The last outbreak of bovine tuberculosis in cattle in the Czech Republic in 1995 was caused by *Mycobacterium bovis* subspecies *caprae*

I. Pavlik[1], F. Bures[2], P. Janovsky[2], P. Pecinka[3], M. Bartos[1], L. Dvorska[1], L. Matlova[1], K. Kremer[4], D. Van Soolingen[4]

[1]Veterinary Research Institute, Brno, Czech Republic
[2]District Veterinary Administration, Zdar nad Sazavou, Czech Republic
[3]Practical Veterinarian, Zdar nad Sazavou, Czech Republic
[4]National Institute of Public Health and the Environment, Bilthoven, The Netherlands

ABSTRACT: The last outbreak of bovine tuberculosis in cattle in the Czech Republic was detected in 1995. Signs of diarrhoea, weight loss and occasional coughing appeared in one 14-year-old cow after giving birth for the thirteenth time. Two months after these symptoms had been observed, it had to be slaughtered and numerous tuberculous lesions were found in its lung tissue, including the pleura. Within three months after the confirmation of the infection and consecutive *intra-vitam* and *post-mortem* diagnostics, all 28 remaining head of cattle from the herd (nine cows, seven bulls, six heifers and six calves) and five pigs were slaughtered. Patho-anatomical lesions were detected in all animals indicative of tuberculosis, from which *Mycobacterium bovis* was cultured and identified on the basis of biochemical tests and virulence test in a guinea-pig. The culture of 33 samples of other biological material than tissues (milk and urine of cows, feeding water, scrapings from the shed, fodder and others) resulted in *M. bovis* being detected in three samples (scrapings from shed walls). By the spoligotyping method *M. bovis* subsp. *caprae* was found in six selected isolates originating from two cows, two heifers and two bulls. It may therefore be assumed that there was one source of infection in the herd, which was the first infected old cow. In comparison with 3 176 spoligotypes in the existing database RIVM (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) and literary data it was found that this spoligotype was also found in Sweden, Belgium, Great Britain, Spain, Poland, Germany and the Czech Republic. It was impossible to determine the source of *M. bovis* subsp. *caprae* of the first infected cow on the basis of results from database and from anamnestic data. Green fodder coming from the farmer's pastures near a forest could be considered as a possible source of *M. bovis* from wild ruminants like red deer (*Cervus elaphus*), which was found infected with bovine tuberculosis in another district of the Czech Republic in 1991.

Keywords: spoligotyping; stable environment; pig

The programme of bovine tuberculosis control in the Czech Republic was completed in 1968 (Polak, 1969). In the last decade of the last century, isolations of the causal agent of bovine tuberculosis *Mycobacterium bovis* in animals and human beings in the Czech Republic were only sporadic (Kovarik *et al.*, 1995; Havelkova *et al.*, 1998; Pavlik *et al.*, 1998a,b, 2002b,c,d,e). The last outbreak of bovine tuberculosis in cattle was found in the district Zdar nad Sazavou in 1995 (Pavlik *et al.*, 2001).

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According to the documented data (Table 1), since 1996 bovine tuberculosis has not been detected in animals in the Czech Republic apart from one incident of *M. bovis* (*M. bovis* subsp. *caprae*) in a farmed deer (*Cervus elaphus*) in May 1999 (Statistical data of State Veterinary Services of the Czech Republic; Machackova *et al.*, 2000; Pavlik *et al.*, 2002c,e). This infected red deer was an offspring of a hind caught in the wild and a buck originating from a zoological garden in Ostrava. The source of *M. bovis* subsp. *caprae* infection remains unclear (Pavlik *et al.*, 2002a).

In the district Zdar nad Sazavou, which covers 1 671 km^2 of a total of 78 864 km^2 of the Czech Republic, bovine tuberculosis in cattle was eradicated as early as in 1964 (Juranek, 1965). Since then *M. bovis* has not been diagnosed in this district in any herd nor in any other wild animal (Statistical data of State Veterinary Services of the Czech Republic, Pavlik *et al.*, 1998a,b, 2002e).

