

ISOLATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* FROM ZOO ANIMALS

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Summary. The article presents the results of cultural study of faeces samples from zoo animals and water samples. Five cultures were isolated from zoo animals (*Lama glama*, *Camelus bactrianus*, *Ammotragus lervia*, *Elaphurus davidianus*, *Bos frontalis frontalis*) and one culture from water sample. Culture-morphological, tinctorial, biochemical and biological properties of epizootic cultures of mycobacteria isolated from zoo animals were studied. It was the reason to attribute isolates to *M. avium* subsp. *paratuberculosis*. The results of conducted research indicate circulation of *M. paratuberculosis* among zoo animals.

Keywords: paratuberculosis, zoo ungulates, *Mycobacterium avium* subsp. *paratuberculosis*, insulation, cultivation, biological test.

Introduction. Paratuberculosis (Enteritis paratuberculosis, John's disease) — a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), characterized by granulomatous enteritis, temporary in the beginning and permanent afterwards diarrhea, progressive depletion, reduce of productivity and animal's death. Domestic ruminants, mainly cattle and sheep, are most susceptible to this disease. Buffaloes, camels, goats, deer, yaks rarely get sick. The range of susceptible to paratuberculosis animals is not limited by ruminant species. MAP has also been isolated from primates, rabbits, cats, foxes, badgers, bears, raccoons, rats, wood mice (Vansnick, 2004). Moreover, MAP is the cause of disease and mortality of many species of wild hoofed animals kept in zoos or endangered species. According to some researchers, species and breed of animals play not-significant role at risk of infecting. The differences in the overall infecting between species can be determined by the level and time of MAP exposure, resistance and susceptibility to the pathogen or other factors. According to the American Zoo and Aquarium Association one third of US zoos have at least one case with clinical paratuberculosis form since 1995. In a study by Weber and Gürke (1992) stated that MAP culture were isolated from fecal samples from 16.1% of the zoo ruminants. Infection was submitted in dwarf goats, moufflon, alpine ibex and Cameroon sheep (Manning, 2001).

In 1999, problem of MAP spread among camelids in the Antwerp Zoo appeared after MAP culture isolation from faeces of okapi (Vansnick, 2004). The presence of MAP in feces and ileocecal lymph nodes in alpaca and camel were confirmed by PCR and by the cultural method in Germany (Burton et al., 2001). According to the large-scale study conducted in 1991–2007 at the San Diego Zoo (United States), MAP has been direct or indirect cause of death of many ungulates.

Paratuberculous infection was confirmed in 74 animals born before 1991 of 30 different species and 71 bovine animals born or imported from other zoos until 1991 in the research conducted on 3 298 animals representing 131 species. The main risk factors are the specific content of animals in captivity (limited space and high concentration), the exchange of animals with other zoos, stress during transportation. The true infections may remain unnoticed for years because of the long incubation period and the discontinuous MAP isolation even when prevention programs are carried out.

Thus in 2007 paratuberculosis was diagnosed in two David deer born in 1994 and 1997 from negative female reacting negative in cultural study of feces for more than 10 years. The infection source was unknown. These data demonstrate the importance of a long-term observation and testing of zoo animals on paratuberculosis (Münster et al., 2013).

As for the Ukrainian zoos, animal's research on paratuberculosis is not carried out and epizootic situation of the disease remains unknown.

The aim of the work was to study the fecal samples of zoo's ungulates and water samples on paratuberculosis.

Materials and methods. Fecal samples were collected for bacteriological examination from cloven-hoofed (*Camelus bactrianus*, *Lama glama*, *Vicugna pacos*, *Ammotragus lervia*, *Elaphurus davidianus*, *Bos frontalis frontalis*) and hoofed (*Equus ferus przewalskii*, *Equus asinus*, *Equus asinus asinus*, *Hippotigris*) animals. Water samples from the reservoir located at the zoo were also collected.

Fecal and water samples was treated with 0.9% sodium N-cetylpyridinium chloride. Suspensions were seeded on the developed in NSC 'IECVM' culture

media with and without growth factor, alcoholic extract of *M. scrofulaceum*, and on the culture media for *MAP* cultivation containing low-moor peat extract and 0.5% citric-ammonium ferric. Seeded cultures were cultivated in an incubator at 37.5 ± 0.5 °C for 6 months. The cultural-morphological, biochemical and biological properties of isolated and adapted culture of mycobacteria with sufficient amount of bacterial mass was studied.

