

Influence of new frost-resistant disinfectant on the ultrastructural organization of atypical mycobacteria

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The article presents the results of experimental studies of *M. kansasii*, *M. gordonae*, *M. xenopi*, *M. flavescens* ultrastructure in normal conditions and after exposure of new anti-frost disinfectant. A glutaraldehyde is an active substance, and a sodium formate used as an antifreeze. It was shown that occurred changes are characterized by destruction of microcapsule, cell wall and cytoplasmic membrane, formation of vacuoles and osmiophil inclusions in the cytoplasm of mycobacteria cells after an exposure of the frost-resistant disinfectant. A dissolution of the microcapsule and cell wall was noted under the action of a disinfectant on *M. kansasii*. Cells partially lost the cytoplasmic membrane. The cytoplasm had the appearance of dark finely granular inclusions. The nucleoid region was not clearly visible. The appearance of vacuoles in the cytoplasm of cells that had a low electron density was observed in the test culture of *M. gordonae* after application of the disinfectant. The effect of disinfectants on *M. xenopi* causes the destruction of the microcapsule and cell wall in almost all microbial cells. The cytoplasm contained small granular substances of different electron densities. The complete disappearance of the microcapsule and cell wall was observed in the culture of *M. flavescens* after action of disinfectant. It was lead to release of the cytoplasm. The cytoplasm gains density and contains vacuoles, as well as a fine-granular substance. The nucleoid region is blurred and densified. The developed disinfectant composition can be used in the complex of veterinary and sanitary measures for the control and prevention of tuberculosis of farm animals at ambient temperatures up to minus 20°C.

Keywords: Atypical *Mycobacterium*; Electron microscopy; Ultrastructure; Cell wall; Nucleoid; Disinfectant; Antifreeze; Low temperature

Introduction

Tuberculosis is a serious public health problem worldwide, despite a regular slow decline in incidence over the past decade (Sulis et al., 2014; Reichman, 2017). The main reason of high level of morbidity and mortality from tuberculosis is an increase the multiresistant mycobacteria strains among the population in Ukraine (Pavlenko et al., 2018). However, there is no correlation between the incidence rates for tuberculosis in cattle and humans.

Today this issue is still very acute and requires constant monitoring despite the success achieved in eradication of tuberculosis in farm animals (Paliy, 2013; Raphaka et al., 2018; Carrisoza-Urbina et al., 2019). This is reasoned by the fact that tuberculosis is a zoonanthropous disease, which has important epidemiological and epizootological significance (Villarreal-Ramos et al., 2018). The urgent problem of modern medicine is mycobacterioses, the etiological factor of which is non-tuberculous atypical mycobacteria. Atypical mycobacteria are saprophytes, a common inhabitant widely distributed in the environment in contradistinction to tuberculosis mycobacteria that are obligate pathogens. These microorganisms have potential pathogenicity and can cause the development of various pathological processes in the macroorganism (Cook, 2010; Henkle & Winthrop, 2015; Koh, 2017). Atypical mycobacteria are isolated in animal husbandry, which cause para-allergic reactions to tuberculin (PPD) for mammals in farm animals (Gcebe, 2013; Basybekova et al., 2018).

A large number of disinfectants are used in order to destroy mycobacteria in the environment, which belongs to different chemical groups and have a composition (Best et al., 1990; Zavgorodnii et al., 2013; Shinoda et al., 2016). The most common chemical disinfectants used in the complex of anti-TB veterinary and sanitary measures include chlorine-containing (Le Dantec et al., 2002; Paliy, 2018; Paliy et al., 2019), aldehyde-containing (Rutala et al., 1991; Paliy et al., 2015, 2018) and acidic (Paliy & Dubin, 2016; Bondarchuk et al., 2019) disinfectants. It should be noted that the resistance of microorganisms to the same disinfectant varies within one species, which also needs to be taken into an account when planning anti-epizootic measures (Paliy, 2014).

A number of research studies have found that most pathogenic and opportunistic microorganisms can withstand cold temperatures for a sufficiently long time (Ukhovskiy et al., 2019). Therefore, decontamination and quarantine activities have their own peculiarities during a winter season. Temperature of the environment and of the treated surfaces is one of important factors that affect the disinfection efficiency. The disinfecting effect of some biocidal agents is reduced at sub-zero ambient temperatures. This occurs because the diffusion rate of the chemical molecules between the disinfectant working solution and the disinfectant substrate is reduced at low temperatures, as well as conditions of the relationship between the microbial cell and the disinfectant are worsened (Zavgorodniy et al., 2013). The search for new disinfectants, whose bactericidal activity will not be reduced when used at low ambient temperatures during the cold season, is now an important issue (Paliy et al., 2020).