The method used to study the molecular epidemiology of bovine tuberculosis is known as spoligotyping (spacer oligotyping) (Kamerbeek *et al.*, 1997). It is based on DNA polymorphism in chromosomal locus in *M. tuberculosis* complex strains characterised by the presence of conservative direct repeats, interspaced by unique spacer sequences (Hermans *et al.*, 1992). This typing method can be used both to recognise particular sub-species within the *M. tuberculosis* complex and to distinguish isolates on the strain level (Aranaz *et al.*, 1996, 1998, 1999; Kremer *et al.*, 1999; Dvorska *et al.*, 2001; Rastogi *et al.*, 2001; van Soolingen, 2001),therefore it is possible to distinguish the following species, subspecies and types of strains using this method: *M. tuberculosis* (Thierry *et al.*, 1990), *M. tuberculosis* Manila genotype (van Soolingen *et al.*, 1995), *M. tuberculosis* Beijing genotype (van Soolingen *et al.*, 1995), *M. tuberculosis* seal genotype (Zumarraga *et al.*, 1999), *M. africanum*

Table 1. Occurrence of *M. bovis* infection in animals in the Czech Republic during 1990–2001 (from: Pavlik *et al.*, 1998a,b, 2002b,c,e; Machackova *et al.*, 2000); statistical data from State Veterinary Administration, Prague, Czech Republic)

Year	*Mycobacterium bovis*					
	total number of out- breaks	cattle farms		other animals than cattle		
		small <9 cows	large ≥10 cows	number	species	origin
1990	0	0	0	0		
1991	2	1	1	1	red deer[1]	nature
				1	European wild goat[2]	game park
1992	2	0	2	1	Bactrian camel[3]	circus
1993	0	0	0	1	bison[4]	ZOO-A
1994	2	0	2	1	tapir[5]	ZOO-B
1995	1	1	0	2	tapir[5]	ZOO-B
				5	domestic pig[6]	farm[6]
1996	0	0	0	0		
1997	0	0	0	0		
1998	0	0	0	0		
1999	0	0	0	1	red deer[1]	farm
2000	0	0	0	0		
2001	0	0	0	0		
Total	7	2	5	13		

Explanations:

[1]red deer (*Cervus elaphus*), [2]European wild goat (*Capra aegagrus*), [3]Bactrian camel (*Camelus ferus*), [4]bison (*Bison bison*), [5]tapir (*Tapirus terrestris*), [6]domestic pig (*Sus scrofa* f. *domestica*) reared on infected farm and fed cow's raw milk

(Viana-Niero *et al.*, 2001), *M. bovis* (Karlson and Lessel, 1970), *M. bovis* BCG (van Soolingen *et al.*, 1992), *M. bovis* subsp. *caprae* (Gutierrez *et al.,* 1997; Aranaz *et al.*, 1999; Niemann *et al.*, 2002), *M. microti* type vole and *M. microti* type llama (van Soolingen *et al.*, 1998), and *M. canettii* (van Soolingen *et al.*, 1997).

The first *M. bovis* subsp. *caprae* isolates were characterised biochemically and genetically from caprine pathological tissue samples isolated in Spain (Aranaz *et al.*, 1999). The only obvious biochemical character that differentiates the *M. bovis* subsp. *caprae* subtype from other *M. bovis* isolates is susceptibility to pyrazinamide (PZA), which is due to the lack of a single point mutation in the *pnc*A gene (Niemann *et al.*, 2002). However, susceptibility to PZA among clinical isolates of *M. bovis* was reported previously and can now be explained by a PZA-susceptible subspecies of *M. bovis* (Niemann *et al.*, 2002). These subtypes are reliably differentiated by spoligotyping: *M. bovis* subsp. *caprae* is characterised by the absence of spacers 1, 3 to 16, 39 to 43. *M. bovis* is characterised by the absence of spacers 3, 9, 16 and 39 to 43 (Dvorska *et al.*, 2001).

The aim of our study was to describe the course and control of bovine tuberculosis in the last outbreak in cattle in the Czech Republic and to identify the causal agent of this infection in greater detail.

