DOI: 10.21005/AAPZ2019.49.1.07

FOLIA POMERANAE UNIVERSITATIS TECHNOLOGIAE STETINENSIS
Folia Pomer. Univ. Technol. Stetin., Agric., Aliment., Pisc., Zootech. 2019, 348(49)1, 71–78

Olena GAVRILINA, Natalia ALEKSEEVA, Svetlana POZMOGOVA[1], Volodymyr KOSTYUK[2]

INDICATION OF MYCOBACTERIA OF MAMMALS AND BIRDS AT THE ZOO

Dnipro State Agrarian and Economic University, Dnipro, Ukraine
[1]National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine», Kharkiv, Ukraine
[2]National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine

Abstract. The results of allergic, bacteriological and biochemical studies in the diagnosis of mycobacterial infections of mammals and birds in the zoo of the municipal enterprise «T. H. Shevchenko park of Culture and Rest» Dnipro city are described. Comprehensive epizootic research was aimed at the exclusion of dangerous pathogenic mycobacteria. Zoological zones of pathogenic mycobacteria were not recorded in the environment and in animal organisms, but in the ground of enclosures and faeces we identified three cultures of acid-fast microorganisms, one of them was identified as M. flavescens, indicating the circulations of mycobacterial agents in internal and external environment of animal biotope of zoo.

Key words: mycobacterial infections, animals of the zoological collection, allergic, bacteriological and biochemical research, diagnosis of mycobacterioses.

INTRODUCTION

Currently, considerable attention is given to the fight against infectious diseases of animals throughout the world. The effective veterinary and sanitary measures are used in the articles, the system of prevention and diagnosis on using the new modern methods is develop and improve (Makarova 2007; Alekseeva 2009; Lecu et al. 2011).

Among zooanthroponotic diseases tuberculosis and mycobacterioses in their social and economic importance occupy a special place so studies made to solve numerous problems associated with infectious pathology are certainly topical (Stetter et al. 1995; Maslow 1997; Zavgorodniy et al. 2006).

It is especially necessary to highlight the problem of diagnosis of mycobacterial infections of animal in the zoological collections, which are one of the most important components of conservation system, restoration of rare and endangered wildlife species. Zoological collections of animals (zoos, naturalistic centers of children and youth students, circuses), usually are in the large cities and serve as cultural and educational institutions. These institutions are visited by a lot of people (generally with children).

Corresponding author: Olena G. Gavrilina, Dnipro State Agrarian and Economic University, Sergey Efremov 25, Dnipro, 49600, Ukraine, e-mail: elgen@i.ua

At the same time the concentration of a variety of animals on restricted areas poses difficult task for veterinary experts of development and improving the diagnosis and prevention of various infectious diseases, particularly tuberculosis and mycobacterioses (Alshinetskiy 2004; Rastogi et al. 2001).

The purpose: The main objective of the research is to determine the welfare of mammals and birds regarding the infections of mycobacterial etiology in the zoological collection of zoological zone of the municipal enterprise «T. H. Shevchenko park of Culture and Rest» Dnipro city.

MATERIAL AND METHODS

The work is carried according to the research topics of the department of Epizootology and infectious animal diseases of Dnipro State Agrarian and Economic University of "Development of prevention system and struggle against tuberculosis of animals" (number of state registration is 0110U2413). Studies were conducted in zoological zone of the municipal enterprise «T. H. Shevchenko park of Culture and Rest», teaching and research laboratory of the department of Epizootology and infectious animal diseases at Faculty of Veterinary Medicine of Dnipro State Agrarian and Economic University and department for tuberculosis and brucellosis stud National scientific center «Institute of Experimental and Clinical Veterinary Medicine». A total of 44 mammals and 27 birds were examined.

The complex method of research with using allergic, bacteriological and biochemical methods of diagnosis is used for determination of epizootic state of research object, such as: allergic tests were performed with using the intradermal tuberculin test and bacteriological examination of fecal tests and material from environmental objects with enclosures and cages with different types of zoo mammals and birds.

For the tuberculin test we used "Purified tuberculin derivative (PPD) for mammals in a standard solution" and "Purified tuberculin derivative (PPD) for poultry in standard solution" of SE "Sumy biofactory".

Tuberculin was injected intradermally with non-needle mechanical injectors BI-7M of LLC "Asol Company" and the insulin syringe of 1cm^3 capacity in a volume of 0.1 cm^3 at a dose of 5000 IU and 2500 IU, respectively.

Allergen was injected to goats and sheep inside palpebral part in the lower eyelid; to ponies, deer and a donkey into the skin in the upper third of the stomach; to foxes and a raccoon dog in the skin in the area of the inner part of a thigh; to bantam hen into the beard; to Muscovy ducks and a wild duck into submaxillary fold; to pheasants, peacocks, buzzards and a guinea fowl in the area of the outer surface of the tibia at 1 to 2 cm above the ankle joint.

