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ISOLATION OF *MYCOBACTERIUM AVIUM* subsp. *PARATUBERCULOSIS* FROM MOUFLON IN BULGARIA

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Abstract. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis (John's disease) mainly in large and small domestic and wild ruminants, and suspected causative agent in human Crohn's disease. In Bulgaria, paratuberculosis is still poorly researched in both groups of ruminants. We present results of the first in-depth study of mouflon, grown free in one hunting reserve in the Western region of the country. The aim was to prove the presence of MAP in diagnostic materials from regularly hunted or dead mouflon suspected for paratuberculosis. Small intestine and mesenteric lymph nodes (MLN) from 12 hunted and 4 dead mouflon and 10 faecal samples (Fc) were studied in the period of 2009–2013. Typical for paratuberculosis pathomorphological lesions were observed in four mouflon (of 16 examined). The intestinal wall was thickened, strongly folded and soft, with severe hyperemia. The MLN were enlarged, soft, with marbled appearance. The affected section of the ileum showed hyperplasia of the mucous corion and submucosa with diffuse infiltration of epithelioid cells. Lymphadenopathy with atrophy of T and B lymphocytes areas was observed in the mesenteric lymph nodes. For bacteriological isolation of MAP, the tissue and faecal samples were decontaminated with NALC-NaOH, cultured in Middlebrook 7H9 Broth and on Herrold's medium. The Ziehl–Neelsen stained smears and isolates were examined microscopically for acid-fast bacteria. Presence of MAP was observed in tissue samples of 4 (25%) mouflon and in 2 (20%) faecal samples. The same samples were confirmed by the IS*900* PCR for the presence of specific for MAP fragments with a commercial amplification kit. The cases of paratuberculosis found at different times in the free-living mouflon in our study prove that the disease exists in Bulgaria and highlight the need for more serious control of the disease among wild and domestic ruminants.

Key words: *mouflon, paratuberculosis, pathomorphology, microbiology, PCR.*

ВЫДЕЛЕНИЕ *MYCOBACTERIUM AVIUM* subsp. *PARATUBERCULOSIS* ИЗ МУФЛОНА В БОЛГАРИИ

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Резюме. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) является этиологическим агентом паратуберкулеза (болезнь Джона), главным образом, у крупных и мелких домашних и диких жвачных животных и предполагаемым возбудителем болезни Крона у человека. В Болгарии паратуберкулез все еще плохо

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изучен в обеих группах жвачных животных. В данной работе представлены результаты первого углубленного исследования муфлонов, выращенных в охотничьем заповеднике в западном регионе страны. Целью исследования было доказать наличие MAP в диагностическом материале от муфлонов при подозрении на паратуберкулез. В период 2009–2013 гг. были исследованы образцы из тонкого кишечника и мезентериальных лимфатических узлов (МЛУ) 12 убитых во время охоты и 4 павших муфлонов и 10 образцов фекалий. Типичные патоморфологические поражения, характерные для паратуберкулеза, наблюдались у четырех муфлонов (из 16 обследованных). Стенка кишечника была толстой, сильно скрученной и мягкой, с выраженной гиперемией. МЛУ были увеличеными, мягкими, имели мраморный внешний вид. На пораженном участке подвздошной кишки наблюдались гиперплазия слизистой оболочки и диффузная инфильтрация эпителиоидными клетками подслизистой оболочки. В мезентериальных лимфатических узлах наблюдалась лимфаденопатия с атрофией в области Т- и В-лимфоцитов. Для бактериологического выделения MAP образцы тканей и фекалий дезактивировали NALC-NaOH, культивировали в бульоне Миддлбрук 7H9 и на плотной среде Херролда. Мазки, окрашенные по Цилю–Нильсену, и изоляты, исследовали под микроскопом на наличие кислотоустойчивых бактерий. Присутствие MAP наблюдалось в образцах ткани 4 (25%) муфлона и в 2 (20%) образцах фекалий. Присутствие MAP в тех же самых образцах было подтверждено с помощью ПЦР IS*900* с коммерческим набором для амплификации специфического для MAP фрагмента. Случаи паратуберкулеза, обнаруженные в нашем исследовании в разное время у свободно живущих муфлонов, показывают, что MAP циркулирует в Болгарии и подчеркивают необходимость более строгого контроля заболеваемости паратуберкулезом среди диких и домашних жвачных животных.