MATERIAL AND METHODS

Characteristics of the farm

On February 7, tuberculous lesions were detected in the respiratory tract of a slaughtered cow. The next day, 28 head of cattle (later, on March 22, 1995 another calf was born), five pigs, ten rabbits and one medium-sized five-year-old dog of undefined breed were secured on the farm for observation. Before the outbreak was diagnosed, milk was daily delivered to a dairy. Annually, the farmer produced about five fattened bulls for slaughter. The remaining milk was fed to pigs on the farm, out of which on average five animals were slaughtered annually in a domestic slaughterhouse. The cattle herd of three to five cows came into existence many years ago. All animals were originally bred on the farm. From 1987, eight heifers and one four-year-old cow were bought. In the years 1993 to 1994 the shed was extended and rebuilt.

Origin of the infected cow in the herd. Slaughtered cow No. 24213 with a tuberculous finding in the thoracic cavity (lung tissue, pleural lymph nodes and pleura) descended from the original herd. It was the oldest cow that gave birth to 13 calves (eight bulls and five heifers) with time intervals of 11 to 12 months in between.

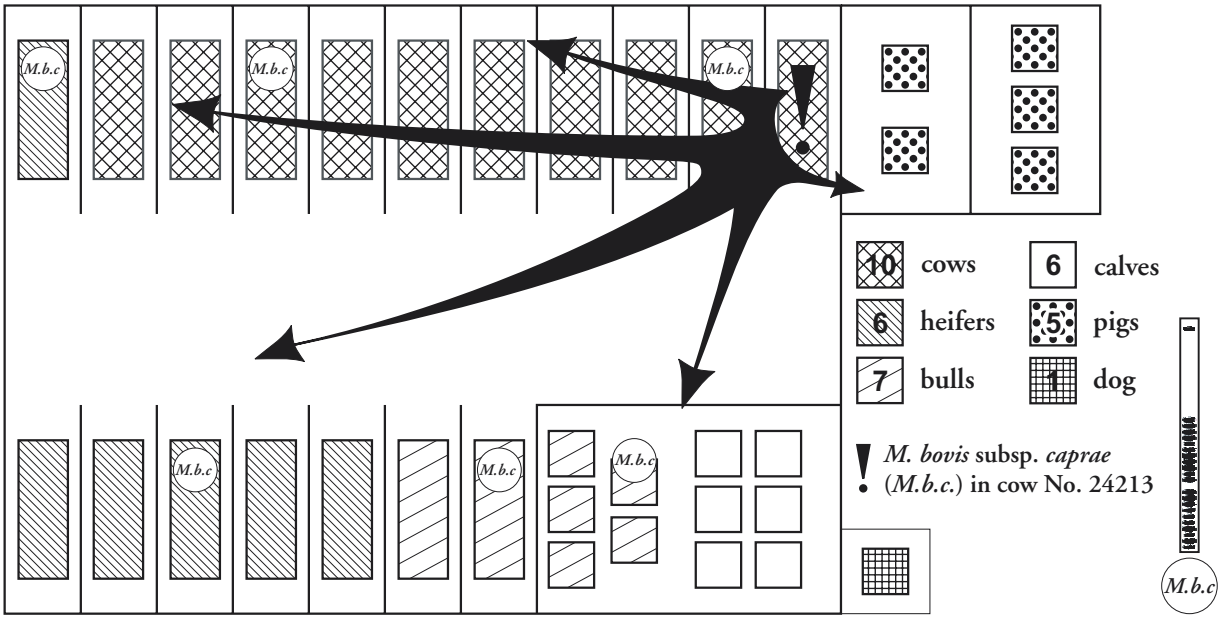

Figure 1. Spread of bovine tuberculosis on an infected farm and spoligotyping results of selected isolates

Table 2. *Intra-vitam* and *post-mortem* diagnostics of bovine tuberculosis in cattle from the outbreak described

