

Risk assessment of mycobacterial infections (human tuberculosis and avian mycobacteriosis) during anatomical dissection of cadavers

M. BARTOS¹, H. PAVLIKOVÁ^{2,3}, L. DVORSKA¹, R. HORVATH⁴, M. DENDIS⁴, P. FLODR⁵, Z. KOLAR⁵, R.T. WESTON^{1,6}, L. PAC², L. MATLOVA¹, I. PAVLIK¹

¹Veterinary Research Institute, Brno, Czech Republic

²Masaryk University, Brno, Czech Republic

³Veterinary and Pharmaceutical University, Brno, Czech Republic

⁴Genex CZ, Ltd., Brno, Czech Republic

⁵Palacky University, Olomouc, Czech Republic

⁶LaTrobe University, Melbourne, Australia

ABSTRACT: The aim of this work was to study the presence of mycobacteria in tissue samples from four cadavers fixed with formalin, and tissue samples from a recently deceased unpreserved individual, who had a history of human tuberculosis infection, undergoing a *post mortem* (cause of death not related to tuberculosis). All were examined for the presence of tuberculous lesions and the specific presence of *Mycobacterium tuberculosis* complex (MTC) and *M. avium* complex (MAC) members by microscopy, culture, and PCR analysis of four genomic elements (IS6110, *mtp40*, IS901, and IS1245). Microscopy examination after the Ziehl-Neelsen staining and culture examination for the presence of mycobacteria were negative in all 22 tissue samples from the four embalmed cadavers. PCR analysis of IS6110 and *mtp40* was positive in tissue samples of tuberculous lesions from the lungs of two embalmed cadavers, and from intact kidney tissue of one of these cadavers. Microscopy and culture examinations of liver and spleen tissues from the unpreserved cadaver were positive for mycobacteria. PCR analysis, specific for *M. avium* subsp. *avium*, was positive in both tissue samples with, and without tuberculous lesions.

Keywords: anatomy education; disinfection; avian tuberculosis; risk assessment; health and safety; zoonosis

Most cadavers used for educational anatomical dissection are obtained from asylum centres. It is possible that some of these cadavers may have a history of tuberculosis infection and consequently, personnel participating in the dissection of these cadavers are exposed to a risk of contracting profession-related mycobacterial infections. The incidence and prevalence of *Mycobacterium tuberculosis* and *M. bovis* infections are very low in human and animal populations within the Central European countries: Croatia, Czech Republic, Hungary, Poland, Slovakia, and Slovenia (Pavlik et al., 2002a,b, 2003, 2005a,b; Erler

et al., 2004; Pavlik, 2006). Of the human infections reported, most are from individuals exposed to poor living conditions such as homelessness, drug addiction, and alcoholism (Grange, 1996; Trnka et al., 1996). Moreover, the expanding pandemic of HIV/AIDS and other immuno-compromising diseases more commonly affect this section of society (Dvorska et al., 2001). Therefore, it is proposed that it is more likely that individuals with this type of background may harbour undiagnosed tuberculosis infections, caused by the members of the *M. tuberculosis* complex (MTC) and/or the *M. avium* complex (MAC).

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Application of Polymerase Chain Reaction (PCR) with primers derived from specific insertion sequences (IS) of mycobacteria, has previously been used to confirm tuberculous infection in archaeological findings, specifically from bones and mummified soft tissues (Salo et al., 1994; Gerszten et al., 2001; Fletcher et al., 2003a,b). IS6110 is an insertion sequence found specifically in *MTC* members (*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. caprae*, *M. bovis* BCG, *M. microti*, *M. pinnipedii*, and *M. canettii*), and can be detected in tuberculous lesions from the lung tissue of infected individuals (Pavlik et al., 2000a; Dvorska et al., 2001; Aranaz et al., 2003; Cousins et al., 2003; Proding et al., 2005).

During anatomical dissection large scale manipulation of the body occurs. Formaldehyde (formalin) may not be sufficient for the complete elimination of viable mycobacteria, especially in tissues with tuberculous lesions (commonly located in the lungs), that contain a high concentration of viable mycobacteria (Kappel et al., 1996). Other tissues, such as calcified tubercles, bone marrow and meninges, which are difficult to preserve with a standard fixative solution (for more details see the Material and methods section), may also harbour viable mycobacteria. Therefore, in cases of undetected mycobacterial infections, personnel, teachers, and students are presented with a risk of infection by mycobacteria (Sterling et al., 2000; Burton, 2003).

