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Morphometric and Densitometric Indicators of *Salmonella* Biofilm under the Exposure of a Disinfecting Preparation

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Objective: Study of morphometric and densitometric parameters of biofilms of microorganisms under the influence of a disinfectant.

Materials and methods: To study the morphology of biofilms in vitro, bacteria were cultivated at 37 ° C for 24 hours. When preparing preparations for optical microscopy, fixation was performed with a mixture of alcohol: ether – 1 : 1 for 10 minutes, stained with 0,5 % gentian violet solution - 2 minutes. The optical density of biofilms was determined by the degree of binding of crystal violet, the optical density was determined in an Immunochem–2100 microplate photometric analyzer (HTI, USA) at a wavelength of 490 nm. To obtain representative information, optical microscopy was performed by random selection of the field of view of a microscope model "BIOMED MS-1 Stereo" (Russia).

Results: When studying morphometric parameters after 48 hours of cultivation, 37 ° C with optical microscopy of preparations stained with gentian violet, general patterns of the formation of the heterogeneous structure of *S. enteritidis* biofilms were revealed: adhesion; fixation; maturation; growth; dispersion. Based on the analysis of densitometric indicators of microorganism cultures, it was established that after 48 hours of cultivation, the values of the absolute values of the optical density of microorganisms: OD_s – 0,395±0,11, the intensity of biofilm formation – I ≥ 0,2–0,3, *S. enteritidis* microorganisms are moderate producers biofilms. Indicators of optical density of *S. enteritidis* under the influence of the drug "Ecodes", 0,10 %: OD_s – 0,383±0,10 intensity of biofilm formation – I ≥ 0,2–0,3, cultures of microorganisms *S. enteritidis* – moderate producers of biofilms. Accordingly, when exposed to concentrations of 0,15 and 0,25 %: OD_s – 0,281±0,09 – 0,204±0,09, the intensity of biofilm formation – I ≥ 0,1–0,2, microorganisms *S. enteritidis* are weak producers of biofilms.

Conclusion: During the formation of *S. enteritidis* biofilms, general patterns of the formation of the heterogeneous structure of S. enteritidis biofilms were revealed: adhesion; fixation; maturation; growth; dispersion. The frequency of occurrence of clusters - aggregation of microorganisms, united by a layer of the extracellular matrix, significantly decreased under the

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influence of various concentrations of the disinfectant "Ecodes".

Key words: Salmonella, biofilm, morphometric indicators, densitometric indicators, disinfectant.

Introduction

The ability to form biofilms, the emergence of resistant forms of microorganisms due to the synthesis of exopolysaccharides, significantly reduce the effectiveness of chemotherapeutic and disinfecting drugs [1]. Colonization resistance of the mucous membrane of the respiratory, digestive, reproductive system ensures the regulation of chronic forms of the course of the infectious process, providing protection against the formation of biofilms of pathogens, including *Salmonella spp.* [3, 16]. Direct correlations have been established between the dispersion of biofilms and the proliferation of microorganisms into the epithelial and connective tissue layers of the dermis [15].

To develop effective measures to combat and prevent infectious pathology, aimed at preventing animal diseases and obtaining safe livestock products, the priority task is to find effective disinfectants that reduce the adhesive properties of microorganisms, disruption of intercellular information exchange, blocking the synthesis or destruction of the polymer matrix, which determined the relevance research topics.

The aim of the work is to study the morphometric and densitometric parameters of biofilms of microorganisms when exposed to a disinfectant.

Materials and Methods

This work with certified *Salmonella enteritidis* ATCC 13076 does not require approval from the Ethics Committee.

The study of biofilms and phenotypic traits was carried out using a certified strain (ATCC): *Salmonella enteritidis* ATCC 13076 [5].

The experiments used meat-peptone broth, "*Nutient Broth*" (HiMedia, India); disinfectant the drug "Ecodez" (JSC NPO *"Novodez"*, Russia) in various concentrations. Organisms were cultured at 37 ^{o}C, 48 hours – control; when exposed to the drug – experience.

For microscopic studies microorganisms were cultured on glass slides placed in petri dishes with 20,0 ml BCH and 5,0 ml suspension of 18-hour cultures of microorganisms in a concentration of 10^5 cfu / ml at 37 ^{o}C, 48 h, fixed with alcohol: ether (1: 1) – 10 min [3]. The samples under study were stained with a 0,1 % gentian violet solution, 0,5 % methylene blue, 0,5 % trypan blue, 0,1 % acridine orange, 0,1 % Congo red aqueous solution, and an aqueous crystal violet solution at a dilution of 1: 2000, according to Gram, "*Gram-color-stain set for the Gram staining method"* (BioVitrum, Russia).