Electron-microscopic studies of mycobacteria are an urgent task of modern science after the action of various adverse factors (Kumar et al., 2015; Kassich et al., 2017). Study of disinfectants action mechanisms on mycobacteria will make it possible to create scientifically based rational schemes for the devitalization of microorganisms in the environment, as well as to approach the problem of the targeted effect of the drug on individual cellular elements.

The aim of the work was to study ultrastructural changes in mycobacterial cells after exposure of a new frost-resistant disinfectant.

Materials and methods

Test cultures of atypical mycobacteria classified by E. Ranyon (1959) were the subject of studies: Group I (photochromogenic mycobacteria) – *Mycobacterium kansasii* (strain 11 – P (1)), a museum strain, non-pathogenic for laboratory and farm animals; Group II (scotochromogenic mycobacteria) – *Mycobacterium gordonae* (inventory No. 47), a museum strain non-pathogenic for laboratory and farm animals; Group III (non-photochromogenic mycobacteria) – *Mycobacterium xenopi* (no inventory No.), museum strain, non-pathogenic for laboratory and farm animals; Group IV (fast-growing mycobacteria) – *Mycobacterium flavescens* (inventory No. 119), a museum strain, non-pathogenic for laboratory and farm animals.

An aldehyde-containing agent was used as a disinfectant. Working solutions of the disinfectant were prepared on the basis of antifreeze (sodium formate) (Paliy et al., 2017). Initially conducted experiments have shown the efficiency of this disinfectant at the temperatures up to minus 20°C. Experimental studies were carried out in the specialized laboratories of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv, Ukraine). The experiments for obtaining of inactivated mycobacteria cultures were carried out using the suspension method of study according to the current methodological approaches (Zavgorodniy et al., 2007) at temperature of minus 20°C. Special temperature conditions were simulated using a freezer. The preparation of an experimental material and an electronic microscopy studies have been carried out according to the generally accepted methodology:

- mycobacteria samples were fixed in a 2.0% solution of glutaraldehyde in phosphate buffered saline (PBS) at pH of 7.4 during 2 hours at 4°C after exposure to disinfectant. Samples were washed twice with PBS to remove glutaraldehyde after fixation. The cell suspension was placed in the protein matrix (10.0% serum albumin) after the fixation in order to preserve the cell population during various electron-microscopic manipulations;

- postfixation was carried out with a 1.0% solution of osmium tetroxide (OsO₄) prepared on phosphate buffer. The fixation period was 1-2 hours at a temperature of 4 C. The material was washed three times with PBS to remove osmium tetroxide;

- dehydration of samples was performed with the use of ethanol at increasing concentrations: 30°C, 50°C – for 15 minutes, 70°C – 30-60 minutes (4 C), further at 80°C, 96°C and three times in absolute ethanol at 15 minutes at room temperature. Samples were placed in absolute acetone three times for more thorough dehydration;

- pouring epoxy resin into samples. The samples were impregnated at room temperature in a mixture: absolute acetone + epoxy resins (Epon-Araldite) in the following concentrations: acetone: resin – 1:3 (2-3 hours); standard resin mixture for pouring – 2-3 hours.

Samples were placed in flooded polyethylene capsules made of Epon-Araldite resin after soaking and exposed for 6-12 hours at room temperature. Subsequently, the capsules were amenable to polymerization at 56 C for 48 hours. The experimental material was removed from the thermostat after polymerization, the quality of polymerization was checked. Then the material was prepared for research. The block with samples was designed in the form of a pyramid for the manufacture of slices.

The ultrathin sections obtained on the ultra-microtome UMPT-7 were collected on palladium mesh and contrasted with a saturated aqueous solution of uranyl acetate and a solution of lead citrate. The ultrastructure of mycobacterial cells was studied using a PEM-125K electron microscope at an accelerating voltage of 75 kV, equipped with a SAI-01A image analysis system (AO "SELMI", Sumy) using a DX-2 CCD camera and "KAPPA" software package, Germany.

Results and Discussion

Mycobacterium kansasii, *M. gordonae*, *M. xenopi*, *M. flavescens* were used in studies that had typical cultural and biological properties (Figure 1).

