of cultures – 0,8 mg/mL. Practically similar MBC was also in enrofloxacin to the data of planktonic cultures S. aureus.

MBC of antibiotics of tetracycline and gentamicin on S. aureus cells began with 0,4 mg/mL. The action of such concentration of antibiotics of tetracycline and gentamicin stopped the development of 14,3 and 10,4% of S. aureus cultures, respectively. In the other part of microbial cultures, MBC of tetracycline and gentamicin increased uniformly and at concentrations of 1,5 mg/mL inhibited the development of the largest number of S. aureus plankton cultures of 42,8 and 54,2%. The highest MBC of antibiotic data, acting on S. aureus cells, was 12,5 mg/mL, at this concentration of tetracycline and gentamicin, growth of 21,4 and 10,4% of microbial cultures, respectively, was inhibited.

The second part of work was to determine the minimum biofilm eradication concentrations of above-mentioned antibiotics on S. aureus, the results of studies are shown in Table. 2.

Table 2: MBECs antibiotics against S. aureus

Antibiotic Concentrations, mg/mL	Tetracycline		Enrofloxacin		Amoxicillin / Clavulonic Acid		Gentamicin	
	n =21	%	n =24	%	n=17	%	n=16	%
100	2	9,5	0	-	0	-	1	6,2
50	11	52,4	0	-	0	-	3	18,7
12,5	6	28,6	2	8,3	0	-	8	50,0
6,2	1	4,7	1	4,2	0	-	3	18,7
3,1	1	4,7	14	58,3	2	11,7	1	6,2
1,5	0	-	5	20,8	8	47,1	0	-
0,8	0	-	1	4,2	2	11,7	0	-
0,4	0	-	1	4,2	4	23,5	0	-
0,2	0	-	0	-	0	-	0	-
0,1	0	-	0	-	1	5,9	0	-
0,05	0	-	0	-	0	-	0	-

From data of Table 2 it is seen that MBECs of antibiotics on cells of Staphylococcus aureus, which are formed in a biofilm, is several times higher, compared with planktonic forms of data of cultures. Among the four tested antibiotics, tetracycline, enrofloxacin, amoxicillin with clavulanic acid and gentamicin, amoxicillin with clavulanic acid was the most effective in influencing S. aureus cells in a biofilm. In 5,9% of cultures of S. aureus MBECs of amoxicillin with clavulanic acid was 0,1 mg/mL, and in more than half of cultures – 58,8% MBECs was 1,5 to 3,1 mg/mL. That is, more than half of cultures of S. aureus MBC on biofilm forms were 7,5 times larger, compared with MBC on planktonic forms.

In fluoroquinolone – enrofloxacin, the smallest MBECs, acting on biofilm forms of S. aureus, was 0,4 mg/mL, which is 8 times higher the concentration compared to that acting on planktonic cells. The highest MBECs of enrofloxacin on S. aureus cells in a biofilm was 12,5 mg/mL, which is 15 times higher than concentration on planktonic forms. Practically, in the majority (58,3%) of cultures S. aureus, which is formed in biofilm, MBECs was 3,1 mg/mL, in such a number of planktonic cultures (59,3%) MBC was 0,2 mg/mL, which in 15,5 times higher concentration.

Antibiotics tetracycline and gentamicin were the least effective in influencing the biofilm forms of S. aureus. The lowest MBECs of antibiotic data that acted on 4,7 and 6,2% of biofilms, S. aureus was 3,1 mg/mL, which was 7,75 times higher than the concentration that stopped the growth of S. aureus planktonic cells. MBECs of tetracycline and gentamicin from 12,5 to 50 mg/mL inhibited the growth of 70-80% of S. aureus cultures in a biofilm that was 8 times higher than that on planktonic forms. The highest MBECs of antibiotic data, which stopped the development of S. aureus biofilm forms, reached 100 mg/mL.

It is known that bacteria in a biofilm during the action of antibacterial agents may be in a persistent form with a low metabolism. Table 3 shows the results of studies on the quantitative content of S. aureus cells in a biofilm after exposure to antibiotics in the MBC determined on planktonic forms.

Table 3: Influence of antibiotics on the number of bacteria in the biofilm (lg CFU/cm^2, x ± SE)

Antibiotic Concentrations, mg/mL	The number of S. aureus cells in the biofilm after the action of antibiotics in MBC determined on planktonic forms			
	Tetracycline, n =11	Enrofloxacin, n =14	Amoxicillin / Clavulonic Acid, n=8	Gentamicin, n=48
12,5	2,8±1,2	-	-	2,8±1,4
6,2	3,6±2,5	-	-	2,8±1,7
3,1	5,5±4,5	-	-	4,3±3,2
1,5	5,9±4,8	-	-	4,9±3,8
0,8	7,0±5,9	2,1±1,5	1,8±1,3	6,0±4,9

0,4	7,2±5,5	4,6±3,5	4,2±3,1	6,3±5,1
0,2	-	5,1±4,0	4,9±3,9	-
0,1	-	5,9±4,8	5,8±4,7	-
0,05	-	6,1±5,0	5,9±4,9	-
Before the antibiotic effect	9,0±7,9	9,0±7,9	9,0±7,9	9,0±7,9

As can be seen from the data in Table 3 MBC of antibiotics, which affects planktonic forms, does not destroy microbial cells in a biofilm. There is a gradual increase in the number of microbial cells in the biofilm composition as the antibiotic concentration decreases. The number of cells in a biofilm, formed by S. aureus at the highest action of MBC on the plankton of culture of tetracycline, was, on average, 250 thousand times less than the number at the lowest concentration of this antibiotic. The number of S. aureus in the biofilm at the lowest action of MBC was only 25 times less, compared with the number of cells in the biofilm in the control. This indicates that the staphylococcus aureus, being present in the matrix of biofilm, is practically inaccessible to the action of antibiotics, despite the high sensitivity of planktonic cells to these antibiotics.

