Mycobacterium caprae infection in cattle and pigs on one family farm in Croatia: a case report

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ABSTRACT: An outbreak of tuberculosis among bovines and pigs caused by *Mycobacterium caprae* is described in this paper. After tuberculin skin tests with bovine purified protein derivates (PPD) six cattle and one sow, own by a small family farm, tested positive whilst three pigs were suspected in 2004. All animals were euthanised and checked for gross pathological lesions. Generalised lesions were found in five cattle and two sows; however one calf and two gilts had lesions that were localised in the submandibular lymph nodes. Mycobacteria were isolated from tissue samples of six cattle and four pigs. Mycobacterial isolates were identified using classical biochemical tests and molecular methods (PCR, GenoType MTBC) as *M. caprae*. Mycobacterial Interspersed Repetitive Unit (MIRU) typing of isolated mycobacteria showed an identical number of repeats in 12 different loci. Results of the research confirmed the domination of *M. caprae* among infected cattle in Croatia; however this paper was the first to confirm a case of *M. caprae* in pigs. The source of the infection was not found.

Keywords: swine; zoonosis; epidemiology; *Mycobacterium tuberculosis* complex; food safety

Tuberculosis is an infectious disease occurring in several animal species including domestic and wild animals, as well as humans. From an economical point of view, bovine tuberculosis in cattle is of the greatest importance, in comparison to other animal species. Infected animals temporarily or permanently shed mycobacteria by means of excretion. The causal agent of bovine tuberculosis causes infection in cattle by entering the host via a respiratory or oral route (Menzies and Neill, 2000).

Mycobacterium bovis and *M. caprae* are primarily a cattle pathogen; however, they have been isolated from goats, camels, horses, pigs, dogs, and cats amongst other animals including human being (Lepper and Corner, 1983; Erler et al., 2004; Prodinger et al., 2005; Thoen et al., 2006). Pigs usually acquire infection by the consumption of unpasteurised milk or by-products of milk processing from infected cows and unsterilised by-products from slaughterhouses. An incident was recorded on a family farm in the Czech Republic, where *M. caprae* was transmitted from cow's milk to domestic pigs by ingestion (Pavlik et al., 2002).

Bovine tuberculosis is an economical and public health threat in developing countries. In countries with no systematic bovine tuberculosis eradication control programmes, most cases of infection are diagnosed in young children. This is the result of raw milk being drank and is often manifested in cervical lymphadenopathy (Thoen et al., 2006).

Although bovine tuberculosis has been controlled for several decades in developed countries, complete eradication has not been achieved. Active animal tuberculosis outbreaks represent possible

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sources of infection for animal and human populations (Ayele et al., 2004; Thoen et al., 2006). Systemic control of bovine tuberculosis in Europe and North America has succeeded in eradicating or reducing the infection of cattle and the human populations, through the elimination of positive skin testing reactors for bovine tuberculin and the pasteurisation of milk (Milian-Suanzo et al., 2000; Thoen et al., 2006).

Systematic eradication control programmes have been implemented in former Yugoslavia to fight tuberculosis due to its spread among animals and the zoonotic character of the disease (Kucel and Tunkl, 1946). The first systematic attempts to eliminate bovine tuberculosis in Croatia were carried out in 1946. Success was achieved and a follow-up campaign was started in 1953 (Tunkl, 1954).

Reported status and incidence of bovine tuberculosis in Croatia and other central European countries (Slovakia, the Czech Republic, Slovenia, Poland and Hungary) has shown good results of control programmes not only in domestic but also in wild animals (Cvetnic et al., 2000a,b; Milian-Suanzo et al., 2000; Machackova et al., 2003; Pavlik et al., 2005; Pavlik, 2006; Trcka et al., 2006). The last confirmed case of bovine tuberculosis in pig in Croatia was described in 1972 (Aleraj et al., 1972). Later investigations in pigs found infections caused especially by members of *M. avium* complex (*MAC*) and opportunistic mycobacteria i.e. *M. fortuitum* and *M. chelonae* (Cvetnic et al., 1998, 2006; Matlova et al., 2005). In 1992 bovine tuberculosis was detected in wild boar in Croatia (Machackova et al., 2003).