A number of researchers established the fact that high resistance of mycobacteria to chemical and physical factors is mainly associated with the complex structure of the cell wall and the lipid-polysaccharide complex in the microbial cell (Jarlier & Nikaido, 1994; Jackson, 2014; Alderwick, 2015). The cell wall is a complex tripartite structure containing a large amount of lipids (approximately 30.0-40.0% of the total weight) (Rastogi et al., 2001). The electron-microscopic organization of mycobacteria is presented below (Figure 2).

A layer of average electron density of uniform thickness with a fringed outer surface is detected on the surface of mycobacteria during the electronic microscopy of control samples that are not exposed for a disinfectant (Figure 2). This layer is located near each cell separately or about several cells at the same time. Some scientists believe that this layer is similar to the microcapsule of a number of gram-positive bacteria (Niederweis et al., 2010; Kalscheuer et al., 2019). Other scientists call it a diffusive layer (Imaeda et al., 1968), or consider it as adsorbed components of the nutrient medium, or destroyed mycobacteria (Maitra et al., 2019).

Atypical mycobacteria have much in common with the submicroscopic organization of tuberculosis pathogens. The thickness of the cell walls of different types of mycobacteria is also approximately the same. Exceptions are certain polymorphic forms (Claeys & Robinson, 2018). Osmiophilic material was detected as an electron-dense substance on the surface of the *M. flavescens* cell wall. This material has the form of osmiophilic filaments. It is believed that this material consists of lipopolysaccharides and acts as a protective capsule of mycobacteria, which protects them from lysosomal macrophage enzymes and antimicrobial agent's activity (Kalscheuer et al., 2019). The outer layer of the cell wall is the following structural organization of mycobacteria which is visible as a osmiophobic gap.