Ключевые слова: *муфлон, паратуберкулез, патоморфология, микробиология, ПЦР.*

Introduction

Paratuberculosis is one of the oldest diseases in animals, described in 1829 in England and studied by John and Froitam in 1895. They first detected acid-alcohol-resistant bacillus in the intestine of a cow with chronic diarrhea. Twort and Inggram (1912) obtained a pure bacterial culture and gave a general description of the disease, named "John's disease" [8]. At the suggestion of Thorel, the etiological agent was named *Mycobacterium avium* subsp. *paratuberculosis* (MAP) [24].

The isolation of MAP from the blood culture of patients, from breast milk and patients with ulcerative colitis gives reason to accept MAP as one of the etiological factors in Crohn's disease [6, 15, 16, 17]. This shows zoonotic nature of MAP, although the question still remains controversial [18, 21].

Etiological agent of the disease, *M. paratuberculosis* is small, thin, Gram-positive, acid-resistant rod. The bacteria are immobile, do not form spores and capsules, pink stained by Ziehl–Neelsen. MAP grows on an enriched culture media and is very resistant to drying, low temperatures and disinfectants [22, 24, 26]. MAP is a facultative aerobic bacterium belonging to *Mycobacterium avium* complex [3, 8, 17, 24]. It is an obligate intracellular pathogen that can not replicate outside of animal and human hosts [2, 16, 18]. Characteristics distinguishing MAP from other Mycobacterium species, include its extremely slow growth, its inability to produce the necessary for its growth mycobactin and possession of 14–18 copies of the IS*900* insertion element in the MAP genome [4, 5, 20, 22].

Paratuberculosis (John's disease) affects mainly the young domestic and wild ruminants, including mouflon (Ovis aries musimon; wild sheep), most commonly causing hypertrophic enteritis. Diagnosis of John's disease is a difficult and very long process. In wildlife, paratuberculosis usually occurs subclinically without visible symptoms. The affected animals spread the agent in the environment through faeces and so contaminate soil, pastures and water and may be the source of infection for other animals [2, 10, 11, 18, 20].

The paratuberculosis in wild animals was first described in 1922 by Jarmy in an antelope in a zoo, and in 1949 by Dorofeev and Kalacheva in deer populations [3]. The disease in captive and free-range mouflon and other wildlife is described in many countries in Europe and worldwide. According to OIE (World Organisation for Animal Health) documents over the last decades, 44% of member states have reported the presence of paratuberculosis and in 2004 is categorized in sheet B as a disease with serious economic and health consequences [18].

In Bulgaria paratuberculosis in wild animals has been poorly studied. There are no official data on the prevalence of the disease in both domestic and wild ruminants. Because its rare clinical manifestations, the incidence of subclinical forms of the disease is not investigated or monitored. Only single cases in cattle of private farms were described [12]. In the period 2009–2013 was con-

ducted the first in-depth study of diagnostic materials from the hunted and dead wildlife, grown freely in one hunting area in the northwestern region of the country [22].

The aim of this study is to prove the presence of MAP in diagnostic material from legally hunted or dead mouflon suspected for paratuberculosis, which may be a risk for spreading the disease in the population of other wild and domestic ruminants in contact with infected animals or contaminated environment.

Materials and methods

Collection of samples. Samples from proximal and distal parts of the ileum (IL) and mesenteric lymph nodes (MLN) of 12 regularly hunted and 4 found dead mouflon were studied for visible macroscopic lesions of paratuberculosis. Faecal samples (Fc) from 8 of killed and 2 dead animals were collected and examined too.

Histopathologic examination. The examinated tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin and processed routinely for histopathologic examination [12]. Sections (5 μm thick) were stained with Hematoxilin-Eosin (H&E).