Based on these theories, the aim of our study was to assess whether a commonly used preserving agent (formalin) would completely abolish mycobacteria from various tissues. This investigation involved the methods of pathology, histology, direct microscopy, culture examinations, and PCR analysis of *mtp40*, IS6110, IS901, and IS1245, to diagnose the presence of mycobacteria in tissues with, and without tuberculous lesions from different body parts of five cadavers.

MATERIAL AND METHODS

Sample origin and conventional examinations

Tissue samples were examined from four cadavers that were embalmed with formalin. Cadaver No. 1 had a record of lung tuberculosis caused by *M. tuberculosis*, cadaver No. 2 had a record of lung

cancer, and cadavers Nos. 3 and 4 had no record of tuberculosis infection. The embalming procedure involved at least 6 months of total immersion in a formalin preserving solution, a standard embalming practice. The composition of the preserving solution was 100 g of salicylic acid, 250 g of potassium nitrate, 650 g of sodium acetate, 100 ml of formaldehyde, 1 000 ml of glycerol, and 3 000 ml of 96% ethanol *cum benzino* in 10 l of water. Tissue samples from a fifth cadaver were collected during a *post mortem* (not preserved in formalin) from a man who was successfully treated for lung tuberculosis caused by *M. tuberculosis* in his youth, but subsequently died from sporotrichoid leucosis.

Tuberculous lesions were evaluated by a pathology examination of all tissue samples. The presence of viable mycobacteria was assessed by culture after HCl-NaOH decontamination (Fischer et al., 2000), and microscopy using homogenised tissue samples and staining using the Ziehl-Neelsen (Z-N) technique. Tissue samples were homogenised by the Stomacher (Kleinfeld Labortechnik, Gehrden, Germany) and after decontamination, 40 µl aliquots were dispensed into the following medium: two solid egg-based agar media according to Stonebrink, two solid egg yolk agars according to Herrold, and two liquid serum media according to Sula. Mycobacterial growth was checked after the first week, and then every second week for three months. Furthermore, twelve different tissue samples from cadaver No. 5 (Table 1) were examined by histology and stained using the haematoxylin-eosin and Z-N methods.

DNA isolation

DNA was isolated in duplicate from each tissue sample according to Walsh et al. (1991), with the DNA captured onto Chelex-100. Stringent precautions were taken to avoid cross-contamination. Clean protective clothing was worn, with frequent glove changes. A separate pre-PCR clean room with "HEPA filtered air" was used for the preparation of PCR solutions, with a dedicated set of pipettes and protective clothing. Handling of PCR products was performed in second, separate clean room. Pipettes and laboratory surfaces were cleaned with liquid detergents (DNA-OFF, Applied Biosystems, USA), rinsed with ultrapure water, and dried with ethanol before use. Sterile tubes and plugged tips were used.

Table 1. Detection of mycobacteria and mycobacterial DNA in the tissue of five human bodies