On one Croatian pig farm, infected with bovine tuberculosis, Tunkl (1952) found 22.8% of positive reactors to bovine tuberculin in pigs. Francetic et al. (1958) diagnosed bovine tuberculosis in 13 pig's lymph nodes. The most recent case of bovine tuberculosis in domestic pig was described in Croatia in 1972 (Aleraj et al., 1972) and in wild boar in the Forestry Pozega in 2004 (Pavlik et al., 2005). Subsequent findings of tuberculous lesions, especially in lymph nodes, were caused by members of *MAC* and/or by opportunistic mycobacteria (Cvetnic et al., 1998, 2006).

Bovine tuberculosis was diagnosed in wild boar lymph nodes in Croatia, Slovakia and Hungary between the years 1983 to 2001; the source of the infection in Croatia remains unclear. In Slovakia and Hungary, the wild boar populations were infected by contact with infected cattle and/or sheep in pastures (Machackova et al., 2003). Bollo et al. (2000) described tuberculous lesions in wild boar in Italy which was caused by members of the *M. tuberculosis* complex (*MTC*). Parra et al. (2003) found *M. bovis* in pigs of Iberian breed and in wild boar. Bovine tuberculosis was diagnosed in pigs in Poland with a history of contact with infected cattle with bovine tuberculosis. In other animals than cattle, bovine tuberculosis was confirmed in sheep and dogs and relatively often in different species of wild animals in zoological gardens (Hermoso de Mendoza et al., 2006).

MTC species *M. tuberculosis, M. bovis, M. africanum* and *M. microti* cause human tuberculosis (Wayne and Kubica, 1986; Spargo et al., 1993). *M. canettii* was rarely diagnosed in humans and has recently been added to the *M. tuberculosis* complex (Van Soolingen et al., 1997). *M. pinnipedii* was described in 2003 (Cousins et al., 2003) and one case of human infection has been published (Thoen et al., 2006). According to novel taxonomy *M. bovis* was divided to *M. bovis* subsp. *bovis* and *M. bovis* subsp. *caprae*; the currently accepted taxonomy for these two members of *MTC* is *M. bovis* and *M. caprae* (Aranaz et al., 2003). The major phenotype characteristic for these two members is the susceptibility to pyrazinamide (PZA) (Aranaz et al., 1999; Niemann et al., 2000). *M. bovis* and *M. caprae* can only be differentiated by molecular biology methods like spoligotyping and MIRU analysis (Kremer et al., 1999; Erler et al., 2004; Prodinger et al., 2005).

Using this taxonomy, *M. caprae* was confirmed among *MTC* isolates from infected cows in Croatia in 2001. The same paper also stated *M. caprae* as the dominant species in cattle in central European countries (Erler et al., 2004). Prodinger et al. (2002) described infections caused by *M. caprae* in cattle, humans and red deer (*Cervus elaphus*) in Austria. Kubica et al. (2003) confirmed in German patients that 69% of cases of bovine tuberculosis were caused by *M. bovis* while 31% of isolates were identified as *M. caprae*.

The aim of the study was to describe the spread of *M. caprae* in cattle and pigs from an outbreak on a small farm in Croatia.

MATERIAL AND METHODS

Based on the guidelines of the State Veterinary Administration, skin testing with bovine tuberculin must be performed on all cattle populations in a

particular country, every three years. Positive reactions to bovine tuberculin were detected in 2004 on a small family farm.

Anamnestic data of farmer's family

The small family farm belonged to a 71 year old man and 68 year old woman. All seven cattle (3 cows, 1 heifer, 1 bull and 2 calves) were of Simmental breed and the seven crossbreed pigs (2 sows, 2 gilts and 3 fattening pigs) were bred together on this farm with the cattle. All animals were aged between three months and three years.

