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The Effect of Antimicrobial Drugs Tylocolinum, Tetragold and Cidisept-o on *Escherichia coli* Ultrastructure

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Abstract

As a result of wide antibiotics, sulfonamides and other antimicrobial agents usage for the therapy of the animals with the bacterial infections caused by various causative agents including *Escherichia coli*, many microorganisms gained resistance to the chemotherapeutic agents. New combined drugs are being worked out during recent years, the components of which have various influence mechanisms on the bacterial cell that helps to provide resistance forming control. The results of the researches of the new antimicrobial agents, containing antibiotics in their composition, and non-antibiotic agent influence on the ultrastructure of *Escherichia coli* are represented in this study.

5-hour *Escherichia coli 866* culture was processed by the drugs of the minimum bactericidal (Tylocolinum-0.39 μg/ml, Tetragold-6.25 μg/ml, Cidisept-o-25 μg/ml) and 4-time concentrations during 3 hours. Samples and control culture (without drugs) were fixed by the 2.5% glutaricdialdehyde on the s-Collidine Buffer, dehydrated in the ethanol with rising concentration, filled in epoxies. Ultrathin slices were stained by 2% water solution of uranyl acetate and lead citrate for 10 minutes. Then they were examined with the use of the electron microscope JEM-100 CX II by JEOL.

The research showed deep ultrastructural changes in *Escherichia coli* cells under the antimicrobial agent influence determined by synergistic effect of combined Tylocolinum and Tetragold drugs components, possessing various bacteria influencing mechanisms, and aldehyde that is a component of Cidisept-o.

The electron microscopy usage allows to get unique information about the impact consequences of the traditional improved drugs and new drugs with antimicrobial activity on the bacterial infectious agents.

Keywords: *Escherichia coli*, ultrastructure, cell wall, membrane, cytoplasm, nucleoid, Tylocolinum, Tetragold and Cidisept-o

1. Introduction

Pathogenic *Escherichia coli* is the most frequent causative agent in youngster farm animal diarrheal syndrome and postpartum diseases of the breeding stock (Terekhov, 2002; Gafarov et al., 2002; Misaylov et al., 2002; Kashin et al., 2003; Nezhdanov, 2005). Antibiotics, sulfanilamides and other antimicrobial drugs (Terekhov, 2002; Nezhdanov et al., 2005; Shabunin, 2008) are used extensively in animal therapy of infections caused by *Escherichia coli*. In spite of the positive results got by chemotherapy of the sick animals and chemoprophylaxis of various infectious diseases including caused by *Escherichia coli*, there is a tendency of their (antibiotics) effectiveness decrease during the last years. It's connected with the fact that, on the one hand, long-term usage of the antibiotics of one class leads to steady infectious agent populations forming as a result of their adaptation, on the other hand, infectious disease aetiology is often determined by the resistant forms and species of pathogenic and opportunistic pathogenic microorganisms causing chronic, atypical, latent infectious process with their prolonged persistence in the organisms of the susceptible animals (Kofer et al., 2002; Noa, 2004; Shakhov et al., 2007; Selionova et al., 2009).

Many bacteria gained and still continue gaining antibiotics resistance and compose problematic microorganism groups (Brian, 1984).

Enterobacteriaceae family microorganisms are the most dangerous among opportunistic pathogenic bacteria rapidly gaining multiple drug resistance (*Escherichia coli*, *Proteus spp.*, *Enterobacter spp.* and others) (Sidorenko, 2001; Gorbenko, 2004; Yankovskij, 2005; Glagolev et al., 2006, Pimenov et al., 2006; Docenko et al., 2007; Shponko, 2007; Afonyushkin et al., 2008; Ahmed et al., 2000).

The results of the researches devoted to the resistance development of the bacteria taken from cattle, pigs and human to the fluoroquinolones showed that the resistant strain number increase was observed among the majority of microorganisms but *E. coli* had the highest characteristics (Aarestrup et al., 2000).

Studying the susceptibility of 309 *Escherichia coli* strains of various serological variants taken from pigs with gastrointestinal diseases, the high frequency of their resistance was established to (%): Ampicillin (90.4), Oxytetracycline (88.0), Gentamicin (45.1), Cefazolin (41.1), Ciprofloxacin (28.2), Chloramphenicol (63.5), Nitrofuran (44.8). Various epigenetics of the resistance to antimicrobial drugs was revealed analyzing the susceptibility of the studied *Escherichia coli* strains. The increase of the *E. coli* resistance was registered during 6 years (2001-2006). Resistance to Ampicillin-13.8%, Oxytetracycline-25.8%, Chloramphenicol-59.3%, Nitrofuran-70.0%. The decrease of their resistance to Chloramphenicol and Nitrofuranwas marked in 2007-2008. During the same years the resistance to Ampicillin and Oxytetracycline was on the same level. The increase of the *E. coli* resistance level was also marked in the period from 2001 to 2004. The increase of *E. coli* resistance level to Gentamicin-79.4%, Cefazolin-68.9%, Ciprofloxacin-54.3%. During the next years (2005-2008) their resistance decrease took place at the level registered in 2001 (Shakhov et al., 2011).

