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BACTERIAL BIOFILMS FORMATION OF CATTLE MASTITIS PATHOGENS

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Summary. The results of the microbial biofilms formation by subclinical and clinical forms of cattle mastitis agents were presented. It was determined, those in cattle with subclinical form of mastitis 2.5 times more strains of *Staphylococcus aureus* were allocated. It is occurred, that biofilm density compared to the clinical form of mastitis demonstrates such level of range. In addition, staphylococci, which are the causative agents of cattle mastitis in 1.4–1.7 times more often form the dense biofilm in comparison to streptococci.

The development of the dense staphylococcal biofilms provides their long-term existence on the teats skin and in the breast of carrier's cattle with subclinical form of mastitis. This helps to transform these animals into the pathogen reservoir. Anti-epizootic measures should always be conducted among the animals with clinical form of mastitis for the preventing of infection process chronic transformation. At a subclinical form of mastitis bacteria are located in biofilm matrix and the antimicrobial effect will be less effective.

Keywords: biofilms, cattle, mastitis, *Streptococcus*, *Staphylococcus*

Introduction. The new theory has been formed in the recent years about ecological regularities of microorganisms existence, especially their relationship with the environment, humans and animals body. The main discoveries in this area are associated with learning of microorganism's ability to form biofilms on surfaces of biogenic and abiogenic origin (Flemming and Wingender, 2010). Biofilms is a living set of one or more types or families of the bacteria that are constantly update, is attached to the biogenic or abiogenic surface and surrounded by the polysaccharide matrix (Donlan and Costerton, 2002). Matrix — a mixture of exopolysaccharides, proteins, nucleic acids and other inorganic substances, which protects the bacteria from environmental factors (Costerton, Stewart and Greenberg, 1999; Mah and O'Toole, 2001). Microorganisms in a biofilm 'communicate' to each other about the development, maturation and destruction of the biofilm using secretory mediators, which play an important role in their social behavior (quorum sensing — QS) (Davies et al., 1998). Pores and channels penetrate the biofilms through this structures microorganisms gets the flow of nutrients and exchange metabolic products (Stoodley, deBeer and Lewandowski, 1994).

Accumulated knowledge indicates that bacteria in biofilms are physiologically different from the microbial cells of the same population in free (planktonic) state. Microorganisms generated in biofilms demonstrate increased resistance to antimicrobial agents and cells of the immune system of a living organism (Behlau and Gilmore, 2008; Gilbert, Das and Foley, 1997; Lewis, 2000). The inhibiting of bacterial biofilms formation to this day remains a problem. Microorganisms in the biofilm do not change their individual sensitivity, but better survive under antibiotics action in dose that exceeds the minimum inhibitory concentration (Stewart and Costerton, 2001).

Many bacterial pathogens in animal's body could potentially form biofilms. Caused by them diseases often recur are chronic and difficult to treat (Mah and O'Toole, 2001). The subclinical forms of mastitis are the typical cattle diseases, caused by microorganisms that are able to form biofilm (*Staphylococcus*, *Streptococcus, E. coli*).

The previous our studies have shown (Kukhtyn, 2004) that at dairy farms about 20% of healthy cows carriers *Staphylococcus aureus* on the teats skin and 5% — in the breast. Therefore important now is to examine the factors that are causing the mastitis in cows, as well as environmental peculiarities of mastitis pathogens.

The aim of the study was to determine the ability of cattle mastitis pathogens (isolated from sick and healthy animals) to form the biofilms.

Materials and methods. Work carried out in Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine.

Diagnosis of subclinical mastitis in lactating cows was conducted in accordance with guidelines (Deutz and Obritzhauser, 2003). Cattle considered sick on mastitis when pathogens (*Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli* etc.) were detected

in breast secretion. To indicate microorganisms was made bacterial inoculation on meat of Baird Parker agar, *Streptococcus* Selective Agar and on the Endo agar. The generic and specific identification of microorganisms were carried out according to the test systems 'Staphy-test 16', 'Strepto-test 16' and 'Entero-test 24' ('Lachema', Czech Republic).

To determine the ability of microorganism's to form biofilms in sterile plastic Petri dishes full with 5 cm^3 Hottinger's media the 1 cm^3 of daily bacterial cultures were incubated at 37 °C for 24 h. After incubation, washed three times cups from planktonic (unattached) microorganisms by phosphate buffer dried and fixed biofilm formed with 96° ethanol 10 minutes. Then they were stained with methylene blue solution for 10 minutes, washed by the phosphate buffer, dried and stained with fuchsine solution for 2 minutes. After a double rinsing the biofilms were evaluated visually and morphological issues were studied by the microscopy (Stepanović et al., 2000).

