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Effectiveness of aldehyde disinfectant against the causative agents of tuberculosis in domestic animals and birds

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In recent years conducted studies showed that mycobacteria are the most resistant microorganisms to negative environmental factors, physical and chemical effects. One of the major issues of animal tuberculosis is the correct selection of effective disinfectants. The aim of our studies was to estimate the interaction of aldehyde disinfectant on animal tuberculosis agents. It was found that the disinfectant, contained glutaraldehyde and formaldehyde, exhibits bactericidal properties on *M. bovis* and *M. avium*, as confirmed by culture, molecular genetics and electronic-microscopic research methods. The disinfectant partially destroyed the integrity surface of cellular structures in *M. avium*. The cytoplasm acquires density and contains vacuoles and finely granular substance. The area of nucleoid is blurred. In *M. bovis* cells the preparation causes destruction of cell surface structures on different poles, leading to the release of the cytoplasm and consolidation of the nucleoid area. Biocidal activity of the aldehyde disinfection preparation consists in the destruction of Mycobacterium surface structures, formation of vacuoles and osmiophilic granules in the cytoplasm. The aldehyde disinfectant impacts on mycobacterial cells with the comprehensive irreversible changes in structural elements that lead to the complete destruction of microorganisms.
Keywords: mycobacterium; electron microscopy; DNA; disinfectant; concentration; exposure

Introduction

According to the Bergey's Manual of Systematic Bacteriology, mycobacteria belong to the order Actinomycetales family *Mycobacterium* and genus *Mycobacteriaceae* (Skerman et al., 1980). Today in the world more than 300 different species of mycobacteria are described and 20 species that are pathogenic for humans were studied (Ellis et al., 2002; Shinnick et al., 1994). In the recent years, there are changes in the biology of pathogens and manifestation of tuberculosis infection. Long passages of Mycobacterium cultures, antibiotics and UV radiation action on them can lead to the phenomenon of dissociation of pigment and non-acid forms (Erohin et al., 2003), L-transformation cell and formation of ultrasmall forms of Mycobacteria which are capable to reversion (Fischer et al., 1968). One of the issues that need to be resolved today is systematic evaluation of sensitivity of reference and circulating strains of microorganisms to disinfectants (Shkarin et al., 2008). It is known that mycobacteria are the most resistant microorganisms to the effects of negative environmental factors and physical and chemical effects (Paliy et al., 2016; Paliy, 2018). It was found that epizootic culture of *M. bovis* is more resistant to disinfectants than the reference strain. The most stable to the bactericidal activity of disinfectants from various chemical groups are *M. scrofulaceum*, *M. intracellulare*, *M. fortuitum* and *M. avium* (Paliy, 2014).

Several researchers have concluded that the high resistance to chemical, physical factors mostly related to the complex structure of the cell wall and lipid-polysaccharide complex in microbial cells (Barrow et al., 1980; Jarlier et al., 1994). *Mycobacterium tuberculosis* and atypical mycobacteria are highly adaptive due to its plasticity properties to the action of antibiotics and placed in ascending order: *M. bovis*, *M. tuberculosis*, *M. avium*, *M. fortuitum*, and the acquisition of a stable resistance change of Mycobacterium accompanied by some differential phenotypic properties (Dyachenko et al., 2009).

Published data from electron microscopic studies of Mycobacterium after antimicrobial action of various chemical groups are rather limited, which gave us the basis to select the appropriate scientific field work.