The main cause of forming bacteria resistance to many used antimicrobial drugs is receiving R-plasmid from other bacteria during conjugation, carrying in themselves resistance gene to antibiotics. They are capable of passing from one bacterium to another, forming the resistance of the whole population (Tatarchuk, 2006; Linde et al., 1998; Schwarz & Werckenthin, 1998; Schwarz & Chaslus-Dancla, 2001).

R-plasmids often contain stability genes to several types of antibiotics giving multiple cross-resistance (poly-resistance) to microorganisms-recipients. As a result of introducing one antibiotic the resistance is formed not only to it but also to a number of antimicrobial drugs that were not used in this concrete case (Stegnij & Krasnikov, 2001).

In recent years combined drugs with components of broad antimicrobial spectrum and different mechanism of action are developed (Gorbenko, 2004; Shabunin, 2008). This is due to the emerged antibiotic resistance of *Escherichia coli.* As an alternative to antibiotics, non-antibiotic remedies are also offered (Belayev, 2008).

The aim of this research was to study the effect of the new combined antimicrobial drugs Tylocolinum and Tetragold containing antibiotics and non-antibiotic remedy Cidisept-o on the ultrastructure of *Escherichia coli* for the therapy of the animals with gastrointestinal and respiratory diseases of bacterial aetiology.

2. Study Materials and Methods

In this study we used the logarithmic phase culture *Escherichia coli 866* which was incubated in plain broth at 500 000 of microbial cells per 1 ml of medium amid enhancing aeration. The drugs were added to the 5-hour culture in minimum bactericidal concentrations (Tylocolinum-0.39 μg/ml, Tetragold-6.25 μg/ml, Cidisept-o-25 μg/ml) and in concentrations 4 times exceeding the minimum one. The last one was chosen for revealing deeper structural changes in the cells (Zhukov & Navashin, 1975). Incubation period took 3 hours. As a control, drug free *Escherichia coli* culture was used.

To produce electron microscopic agents, testing and control cultures were subjected to centrifugation of 3 000 rotations during 20-minute period. The pellet was scoured 3 times by normal saline. After that bacterial cells were placed into 2.5% glutaricdialdehyde on the s-Collidine Buffer (Ito, Karnovsky, 1965). Then post fixation in ethanol with rising concentration and filling in epoxies were carried out (Newman et al., 1982). To receive reliable results, the studies were made three times.

Slices were obtained with the use of the ultratome LKB III-8802A, contrasted in 2% water solution of uranyl acetate and in lead citrate for 10 minutes (Reynolds, 1963) and were examined with the use of the electron microscope JEM-100 CX II by JEOL with boosting voltage of 80 kV and resolution of 3 angstrom. The magnification power was 10000-60000.

3. Results

3.1 Control Cell of Escherichia coli

The *E. coli* cells had a structure that is typical of gram-negative bacteria.They had typical triple-layered cell walls with undulating surface caused by the bimolecular lipid-protein layer. The cell wall layers, periplasmatic

space and plasma membrane were visible and differentiated. The membrane bore against the cytoplasm all over the surface and was well outlined. The dividing *Escherichia coli* cells also had this typical triple-layered structure. The cytoplasm was homogeneous, the nucleoid was visible and occupied the most part of the cytoplasm. Around this nucleoid an electron-dense layer of the ribosomal unit and the membrane structures were discovered (Figure 1a, b).