For the detection of uncultured microorganisms, we used media containing components

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for cell wall repair and reversal of L–forms [4].

The optical density *(Optical Density* – OD*) of* biofilms was determined by the degree of binding of crystal violet (Himedia, India) at a wavelength of 580 nm in anmicroplate photometric analyzer *Immunochem-2100* (HTI, USA). The test samples (control and experiment) were introduced into the wells of a 96-well plate (Medpolymer, Russia) and cultured at 37 ° C for 48 h. Then the liquid was removed, the wells of the plates were washed three times with 200 μL of phosphate-saline solution (pH 7,3). At each washing step, the plates were shaken for 5 minutes. Fixation was performed with 150 μl of 96,0 % ethanol for 15 min, then the wells were dried at 37 °C for 20 min. A 0,5 % crystal violet solution was added to the wells and cultured at 37 ° C for 5 min. The contents of the wells were removed, washed three times with 200 μl of phosphate-saline solution (pH 7,3), and dried. The dye was eluted from the adhered cells with 200 μl of 96,0 % ethanol for 30 min. The studied cultures of microorganisms were differentiated by the intensity offormation: $OD_s \leq OD_c$ biofilm–microorganisms that do not produce biofilm; $OD_c < OD_s \leq (2 \times OD_c)$ –poor biofilm producers; $(2 \times OD_c) < OD_s \leq (4 \times OD_c)$ – moderate producers of biofilms; $(4 \times OD_c) < OD_s$ are strong biofilm producers [8].

The experimental data were subjected to statistical processing using the "Statistika" program for PC Microsoft Excel 2007.

Results and Discussion

Morphometric parameters

After 48 h of cultivation, 37 °C, optical microscopy of preparations stained with gentian violet and methylene blue revealed general patterns of formation of heterogeneous biofilm structures of the studied microorganisms: adhesion; fixation; maturation; growth; dispersion.

Microscopic examination (≥90 % of the field of view) revealed adhesion and fixation to the substrate (cover glass) of rod-shaped Salmonella. Bacterial cells were united by an intercellular matrix of varying color intensity and formed short or long chains. In addition, areas of the diffuse layer of bacteria were identified, which had a typical shape and size for the species.

Densely packed and intercellular matrix groups of cells attached to the surface formed closed structures of various sizes, due to the cells and extracellular matrix attached to the substrate, microcolonies united by the extracellular matrix were formed (Fig. 1).

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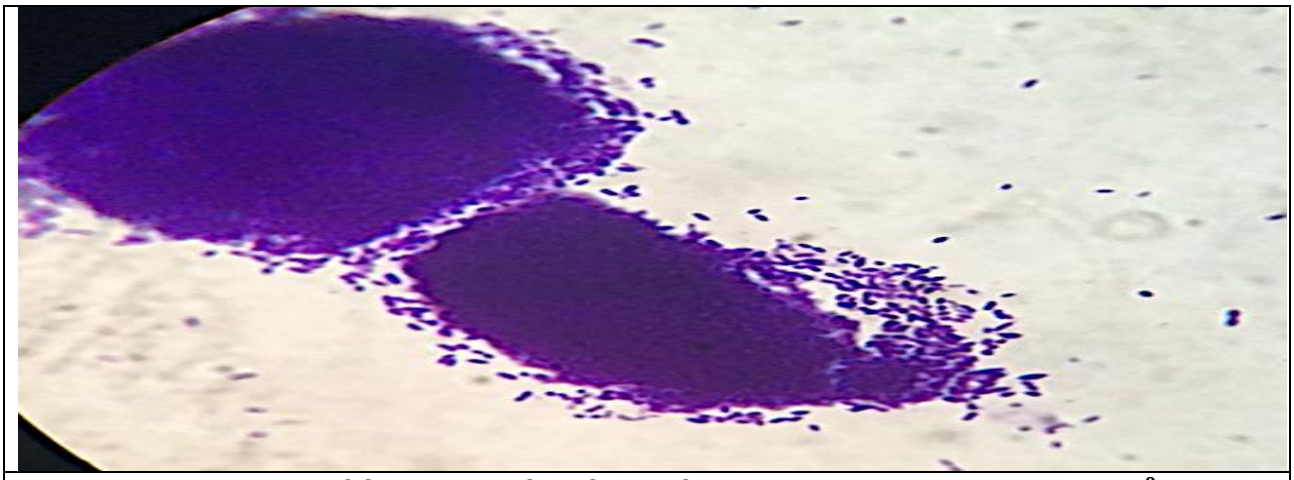