Materials and methods

The disinfectant that contains glutaraldehyde (25 %) and formaldehyde (37 %) were used during experiments as was describe before (Zavgorodniy et al., 2012). Predefined bactericidal activity of disinfectants was estimated by suspension on the test culture fast-growing atypical *M. fortuitum.* In the next stage of research, the following strains of *M. bovis* and *M. avium* were used:
- *M. avium* (strain *IECVM UNIEV*), that was obtained in the Laboratory for Tuberculosis at the NSC "IECVM" as the reference culture by selection in the 1999, pathogenic for rabbits, pigs and chickens. The culture was incubated during 14-21 days on Pavlovsks glycerine potatoes media at (37.5±0.5) °C.
- *M. bovis* (strain *Vallee*), that was obtained from the State Research Institute of Standardization and Control of Medical Biological Agents named A. Tarasevich in 1990, pathogenic for cattle and laboratory animals. The culture was incubated during 30–45 days on Pavlovsks glycerine potatoes media at (37.5±0.5) °C.
Experiments were performed according to the conventional techniques (Zavgorodniy et al., 2007). The study of culture filtrate samples for the *M. bovis* genetic material was performed by polymerase chain reaction (PCR) (Stegniy et al., 2010). DNA extraction was performed using sorbental method (Boom et al., 1990). PCR was performed using commercial kit«PCR-Core» and primers JB21/22 (for the identification of genetic material of *M. bovis*) (Rodriguez et al., 1995). The reference *M. bovis* strain (*Vallee)* was used as a positive control, as a negative control – Tris EDTA buffer.
Structural changes that occur in microbial cells under the influence of disinfectant were studied by electron microscopy of ultrathin sections of mycobacterial cells in accordance with conventional methods (Uikli, 1975). During electron microscopic examination of bacterial test cultures of *M. bovis* and *M. avium* separately transferred using bacteriological loop in the experimental and control flasks with the volume of 20 cm^3.
After weighing in research flasks aqueous solutions of disinfectant at the rate of 1 cm^3 solution on 100 million Mycobacterial cells were inserted. Electron microscopic studies were provided to mycobacteria culture under aldehyde disinfectant. In the control flasks sterile isotonic solution were added instead of disinfectant solutions in an appropriate amount. After a certain exposure the preparation solution was removed and sterile isotonic solution was added, and the test cultures of Mycobacterium were pre-fixed. Fixation of the samples was performed using 2.0 % glutaraldehyde solution in phosphate-buffered saline (PBS) at pH 7.4 during 2 hours at 4.0 °C. After the fixation specimens were washed twice with PBS to remove glutaraldehyde. The suspension of cells after fixation was treated by 10 % solution of serum albumin to maintain cells during different electron microscopic manipulations.
The samples were post fixed for 1-2 h in 1% OsO_4 for 1-2 h, stained with uranyl acetate (0,25%) for 1 night, dehydrated, and embedded in epoxy resin. Thin sections (50 to 60 nm) were stained with uranyl acetate and lead citrate, being then examined with a PEM-125K electron microscope. Visualization was done using CCD camera DX-2 and software package "KAPPA" (Germany). Statistical analysis was performed by Mann–Whitney *U* test (Mann et al., 1947).

Results and discussion

At the first step the bactericidal activity of the disinfectant was estimated by suspension on the test cultures of fast-growing atypical *M. fortuitum* with the concentration of 0.5, 1.0, 1.5, and 2.0% for the exposure of 1, 5, and 24 hours (Table 1).

Table 1. Bactericidal activity of disinfectant against *M. fortuitum* (M±m, n=4)

Applicability		Number of colony forming units (CFU)		
Concentration. %	Exposition. hours	Disinfectant	Negative control	Positive control
0.5	1	149.0±1.2	–	145.0±2.0 (the average exposure for 1 hour); 142.7±1.0 (the average exposure for 5 hours); 139.8±2.1 (the average exposure for 24 hours)
	5	125.3±2.1*	–	
	24	120.1±3.5*	–	
1.0	1	95.5±3.7*	–	
	5	90.5±2.0*	–	
	24	69.4±8.4*	–	
1.5	1	39.4±9.8*	–	
	5	19.1±3.6*	–	
	24	8.9±0.8*	–	
2.0	1	10.9±2.7*	–	
	5	4.0±0.3*	–	
	24	–	–	

Notes: «–» –absence of the growth; * – $p < 0,001$.

The analysis of the results was shown that the disinfectant characterized by sub-bacterycidal and bacteriostatic activity against *M. fortuitum* by concentration from 0,5 till 1.5 %, for exposure 1-24 hours and at a concentration of 2,0 % by exposure 1-5 hours, and destruction of the mycobacterial cells was observed at the concentration of 2,0% after 24 hours.
The next step was to conduct experiments with cultures of *M. bovis* and *M. avium* on different test facilities (lawn, wood, tile, metal, and glass), considering their biological characteristics (Table 2).