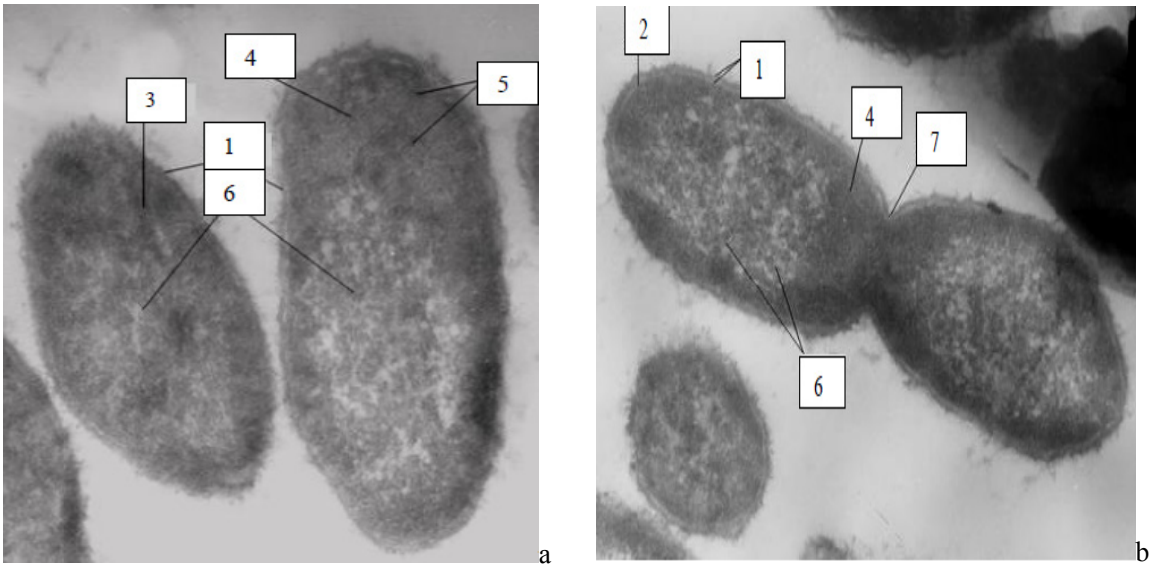

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Figure 1. *E. coli* cells (control). 1-cell wall; 2-plasma membrane; 3-mesosomal structures; 4- cytoplasm; 5-ribosomes; 6-nucleoid; 7-dividing constriction. Magnification -29 000

3.2 The Effect of Tylocolinum on E. coli

As a result of the effect of minimum bactericidal concentration of Tylocolinum during the 3-hour period, about 48% of *Escherichia coli* cells suffered changes. They increased in size. The cell wall lost distinct boundaries of its layers and in some parts the plasma membrane was not detected. At some cellular poles, the cell wall flaking with emission of its components to the surface was observed. In individual cases, local lysis of the plasma membrane with emission of the plasma membrane contents to the intercellular space (Figure 2a) was observed.

The nucleoid merged with the electron-dense layer of the cytoplasm that surrounded it, the loss of homogeneous structure, the electron-transparent sections and darker and denser components were distinguished (Figure 2a). In a majority of cells, cytoplasm took the homogeneous form of an average electron density, ribosomal unit and membrane structures differentiated weakly. In sections of plasma membrane and cell wall outer layer local lysis, electron-reduced sections of cytoplasm were observed (Figure 2b). The cell division was interrupted, the cell wall was not detected in the site of constriction generation.

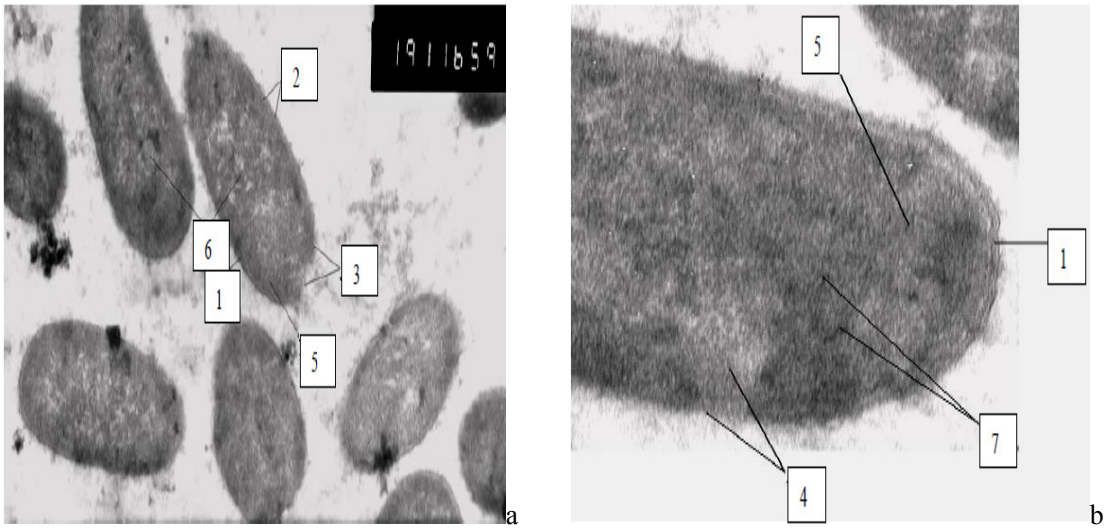

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Figure 2. Effect of Tylocolinum on *E. coli* cells (minimum bactericidal concentration). 1- cell wall; 2-plasma membrane and its melted section; 3- cell wall lysis, its contents emission to the intracellular space; 4-cell wall and cytoplasm lysis; 5-cytoplasm; 6- nucleoid. 7-mesosomal structures. Magnification-19 000 (2a), 60000 (2b)

As a result of the effect of the 4-time exceeding minimum bactericidal concentration of Tylocolinum, about 75% of *Escherichia coli* cells suffered deep structural changes. The destructive effect of the agent on cells differed. In some cases, it affected only cell walls and changed inner structures insignificantly. However, in other cells major changes were detected both on its surface and in the inner structures (Figure 3a).