Fig. 1. Intensity of formation of biofilms of microorganisms *S. enteritidis*, 37^{o} C, 48 h: architectonics of biofilm in the form of a dense network, consisting of gram-negative rod-shaped cells surrounded by an intercellular polymer matrix. Gentian violet. Ey. 10, le. 100, immersion

The formation of a three-dimensional structure of biofilms in the form of a dense network consisting of gram-negative bacteria surrounded by an intercellular polymer matrix occurs due to the synthesis of the extracellular matrix.

With the destruction of the intercellular matrix and the separation of bacterial cells from microcolonies, the dispersion was revealed in the form of isolated branched structures colonizing areas of the substrate free of microorganisms.

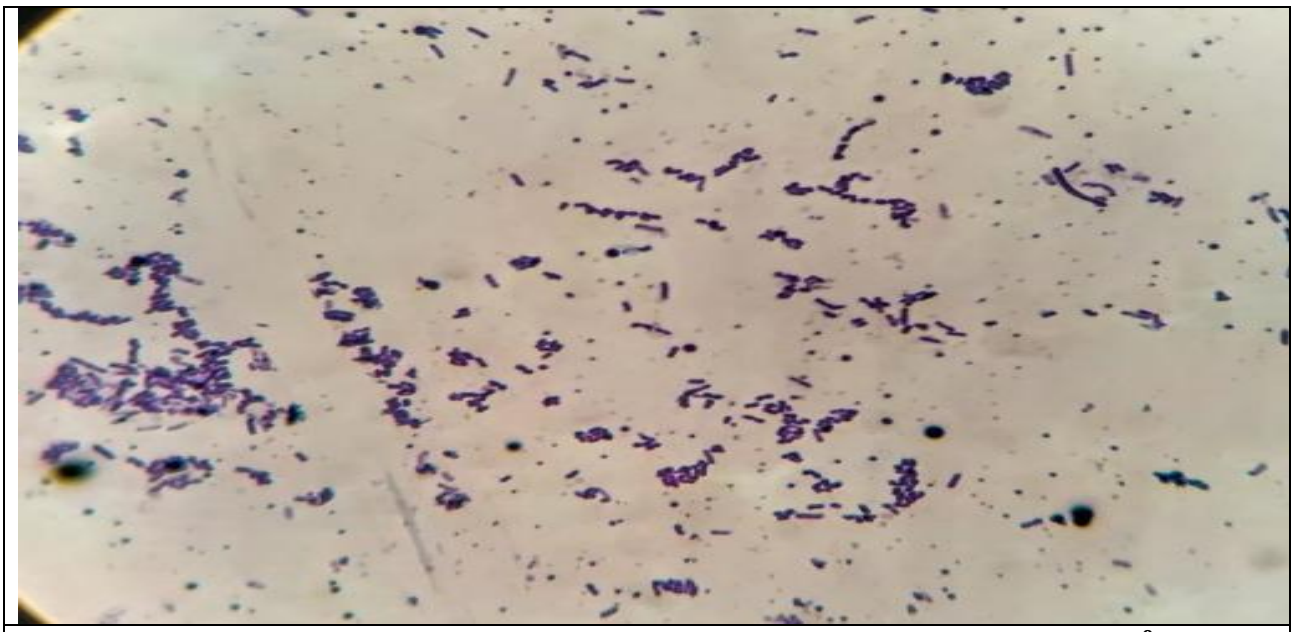

Fig. 2. Intensity of formation of biofilms of microorganisms *S.enteritidis*, 37^{o} C, 48 h: architectonics of biofilm in the form of a dense network, consisting of gram-negative rod-shaped cells surrounded by an intercellular polymer matrix. Gentian violet. Ey. 10, le. 100, immersion

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Densitometric indicators

Based on the analysis of the densitometric parameters of microorganism cultures, it was found that after 48 hours of cultivation, the values of the absolute values of the optical density of microorganisms: OD_s – 0,395±0,11, the intensity of biofilm formation – $I \geq$ 0,2–0,3, microorganisms *S. enteritidis* – moderate biofilm producers (Table 1).