Table 2. Bactericidal activity of disinfectant (2.0 %) against *M. bovis* and *M. avium* using different materials (n=3)

Culture	Exposition, hours	Material	Experiment	Controls	
				Negative	Positive
Mycobacterium bovis	5	lawn	+		++++
		wood	+	–	++++
		tile	–	–	++++
		metal	–	–	++++
		glass	–	–	++++
	24	lawn	–	–	++++
		wood	–	–	++++
		tile	–	–	++++
		metal	–	–	++++
		glass	–	–	++++
Mycobacterium avium	5	lawn	+		++++
		wood	+	–	++++
		tile	–	–	++++
		metal	–	–	++++
		1. glass	–	–	++++
	24	lawn	–	–	++++
		wood	–	–	++++
		tile	–	–	++++
		metal	–	–	++++
		glass	–	–	++++

Notes: «–» – absence of colony growth; «+» – growth up to 10 colonies; «++++» – growth more, than 50 colonies.

It should be noted that the preparation disinfects different test objects contaminated by mycobacteria at the concentration of 2,0% during 24 hours of exposure (U = 0, $p > 0.05$).
Obtained results from the definition of the disinfectant bactericidal activity against *M. bovis* were confirmed by PCR. For this purpose, examined samples of culture material were studied for the presence of genetic material of *M. bovis* disinfectant at the concentration of 1.0 and 2.0% after 24 hours of exposure (Fig. 1).

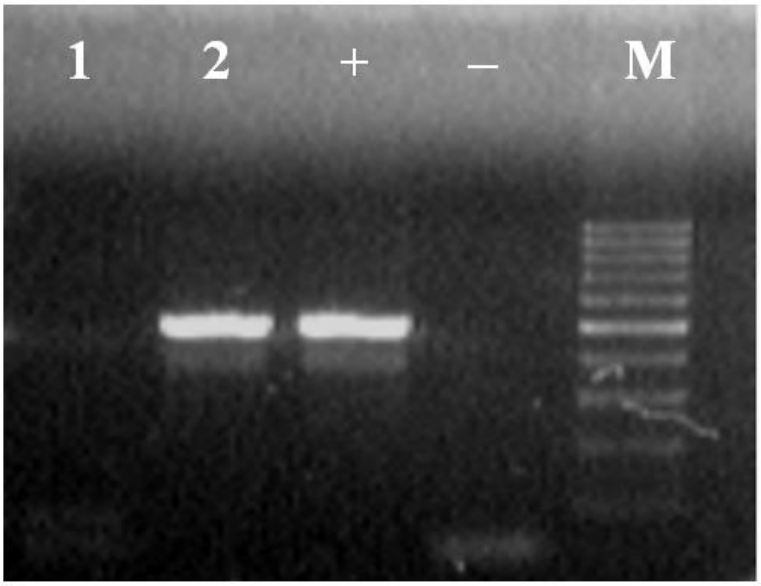
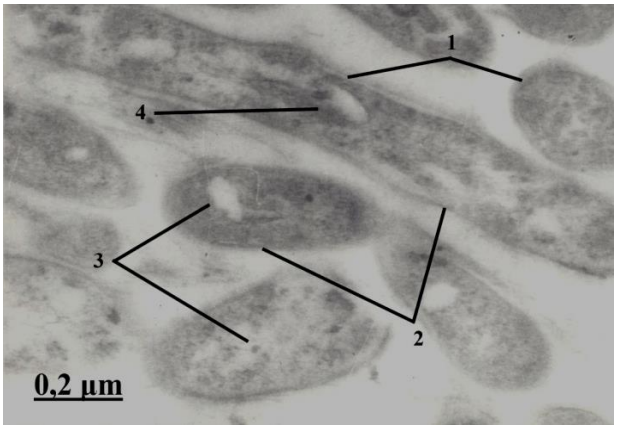
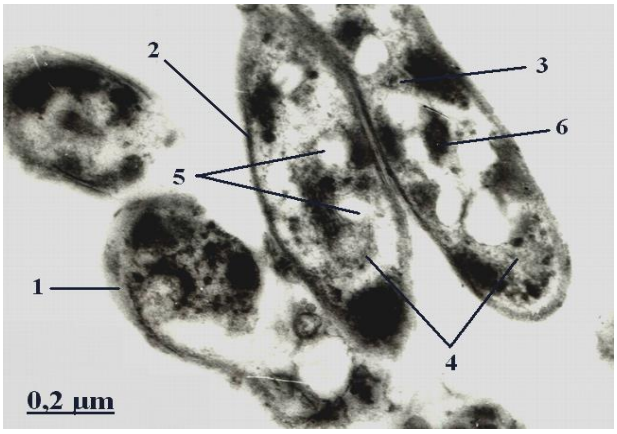