Human body			TBC ^a	Z-N	Cul-	IS6110	IS element	
No.	anamnesis	tissue samples	lesions	AFR ^b	ture ^c	(<i>mpt40</i>)	901	1245
1	Lung TBC	lungs (left; tuberculous focus)	+	–	–	+	–	–
		lungs (left; surrounding tissue)	–	–	–	–	–	–
		lungs (right; intact tissue)	–	–	–	–	–	–
		thoracic wall (intact tissue)	–	–	–	–	–	–
		pleura (intact tissue)	–	–	–	–	–	–
2	Lung carcinoma	lungs (right; tuberculoid foci)	–	–	–	–	–	–
		lungs (right; surrounding tissue)	–	–	–	–	–	–
		lungs (left; intact tissue)	–	–	–	–	–	–
		thoracic wall (intact tissue)	–	–	–	–	–	–
		pleura (intact tissue)	–	–	–	–	–	–
3	Not known	lungs (left; tuberculous focus)	+	–	–	+	–	–
		lungs (left; surrounding tissue)	–	–	–	–	–	–
		lungs (right; intact tissue)	–	–	–	–	–	–
		liver (intact tissue)	–	–	–	–	–	–
		spleen (intact tissue)	–	–	–	–	–	–
		kidney (left; intact tissue)	–	–	–	+	–	–
4	Not known	lungs (left; tuberculous focus)	+	–	–	–	–	–
		lungs (left; surrounding tissue)	–	–	–	–	–	–
		lungs (right; intact tissue)	–	–	–	–	–	–
		liver (intact tissue)	–	–	–	–	–	–
		spleen (intact tissue)	–	–	–	–	–	–
		kidney (left; intact tissue)	–	–	–	–	–	–
5	Lung tuberculosis in the youth and sporotrichoid leucosis	lungs (right; intact tissue)	–	–	Nt	–	+	+
		lungs (left; intact tissue)	–	–	Nt	–	+	+
		liver (miliar tuberculosis)	+	+	+	–	+	+
		spleen (miliar tuberculosis)	+	+	+	–	+	+
		kidney (right; intact tissue)	–	–	Nt	–	+	+
		myocardium (intact left ventricle)	–	–	Nt	–	+	+
		myocardium (intact right ventricle)	–	–	Nt	–	+	+
		kidney (left)	–	–	Nt	–	+	+
		cerebral cortex (intact tissue)	–	–	Nt	–	+	+
		skin from forearm (intact tissue)	–	–	Nt	–	+	+
		pancreas (intact tissue)	–	–	Nt	–	+	+
		spinal bone marrow (intact tissue)	–	–	Nt	–	+	+

^atuberculous lesions evaluated by pathology examination^bacid-fast rods evaluated by histology examination and Ziehl-Neelsen staining^cculture examination of tissue samples

Nt = not tested

Amplification of DNA sequences

MTC. The PCR mixtures were prepared from HotStar TaqTM Master Mix (Qiagen, Germany). Amplification with various sets of primers was used for the detection of specific DNA sequences. The IS6110 specific for MTC members was amplified by the one-tube nested PCR system, according to Cave et al. (1991). The gene *mtp40* encoding protein, specific for MTC members, was amplified by a highly sensitive PCR technique according to Del Portillo et al. (1991) and modified to run for 60 cycles.

MAC. The *M. avium* subsp. *avium* of serotypes 1, 2 and 3, and *M. avium* subsp. *hominissuis* of serotypes 4 to 6, 8 to 11, and 21, were typed according to the presence of IS901 and IS1245 (Bartos et al., 2006).

Amplification of IS901 was performed by one-tube-nested PCR, using the outer primers IS901-NP1: 5'-TTA ACA CGA TGA GTC ATG CG-3', IS901-NP2: 5'-GCT TAT CGA TGT CCT TGA TC-3', and inner primers IS901-NP3: 5'-GTA CCC GGC GAA GAC CTG G-3' and IS901-NP4: 5'-AAG TCC AGC AGC CGT GCT G-3'. The primers were designed by the program GeneBase (Applied Maths, Belgium). The PCR mixtures were prepared from HotStar TaqTM Master Mix (Qiagen, Germany), by adding 10 pmol of each primer. The DNA was denatured by incubation at 94°C for 15 min, followed by 16 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 45 s, and elongation at 72°C for 3 min. The second set of one-tube nested PCR was done by repetition of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 68°C for 45 s, and elongation at 72°C for 2 min. The samples were then incubated at 72°C for 3 min to allow complete elongation of the final PCR products. The specific amplicons from the outer and inner primers were 510 and 377 bp, respectively.

For the detection of IS1245, the primers described by Bartos et al. (2006) were used. The PCR mixtures were prepared from HotStar TaqTM Master Mix (Qiagen, Germany), by adding 10 pmol of each primer. The DNA was denatured by incubation at 94°C for 15 min, followed by 60 cycles of denaturation at 94°C for 1 min, primer annealing at 66°C for 45 s, and elongation 72°C at 2 min. The samples were then incubated at 72°C for 2 min to allow complete elongation of the final PCR products. The specific amplicon was 427 bp.