People living on the farm were subjected to epidemiological and laboratory observation using a tuberculin skin test with Mantoux (Statens Serum Institute, Copenhagen, Denmark), radiological and bacteriological examinations. Tuberculin skin tests were negative, radiology findings were normal and no family members who had been on the farm were found to be infected with bovine tuberculosis.

Examination of animals

Tuberculin skin test. According to Croatian legislation the intradermal tuberculin monotest (0.1 ml *per dosis*) bovine (50 000 IU/ml) purified protein derivate (PPD) is prescribed for detection of infected cattle. Repeated comparative testing using avian (20 000 IU/ml) and bovine (50 000 IU/ml) PPD must be simultaneously carried out on reactors and suspected animals eight weeks after the initial tuberculin skin test. All 14 animals were skin tested with bovine and avian tuberculin (Pliva, Zagreb, Croatia).

Bacteriological examination. All animals from the infected farm were slaughtered, gross pathological examination was carried out and samples of the parenchymatous organs and lymph nodes were collected for laboratory examination. Tissue samples from animals were cut into pieces, decontaminated, homogenised and inoculated on Löwenstein-Jensen, Stonebrink and liquid Middlebrook nutrient media.

Identification of mycobacterial isolates by Polymerase Chain Reaction

DNA isolation from cultivated mycobacteria. A loop of mycobacteria containing one to three CFU was suspended in 50 µl of distilled water, warmed at 99°C for 20 min and periodically mixed in a thermo mixer. The suspension was centrifuged at 16 000 rpm for 5 min and then cooled to room temperature. For further examination the supernatant was used.

Identification of cultivated mycobacteria. Identification was carried out using primers TB1: 5'- GAG ATC GAG CTG GAG GAT CC-3' and TB2: 5'- AGC TGC AGC CCA AAG GTG TT-3'. The reaction mixture contained Amplitaq Gold DNA polymerase, (1 U/sample; Applied Biosystems, USA), 4 µl of each 200 µM deoxinucleotide, 5 µl of buffer, 4 µl of $MgCl_2$ (Applied Biosystems, USA) and 5 µl of supernatant containing the DNA from the isolated mycobacteria. PCR cycles were set up as follows:

– initial polymerase activation for 5 min at 95°C
– 33 cycles of amplification were set at: 30 s at 94°C, 30 s at 60°C, 60 s at 72°C
– chain elongation for 5 min at 72°C
– the reaction was stopped at 4°C (GeneAmp PCR System 2700, Applied Biosystems, USA)
– the expected size of the multiplied fragment using the above mentioned primers was 383 bp (Hance et al., 1989)

M. tuberculosis complex isolate identification

Biochemical identification. Selected biochemical tests including the niacin test, nitrate reduction, Tween hydrolysis, culture on Stonebrink medium, susceptibility to thiophen-2-carboxylic acid hydrazide (TCH) and PZA and growing in Lebec medium were used (Wayne and Kubica, 1986).

PCR. Part of the insertion sequence of IS*6110* specific for members of *MTC* was multiplied using the primers IS1: 5'-CCT GCG AGC GTA GGC GTC GG and IS2: 3'-CTC GTC CAG CGC CGC TTC GG (Eisenach et al., 1990). The reaction mixture contained the same ingredients as mentioned above to a final volume of 50 µl. The multiplication procedure was similar except for:

– 33 cycles of amplification set at: 20 s at 94°C, 20 s at 58°C, 20 s at 72°C
– chain elongation for 2 min at 72°C
– the reaction was stopped at 4°C (GeneAmp PCR System 2700, Applied Biosystems, USA)
– the expected multiplication product size using such a procedure was 123 bp. Visualisation of multiplication products in both procedures was performed via electrophoresis in 2% agarose gel

using a UV transilluminator and camera (Bio-Capt, Vilbert Lourmat, France).