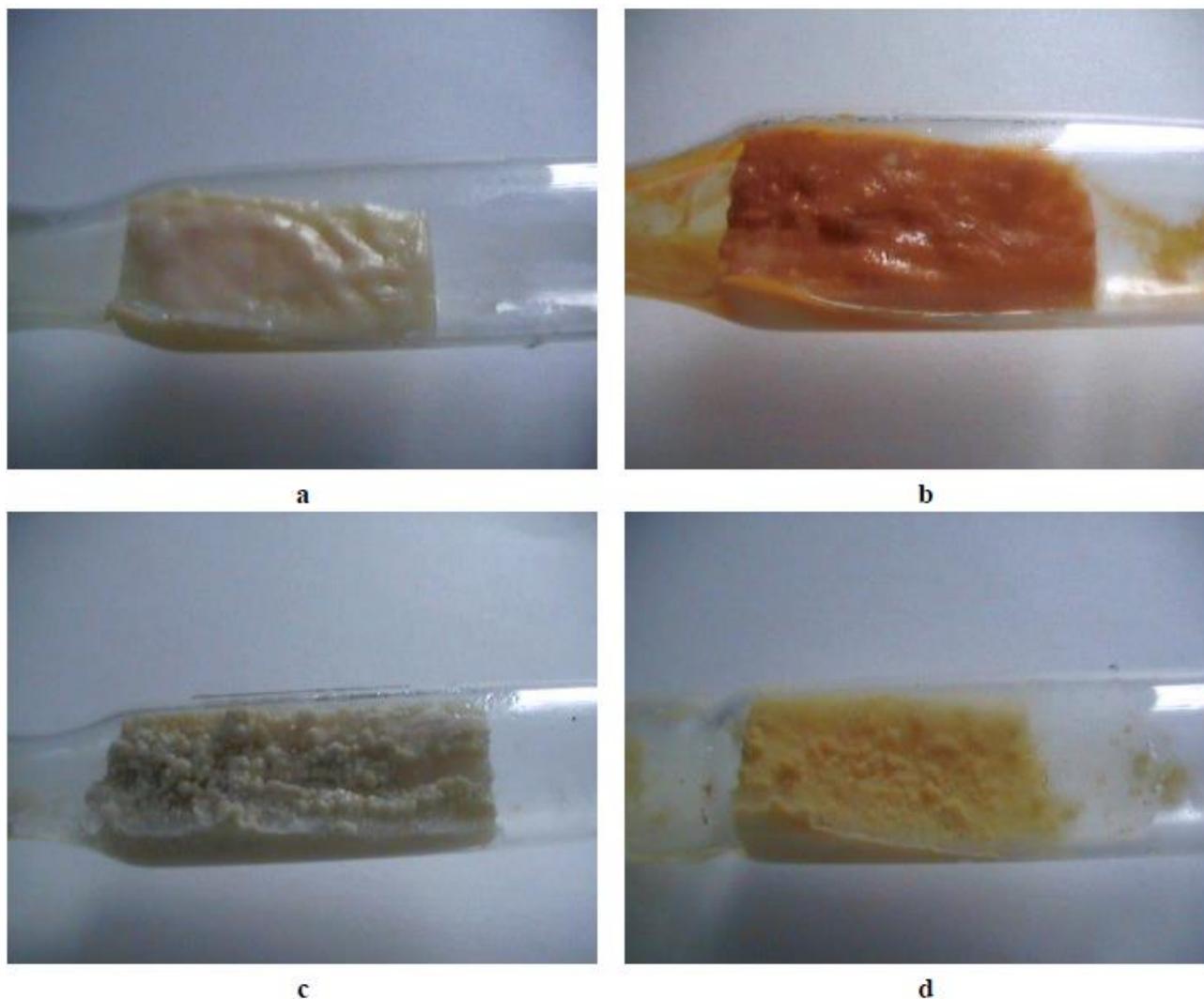


Figure 1. The growth of mycobacteria on the nutrient medium of Pavlovsky. **a)** *M. kansasii*; **b)** *M. goodnae*; **c)** *M. xenopi*; **d)** *M. flavescens*.

The inner layer of the cell wall consists of two thin layers. It has been found that they contain mycolic acid (Abrahams & Besra, 2018). Other researchers found that the inner layer of the cell wall adheres closely to the cytoplasmic membrane and forms a single structure with it in young mycobacterial cells (Alsteens et al., 2008). It was determined that the cell wall of atypical mycobacteria is thicker than in tuberculosis pathogens due to their pronounced polymorphism (Vincent et al., 2018).

The cytoplasmic membrane is located under the cell wall, which is visible as a single-loop formation. It has a three-layer structure in partially lysed mycobacteria (Alsteens et al., 2008). It is believed that the structure of the cytoplasmic membranes includes lipoprotein complexes, it is associated with various enzyme systems in general and oxidation-reduction system in particular. The cytoplasmic membrane is the main osmotic barrier of the cell (Chiaradia et al., 2017). Intracytoplasmic membrane structures, mesosomes, are visible in the cytoplasm of bacteria, which are formed by invagination of the cytoplasmic membrane in the middle of the cytoplasm and have different shape, size, and localization. They take the whole central part in individual cells and have a spiral package. Atypical mycobacteria have a greater number of vacuoles in the cell's cytoplasm that form the cell granulation. The main part of these fine granular inclusions is represented by ribosomes that carry out the synthesis of a specific protein. There are granules in the form of small electron-optical dense inclusions next to them. These inclusions may have a valutin or polymetaphosphate origin.

Mycobacterium nucleoid is presented in the form of granules and conglomerates on ultrathin sections and does not have a membrane. However, it is located away from cell organelles. Thin and coarse osmiophilic DNA strands are visible in the osmiophobic zone. There is a different relationship between the cytoplasmic membrane, the mesosome, and the nucleoid. Thus, in one case, chromatin strands tightly fit the mesosome, but in another case, the cytoplasmic membrane forms membrane structures in the nucleoid (Kriel et al., 2018). We can assume that there is a relationship between the submicroscopic organization of surface structures and the virulence of mycobacteria. Some reports show that pathogenic mycobacteria can form a capsule (Kalscheuer et al., 2019) and have a spore (Traag et al., 2010). This spore plays crucial role in processes of mycobacteria's adaptation to living conditions. However, these issues are controversial and require detailed study. Complex irreversible changes in the structural elements of mycobacteria occur under the action of bactericidal disinfectant on mycobacterial cell in 2.0% of active substance concentration during 24 hours exposure. This leads to the death of microorganisms (Figure 3).