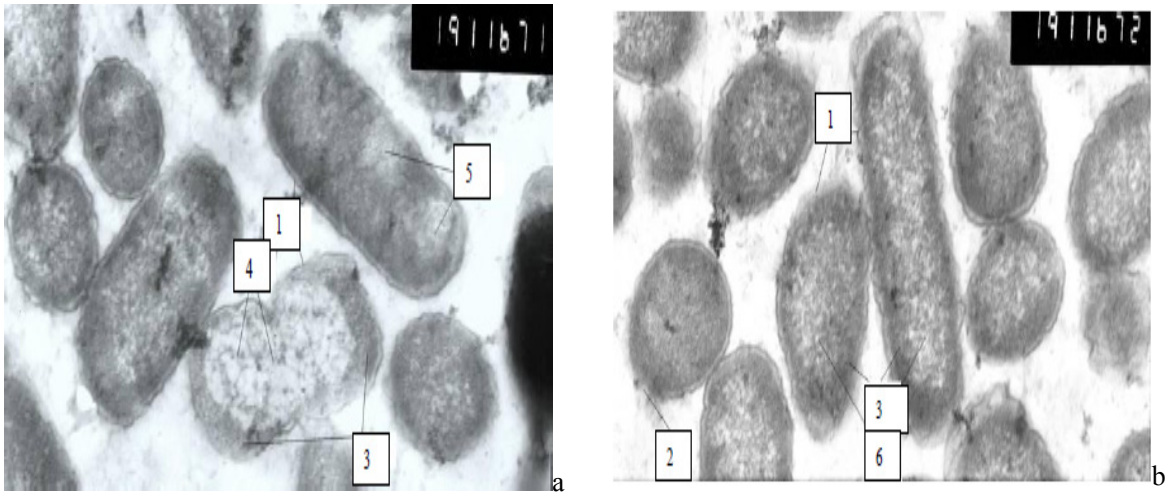

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Figure 3. Effect of Tylocolinum on *E. coli cells* (4-time exceeding of minimum bactericidal concentration) 1-cell wall; 2-cell wall melting, its contents emission to the intracellular space; 3-cytoplasm; 4-cytoplasm and nucleoid lysis, membrane structures; 5-cytoplasm lysis in the cell wall thinning sections;
6-nucleoid lysis. Magnification-19 000

The cell wall thickened all over the surface, in the outer layer irregular shape bulges were formed, at the poles the significant flaking of cytoplasm with complete melting of the plasma membrane was observed. In cytoplasm borne against these sections, electron transparent areas of local lysis were observed. The nucleoid took the form of the light friable small-grained mass with rare more electron-dense osmiophobic sections (Figure 3b). Cells with complete lysis of the nucleoid contents and partly of cytoplasm were detected. In lighter sections membrane structures were detected, small cytoplasm sections were located at the cellular poles (Figure 3a). *Escherichia coli* cell division was inhibited almost completely, in dividing cells the constriction was distinguished weakly.

3.3 The Effect of Tetragold on E. coli

As a result of the effect of minimum bactericidal concentration of Tetragold on *Escherichia coli*, cells increased in size and lost their form. The cell wall thickened, in the outer layer irregular shape bulges were formed, local or complete lysis with emission of its contents to the intercellular space was detected. The plasma membrane flaked of cytoplasm, its melting sections were observed.

The cytoplasm took the form of the small-grained structure of a less electron-density. Membrane and ribosomal components did not differentiate. In the sections of adjoining to the cell wall, wide electron-reduced lysis zones were observed (Figure 4a).

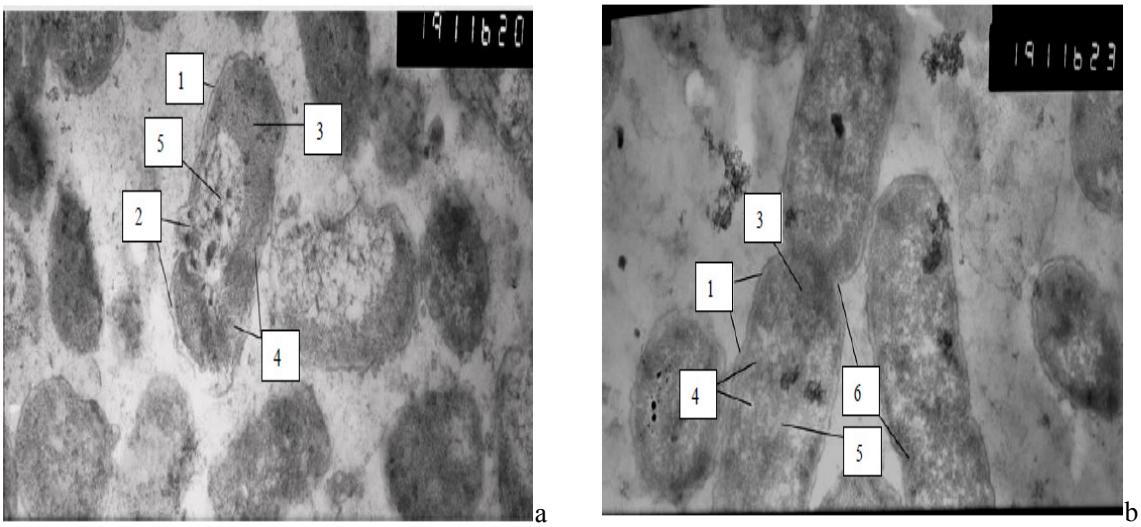

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Figure 4. Effect of Tetragold on *E. coli* cells (minimum bactericidal concentration). 1-cell wall; 2-local and complete melting sections of the cell wall; 3- cytoplasm; 4-cytoplasm sections of less electron density in areas adjoining to the lysed cell wall; 5-lysed nucleoid; 6-constriction between daughter cells. Magnification-19 000