Geno Type® MTBC (Hain Lifescience, Germany). Examination was carried out in order to differentiate between members of the *MTC* (*M. africanum* I, *M. bovis* BCG, *M. bovis*, *M. caprae*, *M. microti*, *M. tuberculosis*/*M. africanum* II, and *M. canettii*) based on the polymorphism of the gene coding for gyrase B. Test procedures included the isolation of DNA from cultured mycobacteria and multiplication using biotinilated primers and reverse hybridisation. Hybridisation included the chemical denaturation of the multiplication product, the hybridisation of one chain biotinilated products using a probe and addition of the conjugate streptavidine alkaline phosphatase and evaluation of colourisation. Differentiation of *MTC* members was performed by the comparison of colourisation in 13 different zones on each membrane along with a standard.

MIRU genotyping. Twelve out of a total of 41 MIRU loci within the genome were chosen to genotype *M. bovis* isolated from bovine and pigs (namely 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40). Mycobacterial DNA was isolated, amplified using HotStarTaq® DNA polymerase (QIAGEN, Germany) and visualised using GelDoc 2000 (BioRad, Germany) after electrophoresis in a 3% agarose gel (Supply et al., 2001).

RESULTS

Intravital diagnostics

Tuberculin skin testing. During the annual tuberculin skin testing using bovine tuberculin, positive reactions were detected on a small family farm with cattle and pigs. Eight weeks later the animals were retested with comparative skin testing using avian and bovine tuberculin. Positive reactions to bovine tuberculin were confirmed in six of the seven cattle and in one of the seven pigs (Figure 1); in three pigs suspected reactions were observed (Table 1).

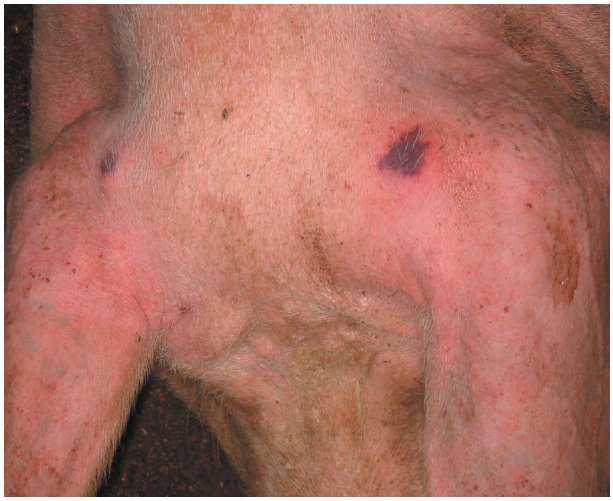

Figure 1. Positive skin reaction in sow No. 80960: on the left reaction to avian tuberculin, on the right reaction to bovine tuberculin (Photo Z. Cvetnic)

Post mortem diagnostics

Gross pathology examination. All 14 animals were slaughtered and the meat inspection revealed mainly generalised tuberculous lesions. Four infected cattle (Figures 2a,b and 3) and two pigs/sows (Figures 4 and 5) had visible tuberculous lesions; tuberculous lesions were localised in only one