Before injecting tuberculin we conducted depilation, plucked out feathers and treated skin at the injection site with 70% solution of ethanol. We kept a skin fold at the injection site between a thumb and a forefinger, measured with calipers and recorded the size. The needle of an insulin syringe was injected by beveled edge outwards at an angle, a pea-shaped bulge "button" occurred in the skin at the injection site.

Accounting of results of goats, sheep, foxes and raccoon dogs was conducted within 48 hours after the injection. Accounting of results of the camel, deer, ponies and donkeys

was conducted within 72 hours, and the birds within 36 hours. Interpretation of the results was carried out according to the current Regulations for prevention and fight of Tuberculosis of animals that was approved by the State Committee of Veterinary Medicine of Ukraine in 03.09.2009, No. 316 and the Regulations for the prevention and elimination of tuberculosis poultry that was approved by the State Committee of Veterinary Medicine of Ukraine in 28.08.2006, No. 64.

Bacteriological studies were used as signaling method for determining welfare concerning mycobacterial infections of zoo mammals and birds. Especially, it was failed to hold allergic tuberculin test as a result of aggression and the impossibility of fixing.

Bacteriological study of fecal tests and material from the objects of the external environment from enclosures and cages was carried out according to “The Guideline for the diagnosis of tuberculosis of mammals and birds” of 1994; it included microscopy, studying of characteristics of culture growth.

Species belonging to the culture of mycobacteria were determined using tinctorial, culture and biochemical tests, in accordance with "Methodological recommendations for specifying the diagnosis of tuberculosis in cattle of well-off farms and determining the species belonging to mycobacterium cultures" (1997).

After microscopic examination and determination of the culture of the purity, the bacterial mass was sown on a dense egg nourishing medium for the cultivation of mycobacteria to accumulate the bacterial mass. Tubers with sown were cultivated in a thermostat at a temperature of 37°C for 21 days. After that, from the grown bacterial mass 1,0 mg/cm^3 of sterile saline solution was prepared. The resulting swab was sown on nutrient media and studied the cultural and biochemical properties.

RESULTS AND DISCUSSION

The follows were studied on mycobacterial infections: 11 goats including mountain goats, which were located in seven enclosures; 7 sheep and 3 male sheep, including 4 Asian lambs (mountain furnace), which were located in three enclosures; 3 ponies, which are located in two enclosures; 1 donkey, 1 camel, 2 deer, 1 llama, which were located in five enclosures; 2 wild boars, which were located in two enclosures; 1 bear, located in a separate enclosure, 4 wolves, which were located in two enclosures; 5 foxes, which were located in three enclosures; 1 raccoon dogs and 2 raccoons-poloskun, which were located in two enclosure and two cages; 4 black, 1 red and 8 white decorative bantams that which were located in one cage with two peacocks; 2 gold pheasants and 3 silver pheasants, which were located in three cages; 2 Muscovy duck, a guinea fowl and a crock duck, which were located in the same cage and 3 common buzzard, which are located in a single cage.

The tuberculin test is the main method of vital diagnosis of tuberculosis of mammals and birds according to the approved normative documents. However, its implementation among wildlife zoo (wild boar, bear, wolves, raccoons-poloskun) is not possible because of the high aggressiveness of the animals and, consequently, the danger that they pose to humans. Fecal tests of such animals and material from objects of environmental were chosen by us from enclosures and cages.

Positive or dubious reactions were not found in any case according to the interpretation of the results of allergic tuberculin tests that were conducted by us. Any clinical manifestations of the disease and thickening of the skin folds more than 2 mm were not observed in all investigated zoo mammals and birds by the allergic method.

For culture study of selected material (n = 72) from the objects of the external environmental (enclosures and cages) and fecal tests (droppings), which were subjected to pretreatment and were plated in 5 test tubes with egg nutrient medium of Mordovian «Nove».

Sowing was performed with bacteriological loop, gently rubbing a sowing material over the entire surface of nutrient medium. We closed the test tubes which were sown with sterile stoppers and placed in a thermostat at 37°C, where the first two days they were kept in an inclined position. The tubes where there was extraneous micro-flora were removed. The sowing was observed within 3 months and checked at least once a week to identify the initial growth of mycobacteria.

Accounting for the growth of isolated cultures were carried out by the following scheme: the period for determination of the initial growth, colony characteristics (shape, surface), texture, pigment-formation, emulgator in the normal saline, tinctorial properties in staining morphology mycobacterium by Ziehl-Neelsen.

Three cultures of acid microorganisms and 72 samples of material from environmental objects (enclosures and cages) and faeces (droppings) of zoo mammals and birds were allocated according to the results of bacteriological study: two culture samples of fecal tests and tests of dry soil of enclosures, where wild boars were kept, one culture from the test with dry soil of the cage number 6, where 2 Muscovy ducks, a guinea fowl and a crock duck were kept.