The cell division process was interrupted, the cell wall components were not detected in the site of constriction generation and undivided daughter bacteria were detected in the form of lobocytes (Figure 4b).

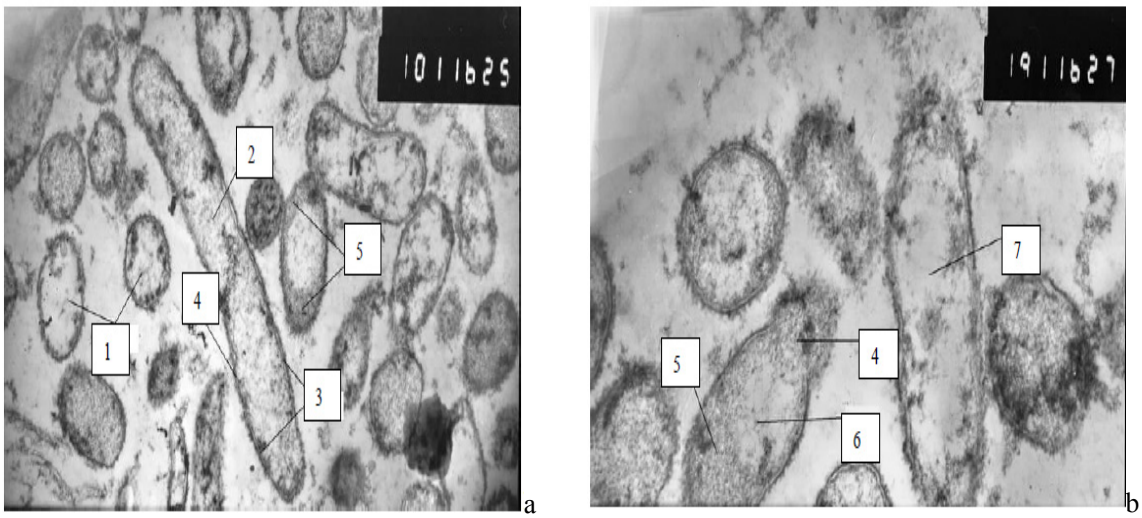

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Figure 5. Effect of Tetragold on *E. coli* cells (4-time exceeding of minimum bactericidal concentration) 1- lysed cells; 2-lysed dividing cell; 3- cell wall remains of the lysed cell; 4-cell wall melting sections; 5-cytoplasm located on the cell periphery; 6- lysed nucleoid electron-transparent section; 7-dead cell. Magnification-10 000 (5a), 19000 (5b)

As a result of the effect of the 4-time exceeding minimum bactericidal concentration of Tetragold, *Escherichia coli* cells suffered deeper structural changes. They decreased in size, thickened and took irregular form. The cell wall had more electron-dense form, lost its undulating triple-layered structure and its components were not observed. All over the cell wall surface, friable material that was the part of the outer layer, entering the intercellular space was detected. In some cells, the cell wall melting with emission of the plasma membrane contents was observed (Figure5b). The plasma membrane was lysed completely or was observed in the form of small sections.

In a majority of cells, the cytoplasm was lysed and on its place wide electron-reduced sections filled with membrane structure remains were formed. In other cells small sections of cytoplasm were located on the periphery and at the poles in the form of the small-grained layer of low electron-density. Mesosomal structures and ribosomal unit were not exposed (Figure 5a, b).

The nucleoid did not differentiate or was lysed completely and on its place the electron-transparent layer was observed.

3.4 The Effect of Cidisent-o on E. coli

As a result of the effect of minimum bactericidal concentration of Cidisent-o on *Escherichia coli*, cells increased in size significantly. The cell wall noticeably thickened due to the outer layer loosening, where irregular shape bulges were detected. In some areas, the outer layer flaked of the cell wall with emission of its contents to the intercellular space. Layers lost distinct boundaries and differentiation. The plasma membrane was not exposed along the entire length, local lysis and its layer separation sections were a subject of observation (Figure 6a).