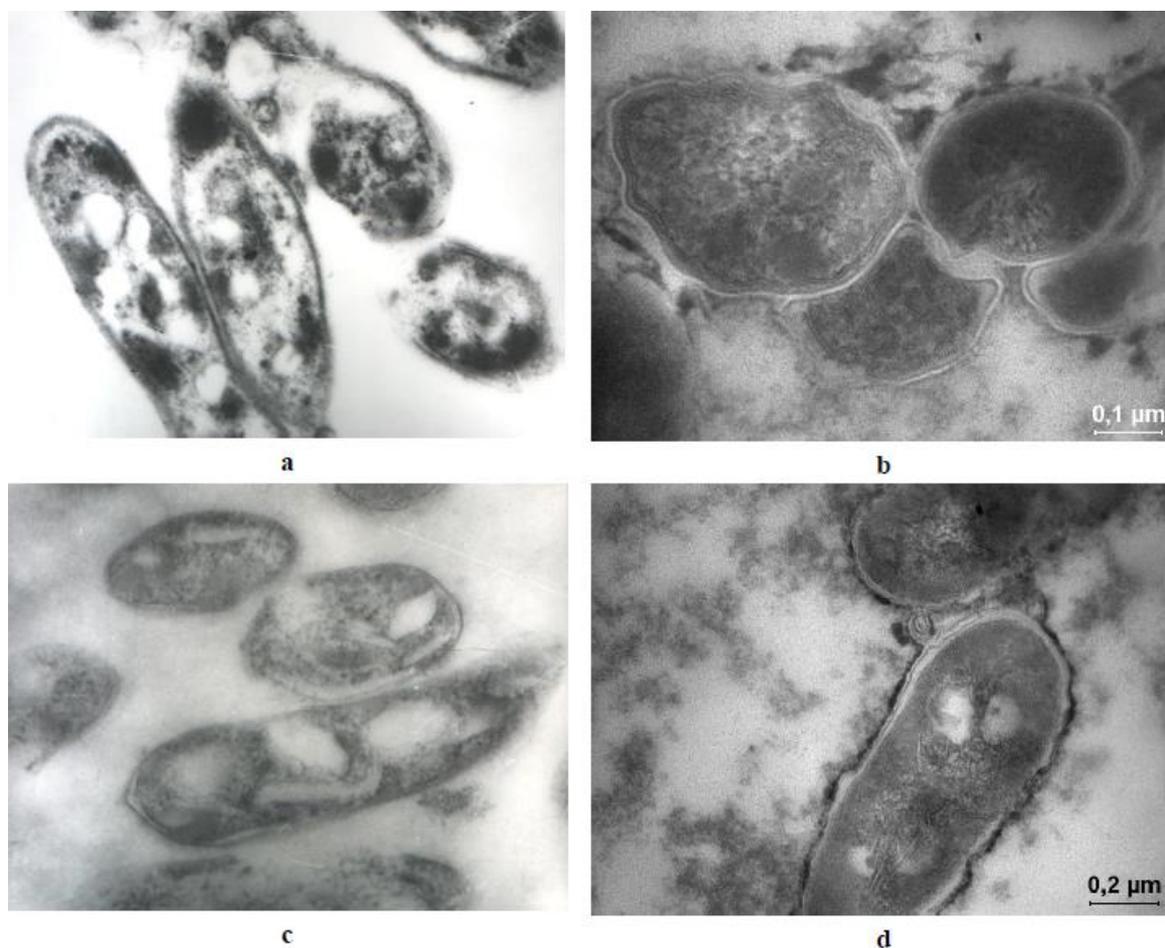


Figure 2. Electron-microscopic organization of mycobacteria. **a)** *M. kansasii*; **b)** *M. gordonae*; **c)** *M. xenopi*; **d)** *M. flavescens*.

The dissolution of the microcapsules and cell wall was observed after disinfectant exposure on *M. kansasii*. Cells partially don't have a cytoplasmic membrane. The cytoplasm has a form of dark finely granular inclusions. The nucleoid region is not clearly visible. The appearance of vacuoles was observed in the cell cytoplasm of test culture *M. gordonae* after application of disinfectant, the cell wall and cytoplasmic membrane are partially blurred and the cytoplasm is represented by small osmiophilic granules. An effect of disinfectants causes the destruction of the microcapsule and cell wall in almost all microbial cells of *M. xenopi*. The cytoplasm contains small granular substances of different electronic density. The complete disappearance of the microcapsule and cell wall of *M. flavescens* culture was observed after the action of the disinfectant which led to cytoplasmic release.

An aldehyde, which contains a frost-resistant disinfectant, interacts with the protein components of membrane structures and cell enzymes due to its high reactivity regarding amino acids, proteins, nucleic acids. It can be assumed that the inactivation of mycobacteria occurs as a result of macromolecular synthesis reduction in bacterial cells.

However, it was also found that the prolonged exposure of chlorine-containing disinfectants on the microbial cell causes irreversible changes in the internal (cytoplasm, nucleoid), as well as external structures (cell wall, cytoplasmic membrane) of mycobacteria (Paliy, 2018). It was found that structural changes are reduced to erosion of the substances of the cell wall as a result of a study of the ultrastructure of mycobacteria after an action of the drug based on quaternary ammonium compounds. A nucleoid practically does not change its structure and retains a finely dispersed fibrillar structure (Paliy, 2013).