Two cultures (culture 1 and 2) appeared to be thin, straight and slightly curved partly acid-fast bacillus (in smears met both red and blue bacillus). For the differentiation of mycobacterium genus and related taxons (pseudomycosis) we additionally applied staining of smears according to Gram's Method, as it turned out cultures accepted Gram staining well, they are gram-positive. In slanting beef-extract agar (BEA) in 2–4 days we saw rounded, smooth, shiny yellowish colonies which in 5–7 days gained orange color, allowing us to attribute them to the genus *Nocardia spp*. One culture (culture 3) had soft, yellowish, oily consistency colonies, and according to Ziehl–Neelsen stain we observed acid-fast (red) short thick bacillus, which allowed us to attribute it to the genus *Mycobacterium*.

Species identification of selected (studied) culture was carried out by studying morphological and phenotypic properties, namely, they studied such features as the rate of growth; the ability to form pigment; ability to grow at different temperatures.

It is found in studying the growth rate (the time required to form visible mature colonies on dense medium) that the primary growth is observed on 12th–16th day, and at transplant – on 4th–7th day. Dedicated culture is proved fast-growing.

Studying the ability to grow at different temperatures was performed by sowing a suspension of studied culture in isotonic solution of sodium chloride (of 0,2 cm^3) into the test tubes with egg medium and incubated sown test tubes at 22, 37 and 45°C. The studied mycobacterial culture grew at temperatures 22 and 37°C and did not grow at 45°C.

By the ability to produce pigment mycobacteria are divided into three groups: photochromogeneous (they produce a pigment only when exposed to light, in the darkness they have a creamy color); scotochromogenous (they produce a pigment from the intense

yellow to orange with growth both in light and in the dark); non-chromogenic (they do not form the pigment, their colonies will only be whitish or creamish).

Suspension of the studied culture of mycobacteria (0.2 cm^3) was sowed in two test tubes with egg nutrient medium to determine photo chromogenic properties and both test tubes were wrapped in aluminum foil (to protect them from light in a thermostat) and incubated at 37°C. One of the test tubes was liberated after the emergence of growth culture from the foil and placed for 2 hours under sunlight, marked and continued incubation. Coloration of culture was checked after 1 to 2 days. If culture becomes yellow coloration and remains creamy in the control, so it is photo chromogenic culture. The studied culture in the light and in the dark was an intense yellow pigment, so that was revealed as scotochromogenous.

The following facts were established for the initial identification of studied culture of mycobacteria:

– primary growth was observed on the 12th–16th day, and at replanting on 4th–7th day, which allowed to include it to fast-growing;
– grows at the temperatures 22 and 37°C, and it does not grow at the temperatures 45°C;
– the pigment produces intense yellow as in the light and in the dark at the ability to form the pigment to scotochromogenous.

Studied mycobacteria culture can be attributed to the IV group by the Runyon classification comparing our obtained results concerning the establishment of the species belonging of studied mycobacteria culture. This group also includes fast-growing mycobacterium of 12 species – *M. smegmatis, M. phlei, M. fortuitum, M. vaccae, M. diernhoferi, M. thamnpheos, M. flavescens, M. peregrinum, M. ulcerans, M. parafinicum, M. chelonei subsp. chelonei, M. chelonei subsp. abscessus*. However, only 8 species of mycobacteria grow at the temperatures 22 and 37°C and they do not grow at the temperature 45°C – *M. fortuitum, M. vaccae, M. diernhoferi, M. flavescens, M. peregrinum, M. parafinicum, M. chelonei subsp. chelonei, M. chelonei subsp. abscessus,* only 3 species of mycobacteria are able to produce the pigment – *M. vaccae, M. flavescens, M. peregrinum.*

It was necessary to conduct biochemical research for the final identification of studied culture, based on the determination of enzyme activity (amidazy, catalase) tolerance to 5% of sodium chloride, restoration of nitrate and teluryt of potassium, absorption of iron. Biochemical properties of three species of studied mycobacteria are presented in the Table 1.

Table 1. Biochemical indicators of species differentiation of mycobacteria

The title of the biochemical test	Types of mycobacteria		
	M. vaccae	*M. flavescens*	*M. peregrinum*
Acquisition of iron (accumulation)	+	–	–
Catalase activity	+	+	+
Amidazy activity	+	+	±
Tolerance to 5% of NaCl	+	+	+
Restoration of nitrates	+	+	+
Recovery of teluryt	+	±	±

According to the data presented in the Table 1 paramount importance belongs for tests for iron absorption for species identification of studied culture of mycobacterium for differentiation, determination of Amidazy activity and recovery of teluryt, as results of other biochemical tests are identical to mycobacteria that are subject to differentiation.