To determine the density of the biofilms formation the 96-well plastic plates were used. The 0.1 cm^3 daily culture of microorganisms was plated to the holes and incubated for 3 hours at room temperature. Then 1 cm^3 of meat agar was added and incubated at 37 °C for 24 h. After incubation, the wells were washed three times with phosphate buffer, dried and fixed biofilm formed 96° ethanol for 10 minutes. Then stained with a solution of 0.1% crystal violet for 10 min, again washed with phosphate buffer and dried. In the hole put 96° ethanol and washed them well. Measured optical density of alcohol wash solution spectrophotometric ally at a wavelength of 570 nm (Stepanović et al., 2000).

Electron microscopic study of the formed microorganism biofilms on abiotic surfaces (glass) was performed using a raster electronic microscopy in the mode of secondary electrons at a voltage of 20 thousand and increasing every 20,000 to 30,000 times.

Results. Microorganisms *S. agalactiae, S. dysgalactiae, S. aureus* and *S. epidermidis* were allocated in dairy farms from healthy animals and cattle with clinical and subclinical forms of mastitis. The ability of these bacteria to form biofilms in conditions *in vitro* was studied. The research results presented in the table.

As the table shows, almost all cultures *S. aureus*, which are marked with subclinical form of mastitis, formed dense biofilm. In clinical form of mastitis number of *S. aureus*, which formed biofilm with the density 38.7±3.4%. The same trend have noted with the presence of other mastitis pathogens, which is characterized by an increase in 2.4–3.4 times the number of selected microorganisms which form biofilms in the subclinical form mastitis animals in comparison with animal micro flora from the clinical form. The research results also certify that the *Staphylococcus*, which are the agents of cattle mastitis in 1.4–1.7 times denser biofilm was formed than by *Streptococcus* (Table 1). This indicates that cow's staphylococcus origin bacterial mastitis will be potentially harder in the treatment.

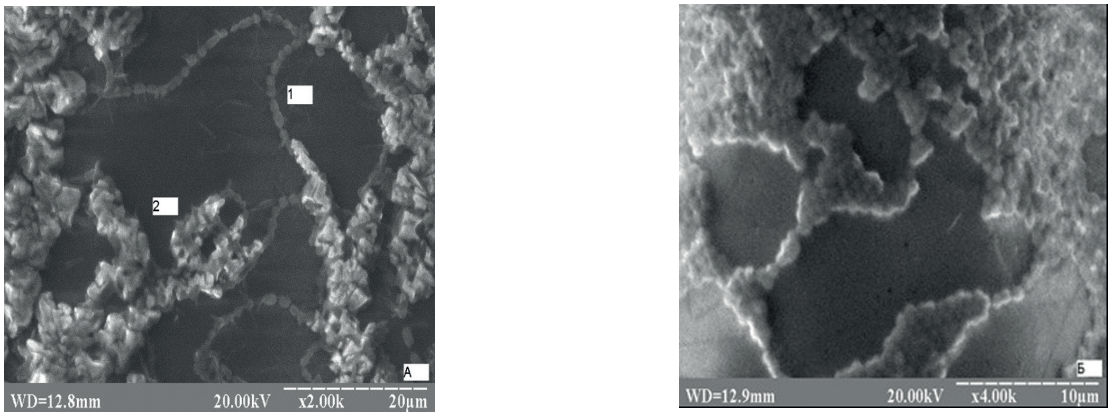
Table 1 – Formation of biofilm by cattle mastitis pathogens, %, M±m, n=80

Type of microorganism	Number of microorganisms which form a dense biofilm in various forms of mastitis and with carriers		
	subclinical	clinical	carriers
S. aureus	97.5±1.6**	38.7±3.4*	94.2±3.2
S. epidermidis	78.3±6.2**	32.4±2.7*	76.3±7.5
S. agalactiae	56.7±2.7	22.3±1.8*	–
S. dysgalactiae	62.5±3.1	18.4±1.5*	–

Note: * — R≤0.01 for subclinical forms of mastitis; ** — R≤0.001 for streptococcal mastitis

The Figure 1 shows the results of electron-microscopic studies *S. aureus* and streptococcus that are in dense biofilm formation.

Discussion and conclusions. The study of *S. aureus* ability for biofilm formation gives us a new look at the cattle mastitis problem. We found that strains of *S. aureus*, which are marked with in cows with subclinical mastitis demonstrated much better colonies forming activity. So at first sight it becomes clear why healthy cows that carriers *S. aureus* (and other staphylococcus) on the teats skin, in the breast, and cows sick on subclinical form of mastitis are less active infection disseminators for some time, compared with sick animals on mastitis clinical form. Obviously, the low ability of cows that are carriers of pathogens, as a source of infection, is because the bacteria carriers are in the biofilm matrix unlike planktonic bacteria that exist in acute clinical mastitis. Staphylococcal biofilm formation provides the long-term existence in animal's carriers. It supports the transform them into a reservoir of the pathogen. Maybe stay of *S. aureus* into biofilm formation in the carrier and in cows suffering from subclinical form of mastitis — is pathogen conservation as a species ensuring on a dairy farm. Cause illness — this is not the main task of microorganisms which are in biofilm formation. The emergence of subclinical forms of mastitis is the manifestation factor of infection. It is well known that the interaction of the microorganism and host depends of his resistance and local and general immunity. *S. aureus* exciting as the biofilm matrix is the reason of practically inaccessible to antibiotics, despite the high sensitivity of planktonic cells to these agents.



