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Case Report

HIGH PREVALENCE OF *MYCOBACTERIUM AVIUM* SUBSP. *HOMINISSUIS* IN A BATCH OF QUARANTINED PIGS IN CROATIA

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Summary: Tuberculosis is a common occurrence in pigs, which are susceptible to infection with both *Mycobacterium (M.) avium subsp. avium* and *M. avium* subsp. *hominissuis*. The aim of this study was to present a case of tuberculosis in a batch of quarantined pigs, imported to Croatia from Austria. Diagnostic examination of 125 quarantined animals included a comparative tuberculin skin test with avian and bovine tuberculin. As 67.2% of pigs showed either positive or suspicious reaction to avian tuberculin, the animals were retested. The results of the second skin test revealed an even higher proportion of reactors (91.1%). Therefore, the State Veterinary Office ordered slaughtering of the animals, and post-mortem examination (gross examination and bacteriology) of the lymph nodes of 121 pigs was performed for diagnostic purposes. Gross tuberculous lesions were observed in 107 pigs (88.59%), mostly in mesenteric lymph nodes (52.1%). *M. avium* subsp. *hominissuis*, identified with molecular methods, was isolated from 113 pigs (93.4%). This report stresses the importance of effective preventive and control measures which are necessary on farms raising animals for breeding purposes.

Key words: swine; tuberculosis; lymph nodes; tuberculin skin test; PCR

Introduction

Swine tuberculosis (TBC) is a chronic infectious disease characterised by inflammatory changes prone to calcification in various body sites, mostly in the digestive system. There is no mycobacterial species specific to pigs, like *Mycobacterium (M.) tuberculosis*, to be found in humans, *M. bovis* in bovines or *M. avium* in poultry and birds. However, pigs are susceptible to infection caused by members of the *M. tuberculosis* and *M. avium* complex, and by opportunistic mycobacterial species, e.g. *M. fortuitum* and *M. chelonae* (1, 2).

Systematic control programmes for bovine TBC and reduced incidence of human TBC decreased the importance of *M. bovis* and *M. tuberculosis* infections in pigs (3). The possibility of infection caused by *M. caprae* exists in pigs fed with raw milk of infected cows (4). However, infections caused by *M. avium* complex are the most common in pigs and have been described worldwide (5-16). Environment (water, soil, sawdust, feedstuff, birds etc.) is a risk factor for human and animal infections caused by the *M. avium* complex, comprising *M. avium* and *M. intracellulare* (1, 17-20).

M. avium is currently divided into four subspecies, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis* (21). *M. avium* subsp. *avium* is the causative agent of avian TBC. Primarily affecting birds, it may also infect other animal species. It comprises serotypes 1, 2 and 3; its genome contains mobile elements IS*901* and IS*1245*. *M. avium* subsp. *hominissuis* was proposed to distinguish organisms found in humans and pigs from those isolated from birds (22). Predominantly found in the environment, *M. avium* subsp. *hominissuis* isolates are weakly

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virulent for birds but are frequently encountered in tuberculous lesions in different animals, especially pigs (23). This subspecies includes serotypes 4-6, 8-11 and 21 and is of the IS*901*-, IS*1245*+ genotype. Pigs are susceptible to infection with both *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*. The prevalence seems to be correlated with the presence of certain subspecies in the environment. However, *M. avium* subsp. *avium* is considered a much more serious pathogen for pigs than *M. avium* subsp. *hominissuis* (13).

The aim of this case report was to present the results of the skin tuberculin test, necropsy, isolation and identification of *M. avium* subsp. *hominissuis* performed as a part of quarantine procedures in breeding pigs imported from Austria.

Materials and methods

Anamnestic data

At the beginning of March 2006, 120 gilts and one boar of Edelschwein race from farm A, two boars of the same race from farm B and two boars of the same race from farm C were imported from Austria. The pigs intended for breeding were from six to nine months of age, weighing between 90 and 130 kg. The pigs were transported from Austria and placed in a quarantine unit of the farm near the town of Petrinja (County of Sisak-Moslavina). The quarantine conditions complied with the standards prescribed by the State Veterinary Office of the Republic of Croatia. Diagnostic examination of the quarantined animals was in accordance with the current legislation prescribed by the same government body.