To study the assimilation (accumulation) of iron we put the suspension of studied mycobacteria culture into six test tubes with egg nutrient medium for the cultivation of mycobacteria; into three test tubes we added 0.5 cm^3 of sterile 2% solution of lemon ammonia iron and not putting into the other three, we kept it in a thermostat and watched the beginning of the growth of the colonies. The reaction is considered positive if the culture turns brown in the test tubes and in the control tubes the color of colonies is unchanged. The studied mycobacterial culture showed a negative test result, the color of colonies which grew did not change allowing us to exclude the type of *M. vaccae*.

Determining amidase activity of the studied mycobacteria culture was performed by adding amide solutions to the nutrient medium (carbamide, nicotinamide and pyrazinamide) and seeding suspension of a culture with further incubation in a thermostat at 37°C. When there was a growth of colonies we added into the test tubes with the inoculation 0.5 cm^3 of Nessler's reagent and recorded results. The appearance of rusty color in test tubes with culture that is absent in the control tubes indicates a positive result. The studied mycobacteria culture showed a negative test result, which allowed us to exclude *M. peregrinum.*

The conducted in teaching and research laboratory of the department of Epizootology and infectious animal diseases biochemical studies, allowed identifying the selected culture of mycobacteria as a type of *M. flavescens*.

In a control study in the department for the study of tuberculosis and brucellosis NSC «Institute of Experimental and Clinical Veterinary Medicine», the isolated subculture was well grown on a nutrient medium at temperatures of 25°C and 37°C, as well as with 5% sodium chloride and did not show growth at a temperature of 45°C. When cultivated in the dark and in the light, it formed a light yellow pigment, had a small positive catalase activity, as well as a reaction with urea, nicotinamide, pyrazinamide, hydrolyzed tween-80, and accumulated lemon ammonia iron, enzyme reductase. Identical properties have been established in the reference culture of *M. flavescens*, which allowed the studying culture to be classified as *M. flavescens*.

CONCLUSIONS

The mycobacterial infections of mammals and birds in artificial and natural conditions are controlled zoonotic multifactorial infectious pathologies that require permanent epizootic monitoring with using the complex of laboratory and allergic researches. Therefore, the indication of pathogens is not enough informative and transmission of sensitive biological objects is often enough and long without manifestation of infection at the stage latency period or reservation.

In conducting the comprehensive epizootological study of mammals and birds of zoological collection on mycobacterial infection in the ecological biotope of zoological zone of the municipal enterprise “T.H. Shevchenko park of Culture and Rest” we did not register delayed-type hypersensitivity reactions during the intradermal tuberculin test by officinal sensitins of

mycobacteria and did not isolate pathogenic mycobacteria, but from environmental objects and faeces (droppings) we identified 3 cultures of acid-fast microorganisms by culture method: two of which were identified to the type of *Nocardia spp.* and one was identified as the type of *Mycobacterium flavescens*.

REFERENCES

Alekseeva N.V. 2009. Rozrobka ta udoskonalennia zasobiv spetsyfichnoi diahnostyky tuberkulozu ptytsi, in: Extended abstract of candidates thesis. Odessa, [b.w.]. [in Ukrainian]

Alshinetskiy M.V. 2004. Diagnostika tuberkulyoza zooparkovyih zhivotnyih [Diagnosis of tuberculosis of zoo animals]. Veter. Pathol. 1–2, 147–148. [in Russian]

Lecu A., Ball R. 2011. Mycobacterial infections in zoo animals: relevance, diagnosis and management. Inter. Zoo Yearbook 45(1), 183–202.

Makarova M.V. 2007. Ne-tuberkuloznyie mikobakterii: klassifikatsiya, epidemiologiya, patologiya u lyudey i zhivotnyih, laboratornaya diagnostika [Non-tuberculous mycobacteria: classification, epidemiology, pathology in humans and animals, laboratory diagnostics]. Probl. Tubercul. Lung Dise. 10, 7–17. [in Russian]

Maslow J. 1997. Tuberculosis and other mycobacteria as zoonoses, in: Proceeding American Association of Zoo Veterinarians Annual Conference, Houston, Texas, 110–115.

Rastogi N., Legrand E., Sola C. 2001. The mycobacteria: an introduction to nomenclature and pathogenesis. Sci. Techn. Rev. Office Inter. Epizoot. 20(1), 21–54.

Stetter M.D., Mikota S.K., Gutter A.F., Monterroso E.R., Dalovisio J.R., Degraw C., Farley T. 1995. Epizootic of Mycobacterium bovis in a zoological park. J. Am. Veter. Med. Assoc. 207(12), 1618–1621.

Zavgorodniy A.I., Kalashnik N.V., Alekseeva N.V. 2006. Tuberkulez ptits [Bird tuberculosis]. Veter. Med. 87, 337–344. [in Ukrainian]