RESULTS

Embalmed cadavers

Tuberculous lesions (tubercles, necroses, and calcifications) were found in lungs of cadavers Nos. 1 (Figure 1), 3, and 4 (Table 1). Only a diffuse lesion was found in cadaver No. 2. Histological examination, using the haematoxylin-eosin staining technique was attempted, but severe tissue degradation made subsequent evaluation of tissue structures impossible and there was no detection of acid-fast rods (AFR), which are indicative of the presence of mycobacteria. Microscopic examination of homogenised tissue samples, prior to decontamination (for culture), and stained according to Ziehl-Neelsen, were also negative for AFR. Culture examinations for *M. tuberculosis* in all 20 tissue samples were negative, however, mycobacterial DNA specific for MTC was detected by the presence of IS6110 and *mtp40*, by PCR analysis of tissue samples taken from lung tissue with tuberculous lesions from cadavers Nos. 1 and 3 (Table 1, Figures 2 and 3).

Unpreserved cadaver

A total of 12 different tissue samples were examined from the cadaver No. 5 (Table 1). Tuberculous lesions and AFR were present in the liver and spleen, from which *M. avium* subsp. *avium*, of genotype IS901+ and IS1245+, was isolated. IS901 (Figure 4) and IS1245 (Figure 5) were detected in all tissue samples, whilst *mtp40* was only detected in spleen tissue (Table 1).



Figure 1. Lung with tuberculous focus (Photo I. Pavlik)

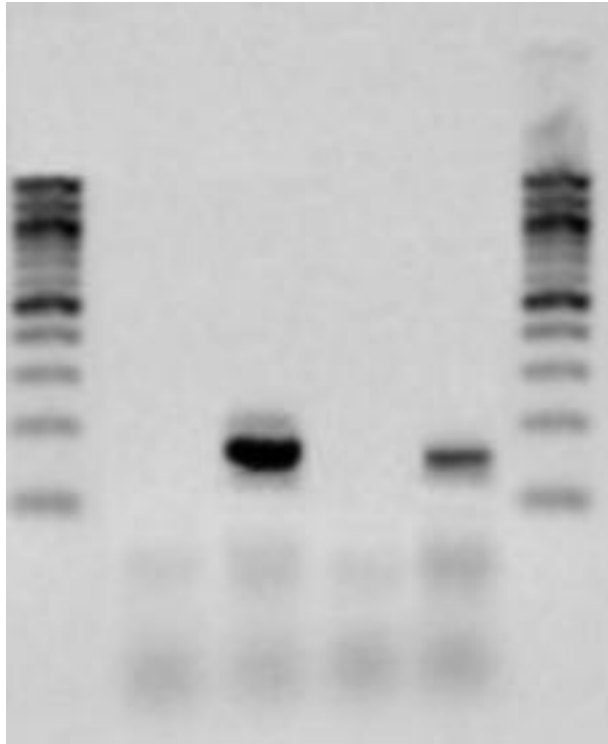


Figure 2. Amplification of *IS6110* by nested PCR of tissue from cadaver No. 1. The amplicon length was 197 bp. Samples from left to right: 100 bp ladder, negative control, positive control, un-lesioned lung tissue (negative reaction) and tuberculous lung tissue (positive reaction), 100 bp ladder (New England Biolabs, USA)

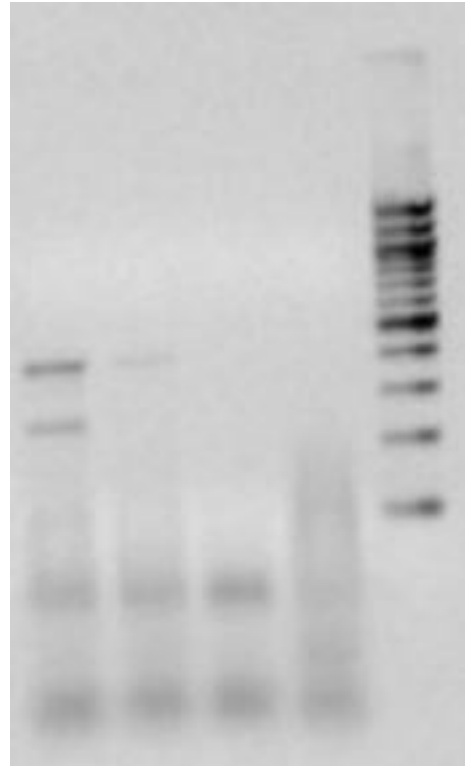


Figure 3. Amplification of gene *mtp40* by PCR of spleen tissue sample from cadaver No. 3. The amplicon length was 396 bp. Samples from left to right: spleen, positive control, negative isolation control, negative PCR control, 100 bp ladder (New England Biolabs, USA)