Tuberculin skin test

All 125 pigs were tuberculin tested with 0.1 ml of avian PPD (20,000 IU) and bovine PPD (50,000 IU; VETERINA d.o.o., Kalinovica, Croatia) administered intracutaneously at auricular basis. The tuberculins were administered simultaneously: avian in the left and bovine in the right ear. Skin reactions were evaluated after 48 and 72 hours according to manufacturer's recommendation. Reaction was negative if no or only pea-sized oedema without redness appeared at the site of application. Reaction characterised by oedema of approx. 2 cm in diameter and visible redness not surrounded with a red circle was regarded as suspicious. Reaction was positive if the site of application was oedematous (2-5 cm in diameter), surrounded by a circular red zone with warmer, purple-red coloured centre and covered by scab. Since a high percentage of pigs were positive at the first skin tuberculin test carried out on 14 March 2006, the State Veterinary Office recommended a repeated skin test which was performed on 30 May 2006.

Gross examination

Due to the high percentage of pigs with positive and suspicious reactions in the second test, the State Veterinary Office ordered the slaughter of 121 pigs (118 gilts and three boars). During the quarantine period two gilts died. Two negative boars were not slaughtered, due to their high genetic value. Slaughtered pigs were checked for gross lesions, and lymph nodes (*ln. submandibularis, ln. mesenterialis, ln. inguinalis, ln. mediastinalis, ln. hepaticus, ln. ileocaecalis*) were collected for investigation.

Bacteriology

For microscopy, lymph node smears were Ziehl-Neelsen (ZN) stained and checked for the presence of acid-fast bacilli (AFB). Lymph nodes were then homogenised, concentrated, decontaminated with NALC-NaOH, inoculated on standard nutrient media (Löwenstein-Jensen with pyruvate, Löwenstein-Jensen with glycerine, Stonebrink and Middlebrook 7H10) and incubated at 37°C for two months. Media were checked for growth of mycobacteria at weekly intervals. The colonies were ZN stained; positive colonies were subcultured and identified by biochemical and molecular methods (25-27).

Identification of isolates by biochemical and molecular methods

ZN positive colonies were identified according to colony morphology, growth temperature and pigment production. Biochemical tests included the following enzymatic activities: nitrate reductase, catalase, Tween 80 hydrolysis, amidase, arylsulfatase, pyrazinamidase and urease (25).

The isolates were identified as members of the genus *Mycobacterium* also by amplification of DNA sequence containing the gene coding for 65 kDa antigen common for all mycobacteria. The primers TB1 (5'-GAG-ATC-GAG-CTG-GAG-GAT-CC-3') and TB2 (5'-AGC-TGC-AGC-CCA-AAG-GTG-TT-3') were used to amplify a 383 bp fragment (26). Subsequent-

ly, the isolates were subjected to identification with the commercially available identification kit GenoType *Mycobacterium* CM (Hain Lifescience, Nehren, Germany) which enables identification of mycobacteria commonly found in clinical samples. In order to differentiate *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*, the isolates were tested by PCR using the primer set P1 FR300 (5'-CAG-CCA-GCC-GAA-TGT-CAT-CC-3') and P2 FR300 (5'-CAA-CTC-GCG-ACA-CGT-TCA-CC-3') described previously by Kunze et al. (27). Amplification product size depends upon the presence or absence of IS*901*. IS*901*+ positive isolates (*M. avium* subsp. *avium*) show an amplification product of 1700 bp, while IS*901*- isolates give amplicons of 300 bp (*M. avium* subsp. *hominissuis*).

PCR results were detected by electrophoresis in 2% agarose gels and visualised by UV transilluminator and camera (Bio-Capt, Vilbert Lourmat, France).

Results

Tuberculin skin test

Among 125 quarantined pigs tested in the first skin test, positive reactions to avian tuberculin were observed in 44 pigs (35.2%), while 40 pigs (32%) were regarded as suspicious reactors. In the second test, which included 123 pigs, the number of reactors and intensity of reactions increased in comparison to the first test (Table 1). All positive pigs originated from farm A. An example of positive reaction is shown in Figure 1.