Bacterial cultures. The isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from tissue samples (TS) and faeces (Fc) was carried out with commercial kit DNA-Sorb-B (Sacace Biotechnologies, Italy). The tissue smears stained with commercial Ziehl–Neelsen (ZN) Color Kit (Liofilchem, Italy) were observed microscopically. Briefly, the tissue and faecal samples were homogenised in saline solution and decontaminated with NALC-NaOH (N-acetyl-L-cysteine-sodium hydroxide). The sediment obtained after the sample treatment was resuspended in phosphate buffer. 100 μl of each sample were cultured in 2 tubes with Herrold's medium with Mycobactin J and pyrovate (Remel-Kansas, USA) and Middlebrook 7H9 Broth with polysorbate 80 (Becton&Dickinson, USA). Bacteriological study was conducted on 16 samples of IL, 10 of MLN and 10 of Fc. The samples were grown at 37°C from 3 to 6 months.

PCR detection assay. For isolation and amplification of MAP's DNA from tissue samples and faeces, both DNA-Sorb-B kit and respectively M. paratuberculosis Vet DNA amplification kit (Sacace Biotechnologies, Italy) were used. To confirm the strains identified bacteriologically as MAP, the IS*900* PCR-based technique was carried out. DNA kit's control and DNA from MAP 7072 (clinical isolate from deer) were used as positive controls. The size of the PCR products was determined by a DNA marker of 100 bp.

Results

Typical for paratuberculosis pathomorphological lesions were observed in 25% of mouflon (4 out of 16 examined). In affected animals, hyperplastic changes were observed in the proximal and distal segments of the ileum. The intestinal wall was thickened, strongly folded and soft, with severe hyperemia (fig. 1, see cover II). Intestinal content was scanty with watery to thick whitish consistency (fig. 2, cover II). The MLN were enlarged, soft, with marbled appearance. Their capsule and cut surface had an intensive yellowish-cream color, without bleeding, abscesses or caseous foci.

Histologically the affected section of the ileum showed hyperplasia of the mucous corion and submucosa with diffuse infiltration of epithelioid cells. In the altered parts of the ileum diffuse noncaseating granulomatous enteritis was observed. The normal structure of the intestinal mucosa was altered. The lamina propria was markedly expanded by lymphocytes and scattered macrophages with foamy cytoplasm and absence of Langhans giant cells Intestinal villi were shortened and thickened. Atrophy of the intestinal crypts was observed due to compression from the mononuclear inflammatory cells. The microscopic examination of lymph nodes from mouflon revealed extracellular deposition of amyloid in the form of an amorphous eosinophilic substance. Its accumulation is high and exerts strong pressure on the lymph tissue, causing severe atrophy of T and B lymphocyte areas. The lymph node cortical and para-cortical zones are mainly affected (fig. 3, cover II).

The bacteriological study of MLN and IL tissue samples and faeces showed primary growth of single colonies at 5–6 weeks of the culturing on the selective Herold's medium. In the presence of specific growth we observed clearly separated, very small, convex, soft, colorless, translucent and humid colonies. In abundant growth colonies remained small, round, smooth and glossy (fig. 4, cover II). On the third month of culturing on the solid medium the colonies became opaque, gray-whitish, rough and dry. On the Middlebrook 7H9 Broth bacteria have formed a thick, whitish veil. MAP was isolated from tissue samples of the ileum and MLN of 3 hunted and 1 dead mouflon with pathomorphological lesions and from 2 faecal samples. Other tissue samples and faeces, showed no growth on culture media and after 6 months cultivation (tabl.). The microscopic observation of Zieh–Neelsen (ZN) colored smears found immobile, short pink-red colored rods, clustered on heaps or located severally (fig. 5, cover II). The presence of MAP in the same samples was confirmed by IS*900* PCR kit. PCR analysis showed a presence of specific 209-bp DNA fragment (fig. 6). The other samples were PCR negative (tabl.).