DISCUSSION

Cadavers for anatomical dissection are commonly obtained from asylum centres, and the disease history of these individuals is usually not available. Therefore, bodies of such individuals may be infected with undiagnosed mycobacterial disease, such as tuberculosis. *M. bovis* in particular, can occur more often in older individuals who have previously worked in the agricultural industry (Pavlik et al., 2003; Prodinger et al., 2005). Patients with compromised immune systems are also particularly susceptible to mycobacterial infections, including those caused by *MAC* members (Pavlik, et al., 2000b; Dvorska et al., 2002; Mijs et al., 2002). Dissections of cadavers with the above life histories, but with undiagnosed microbial infection, pose a risk to professionals and students performing dissections.

The presence of the DNA from *MTC* (positive for *IS6110*) and *M. avium* subsp. *avium* (positive for *IS901* and *IS1245*) in the tissues of three of the cadavers in our study should not be underestimated (Table 1). The DNA detected by PCR may represent intact, but dead mycobacteria, of which the phospholipids' particles of the bacterial wall are capable of irritating the humoral immune system. It has been shown that mycobacteria may survive in human bodies after death (Nolte, 2005), and survive in tissues after long-term preservation (Thoen and Bloonm, 1995; Grange, 1996; Gerston et al., 1998, 2004; Barnes et al., 2000; Park et al., 2003). It is also possible that this DNA may represent viable mycobacteria of this kind, residing in calcified tissue of parenchymatous organs, or in the bone marrow of recovered individuals.

The PCR positive tissues cultured from all four dissected bodies were negative (Table 1), perhaps

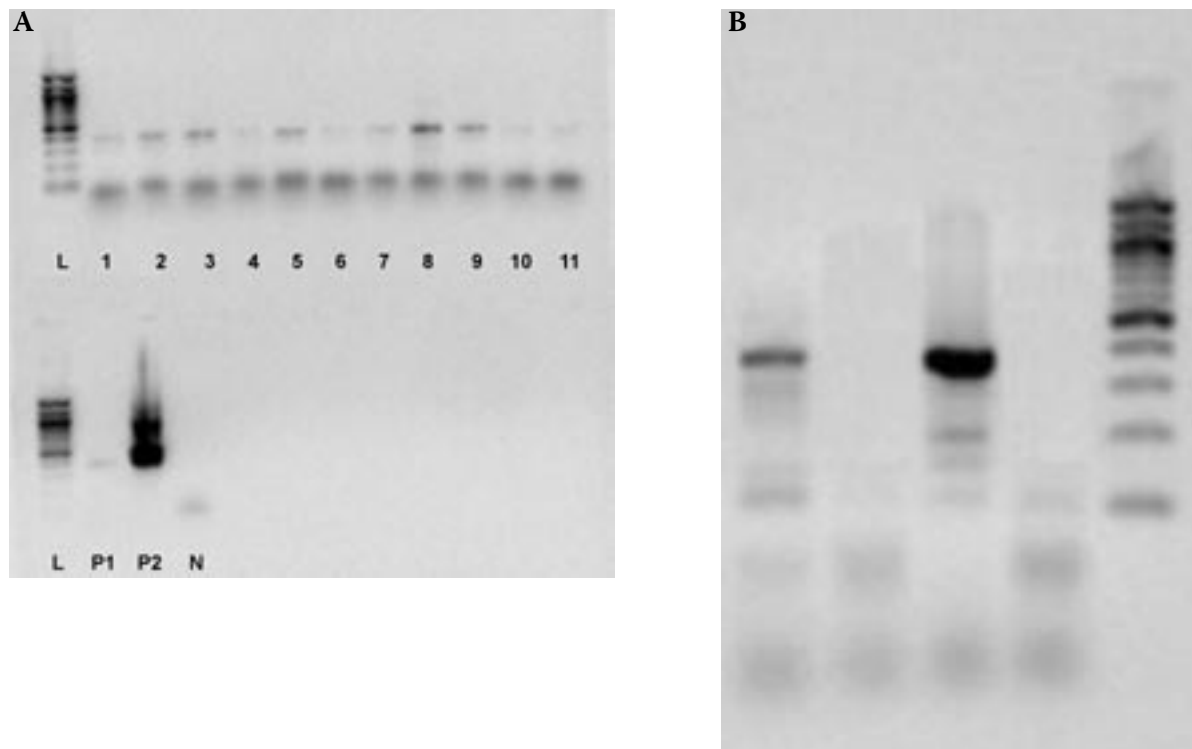
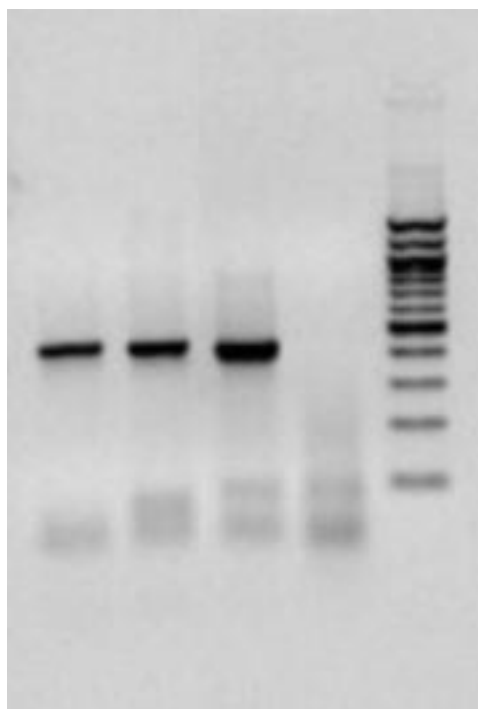