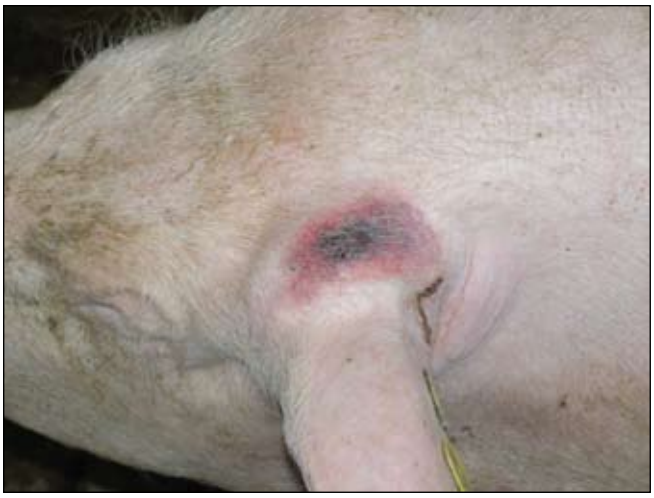

Figure 1: Positive reaction to avian tuberculin in a pig

Gross pathology examination

A total of 121 slaughtered pigs were inspected for gross lesions at the slaughterhouse. Visible granulomatous changes characteristic for TBC were found in 107 pigs (88.59%). Submandibular and mesenteric lymph nodes were moderately augmented with incorporated yellow foci of 3-10 mm in diameter (Figure 2). Calcified granules were visible in the caseous contents of dissected nodes. Mesenteric lymph nodes alone were visibly changed in 63 pigs (52.1%) and both mesenteric and submandibular nodes in 35 pigs (28.9%). Changes in other lymph nodes were rarely observed (Table 2).

Table 1: Results of tuberculin skin tests performed in quarantined pigs

Date of test	No. of tested pigs	Positive reaction		Suspicious reaction		Total reaction (+, +/-)		Negative reaction	
		No.	%	No.	%	No.	%	No.	%
16 March 2006	125	44	35.2	40	32	84	67.2	41	32.8
30 May 2006	123	95	77.2	17	13.8	112	91.1	11	8.9

Table 2: Localisation of necropsy changes found in investigated lymph nodes of 121 slaughtered pigs

No. of pigs	Location of changes				
	SLN	MLN	SLN + MLN	SLN + MLN + PLN	Total
121	7	63	35	2	107
100%	5.8	52.1	28.9	1.7	88.5

Legend: SLN – submandibular lymph node, MLN – mesenteric lymph node, PLN – portal lymph node

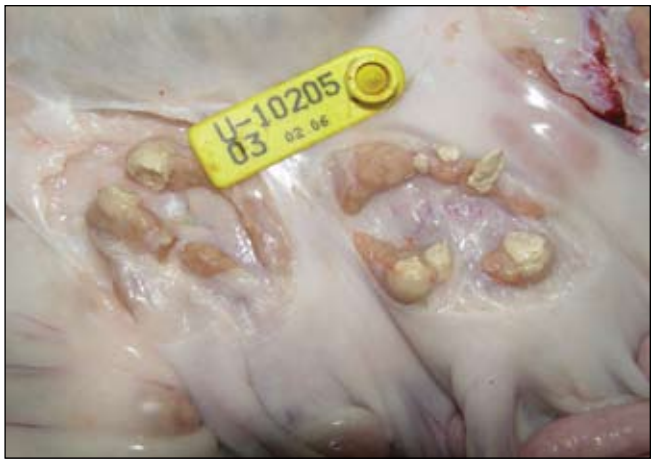

Figure 2: Tuberculous lesions in a mesenteric lymph node of a swine at slaughter

Bacteriology and identification of the isolates

Mycobacteria were isolated from the lymph nodes of 113 swine (93.4%). All isolates were identified as members of the genus *Mycobacterium*. Using the GenoType *Mycobacterium* CM kit, the isolates were identified as *M. avium*. PCR used for differentiation of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *avium* resulted in amplicons specific for *M. avium* subsp. *hominissuis* only.