MBC of antibiotics amoxicillin with clavulanic acid and enrofloxacin was much more active on S. aureus cells in the biofilm compared to tetracycline and gentamicin. After the action of the highest MBC (0,8 mg/mL) of these antibiotics, the number of cells in the biofilm was 1,8±1,3 and 2,1±1,5 lg CFU/cm^2, respectively. Also, we note the smallest number of S. aureus cells in the biofilm after the action of the lowest MBC of amoxicillin (5,9 lg CFU/cm^2), as compared to the effect of antibiotics.

DISCUSSION

It is now well established that chronic inflammatory processes, in particular mastitis, are caused by microorganisms in a biofilm and traditional etiotropic antibacterial therapy is ineffective [15, 18]. Despite the sensitivity of planktonic forms of bacteria – mastitis causative agents to antibiotics [19]. Therefore, scientists are searching for effective methods and developing new agents that would influence the bacteria in biofilms to effectively treat inflammatory processes, including mastitis [21].

Our studies have shown that among the four tested antibiotics, tetracycline, enrofloxacin, amoxicillin with clavulanic acid and gentamicin, the lowest MBC on planktonic cells of S. aureus was found in amoxicillin with clavulanic acid and enrofloxacin, which ranged from 0,05 to 0,8 mg/mL. In antibiotics of tetracycline and gentamicin, MBC on planktonic cultures ranged from 0,4 to 12,5 mg/mL. Studies [10, 19] also indicate a high sensitivity (73,4-100%) of microflora, isolated at mastitis to antibiotics of aminoglycosides and fluoroquinolones.

These studies coincide with numerous data on the need to determine the sensitivity of microflora to antibiotics in the treatment of mastitis. However, despite the significant sensitivity of planktonic forms of bacteria isolated with mastitis to antibiotics, a positive result in the treatment is not always achieved [5, 6], as in the pathogenesis of chronic subclinical mastitis the leading role belongs to the biological forms of bacteria [2].

In determining the effect of **MBECs** antibiotics on biofilm forms of bacteria, it was found that MBC on S. aureus cells in a biofilm was several times higher than that which was on planktonic forms of microorganisms. Also, the increased resistance of bacteria in a biofilm, isolated from mastitis in cows to antibiotics, was reported by other scientists [15].

According to our data, 5,9% of S. aureus cultures MBC of amoxicillin with clavulanic acid was 0,1 mg/mL, and in more than half of cultures – 58,8% MBC was 1,5 to 3,1 mg/mL. That is, more than half of cultures of S. aureus **MBECs** forms were 7,5 times higher, compared with MBC on planktonic forms. Practically similar data are also used in the determination of MBC enrofloxacin on S. aureus biofilm. It was also noted that MBC of antibiotics amoxicillin with clavulanic acid and enrofloxacin was much more active on S. aureus cells in the biofilm compared to tetracycline and gentamicin. After the action of the highest MBC, which acted on planktonic forms of amoxicillin and enrofloxacin (0,8 mg/mL), the number of cells in the biofilm was 1,8±1,3 and 2,1± 1,5 lg CFU/cm^2, respectively, and for the action of the highest MBC of tetracycline and gentamicin, the number of S. aureus in the biofilm was 2,8 ± 1,6 CFU/cm^2.

The more effective action of amoxicillin with clavulanic acid and enrofloxacin on biofilm forms of S. aureus can be explained by comparing the molecular weight of these antibiotics. The molecular weight of amoxicillin with clavulanic acid is 365,4/199,16 g/mol, while enrofloxacin is 359,4 g/mol. At the same time, the antibiotics tetracycline and gentamicin had a molecular weight of 444,4 and 477,6 g/mol, respectively, which was 1,3 times higher than that of amoxicillin and enrofloxacin. Due to the lower molecular weight of antibiotic, it is easier to diffuse through the pores and channels of biofilm and effectively reduce the number of microbial cells. The relationship between molecular weight and the effect on biofilm is reported by other scientists who conducted studies in vitro [21].

Thus, summing up the study it can be argued that the bacteria, isolated at subclinical forms of mastitis of cows, have the ability to form a biofilm of high density, which complicates the effectiveness of antimicrobial therapy of the disease and determines the chronic nature of its course. Therefore, in order to substantiate the effectiveness of mastitis treatment, it is necessary to select such antimicrobial concentrations, which effectively acts on microbial cells, formed in the biofilm.

CONCLUSION

It was found that among the four tested antibiotics, tetracycline, enrofloxacin, amoxicillin with clavulanic acid and gentamicin, the lowest MBC on planktonic cells of S. aureus was found in amoxicillin with clavulanic acid and enrofloxacin, which ranged from 0,05 to 0,8 mg/mL. In antibiotics of tetracycline and gentamicin, MBC on planktonic cultures ranged from 0,4 to 12,5 mg/mL.

It has been found that **MBECs** of antibiotics on S. aureus cells, which are formed in biofilm, are several times higher than those of planktonic forms of culture data. The most effective exposure to S. aureus cells in the biofilm was amoxicillin with clavulanic acid. In more than half of cultures of S. aureus, **MBECs** on biofilm forms was 7,5 times higher, compared with MBC on planktonic forms.

After the action of the highest MBC that affects planktonic forms of amoxicillin with clavulanic acid and enrofloxacin (0,8 mg/mL), the number of cells in the biofilm was 1,8±1,3 and 2,1±1,5 lg CFU/cm^2, in accordance.

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