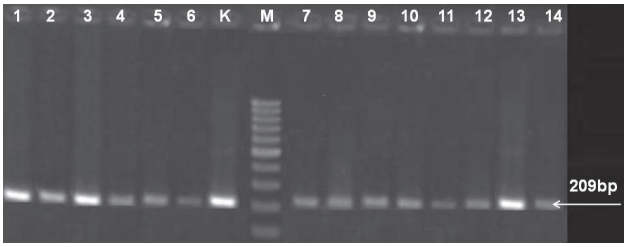

Figure 6. 209 bp-PCR products from bacterial isolates and tissue samples from the mesenteric lymph nodes (MLN) and small intestines (SI) of mouflons

1–3 — bacterial isolates from MLN; 4–6 — bacterial isolates from the small intestine; K — positiv control MAP 7072 (clinical isolate from deer); M — molecular weight marker; 7–8 — fecal samples; 9–11 — tissue specimens ofMLN; 12, 14 — tissue samples of the intestine; 13 — DNA kit's control

Discussion

In wildlife disease usually remains hidden and animals are only carriers and emitters of mycobacteria, which hinders the diagnosis of paratuberculosis. In the examined samples pathoanatomical changes were observed in the small intestine and mesenteric lymph nodes. Proliferation of intestinal mucosa varied significantly from less pronounced to clearly visible changes. Similar lesions in domestic and wild ruminants with paratuberculosis were described by many authors [3, 7, 8, 9, 12, 14, 20, 26].

The histological examination is the first opportunity for detection of subclinical cases of the disease and in wild animals it is very important. In the altered parts of the intestinal mucosa we observed infiltration of a variable numbers of diffusely located macrophages, lymphocytes and plasma cells significantly expanding lamina propria, thickened intestinal villi and necrotic debris in intestinal crypts. In the MLNs we observed lymphadenopathy. Many authors reported similar histological findings in sheep, goats, cattle and wild animals suffering from paratuberculosis [2, 7, 12, 14, 26]. In the affected tissues, some of authors observed also the presence of a single or large amount of multinucleated Langhans giant cells. We have not found giant cells, but only diffuse deposition of an amorphous, eosinophilic extracellular substance and atrophy of the T and B lymphocyte areas. Such results are not unusual and depend on the species and age of the animals, the development stage and intensity of the infection as well as the prevalence of MAP in the wildlife.

After microscopic examination of tissue and faecal smears we observed pink-red rods located singly or clustered on heaps, resembling morphology many other mycobacteria. Therefore, the finds can only direct us to the disease. The bacteriological method, although time consuming, still remains a "gold standard" for detection of MAP because it does not give false-positive results [11, 18, 19]. Unlike other mycobacteria, MAP is best developed for the nutrient medium containing mycobactin J, a growth factor that bacteria can't produce on their own [1, 3, 18, 26]. Such is the Herald's medium, which we have also successfully used. The infectious agent was isolated from the altered tissues of the IL and MLN of 4 (25%) mouflons. The isolation of MAP from faecal samples is also an important moment in the diagnosis of the disease. Although the method is considered to be highly specific and sensitive test to detect infected animals in herds [11], it has limited sensitivity for subclinically infected and free-living wild animals. In our study MAP was isolated from only two faecal samples (20%). Established bacteriological findings corresponded to those described by other

Table. Overview of data obtained from mouflon`s samples and types of analyses

Animal species	Tested animals	MAP positive animals (No.)	Type of sample	Analyzed samples	Type of analysis				
					Autopsy findings (+)	Histology findings (+)	Microscopy (+)	Culture (+)	PCR (IS*900*) (+)
Mouflons	hunted 12	3	SI	12	3	3	3	3	3
			MLN	10	3	3	3	3	3
			Fc	8			2	2	2
	dead 4	1	SI	4	1	1	1	1	1
			MLN	4	1	1	1	1	1
			Fc	2			0	0	0
Total No.	16	4	Ts/Fc	30/ 10	8	8	10	10	10