Figure 4. **A.** Amplification of *IS901* by nested PCR of tissue samples from cadaver No. 5. The amplicon length was 377 bp. Lower line: L = 100bp ladder (New England Biolabs, USA), 1 = liver, 2 = lung (right), 3 = lung (left), 4 = myocardium of left heart ventricle, 5 = myocardium of right heart ventricle, 6 = kidney (right), 7 = kidney (left), 8 = cerebral cortex, 9 = skin from forearm, 10 = pancreas, 11 = spinal bone marrow. Upper line: L = 100 bp ladder (New England Biolabs, USA), P1 = positive control 1, P2 = positive control 2, N = negative control. **B.** Amplification of *IS901* by nested PCR of spleen tissue sample from cadaver 5. The amplicon length was 377 bp. Samples from left to right: spleen, negative isolation control, positive control, negative PCR control, 100 bp ladder (New England Biolabs, USA)



due to the likelihood that the decontamination procedure NaOH-HCl (Fischer et al., 2000) was able to abolish positive cultures, even if viable mycobacteria may have been present.

The importance of tissue samples from fixed cadavers, positive for *IS6110* specific for *MTC* is uncertain. However, the possibility remains, that viable mycobacteria are indeed present in the fixed cadavers examined, despite the negative microscopic and culture examinations. Sampling tissue for subculture may not have been extensive enough to cover all sections of the cadaver to ensure all kinds of tissue were thoroughly represented.

The presence of mycobacteria was not demonstrated in microscopic or culture examinations of

Figure 5. Amplification of *IS1245* by high sensitivity PCR of spleen tissue from cadaver 5. The amplicon length was 427 bp. Samples from left to right: spleen, positive control 1, positive control 2, negative PCR control, 100 bp ladder (New England Biolabs, USA)

tuberculous lesions from cadavers Nos. 1, 3, and 4 (Table 1), which signifies either very low levels, or complete elimination of viable mycobacteria. Fragments of mycobacterial DNA may remain in the preserved tissue and skeletal fragments of individuals who were once infected with mycobacteria. However, the size of these fragments rarely exceeds 200 bp (Paabo, 1987), and makes detection of this DNA by PCR amplification very difficult. PCR amplification of IS6110 has been shown to be a very sensitive and useful assay for these kinds of samples (Salo et al., 1994; Fletcher et al., 2003a,b). IS6110 PCR analysis revealed the presence of DNA of *MTC* from cadavers Nos. 1 and 5, which correlates with the anamnesis data (Table 1). It is not clear why a single copy *mtp40* locus was found in the spleen of the non-embalmed body No. 5 when a multicopy locus IS6110 was not found.

Based on our results presented in Table 1, we would like to reiterate the importance all the regulations and rules related to sanitary procedures for the protection of staff and students working with cadavers with tuberculous lesions in the parenchymatous organs.

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Corresponding Author:

Prof. MVDr. Ivo Pavlik, CSc., Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 533 331 601, fax +420 541 211 229, e-mail: pavlik@vri.cz