Fig. 1. The electrophoregram of PCR products using primers JB21/22: 1 – disinfectant (2.0 % – 24 h); 2 – disinfectant (1.0 % – 24 h); «+» and «–» – positive and negative control samples respectively; M – molecular weight marker M100.

According to the results, after the action of disinfectant at the concentration of 2,0 % amplification was not observed that can indicate the degradation of Mycobacterium DNA under formaldehyde impact (Schander et al., 2003). However, at the concentrations of 1,0 % the amplicons were successfully synthesized. Next stage of the studies was examination of the ultrastructural changes in mycobacteria after bactericidal activity of aldehyde disinfectant. As a control ultra-thin organization of mycobacteria were studied in normal conditions (Fig. 2).



A: 1 – microcapsule; 2 – cell wall; 3 – cytoplasm; 4 – nucleoid.



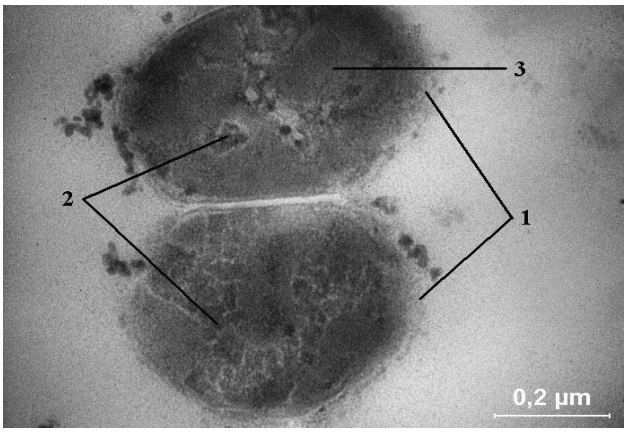
B: 1 – microcapsule; 2 – cell wall; 3 – cytoplasm; 4 – mesosomes; 5 – vacuoles; 6 – nucleoid.

Fig. 2. Ultra-thin organization of *M. bovis* (A)and *M. avium* (B) in normal conditions.

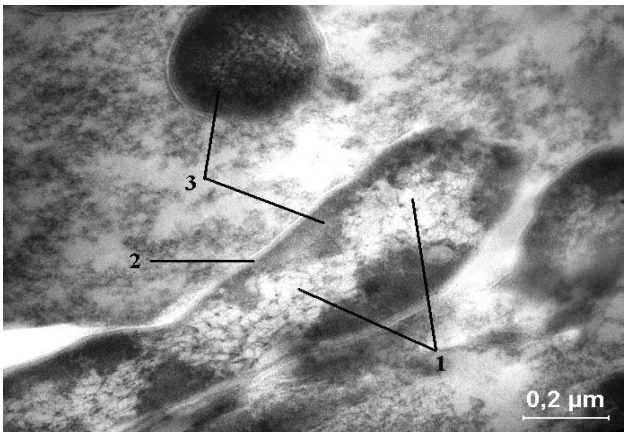
Analysis of the results demonstrated that mycobacterial cells have a high degree of polymorphism and, depending on the cut plane, are round, oval or rod-shaped form. Microcapsules are presented by osmiophilic material, sometimes combining several bacteria. Between the microcapsules and cell wall osmiophilic layer was visible. The cell wall appears as a single-contour structure of monolayer, which is adjacent to the cytoplasmic membrane. Cytoplasmic membrane in our study was a single-layer structure and consisted of a homogeneous electron-dense material. Between the cytoplasmic membrane and cell wall placed electronic transparent layer which is not always detected. Cytoplasm was dense, osmiophilic and granular. It was reviewed osmiophilic tight formations which include microgranular inserts and amorphous substance of the average electron density (volutin granules). Also, there are vacuoles and granules of various sizes, well-developed membrane structures (mesosomes). Nuclear zone of the bacteria did not have the bounding membrane. Nucleoid located in the center of the cell as the osmiophobic area with the diffuse DNA fibrils.