The experiments determined the main structural changes in mycobacterial cells after the exposure of frost-resistant disinfectants, which confirms its effectiveness when used in the general complex of anti-tuberculosis measures at low ambient temperatures. The

prospect of further research is in a comparative study of ultrastructural changes of microbial cells after the action of bactericidal concentrations of disinfectants of various chemical origins.

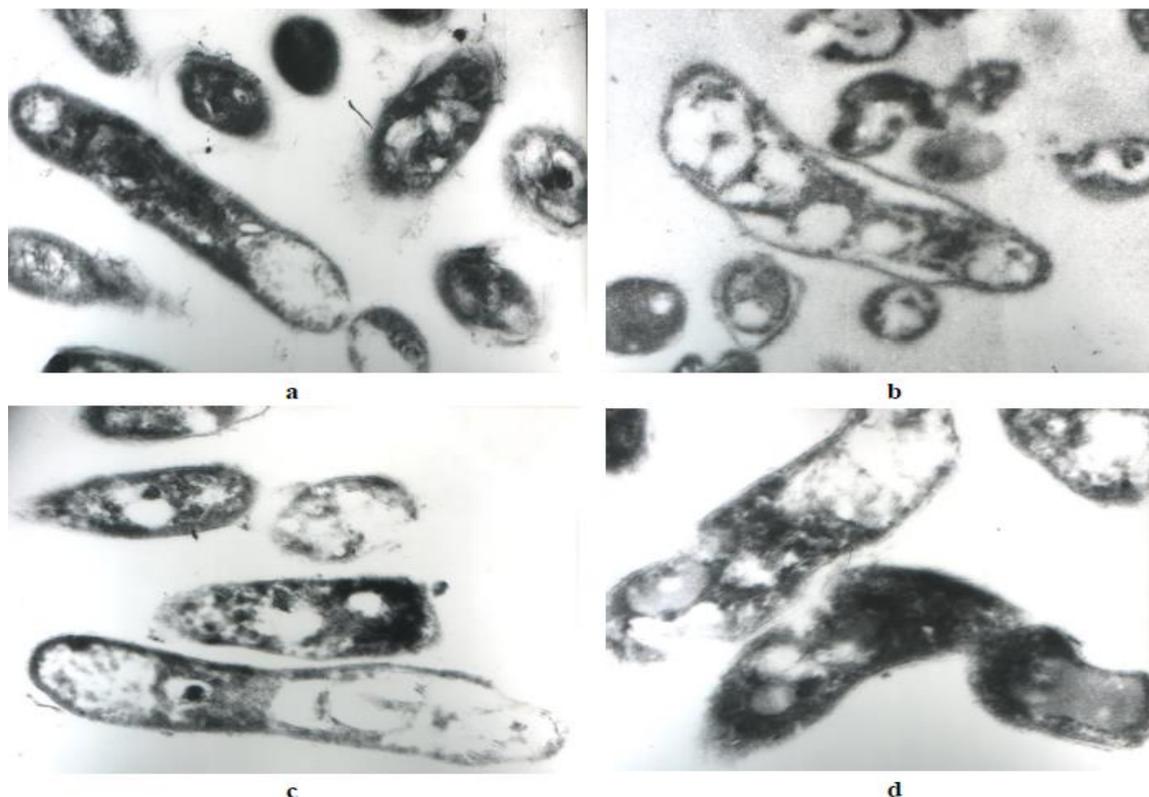


Figure 3. Ultrastructural changes in mycobacterial cells after the exposure of a disinfectant. **a)** *M. kansasii*; **b)** *M. goodii*; **c)** *M. xenopi*; **d)** *M. flavescens*.

Conclusion

Bactericidal properties of new frost-resistant disinfectant composition regarding *M. kansasii*, *M. goodii*, *M. xenopi* and *M. flavescens* have been confirmed by electron microscopy studies. Glutaraldehyde is an active substance of mentioned composition, and sodium formate is used as an antifreeze. Changes that occur in atypical mycobacteria after the exposure of an aldehyde frost-resistant disinfectant are characterized by the destruction of the microcapsule, cell wall, cytoplasmic membrane, and by the formation of vacuoles and osmiophilic inclusions in the cytoplasm of cells. The developed disinfectant composition can be used in the general complex of veterinary and sanitary measures for control and prevention of tuberculosis in farm animals at ambient temperatures up to minus 20°C.

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