Animal		Date of		Skin test (mm)					
category	No.	birth	emergency slaughter	28.10.1994		8.2.1995		21.3.1995	
				BT	AT	BT	AT	BT	AT
Cow	24213	8.6.1981	7.2.1995	0	0	nt	nt	nt	nt
	06575	13.7.1987	3.4.1995	0	0	17.0	6.0	26.0	11.0
	43632	8.5.1988	3.4.1995	0	0	13.0	3.0	12.0	3.0
	64781	6.5.1990	3.4.1995	0	0	9.0	8.0	2.5	1.0
	46318	21.12.1988	3.4.1995	0	0	4.0	13.0	10.8	8.0
	41218	2.12.1988	3.4.1995	0	0	13.0	8.0	16.0	8.0
	61032	?.7.1987[1]	3.4.1995	0	0	15.0	5.0	12.5	5.0
	46526	6.2.1989	3.4.1995	0	0	max.	4.0	27.0	6.0
	4500	23.5.1988	22.2.1995	0	0	12.0	3.0	nt	nt
	09231	?.7.1988[2]	3.4.1995	0	0	9.0	2.0	9.0	4.0
Heifer older than 6 months	16836	2.4.1994	3.4.1995	nt	nt	28.0	6.0	8.0	6.5
	16837	?.6.1994	22.2.1995	nt	nt	11.0	3.0	nt	nt
	37817	?.8.1993[3]	1.3.1995	nt	nt	16.0	16.0	nt	nt
	1	?.5.1994	22.2.1995	nt	nt	11.0	5.0	nt	nt
	2	?.5.1994	3.4.1995	nt	nt	23.0	5.0	23.0	4.0
	3	20.10.1994	3.4.1995	nt	nt	0	0	14.5	1.5
Bull for fattening (more than 300 kg)	1	?.11.1993	22.2.1995	nt	nt	max.	8.0	nt	nt
	2	?.12.1993	1.3.1995	nt	nt	17.0	1.0	nt	nt
	3	?.2.1994	1.3.1995	nt	nt	23.0	0	nt	nt
	4	?.2.1994	1.3.1995	nt	nt	18.0	6.0	nt	nt
	5	?.4.1994	3.4.1995	nt	nt	15.0	9.0	12.0	3.0
	6	11.3.1994	3.4.1995	nt	nt	19.0	3.0	8.5	2.0
	7	22.4.1994	3.4.1995	nt	nt	28.0	6.0	21.0	9.0
Calf	bull 1	12.8.1994	3.4.1995	nt	nt	19.0	0	20.0	1.3
	heifer 2	2.12.1994	3.4.1995	nt	nt	0	0	13.0	7.5
	heifer 3	7.12.1994	3.4.1995	nt	nt	15.0	4.0	21.0	5.0
	bull 4	23.7.1994	3.4.1995	nt	nt	14.0	4.0	4.5	0.8
	bull 5	21.9.1994	3.4.1995	nt	nt	27.0	4.0	16.0	4.8
	heifer 6	22.3.1995	3.4.1995	nt	nt	nt	nt	nt	nt

Explanations:

BT = bovine tuberculin Bovitubal (14 000 TU/ml, 0.2 ml *pro dosi*, Bioveta Ivanovice na Hane, Czech Republic), **AT** = avian tuberculin Avitubal (14 000 TU/ml, 0.2 ml *pro dosi*, Bioveta Ivanovice na Hane, Czech Republic), **+** positive, **+–** suspicious, **–** negative, **?** = not exactly known, **nt** = not tested, **max.** = non–measurable skin reaction, **ACCU** = ACCU– PROBE (Gen– Probe Incorporated, San Diego, California, USA), ***MTB*** = probe for the detection of *M. tuberculosis* complex species (*M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. bovis* BCG, *M. canetti* and *M. bovis* subsp. *caprae*), ***MA*** = examination with three probes for the detection of *M. avium* com-