There are many vacuoles in the cytoplasm of atypical mycobacteria, while in the cytoplasm of both *M. avium* and *M. bovis* cells osmiophilic rounded inclusion forming grains were found.

At the final stage the changes occurring in microbial cells due to the aldehyde bactericidal activity of the disinfection preparation were determined (Fig. 3).



A: 1 – absence of surface structures; 2 – osmiophilic inclusions 3 – cytoplasm.



B: 1 – vacuole; 2 – cell wall; 3 – cytoplasm.

Fig. 3. Ultra-thin organization of *M. bovis* (A) and *M. avium* (B) after bactericidal activity of the disinfectant.

Disinfectants partially damage the surface integrity of cellular structures in *M. avium*. The cytoplasm gained density and contain vacuoles and finely granular substance. The cell wall and cytoplasmic membrane are partially blurred. The nucleoid area is

blurred. The disinfectant destructed predetermined *M. bovis* cell surface structures on different poles, which led to the release of the cytoplasm. Nucleoid area was condensed. The cytoplasm contains a substance small granular different electronic density. Aldehyde preparation primarily interacts with the protein components of membrane structures of enzymes and cells due to the high reactivity on amino acids, proteins, nucleic acids. The changes, that occur in mycobacteria after the disinfectant application, characterized by the destruction of surface structures (microcapsules, cell wall, cytoplasmic membrane), the formation of vacuoles in the cytoplasm and osmiophilic inclusions.

The mechanisms of Mycobacterium resistance to physical and chemical factors is due to the unique structure of the surface structures of microbial cells. It is proved that there is a direct relationship between the organization structure and surface virulence of mycobacteria (Katz et al., 1972). In our studies a normal mycobacterial cell had a high degree of polymorphism and there were round, oval, rod-shaped forms depending on the cut surface.

It was found that the surface of cells was presented by osmiophilic formations that sometimes combined several bacteria. This layer was identified as microcapsule. It should be noted that this layer is like microcapsules that appears on the surface of gram-positive bacteria and often leads to combining of several cells (Imaeda et al., 1968; Katz et al., 1972). According to our data microcapsules is a protective cover that protects cells from the external factors. These data confirm the study, which identifies the presence of microcapsules as an integral morphological structure of mycobacteria, although it is believed that it can be adsorbed elements of nutrient medium or destroyed mycobacteria (Erohin et al., 1972). There is visible osmiophilic layer between the microcapsules and cell wall, referred to the outer layer of the cell wall. It is a structure which was formed by extracted substance after dehydration and fixation. However, it is believed that this layer is a continuation of microcapsules (Yushchenko, 1968).

The cell wall structure appears as a single-monolayer formation and close locates to the cytoplasmic membrane. It is confirmed that the *Mycobacterium tuberculosis* and atypical mycobacteria cell wall represented by four layers consisting of murein, peptide-lipids and peptide-glycolipids (Imaeda, 1975). However, clearer images of this fact are not represented.

Thus, our studies of Mycobacteria surface structures showed different electronic density of three layers. The outer layer was recognized as microcapsule, the next layer was assigned to the outer layer of the cell wall, and the last – found as the cell wall. Cell cytoplasm was dense, osmiophilic and granular. It contained osmiophilic tight formations with microgranules and amorphous substance average electron density (volutin granules). These granules are specific for the mycobacteria and contain phosphates. Nuclear zone of the bacteria had no tail shell, as noted by other authors (Avakian et al., 1972). Such changes under the influence of the aldehyde chemicals were found in *Pasteurella*, which were caused destructive changes due to oxidative effect to all components of the cell (Shahov, 2011).

Conclusions

The results of the experiments showed that the disinfectant containing glutaraldehyde and formaldehyde, exhibits bactericidal properties on *M. bovis* and *M. avium*, which is confirmed by cultural, molecular genetics and transmission electron microscopy techniques.

Biocidal activity of the aldehyde disinfection preparation characterized by the destruction of Mycobacterium surface structures, formation of vacuoles in the cytoplasm and osmiophilic inclusions. The comprehensive irreversible changes in Mycobacterium structural elements lead to the destruction of microorganisms due to the aldehyde disinfectant action.

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