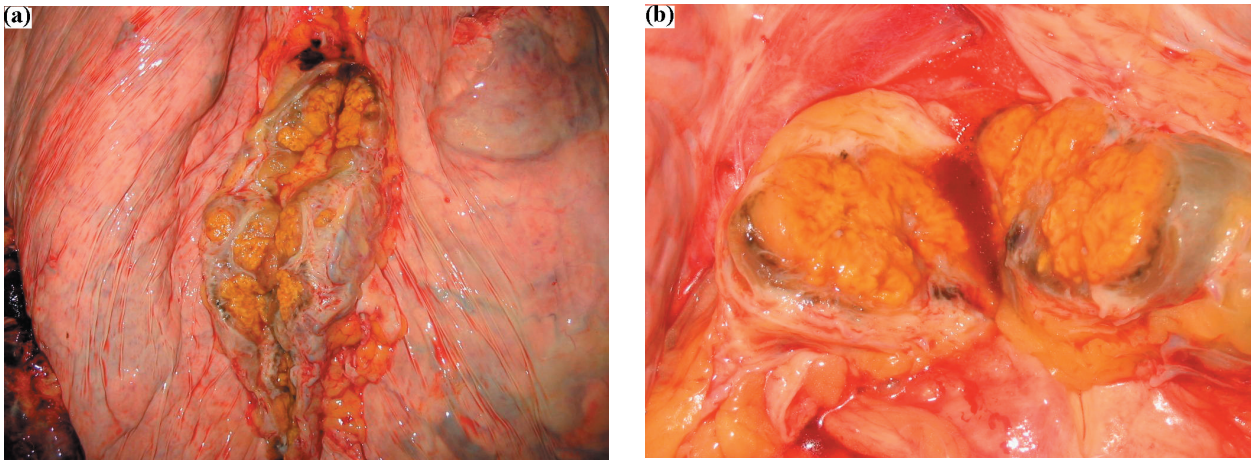

Figure 2. Tuberculous lesion in lungs of cow (Photo Z. Cvetnic). a – cow No. 85514; b – cow No. 65248

Table 1. Skin testing with bovine tuberculin and pathological examinations on the infected farm

Animal			Lymph nodes												Tissue of								
Species/ Cathegory	ID	Skin test[1]	head			mesenteric			mediastinal			portal			lung			liver			udder		
			PA	ZN	C	PA	ZN	C	PA	ZN	C	PA	ZN	C	PA	ZN	C	PA	ZN	C	PA	ZN	C
Cow	85514	+	+	+	+	–	–	–	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–
	65246	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
	65248	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	–
Bull	39134	+	+	+	+	–	–	–	+	+	+	–	–	–	+	+	+	–	–	–	Nt	Nt	Nt
Heifer	39135	+	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	+	+	+	Nt	Nt	Nt
Calf	39136	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
	39859	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
Sow	80960	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
	80962	±	+	+	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Gilt	80964	±	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
	80963	±	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
Pig	80961	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
	80959	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
	80958	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Nt	Nt	Nt
Total	+	7	9	7	9	3	2	3	6	6	6	5	2	3	4	3	4	2	2	2	1	1	1
	±	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	–	4	5	7	5	11	12	11	8	8	8	9	12	11	10	11	10	12	12	12	4	4	4

[1]Skin test with bovine tuberculin (Pliva, Zagreb, Croatia): + postive reaction, ± dubious reaction, – negative reaction

ID = identification number of animal

PA = pathological examination: + tuberculous lesions, ± adenopathy, – without lesions

ZN = detection of acid-fast rods after the Ziehl-Neelsen staining in homogenised tissues before the culture examination

C = culture examination for the presence of mycobacteria

Nt = not taken

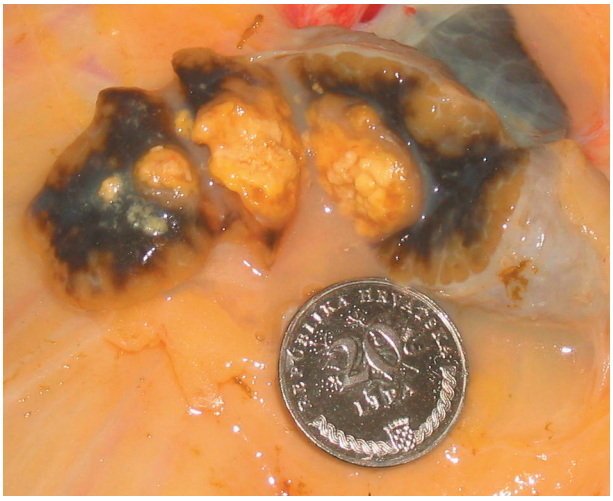

Figure 3. Tuberculous lesion in mesenteric lymph nodes of cow No. 65248 (Photo Z. Cvetnic)

calf and two gilts in submandibular lymph nodes (Table 1).