Examination of respiratory tract						Isolate identification			
Pulmonary tissue			Lymph nodes			IS	ACCU		Spoligotype
PA	BS	*M. b.*	PA	BS	*M. b.*	*6110*	*MTB*	*MA*	
+	+	+ (+)	+	+	+ (+)	+	+	–	nt
–	–	+	+	+	+	nt	nt	nt	nt
–	–	+	+	+	+	nt	nt	nt	nt
–	–	+	+	+	+	nt	nt	nt	nt
–	+	+	+	+	+	nt	nt	nt	nt
–	nt	nt	+	–	+	nt	nt	nt	nt
–	nt	nt	+	–	+	+	+	–	nt
–	nt	nt	+	+	+	+	+	–	nt
–	–	–	+	–	+ (+)*	+	+	–	*M. b. c.*
–	nt	nt	+	–	+*	+	+	–	*M. b. c.*
–	+	+	+	+	+	nt	nt	nt	nt
+	+	+	–	–	+ (+)*	+	+	–	*M. b. c.*
+	+	+	+	+	+	nt	nt	nt	nt
–	nt	nt	+	–	+*	+	+	–	*M. b. c.*
–	–	+	+	+	+	nt	nt	nt	nt
–	nt	nt	+–	–	+	nt	nt	nt	nt
–	–	+ (+)*	–	+	+	+	+	–	*M. b. c.*
–	nt	nt	+	+	+	nt	nt	nt	nt
–	nt	nt	+	+	+	nt	nt	nt	nt
–	nt	nt	+	+	+	nt	nt	nt	nt
–	–	+	+	+	+	nt	nt	nt	nt
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–	nt	nt	+	–	+	+	+	–	nt
–	nt	nt	+–	–	+	+	+	–	nt
–	nt	nt	+–	–	+	nt	nt	nt	nt
–	–	+*	+	+	+	+	+	–	*M. b. c.*
–	nt	nt	+–	+	+	+	+	–	nt

plex species: 1. probe for the identification of *M. avium* and *M. intracellulare* (serotypes 1 to 28), 2. probe for the identification of *M. avium* (serotypes 1 to 3 of genotype IS*901*+, IS*1245*– and serotypes 4 to 6, 8 to 11 and 21 of genotype IS*901*– , IS*1245*+), 3. probe for the detection of *M. intracellulare* (serotypes 7, 12 to 20 and 22 to 28 of genotype IS*901*– , IS*1245*–), **BS** = direct microscopy for the detection of acid fast rods after Ziehl– Neelsen staining, ***M. b.*** = *M. bovis*, ***M. b. c.*** = *M. bovis* subsp. *caprae*, (+) = isolate virulent for guinea-pig in biological trial, [1]first insemination on January 21, 1989, [2]first insemination on November 17, 1989, [3]first insemination on February 2, 1995, *isolate examined by spoligotyping

Breeding and zoo-hygiene status of the herd. Considering the successful rebuilding of the shed and the farmer's long experience, the breeding and zoo-hygiene status of the farm was good. In winter, animals received the farm's own hay, crush and mineral supplements. In summer, hay was replaced by green fodder that was obtained from pastures near a forest. They were not used for cattle grazing because of their distance from the farm. The shed was whitewashed annually. In the fodder preparation room, calves were housed in one cubicle, three large pigs in the second and two smaller pigs in the third cubicle (Figure 1). A hen-house was situated above the pig cubicles that was however empty for several months at the time of the investigation. House sparrows (*Passer domesticus*) often flew back and forth to the farmyard and occasionally they also flew into the fodder preparation room or the shed. All domestic animals were tended by the farmer (a man about 50 years old) and sporadically also by his wife. Their 20-year-old daughter hardly ever looked after the animals.

Diagnostics of bovine tuberculosis

Intra-vitam **diagnostics**. Comparative skin testing was carried out annually using bovine tuberculin (Bovitubal, 14 000 TU/ml, 0.2 ml *pro dosi*, Bioveta Ivanovice na Hane, Czech Republic) and avian tuberculin (Avitubal, 14 000 TU/ml, 0.2 ml *pro dosi*, Bioveta Ivanovice na Hane, Czech Republic). The results of these tuberculin skin tests were always negative. According to the records of the farmer and the town veterinarian, approximately 12 years prior to registering this case, a doubtful reaction to bovine tuberculin occurred in three heifers. Two heifers were then slaughtered for reasons of diagnostic slaughter, with negative patho-anatomical and laboratory examinations of lymph nodes for mycobacteria. The third doubtful reacting heifer was left in the herd (most probably cow No. 24213) due to the consecutive negative comparative skin testing with avian tuberculin. According to the records of the competent District Veterinary Administration (DVA), no patho-anatomical lesions indicating tuberculosis were found after slaughter in cattle and pigs from this herd during the period of at least 10 years. Prior to the slaughter of infected cow No. 24213, the last regular comparative skin testing of all animals older than 24 months was conducted on October 28, 1994 with a negative result (Table 2). When investigating the infection, two subsequent comparative skin tests were conducted in the herd on February 8, 1995 and March 21, 1995. The farmer's family was examined using skin test Mantoux (in March 1995).