Table 1

Densitometric parameters of biofilms of microorganisms, 48 h

Culture of microorganisms	**Optical density (OD)**			
	Control (OD_c)	**Experiment (OD_s)**	**Δ (OD_s-$OD_{c)}$**	**Intensity (*I*)**
S. enteritidis	0,099±0,03	0,395±0,11	0,296±0,14	$I \geq$ 0,2–0,3
Note. *OD is the optical density; OD_C –OD control; OD_S –OD of the investigated sample; I – intensity: the difference between the OD of the test sample (OD_S) and the control (OD_C)*				

Indicators of the optical density of *S. enteritidis* when exposed to the drug "Ecodes", 0,10 %: OD_s – 0,383±0,10 intensity of biofilm formation – $I \geq$ 0,2–0,3, cultures of microorganisms *S. enteritidis* are moderate producers of biofilms. Accordingly, when exposed to concentrations of 0,15 and 0,25 %: OD_s – 0,281±0,09 – 0,204±0,09, the intensity of biofilm formation – $I \geq$ 0,1–0,2, microorganisms *S. enteritidis* are weak producers of biofilms (Table 2).

Table 2

Densitometric parameters of biofilms of microorganisms when exposed to the drug "Ecodez", 48 hours

Concentration of the drug, %	**Optical density (OD)**			
	Control (OD_c)	**Experiment (OD_s)**	**Δ (OD_s-$OD_{c)}$**	**Intensity (*I*)**
0,10	0,096±0,03	0,383±0,10	0,287±0,13	$I \geq$ 0,2–0,3
0,25	0,099±0,05	0,281±0,09	0,182±0,14	$I \geq$ 0,1–0,2
0,50	0,098±0,03	0,204±0,12	0,106±0,15	$I \geq$ 0,1–0,2
Note. *OD is the optical density; OD_C –OD control; OD_S –OD of the investigated sample; I – intensity: the difference between the OD of the test sample (ODS) and the control (OD_C)*				

Conclusion

Analyzing the results of the studies, we state that the evolutionary mechanism of adaptation due to the manifestation and fixation of mutations, intercellular communication, sorption and aggregation of heterogeneous biofilms, cyclic growth modes cause persistence non-cultivated microorganisms in interepidemic and interepizootic periods [4].

When identifying microorganisms from patmaterial from broiler chickens, the frequency of occurrence of *S. Heidelberg* was 23,5 % with an average bacterial concentration of 3,579 log

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CFU / g [10].

Microorganisms *S. enteridisserovar* were the predominant isolated from samples of chicken meat and processed products – 28,5 %, eggs and egg products – 61,5 %. *S. Typhimurium* (35,2 %) and *S. Derby* (18,8 %) predominated in pork samples; *S. Weltevreden* – in seafood samples (19,2 %) [6].

Of 1035 food samples, 147 (14,2 %) isolates of *Salmonella spp.,* Were identified most often detected in fresh meat (28,0 %), ready-to-eat foods (9,0 %), frozen convenience foods (7,1 %) and fresh products (4,5 %). The most common serovars were *Salmonella Enteritidis* (46,3 %; 68/147), *Salmonella Typhimurium* (32,7 %; 48/147) and *Salmonella Derby* (6,8 %; 10/147). The isolates were found to be resistant to sulfisoxazole (93,9 %; 138/147) and sulfamethoxazole (61,2 %; 90/147) [22].

In the study of 558 samples from production facilities of 165 poultry farms, the prevalence of *Salmonella spp.* amounted to 47,9 %. 23 serotypes were identified, of which *S. Kentucky* and *S. Isangi* accounted for 32,9 % and 11,0 %, respectively [12].

A positive correlation (r=0,89) was established between the densitometric parameters of biofilms (OD_{490} – 0,676±0,079 – 0,608±0,110) and the resistance profile of multi-resistant *Salmonella spp.,* isolated in diseases of the digestive system of animals to antibacterial drugs [11].

The antibiotic resistance of bacteria in the form of a biofilm is significantly higher than that of "planktonic" bacteria, the difference between the values of the minimum inhibitory concentration and the minimum biofilm-eliminating concentration of antibiotics ranged from several tens (for *S.intermedius* gentamicin – 38), up to several hundred times (for *S.aureus* ciprofloxacin – 256) [2].