a) *S. agalactiae* b) *S. aureus*

Figure 1. Biofilms formation of mastitis pathogens in abiogenically surface (glass): a) 1 — single bacterium *S. agalactiae* outside biofilm; 2 — cells *S. agalactiae*, which are formed in the biofilm that has a three-dimensional surface and solid polysaccharide matrix; b) strains of *S. aureus*, which are in continuous biofilm

Strains of *S. aureus*, which cause clinical form of mastitis, form weak biofilm or its formation required longer period of time (24 hours). However, cows suffering from clinical form of mastitis infection spread much more active for some time, compared to carriers and cows with subclinical form of mastitis. Therefore, we believe all disease control measures should always conduct among patients with clinical form of mastitis preventing 'chronic' process because during subclinical form of mastitis, microorganisms are in biofilm matrix and the antimicrobial effect will be less effective.

Studies of the cow's mastitis pathogens ability to form biofilm are important for effective anti-mastitis measures on dairy farms and the development of new drugs with specific properties that will act on the microorganisms in biofilm.

Author contributions. M. Kukhtyn, A. Bergilevich, and Yu. Horyuk investigated the ability to form biofilm in staphylococci, V. Horyuk and Ya. Stravskyy studied streptococci. Yu. Horyuk and M. Kukhtyn carried out the analysis of research results.

References

Behlau, I. and Gilmore, M. S. (2008) 'Microbial biofilms in ophthalmology and infectious disease', *Archives of Ophthalmology*, 126(11), pp. 1572–1578. doi: 10.1001/archopht.126.11.1572.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) 'Bacterial biofilms: A common cause of persistent infections', *Science*, 284(5418), pp. 1318–1322. doi: 10.1126/science.284.5418.1318.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. T. and Greenberg, E. P. (1998) 'The involvement of cell-to-cell signals in the development of a bacterial biofilm', *Science*, 280(5361), pp. 295–298. doi: 10.1126/science.280.5361.295.

Deutz, A. and Obritzhauser, W. (2003) *Eutergesundheit und Milchqualität: Krankheiten erkennen, vorbeugen, behandeln*, Stocker. ISBN 9783702009878. [in German].

Donlan, R. M. and Costerton, J. W. (2002) 'Biofilms: Survival mechanisms of clinically relevant microorganisms', *Clinical Microbiology Reviews*, 15(2), pp. 167–193. doi: 10.1128/cmr.15.2.167-193.2002.

Flemming, H.-C. and Wingender, J. (2010) 'The biofilm matrix', *Nature Reviews Microbiology*, 8(9), pp. 623–633. doi: 10.1038/nrmicro2415.

Gilbert, P., Das, J. and Foley, I. (1997) 'Biofilm susceptibility to antimicrobials', *Advances in Dental Research*, 11(1), pp. 160–167. doi: 10.1177/08959374970110010701.

Kukhtyn, M. D. (2004) *Veterinary and sanitary evaluation of cow's milk on the content of Staphylococcus aureus [Veterynarno-sanitarna otsinka moloka koroviachoho nezbyranoho za vmistom zolotystoho stafilokoku]*. The dissertation thesis for the scientific degree of the candidate of veterinary sciences. Lviv: Lviv National Academy of Veterinary Medicine named after S. Z. Gzhytskyj. [in Ukrainian].

Lewis, K. (2000) 'Programmed death in bacteria', *Microbiology and Molecular Biology Reviews*, 64(3), pp. 503–514. doi: 10.1128/mmbr.64.3.503-514.2000.

Mah, T.-F. C. and O'Toole, G. A. (2001) 'Mechanisms of biofilm resistance to antimicrobial agents', *Trends in Microbiology*, 9(1), pp. 34–39. doi: 10.1016/s0966-842x(00)01913-2.

Stepanović, S., Vuković, D., Dakić, I., Savić, B. and Švabić-Vlahović, M. (2000) 'A modified microtiter-plate test for quantification of staphylococcal biofilm formation', *Journal of Microbiological Methods*, 40(2), pp. 175–179. doi: 10.1016/s0167-7012(00)00122-6.

Stewart, P. S. and Costerton, J. W. (2001) 'Antibiotic resistance of bacteria in biofilms', *The Lancet*, 358(9276), pp. 135–138. doi: 10.1016/s0140-6736(01)05321-1.

Stoodley, P., deBeer, D. and Lewandowski, Z. (1994) 'Liquid flow in biofilm systems', *Applied and Environmental Microbiology*, 60(8), pp. 2711–2716. Available at: http://aem.asm.org/content/60/8/2711.full.pdf.