Veterinary hygienic inspection after slaughter. Animals were slaughtered stepwise on February 7, 1995 (first infected cow No. 24213), February 22, 1995 (four animals), March 1, 1995 (four animals) and April 3, 1995 the remaining 20 animals (Table 2). All slaughtered animals were given a veterinary hygienic examination after slaughter according to Directive No. 1/1989 issued by the State Veterinary Administration. Lymph nodes and lung tissue were removed for laboratory examinations from each slaughtered animal.

Post-mortem **diagnostics of mycobacteria**. Tissues of animals (Table 2) and samples from the environment (Table 3) were homogenised, decontaminated using NaOH-HCl and cultured onto one Herrold Egg Yolk Medium, one egg medium of Stonebrink, and one liquid serum medium of Sula using the method described earlier (Fischer *et al.*, 2000). Homogenised samples prior to preparation, as well as all mycobacterial isolates were examined microscopically after staining by the method according to Ziehl-Neelsen for the detection of acid fast rods (Kubin *et al.*, 1986).

Identification of mycobacterial isolates. Mycobacterial isolates (n = 49) originating from cattle (n = 43, Table 2) and from the environment (n = 6, Table 3) were identified by biochemical methods including negative results for niacin accumulation and nitrate reduction as well as susceptibility to TCH. Six isolates (five isolates from cattle and one isolate from the environment) were examined in a biological trial on a guinea-pig (Wayne and Kubica, 1986). DNA of ten isolates was analysed using the method Accu-Probe (Gen-Probe Incorporated, San Diego, California, USA) with probes *M. tuberculosis* complex, *M. avium* complex, *M. avium* and *M. intracellulare* according to the manufacturer's instructions and IS*6110*-targetting PCR. DNA of six selected isolates from cattle (Table 2) was subjected to a spoligotyping method according to methodology described previously (Kamerbeek *et al.*, 1997). One *M. avium* complex isolate from water (Table 3) was identified using the PCR method for the detection of specific insertion sequences IS*901* (Kunze *et al.*, 1991), IS*1245* (Guerrero *et al.*, 1995) and serotyping (Wolinsky and Schaefer, 1973; Süssland and Hrdinova, 1976).

Table 3. Culture examinations of biological material other than tissues from infected cattle

Examined samples*	Number of		
	samples	*M. bovis*	other mycobacterial species
Milk	15	0	0
Cow's urine	7	0	0
Drinking water	3	0	2 × *M. avium* subsp. *avium*
House sparrow (*Passer domesticus*)	2	0	0
Farm produced crush	3	0	1 × *Mycobacterium* sp.
Scrapings from the stable	3	3 (+)	0
Manure	0	0	0
Non-vertebrates	0	0	0
Total	33	3	3
%	100	9.1	9.1

Explanations:

*examinations carried out at VRI Brno (M. Pavlas, J. Bartl) and at SVDI Brno (K. Kovarik), (+) one isolate virulent for guinea-pig in biological trial, *M. avium* subsp. *avium* of genotype IS*901*– and IS*1245*+ and serotype 8

Of 49 isolates of *M. bovis*, six were examined by spoligotyping, out of which all corresponded with the spoligotype of *M. bovis* subsp. *caprae* (Table 2, Figure 1). Three isolates originated from tuberculous lesions and three from unchanged lung tissue and lymph nodes (Table 2).