Discussion

Swine TBC can cause huge economical losses. Pavlik et al. (13) stated that direct losses due to TBC can reach 24% of the total swine price. In order to avoid them, effective diagnostic methods are needed to prevent the spread of the disease. The tuberculin skin test, recommended by the OIE and used worldwide, serves as an indirect diagnostic method for TBC since the infected animals develop hypersensitivity against the causative agent. Post-mortem examinations are used for definitive diagnostics of TBC. Standard identification methods of the causative agent, based on morphological and biochemical characteristics, made the differentiation within *M. avium* difficult. Development of molecular methods facilitated and improved the identification and contributed to the description of new subspecies.

In this study, a high proportion of pigs imported from Austria showed positive reactions to avian tuberculin. In the first tuberculin test, 67.2% of tested pigs were either positive or suspicious reactors, while in the repeated test the proportion increased to 91.1% of tested pigs. The reason for substantial increase of positive reactors (from 35.2% to 77.2%) most probably lies in the fact that the infection in young animals was acute and the organisms needed time to create an immunological response. Some pigs reacted also to bovine tuberculin but with weaker intensity. Subsequent bacteriological examination of specimens from these animals revealed only the presence of *M. avium* subsp. *hominissuis*.

In 121 carcasses of slaughtered pigs, tuberculous granuloma localised in submandibular and mesenteric lymph nodes were found in 107 pigs (88.5%). The changes were most frequently localised in mesenteric lymph nodes (52.1%), followed by both mesenteric and submadibular (28.9%) lymph nodes. Lesions in other lymph nodes were rarely observed. Pavlik et al. (13) reported similar results: 65.3% of granuloma in swine were detected in mesenteric lymph nodes, followed by submandibular (18.6%), both submandibular and mesenteric (15.9%) lymph nodes, while 0.1% of changes were observed in parenchymatous organs.

All isolates from this investigation were identified as *M. avium* subsp. *hominissuis*. This is a common finding in pigs (2, 18). Pate et al. (14) reported domination of *M. avium* subsp. *hominissuis* (60.9%) over *M. avium* subsp. *avium* (33.8%) among mycobacteria isolated from swine in Slovenia between 2000 and 2003. Pavlik et al. (13) described a reverse situation in a different time period (1990-1999), with *M. avium* subsp. *avium* being more prevalent (55.7%) than *M. avium* subsp. *hominissuis* (39.2%). However, in the second part of the 1990s the percentage of isolated *M. avium* subsp. *hominissuis* strains started to increase, which was probably connected to a change in the sources of infection for pigs. One of our previous studies (16) revealed that the majority of mycobacteria isolated from swine in Croatia belonged to *M. avium* complex (95.7%), while other identified species included *M. fortuitum* (3.3%), *M. chelonae* (0.5%) and *M. peregrinum* (0.5%). *M. avium* subsp. *hominissuis* was identified in larger proportion than *M. avium* subsp. *avium* (78.9% versus 21.1%).

Sawdust was used as bedding material on farm A (probably in the farrowing units) of this study. Therefore, the piglets were very likely exposed to high concentrations of *M. avium* subsp. *hominissuis* which is frequently found in the environment (12). Cvetnić et al. (16) found the highest proportions of tuberculin skin test reactors on farms where sawdust or wood shavings were used for bedding in farrowing and

boar units. In one case, the proportion of infected boars reached 70%. Isolation of *M. avium* subsp. *hominissuis* serotype 8 from the lymph nodes of pigs and from sawdust indicated the latter as the most probable source of infection. This has been suggested before (1, 28-30). The development of genotyping methods enabled an insight into molecular characteristics of investigated isolates and confirmation of previously suggested epidemiological links. Matlova et al. (2) managed to demonstrate identical genotypes of *M. avium* subsp. *hominissuis* from sawdust and from clinical samples of pigs, therefore confirming the hypotheses regarding the sawdust being a source of infection for pigs. The same situation was proven also in Croatia by Špičić et al. (31).

Knowledge about the ecology and epidemiology of mycobacteria is of great importance for successful prevention and control of the infections. Susceptibility of swine to various mycobacteria poses a constant risk for outbreaks of clinical disease in swine herds. Therefore, effective preventive and control measures are necessary on farms raising animals for breeding purposes.

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References

1. Cvetnic Z, Kovacic H, Ocepek M. Mykobakterien in der Umwelt und im Futter von Schweinen in Kroatien (in German). Wien Tierärztl Monatscrift 1998; 85: 18–21.