SI — small intestine; MLN — mesenteric lymph nodes; Fc —faeces; Ts —tissue samples (SI + MLN).

authors [2, 20, 23]. The MAP isolation from tissues and faeces of mouflon was confirmed by the PCR which is recommended by the World Organisation for Animal Health [18]. Based on genomic analysis Englund et al. [5] reveal that this element is also present in other mycobacterial species. Harris and Barletta [8], Vansnick et al. [25] noted that most of the used IS*900* PCR primers did not differentiate MAP from other members of the *M. avium* complex because they give identical DNA amplicons. More recent research by other authors describe different variants for detection of paratuberculosis bacteria and most of them are also based on the IS*900* insertion element, which is accepted as a standard molecular marker for MAP [5, 20, 23, 26].

We used an IS*900* amplification kit, recommended as a specific for *M. avium* complex, respectively for MAP [22]. The resulting PCR products coincided with the established specific patomorphological changes and the isolated bacterial culture from the affected tissues of the four mouflons and the two faecal samples, indicating that the amplicons were MAP-specific (tabl.).

The paratuberculosis refers to an "open infection" with large excretion of causative agent in the environment. Contamination of soils, plants, pastures, water and air after excretion of MAP with faeces of naturally infected mouflon was previously reported [10, 19, 26]. The causative agent was isolated from both faeces and skeletal muscles of the tested mouflon [23]. The excretion of MAP into the environment presents a risk of transmission of the disease to other wild and domestic animals and to endanger the people consuming contaminated food and water. This confirms the serious health and economic importance of the disease [16, 18].

The paratuberculosis cases detected over different periods in free-living mouflon in our study shown that the disease exists in Bulgaria. The results indicate the need for further research on hunting reserves in the country and their neighboring cattle farms. This would clarify the prevalence of the disease among wildlife and the potential risk for the domestic ruminants.

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References

1. Ayele W.Y., Svastova P., Roubal P., Bartos M., Pavlik I. Mycobacterium avium subsp. paratuberculosis cultured from locally and commercially pasteurised cow's milk in the Czech Republic. *Appl. Environ. Microbiol., 2005, vol. 71, no. 2, pp. 1210–1214.*
2. Behr M.A., Collins D.M. Paratuberculosis: organism, disease, control. *CAB International, Oxford U.K., 2010, 392 p.*
3. Belchev D. Investigations on paratuberculosis on the cattle and sheep. PhD Thesis. Central Research Veterinary Medical Institute. *Bulgaria: Sofia, 1977.*
4. Collins D.M, Gabric D.M., de Lisle G.W. Identification of a repetitive DNA sequence specific to Mycobacterium paratuberculosis. *FEMS Microbiol., 1989, vol. 60, pp. 175–178.*
5. Englund S., Bölske G., Johansson K.E. An IS*900* like sequence found in a Mycobacterium species other than Mycobacterium avium subsp. paratuberculosis. *FEMS Microbiol., 2002, vol. 209, pp. 267–271.*
6. Golan L., Livneh-Kol A., Gonen E., Yagel S., Rosenshine I., Shpigel N.Y. Mycobacterium avium subsp. paratuberculosis invades human small-intestinal goblet cells and elicits inflammation. *J. Infect. Dis., 2009, vol. 199, no. 3, pp. 350–354.*
7. Hailat N.Q., Hananeh W., Metekia A.S., Stabel J.R., A-Majali A., Lafi. S. Pathology of subclinical paratuberculosis (Johne's Disease) in Awassi sheep with reference to its occurrence in Jordan. *Veterinarni Medicina, 2010, vol. 55, no. 12, pp. 590–602.*
8. Harris N.B., Barletta R.G. Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine. *J. Dairy Sci., 1998, vol. 81, no. 1, pp. 283–288.*
9. Johne H.A., Frothingham L. Ein eigenthuemlicher Fall von Tuberkulose beim Rind. *Deutsche Zeitschrift für Tiermedicin und Pathologie, 1895, vol. 21, pp. 438–454.*
10. Kaevska M., Lvoncik S., Lamka J., Pavlik I., Slana I. Spread of Mycobacterium avium subsp. paratuberculosis Through Soil and Grass on a Mouflon (Ovis aries) Pasture. *Curr. Microbiol., 2014, vol. 69, pp. 495–500.*
11. Kalis C.H.J., Collins, M.T., Barkema, H.W., Hesselink, J.W. Certification of herds as free of Mycobacterium paratuberculosis infection: actual pooled faecal results versus certification model predictions. *Prev. Vet. Med., 2004, vol. 65, pp. 189–204.*
12. Koev K., Dinev I., Atev I. Cases of paratuberculosis in cattle. *Veterinarna Sbirka, 2015, no. 1–2, pp. 34–37.*
13. Lillie R.D. Histopathologic technic and practical histochemistry. 3rd edition. New York: McGraw-Hill, 1965.
14. Manning E.J.B. Paratuberculosis in captive and free-ranging wildlife. *Veterinary Clinics of North America: Food Animal Practice, 2011, vol. 27, no. 3, pp. 621–630.*
15. McNees A.L., Markesich D., Zayyani N.R., Graham D.Y. Mycobacterium paratuberculosis as a cause of Crohn's disease. *Expert Rev. Gastroenterol. Hepatol., 2015, vol. 9, no. 12, pp. 1523–1534.*
16. Naser S.A., Ghobrial G., Romero C., Valentine J.F. Culture of Mycobacterium avium subsp. paratuberculosis from the blood of patients with Crohn's disease. *Lancet, 2004, vol. 364, pp. 1039–1044.*
17. Naser S.A., Schwartz D., Shafran I. Isolation of Mycobacterium avium subsp paratuberculosis from breast milk of Crohn's disease patients. *Am. J. Gastroenterol., 2000, vol. 95, pp. 1094–1095.*