The biochemical properties of adapted subcultures were studied using following tests: hydrolysis of Tween-80, the recovery of potassium tellurite, determining of catalase and amidase activity.

The biological properties of isolated cultures were studied using double (with 7 days interval) intravenous infection of one-month old rabbits with culture suspension at a concentration of 2 mg/cm^3 on a sterile saline. The allergic test of infected animals was conducted every 30 days after infection using avian tuberculin (PPD). The experiments with animals was conducted based on the principles of bioethics.

Results. Four-six 'blind' passages have been made on a media with growth factor before visible growth of colonies from *Lama glama*, *Camelus bactrianus*, *Ammotragus lervia*, *Elaphurus davidianus*, *Bos frontalis frontalis* fecal samples and water samples was obtained. While smear microscopy of isolated cultures ($n=6$) stained by Ziehl-Neelsen method, characteristic clusters of very small acid-resistant sticks, cocci forms and less sticks arranged singly was observed. Primary colonies had the appearance of transparent beads of diameter less than 0.5 mm, but over time, the colony becomes more intense white color.

During further number of passages needed amount of bacterial mass for further study could only be obtained from cultures isolated from the *Elaphurus davidianus*, *Lama glama* and water samples. The remaining three isolates (by *Camelus bactrianus*, *Ammotragus lervia* and *Bos frontalis frontalis*) regardless of the number of performed passages, grew very slowly (4 month) in the form of tiny colonies. Thus, slow growth, dependence on growth factor, specific morphology and microorganism's location in smears was the reason to attribute isolated culture to *Mycobacterium avium* subsp. *paratuberculosis*. Culture obtained from *Elaphurus davidianus*, *Lama glama* and from water samples were adapted to the Pavlovskiy medium and egg medium with 0.5% citric-ammonium ferric. Colonies were white to gray or light

cream color depending on the nutrient medium on which they were cultured. Colonies grew at a temperature $37-40$ °C, had a smooth, moist, eventually folded surface and an oily consistency (S-form), at temperatures of 20 and 45 °C growth was not observed. The cultures did not grow on media containing 5% sodium chloride and sodium salicylate (1 mg/cm^3).

Basing on the study of the biochemical properties it was established that isolates have had a negative reaction with carbamide, catalase, pyrazinamide and weak reaction with nicotinamide, did not hydrolyzed Tween-80, restored potassium tellurite for 21 hours. Only culture from *Elaphurus davidianus* hydrolyzed Tween-80 after 10 hours.

Biological properties of three isolated cultures were studied. The following symptoms was conducted on experimentally infected rabbits while biotest ($n=6$, 2 bodies for each culture): cachexia, growth retardation, diarrhea, atrophy of hind limbs muscles. Rabbits died after 2–3 month. There were positive reactions to the avian tuberculin (PPD). Allergic reaction to mammal tuberculin (PPD) was absent. A big amount of aggregations of very small acid-fast rods were observed during microscopy of fecal samples. Specific for paratuberculosis changes were found in the small intestine, especially in the ileum, jejunum, and ileocecal valve during pathological examination of all animals. Intestinal contents represented light yellow transparent mucus with bubbles of gas. The walls of the intestine were 3–4 times thickened, sometimes with points of hemorrhages. There were areas with transverse and longitudinal furrows mucosa with fine gray-white nodules in the intestine. Serous nodules were also observed on mesenteric lymph nodes and liver. Original cultures were isolated from the lungs, spleen, liver and intestines as a result of seeding of biomaterial on nutrient medium with growth factor. Thus, specific for paratuberculosis lesions reproduced in experimentally infected rabbits confirmed that isolated from *Elaphurus davidianus* and *Lama glama* faeces and water samples cultures are *Mycobacterium avium* subsp. *paratuberculosis*.

Conclusions. Six mycobacterial culture isolates were attributed to *M. avium* subsp. *paratuberculosis* on the basis of study of culture-morphological, tinctorial, biochemical and biological properties. Isolation of *MAP* from fecal and water samples testifies circulation and risk of this infection spread among zoo animals. This fact justifies the need for further research on paratuberculosis.

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