2. Matlova L, Dvorska L, Palecek K, Maurenc L, Bartos M, Pavlik I. Impact of sawdust and wood shavings in bedding on pig tuberculous lesions in lymph nodes, and IS*1245* RFLP analysis of *Mycobacterium avium* subsp. *hominissuis* of serotypes 6 and 8 isolated from pig and environment. Vet Microbiol 2004; 102: 227–36.

3. Aleraj Z, Tunkl B, Karlovic M. Using of UV rays for fast TBC diagnosis and eradication from swine (in Croatian). Praxis Vet 1972; 3: 135–40.

4. Cvetnic Z, Spicic S, Katalinic-Jankovic V et al. *Mycobacterium caprae* infection in cattle and pigs on one family farm in Croatia: a case report. Vet Med – Czech 2006; 51: 523–31.

5. Zorawski C, Karpinski T, Skwarek P, Loda M. *M. intracellulare* serotip 8 (Davis), existing in sawdust as a cause of mass infection of pigs. Bull Vet Inst Pulawy 1983; 26: 1–5.

6. Karlović M, Lojkić M, Bilić V, Kovačić H. The health issues in swine farms (in Croatian). Vet Arh 1985; 55: S9.

7. Gradner IA, Hird DW. Environmental source of mycobacteriosis in a California swine herd. Can J Vet Res 1989; 53: 33–7.

8. Haugegaard J, Knudsen S, Bager, F. Tuberculous lymphadenitis caused by *Mycobacterium avium-intracellulare* in Danish swine herds. In: 12th International Pig Veterinary Society Congress Proceedings. Hague, 1992: 343.

9. Balian SC, Ribeiro P, Vasconcelos SA et al. Tuberculosis lymphadenitis in slaughtered swine from the State of Sao Paulo, Brasil: microscopic histopathology and demonstration of mycobacteria. Rev Saude Publica 1997; 31: 391.

10. Komijn RE, De Haas PE, Schneider MM et al. Prevalence of *Mycobacterium avium* in slaughter pig in the Netherlands and comparison of IS*1245* restriction fragment length polymorphism patterns of porcine and human isolates. J Clin Microbiol 1999; 37: 1254–59.

11. Offermann U, Bodmer T, Audige L, Jemmi T. The prevalence of salmonella, yersinia and mycobacteria in slaugtered pigs in Switzerland (in German). Schweiz Arch Tierheilkd 1999; 141: 509–15.

12. Matlova L, Dvorska L, Bartl J et al. Mycobacteria isolated from environment of pig farms in the Czech Republic during the years 1996 to 2002. Vet Med – Czech 2003; 48: 343–57.

13. Pavlik I, Matlova L, Dvorska L et al. Tuberculosis lesions in pigs in the Czech Republic during 1990–1999: occurrence, causal factors and economic losses. Vet Med – Czech 2003; 48: 113–25.

14. Pate M, Zdovc I, Pirš T, Krt B, Ocepek M. Isolation and characterisation of *Mycobacterium avium* and *Rhodococcus equi* from granulomatous lesions of swine lymph nodes in Slovenia. Acta Vet Hung 2004; 52: 143–50.

15. Pavlik I, Matlova L, Dvorska L, Shitaye JE, Parmova I. Mycobacterial infections in cattle and pigs caused by *Mycobacterium avium* complex members and atypical mycobacteria in the Czech Republic during 2000–2004. Vet Med – Czech 2005; 50: 281–90.

16. Cvetnić Ž, Špičić S, Benić M et al. *Mycobacterium* infections in pigs in Croatia. Acta Vet Hung 2007; 55: 1–9.

17. Martin G, Schimmel D. *Mycobacterium avium* infections in poultry – a risk for human health or not? Dtsch Tierarztl Wochenschr 2000; 107: 53–8.

18. Matlova L, Dvorska L, Ayele WY, Bartos M, Amemori T, Pavlik I. Distribution of *Mycobacterium avium* complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. J Clin Microbiol 2005; 43: 1261–8.

19. Reed C, Von Reyn CF, Chamble S et al. Environmental risk factor for infection with *Mycobacterium avium* complex. Am J Epidemiol 2006; 164: 32–40.