Bacteriological examination and biochemical identification of mycobacterial isolates. Mycobacteria were isolated from 6 cattle and 4 pigs (Table 1). Isolates were grown on Stonebrink medium and CFU were smooth, colourless and eugonic. Biochemical characterisation found niacin tests to be negative, negative nitrate reduction, growth on Stonebrink medium and susceptibility toward PZA. Therefore isolated mycobacteria were designated as *M. bovis*.

PCR identification. Confirmation of mycobacteria was performed by amplification of the 65 kDa antigen specific DNA sequence coding for members of the genus *Mycobacterium*. The amplification product size was 383 bp in all isolates. Further examination using PCR confirmed all isolated strains as members of the *MTC*. The amplification product size was 123 bp (Figure 6). The hybridisation procedure (GenoType) revealed that all isolates belonged to *M. caprae.*

MIRU typing. All isolates analysed by PCR (Figure 6) were of identical MIRU type with the same number of repeats in loci, namely: 2 (2), 4 (3), 10 (6), 16 (4), 20 (2), 23 (4), 24 (2), 26 (5), 27 (3), 31 (5), 39 (2) and 40 (2).

DISCUSSION

Our results confirm the continual presence of *M. caprae* in Croatia first described in cattle isolates in 2001 (Erler et al., 2004). The pathogenicity of *M. caprae* for cattle and for domestic pigs was described (Table 1) as previously in the Czech Republic (Pavlik et al., 2002).

Infected cows shed causal agent of bovine tuberculosis especially through milk and lung expectorations. In main cases, the human population can be infected by consumption of raw milk and cheese produced from raw milk (Thoen et al., 2006). The people living on the farm in this study were not infected. This was perhaps due to a short period of contact with *M. caprae* infection in the reared cattle and pigs, also due to consumption of properly heated (boiled) milk.

In Croatia, only eleven cases of bovine tuberculosis were diagnosed in human patients between the years 1990 to 1997. The prevalence of bacteriologically confirmed bovine tuberculosis in human patients was only 0.02% during that period

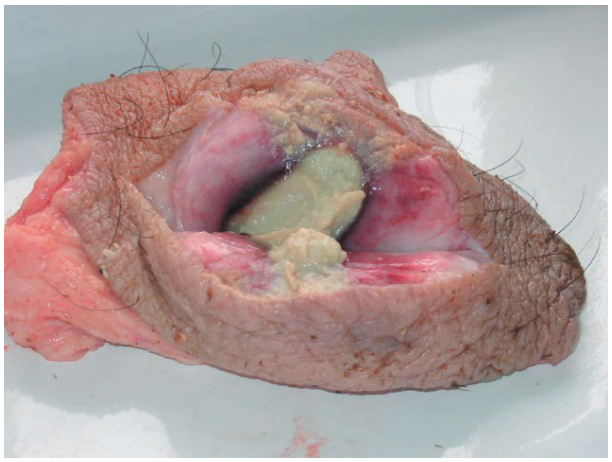

Figure 4. Tuberculous lesion in mammary gland of sow No. 80960 (Photo Z. Cvetnic)