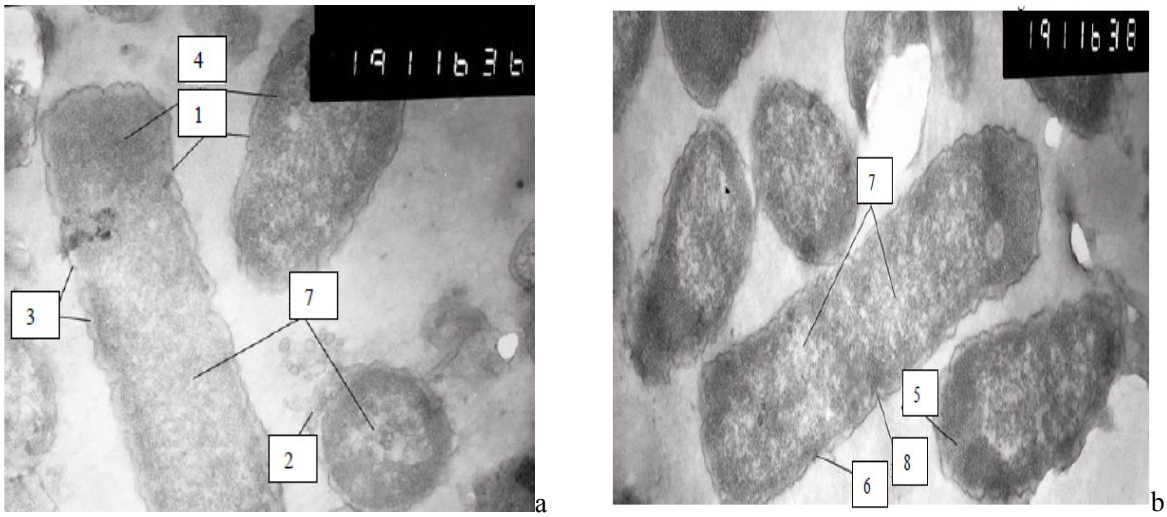

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Figure 6. Effect of Cidisept-o on *E. coli* cells (minimum bactericidal concentration). 1-cell wall; 2-cell wall melting; 3-plasma membrane and its lysed sections; 4-cytoplasm; 5-cytoplasm lysis section; 6- lysed cell wall; 7-nucleotide lysis; 8-constriction between daughter cells. Magnification-19000

The cytoplasm took the grained form with heterogeneous electron-optical density, in the melting parts of plasma membrane and a cell wall lighter reduced lysis sections were observed. On the periphery and at the poles more electron-dense osmiophobic sections were exposed.

Comparing to the control the nucleoid was less electron-dense, large sections of blooming combined with denser membrane structures of irregular form were exposed. The nucleoid and cytoplasm lost distinct boundaries. The ribosomal complex did not differentiate.

Cell division was interrupted, the cell wall was not detected in the site of constriction generation (Figure 6b).

As a result of the effect of the 4-time exceeding minimum bactericidal concentration of Cidisept-o, *Escherichia coli* cells lost their form and increased in size. Long cells formed due to the cell division interruption were exposed.

The cell wall of some bacteria became thin and in a majority of its sections the complete lysis of all its

components with an emission of the contents to the intercellular space was observed. In a majority of cells the cell wall was not detected.

Completely lysed cells were also detected. In these cells, the plasma remains with large sections of blooming were located on the periphery. In the place of the nucleoid, membrane cellular structures of irregular shape were detected. The main part of the cell wall melted completely which caused both the emission of its contents to the intercellular space and the loss of the cell form (Figure 7a, b).