20. Fischer OA, Matlova L, Dvorska L et al. Various stages in the life cycles of syrphid flies (*Eristalis tenax*; Diptera: Syrphidae) as potential mechanical vectors of pathogens causing mycobacterial infections in pigs herds. Folia Microbiol (Praha) 2006; 51: 147–53.

21. Turenne CY, Wallace R Jr, Behr MA. *Mycobacterium avium* in the postgenomic area. Clin Microbiol Rev 2007; 20: 205–29.

22. Mijs W, de Haas P, Rossau R et al. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* to bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. avium*. Int J Syst Evol Microbiol 2002; 52: 1505–18.

23. Pavlik I, Svastova P, Bartl J, Dvorska L, Rychlik I. Relationship beetwen IS*901* in the *Mycobacterium avium* complex strains isolated from birds, animals, humans and the environment and virulence for poultry. Clin Diagn Lab Immunol 2000; 7: 212–7.

24. Luna LG. Manual of histologic methods of the Armed Forces Institute of Pathology. New York: McGraw Hill, 1979.

25. Kent PT, Kubica GP. Public health mycobacteriology: a guide for level III laboratory. Atlanta: US Department of Health and Human Services, Centers for Disease Control, 1985.

26. Hance AJ, Grandchamp B, Levi-Frebauld V et al. Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol Microbiol 1989; 7: 843–9.

27. Kunze ZM, Portaels F, McFadden JJ. Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS*901*. J Clin Microbiol 1992; 30: 2366–72.

28. Reznikov M, Leggo JH, Taffley RE. Further investigation of an outbreak of mycobacterial lymphadenitis at a deep – litter piggery. Aust Vet J 1971; 47: 622–8.

29. Saitanu K, Holmgaard P. An epizootic of *M. intracellulare*, serotype 8 infection in swine. Nord Vet Med 1977; 29: 221–6.

30. Saxegaard F. Serological investigations of *Mycobacterium avium* and *M. avium* like bacteria isolated from domestic and wild animals. Acta Vet Scand 1981; 22: 153–61.

31. Spicic S, Pate M, Katalinic-Jankovic V et al. Molecular epizootiology of *Mycobacterium avium* subsp. *hominissuis* isolated from humans, animals and the environment in Croatia. J Comp Immunol Microbiol Infect Dis 2010 In press

MOČNA RAZŠIRJENOST *MYCOBACTERIUM AVIUM* SUBSP. *HOMINISSUIS* V SKUPINI PRAŠIČEV, KI SO BILI V KARANTENI NA HRVAŠKEM

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Povzetek: Tuberkuloza je pogosti pojav pri prašičih, ki so dovzetni za okužbo z *Mycobacterium (M.) avium* subsp. *avium* kot tudi *M. avium* subsp. *Hominissuis*. Cilj pričujoče raziskave je opis primera tuberkuloze v skupini prašičev v karanteni, ki so bili v Hrvaško uvoženi iz Avstrije. Diagnostična preiskava 150 živali v karanteni je vključevala tuberkulinski kožni test s ptičjim in govejim tuberkulinom. Ker je bila pri 67,2 odstotkih prašičev opazna pozitivna ali sumljiva reakcija, so živali ponovno preiskali. Rezultati drugega kožnega testa so pokazali še večji delež živali, ki so reagirale na antigene (91,1 odstotka). Na podlagi rezultatov je Državna veterinarska uprava odredila zakol živali ter izvedbo posmrtnega pregleda (patologija, bakteriologija) bezgavk 121 prašičev iz diagnostične preiskave. Velike patološke tuberkulozne spremembe so bile opazne pri 107 prašičih (88,6 odstotka), in sicer večinoma v oporkovih bezgavkah (52,1 odstotka). *M. avium* subsp. *hominissuis*, dokazano z molekularnimi metodami, smo izolirali pri 113 prašičih (93,4 odstotka). To poročilo želi poudariti pomembnost učinkovite preventive in kontrolnih pregledov, ki so nujni pri gojenju živali za vzrejne namene.

Ključne besede: prašič; tuberkuloza; bezgavke; tuberkulinski kožni test; PCR