Study of antibiotic resistance of isolates *S. Kentucky* (n = 11), *S. Enteritidis* (n = 4), S. Typhimurium (n = 3), *S. Breanderp* (n = 1), and *S. Newport* (n = 1), isolated from samples from dead birds on poultry farms. showed that 95,0 % of isolates were resistant to penicillin, 85,0 % to norfloxacin and colistin, 75,0 % to gentamicin, 70,0 % to nalidixic acid and 60,0 % to flumequin [18].

Microorganisms *S. enteritidis* isolated from samples of pig waste from pig complexes (n = 87) showed resistance to sulfamethoxazole: in raw waste 40/87 – 46,0 % of resistant isolates, in wastewater samples 34/47 – 85,0 %. Microorganisms were resistant to sulfamethoxazole –5/84 (6,0 %), chloramphenicol: 29/87 (33,0 %); ampicillin –26/87 (20,0 %) [9].

In the study of resistance to antibacterial drugs of 51 *Salmonella spp.* isolates isolated from 379 environmental samples, serovar *Salmonella enterica* 32/51 (62,7 %) showed multidrug resistance, five virulence plasmids were identified, including IncFIIS 17/51 (33,3 %), IncI1α

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12/51 (23,5 %), IncP 8/51 (15,7 %), IncX1 8/51 (15,7 %), and IncX2 1/51 (2,0 %) [7].

When assessing the antibacterial resistance profiles of 31 *Salmonella spp.* isolated from samples on poultry farms, it was found that 94,4 % of isolates *S. Infantis* and 50.0 % of S. *Typhimurium* had low resistance to ceftriaxone, ampicillin and cefepime. The genes of *β*-lactamases producing carbapenemases (blaIMP, blaVIM, blaOXA48, blaKPC, blaNDM) were identified [21].

The adhesive properties of pathogenic enterobacteria are realized through fimbrial structures and afimbrial adhesins, interacting with receptors of epithelial cells, which activates the metabolism of bacterial cells [17]. The toxigenic properties of Salmonella are realized through the production of thermostable and α-hemolysins, which damage the membrane; interacting with membrane receptors and having catalytic activity; injection, penetrating into the target cell with activation in the cytoplasm of eukaryotes [14]. The invasion of microorganisms, including Salmonella, is realized in an active way – interaction with receptors of eukaryotic cells, and in a passive way – phagocytosis [13]. With a decrease in the intensity of biofilm formation: (0,101±0,04–0,113±0,15), an increase in the number of dissociated colonies was revealed: S-form, d = 2,0–5,0 mm; R- form, d≥3,0 mm; M- form, d = 1,5–3,0 mm; d-form, d = 0,2–0,5 mm the number of viable cells decreased [4]. The isolates circulating among the poultry population and isolated from raw materials and poultry products: *Corvallis, Brancaster,* and *Albany,* isolated from poultry, were multiresistant to the studied antibiotics and all strains produced biofilm, 69,3 % were strong biofilm producers [19].

To prevent contamination of food raw materials, *Salmonella spp.* promising is an inactivated trivalent vaccine containing the serovars *Salmonella Enteritidis* (O: 9, serogroup D) and *Salmonella Typhimurium* (O: 4, serogroup B), the serovar *Salmonella Infantis* (O: 7, serogroup C1) and an adjuvant (aluminum hydroxide) for infection of birds *S. Hadar* (O: 8, serogroup C2) $1x10^6$ CFU / g showed an increase in serovar-specific antibodies (34,4%), detected up to 56 weeks of age [11].

Isolates of *S. enteridis* (n = 15), *S. typhimurium* (n = 12), and *S. weltevreden* (n = 8) from poultry meat samples were sensitive (100,0 %) to chloramphenicol and ofloxacin [19].

Under the influence of increasing concentrations of the drugs "Litikase" (250-1000 U) and "Farnesol" (100 – 400 μM), as well as their combinations on biofilms of microorganisms, a decrease in optical density was revealed, microorganisms are weak producers of biofilms (OD_s – 0,102±0,09 – 0,200±0,06), a direct correlation (r=0,87) of morphometric (%) and densitometric indicators (OD_s) was established, reflecting a decrease in the frequency of occurrence of clusters and optical density, respectively. [20].

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Competing Interests

The authors declare that they have no competing interests.

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