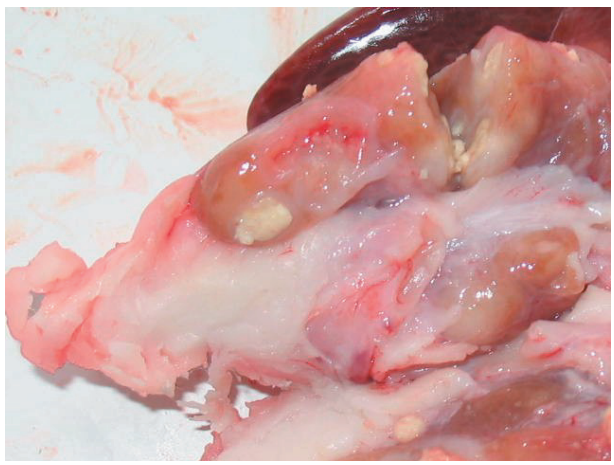

Figure 5. Tuberculous lesion in mesenteric lymph nodes of sow No. 80962 (Photo Z. Cvetnic)

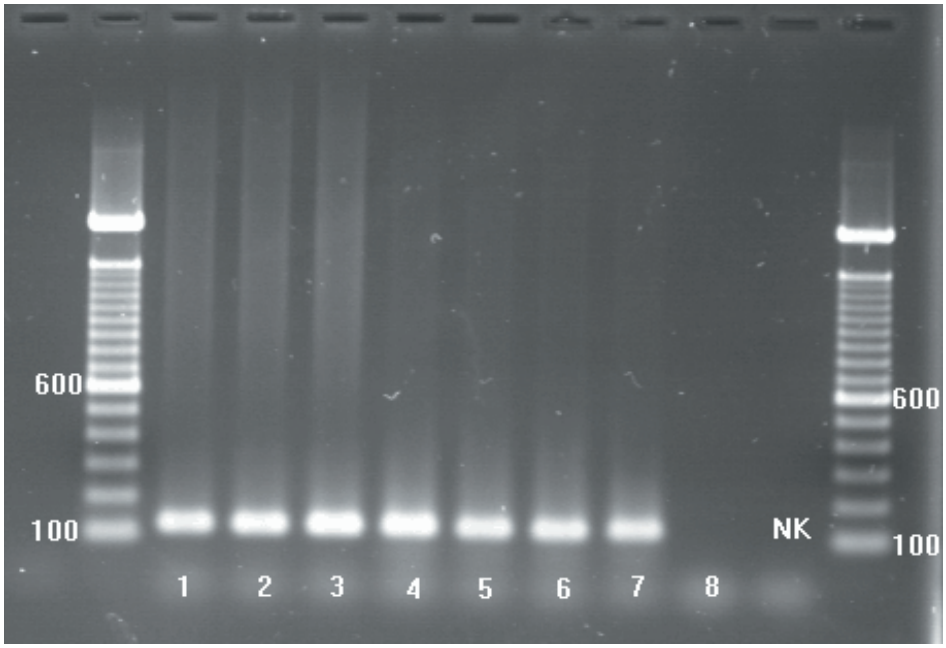

Figure 6. Identification of *Mycobacterium tuberculosis* complex isolates by PCR

Legend (from the left):

Ladder; 1 – isolate from cow No. 85514;, 2 – isolate from cow No. 65246; 3 – isolate from heifer No. 39134; 4 – isolate from sow No. 80962; 5 – isolate from sow No. 80960; 6 – isolate from gilt No. 80964; 7 – *Mycobacterium bovis* (standard); 8 – *Mycobacterium avium* serotype 1; NK – negative control; ladder

(Cvetnic et al., 2000a). Human cases of the infection in regions free of bovine tuberculosis, including central European countries, are usually the result of reactivation of previous infections in elderly people (Pavlik et al., 2003; Thoen et al., 2006). Due to this fact, it is an important preventive measure to examine the farmer and his family during the consecutive years for bovine tuberculosis.

The current epidemiological situation regarding bovine tuberculosis in the human population in Croatia is stabilised. Bovine tuberculosis in wildlife, especially in wild boar, highlights the possibility of the introduction of infection to cattle and pig farms in the near future. The final conclusion of our research is that the control programme for the eradication of bovine tuberculosis in Croatia should be continued.

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