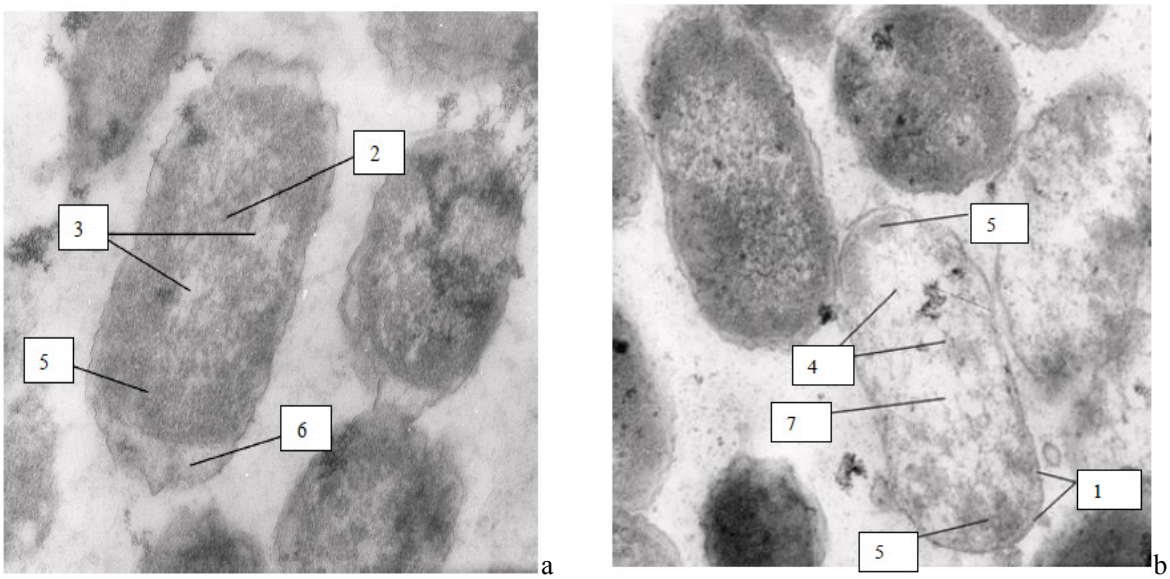

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Figure 7. Effect of Cidisept-o on *E. coli* cells (4-time exceeding of minimum bactericidal concentration). 1-cell wall; 2-nucleoid; 3- lysed nucleoid electron-transparent section; 4-membrane structures on the lysed nucleoid site; 5-cytoplasm; 6- large melting sections of the cell wall in the bacterial pole; 7-dead *E. coli* cell. Magnification-19 000

4. Discussion

The described structural failure of *Escherichia coli* cells depended on the mechanism of action of observed antimicrobial drugs and their concentration.

The identified changes in bacteria ultrastructure provoked by Tylocolinum were caused by the synergistic effect of its components- colistin and tylosin.

Colistin (polymyxin E) belongs to the polymyxin group, polypeptide antibiotics and consists of positively charged polypeptide ring and polypeptide chain terminated with the branched fatty acid connected to it. Colistin molecules bound with the anionic phospholipid layer of the cell plasma membrane. The cell osmotic barrier interruption caused by this process results in the emission of its intracellular components, the hydration increase and the following lysis. Positively charged cyclic peptide bounds electrostatically with anionic phosphatic groups of membrane phospholipids. At the same time, the fatty acid side-chain bounds in its inner hydrophobic sections. Mentioned hydrophobic interactions result in interruption of the membrane lipid structures, the membrane organization gets broken and its permeability increases for both intracellular and extracellular components (Beringer, 2001; Gales, 2001).

In addition, in gram-negative bacteria the colistin interacts with lipopolysaccharides of the cell wall outer layer that provides similar disorganizing effect.

Tylosin belongs to the macrolide class. It blocks the protein synthesis that takes place in bacterial ribosomes 70S. An application site of the agent effect is the 50S ribosomal subparticle. Tylosin bounds with ribosomes in their active center due to the hydrophobic interaction. It prevents both tRNA built into the polypeptide chain of amino acid from emission and the bound of a new one which inhibits the next steps of the protein synthesis.

Tylosin penetrates into the cell by means of the passive diffusion. The antibiotic effect depends on the final saturation of bounding sections. The plasma membrane is a significant barrier on the way toward its effect

application site (Mashkovskij, 2008).

The synergistic effect when partnering with colistin is caused by the primary effect of colistin on the plasma membrane. It accelerates the tylosin penetration into the cell and both agents affect different structures of the bacterial cell simultaneously.

The plasma membrane destruction due to the effect of tylocolin on the microbial cell is caused by divalent cations extrusion of magnesium and calcium that provide membrane stability. At the same time, hydrophobic connections of colistin and lipid molecules result in a loss of recovering possibility of the membrane structural integrity. Revealed heterogeneous electron-optical density of the cytoplasm is the evidence of water-soluble low-molecular components emission from the cell and of the cell hydration increase. The cell wall loss of stiffness and filling of the cell contents result in its size increase.

In a majority of cells, not only optically transparent sections were detected but also electron-dense (hydrophobic) sections that were the conglomeration of ribosome-protein complex that had lost their activity due to the effect of tylosin.

The detected *Escherichia coli* cells changes due to the effect of Tetragold is a result of a combination of colistin, trimethoprim and sulphanilamides that are in the drug composition.

The sulphamilamide structure has a similar form with n-aminobenzoic acid that is the growth factor for some microorganisms. The acid takes part in the folic acid synthesis giving its n-amino benzyl group. On this basis, the folic acid synthesis is inhibited competitively in the presence of sulphanilamides. These agents can serve as alternative substrates in biosynthetic reactions and bound more firmly with catalyzing enzymes. As a result, important intermediary metabolites of the folic acid synthesis don't form. The cell wall is impermeable for these compounds and bacteria cannot make the good deficit from without. Whereas sulfanilamides and n-aminobenzoic acid can easily penetrate through the cells.

Trimethoprim (another antimicrobial agent component) acts as an inhibitor of the dihydrofolatereductase enzyme that participates in the folic acid synthesis. Trimethoprim is a pyrimidine derivate that even in low concentrations reveals a high-selective effect on the bacterial enzyme (Sokolov, 1997; Mashkovskij, 2008).

The sulphanilamide and trimethoprim combination inhibits the folic acid synthesis, its derivates and precursor metabolites completely which affects many metabolic processes. Loss of one-carbon compounds interrupts the protein synthesis due to the methionine deficit and the deficit of purine and pyrimidine bases, thymidine particularly, inhibits the nucleic acid synthesis. DNA replication, its reparation and RNA forming are interrupted which causes the protein biosynthesis process inhibition (Brogden et al., 1982; Huoviven et al., 1995).