18. OIE (World Organisation for Animal Health), Terrestrial Manual, 2014. Chapter 2.1.11. Paratuberculosis (Johne's disease).
19. Pribylova R., Slana I., Kaevska M., Lamka J., Babak V., Jandak J., Pavlik I. Soil and plant contamination with Mycobacterium avium subsp. paratuberculosis after exposure to naturally contaminated mouflon feces. *Curr. Microbiol., 2011, vol. 62, pp. 1405–1410.*
20. Pribylova R., Slana I., Lamka J., Babak V., Hruska K., Pavlik I. Mycobacterium avium subsp. paratuberculosis in a moufon herd without clinical symptoms monitored using IS900 real-time PCR: a case report. *Veterinarni Medicina, 2010, vol. 55, no. 12, pp. 625–630.*
21. Sartor R.B. Does Mycobacterium avium subsp. paratuberculosis cause Crohn's disease? *Gut, 2005, vol. 54, no. 7, pp. 896–898.*
22. Savova T. New methods for diagnostics of infections caused by Mycobacterium avium and Mycobacterium paratuberculosis. PhD thesis. *Bulgaria: Sofia, 2014.*
23. Slaná I., Lamka J., Přibylová-Dziedzinská R. Detection of Mycobacterium avium subsp. paratuberculosis in mouflon muscle tissue. *MASO Int. J. Food Sci. Technol., 2014, vol. 2, pp. 107–111.*
24. Thorel M.F., Krichevsky M., Lévy-Frébault V.V. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of Mycobacterium avium, and description of Mycobacterium avium subsp. avium subsp. nov., Mycobacterium avium subsp. paratuberculosis subsp. nov., and Mycobacterium avium subsp. silvaticum subsp. nov. *Int. J. Syst. Bacteriol., 1990, vol. 40, no. 3, pp. 254–260.*
25. Vansnick E., de Rijk P., Vercammen F., Geysen D., Rigouts L., Portaels F. Newly developed primers for the detection of Mycobacterium avium subsp. paratuberculosis. *Veterinary Microbiology, 2004, vol. 3, no. 100, pp. 197–204.*
26. Zarei Kordshouli F., Khodakaram Tafti A., Haghkhah M. Pathological, bacteriological, and molecular characteristics of natural outbreaks of Johne's disease in goats of Fars Province, Iran. *Int. J. Mycobacteriol., 2016, vol. 5, no 1, p. 202.*

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Иллюстрации к статье «Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from mouflon in Bulgaria» (авторы: T. Savova, R. Petrova, V. Valcheva, M. Bonovska, H. Najdenski) (с. 665–670)

Figure 1. Small intestine of mouflon without pathological changes in the upper part and hyperemic and strongly folded intestinal wall in the lower part

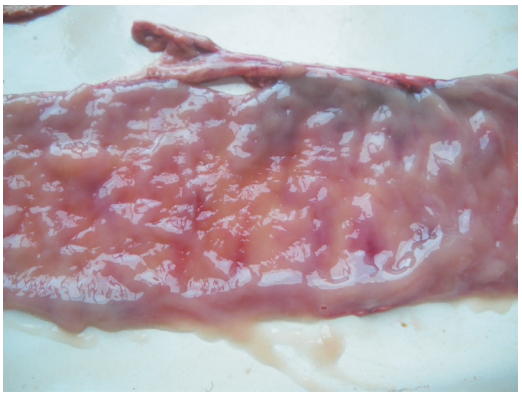

Figure 2. Small intestine incision, covered with muddy whitish mucus substance

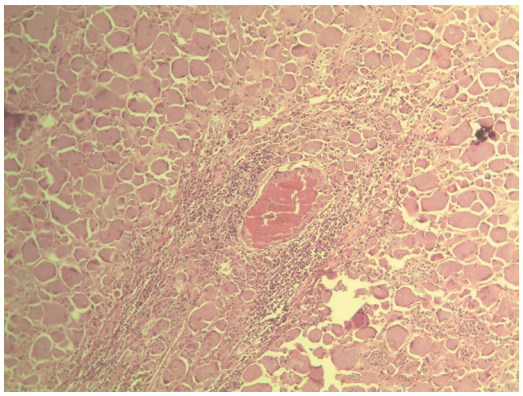

Figure 3. Mesenterial lymph node of mouflon. Extracellular deposition of amorphous eosinophilic substance. XE, ×10

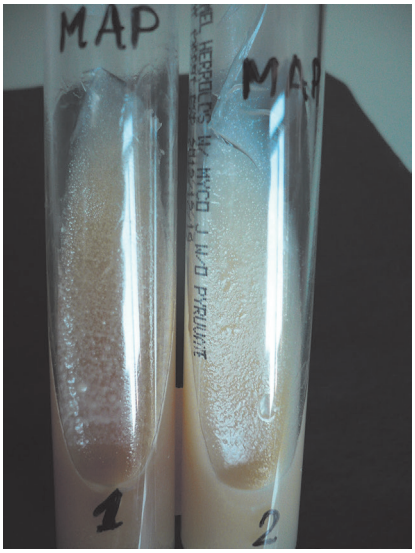

Figure 4. Bacterial growth of MAP on Herrold's medium with Mycobactin J

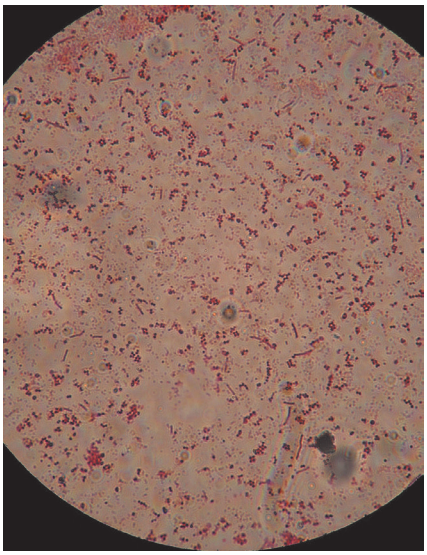

Figure 5. Ziehl–Neelsen colored smear from mesenteric lymph nodes of mouflon