The effect of Tetragold on microbial cells exhibited the structure integrity damage to the cell wall, its components and the plasma membrane even in minimum bactericidal concentrations. The effect of the 4-time exceeding minimum bactericidal concentration was that *E.coli* cells suffered deeper structural and morphological changes. Most bacteria undergo the lysis, on the cytoplasm and nucleoid sites large electron-transparent sections filled with membrane mass were detected. In some cells the plasma remains were located on the periphery and had a low-electron dense granular form. When cell wall melted, the cytoplasm and nucleoid emission to the intercellular space was observed.

Cidisept-o belongs to the aldehyde class and affects directly the plasma membrane of microorganisms, being adsorbed on its phospholipid components. As a strong oxidizing agent, Cidisept-o causes double bonds formation in fatty acids tails provoking loss of the membrane stiffness. The lipid layer becomes more fluid and protein and lipid redistribution takes place. As a result, membrane permeability gets broken and water-soluble essential metabolites, such as phospholipid, purine and pyrimidine compounds and energy exchange enzymes emerge into the intercellular space. The membrane outer surface accumulates redundant negative potential that causes intercellular space water penetration in the cell.

Electron-dense insertions of bacterial cells may become enzyme overactivity sites or the accumulation of ribosomal-protein complex, formed as a response to the drug effect and directed at new protein molecule synthesis promoting cell and membrane structural integrity recovery. The interaction of carbonyl group and protein amino acids results in disruption of native solid connections of protein molecules. The enzyme and its active center tertiary structure changes resulting in decreased or muted activity.

The drug effect on nucleic acid may cause interruption in phospho-dietheric connections between compounds and breach of hydrogen compounds that provide the space structure of nucleic acids.The loss of nucleotides due to the cell wall permeability increase and new compounds synthesis inhibition impede DNA reparation, doubling and RNA formation. Ascertained abundance of damaged and dead *E. coli* cells due to the contact with Cidisept-o

comparing with the effect of Tylocolinum is caused by its impact of a strong oxidizing agent on different structures of microbial cells.

Agent impact impeded *E.coli* cell division. The cell wall forming between daughter cells in the constriction zone was interrupted and as a result, bacteria took form of lobocytes.

5. Conclusion

The submicroscopic study detected significant ultrastructural changes in *E. coli* cells caused by antimicrobial drugs. Denoted differences in the effect on cells of Tylocolinum, Tetragold and Cidisept-o that belong to different antibacterial drug groups are related to the mechanism of action peculiarities attached to the active agents that enter into their composition.

The perfection of the traditional and creation of the new drugs with antimicrobial activity demands the usage of the methods allowing g to provide complex estimation of the mechanisms and the consequences of their impact on the bacterial target cells (Finberg et al., 2004). One of these methods is electron microscopy.

Using it Chavdarova et al. (1977) researching *E. coli* strain morphological changes after a combined influence of Ampicillin and Cefalexin stated that the drugs inhibit dividing constrictions and stop cells division as a result of which highly lengthened threadlike forms appear. It is also shown that morphological changes of the *E. coli* cells at combined usage of the Ampicillin and Cefalexin are mainly determined by Ampicillin impact and Cefalexin most probably intensifies the degree of these changes.

Shabanova et al. (2009) estimated ultrastructural changes of hemolytic and enterohaemorrhagic *E. coli* cells under the influence of diffusing bacteriocins-like materials of lactobacilli in lactobacillus-agar appearing in destabilization of cell wall, periplasmatic space widening, and appearance of the cytoplasm parts with the low electron density in the polar cell sections with the flaky material visualization.

Electron microscopical study of the “Optimax” (made on the basis of alkylamine) biocidal influence on *E. coli* stated that ultrastructural bacteria changes under the drug influence prove total construct damage of the cells. The damage of the bacteria ribonucleo-protein complex excludes their possibility to from resistance to this drug (Didenko et al., 2012).

Studying the influence mechanism of the combined Dioxigenum drug on *E. coli*, ultrastructural and functional changes of their cells under the bactericidal concentration of the drug determined by the synergistic effect of its Dioxydinum and Gentamycin components, possessing different influence mechanisms, were stated (Shakhov et al., 2012).

Electron microscopical studies showed ultrastructural changes in *E. coli* cells under the influence of the combined Dioxinorum drug bactericidal concentration determined by the effect of one of the Dioxydinum components on the cell wall and synergistic effect of Dioxydinum and Norfloxacin possessing various influence mechanisms on genetic apparatus of the bacteria (Shakhov et al., 2013).

Thereby the results of our own researches show that electron microscopy allows to get unique information about the consequences of various antibacterial drugs influence on *E. coli*. Our results can be used in the further researches, measuring known and new-synthesized compounds biocidal effect not only on *E. coli* but also on the other microorganisms.

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