Financial sources for the eradication of the outbreak

As the farmer was not insured, it was necessary to cover most of the losses from other sources. All *intra-vitam* diagnostics carried out by a veterinarian and State Veterinary Diagnostic Institute in Brno were funded from resources of the District Veterinary Service. Examinations undertaken at the Veterinary Research Institute in Brno were covered from research project funds. The final disinfection, disinsectisation and deratisation were funded by a district authority in Zdar nad Sazavou. Bodies and organs of all animals slaughtered on February 7, 1995, February 22, 1995 and March 1, 1995 were condemnt without any financial compensation. All animals slaughtered on April 3, 1995 were purchased for 0.6 EUR/kg (the then average purchasing price was approximately 0.9 EUR/kg) for processing and mycobacteria were grown from lung tissue and pleural lymph nodes. The isolates were identified as *M. bovis* using the method Accu-Probe (probe for the detection of *M. tuberculosis* complex bacteria), IS*6110* PCR, biochemical procedures and a biological trial on guinea-pigs.

Methodology for eradicating the outbreak

Preventive measures from February 8, 1995. On the following day, an anamnestic investigation was conducted directly in the herd. It was announced on the basis of the results that bovine tuberculosis of cattle was suspected and with regard to the serious circumstances, the following control measures were introduced:

– the whole cattle herd, five pigs and one dog were identified as the focus of infection (Figure 1, Table 4)
– all transfers of animals were banned apart from transfer to the slaughterhouse
– skin testing of all species and categories of farm animals was ordered
– selling milk outside of the farm was banned
– milk supply to the dairy (approx. 200 l daily) was not limited (milk was pasteurised instead of 72 to 74°C for 20 to 30 seconds instantaneously 85°C from February 9, 1995)

Table 4. Tuberculin skin testing of other animals than cattle and of the farmer's family

Tested animals and humans	Tuberculin used		
	Bovitubal	Avitubal	Mantoux
Pig (weight 180 kg) No. 1	+	+	nt
Pig (weight 180 kg) No. 2	+	+	nt
Pig (weight 180 kg) No. 3	–	–	nt
Pig (weight 70 kg) No. 1	+	+	nt
Pig (weight 70 kg) No. 2	+	+	nt
Dog (5 years old)	–	nt	nt
Farmer (about 50 years old)	nt	nt	+
Farmer's wife (about 45 years old)	nt	nt	–
Farmer's daughter (about 20 years old)	nt	nt	–

Explanations:

nt = not tested, **Bovitubal** 14 000 TU/ml, 0.2 ml *pro dosi* Bioveta Ivanovice na Hane, Czech Republic, **Avitubal** 14 000 TU/ml, 0.2 ml *pro dosi*, Bioveta Ivanovice na Hane, Czech Republic, **Mantoux** 50 TU/ml, 0.1 ml *pro dosi*

– the farmer was recommended to boil the milk for his own use until the end of the case investigation
– the farmer was provided with adequate instructions about the infection in order to immediately create conditions for the successful control of the infection and to prevent it from spreading

First regional tuberculin skin testing on February 8, 1995. Comparative tuberculin skin testing was carried out in all head of cattle on the second day after slaughter of the first animal with tuberculous lesions. Out of the 27 animals examined in the herd, 25 animals reacted with tuberculin Bovitubal from reaction number 4.0 mm to unmeasurable reaction and 23 animals reacted with tuberculin Avitubal from reaction number 1.0 mm to 16.0 mm. Only one heifer and one calf reacted with neither of both tuberculins (Table 2). All five pigs kept in the adjacent sty were also examined by tuberculin skin testing (Table 4). Positive reactions to both tuberculins were found in two larger and two smaller pigs (visible redness was observed at the place of tuberculin application, central necrosis was found in two animals). Tuberculin skin testing with Bovitubal was negative in the dog. A strong reaction to human tuberculin was found in the farmer (larger than 30 mm) while tuberculin skin testing of his wife and daughter was negative (Table 4).

Diagnostic slaughter on February 22, 1995. On the basis of the results of a skin test and current animal efficiency, four animals were slaughtered on February 22, 1995: one cow, two heifers and one bull. Tuberculous lesions were found in the pulmonary tract of the cow and two heifers. In the bull, no patho-anatomical finding indicating tuberculosis was found, but acid fast rods were found by direct microscopy of the pulmonary lymph nodes. *M. bovis* was identified in the examined material from all four animals (Table 2).

Diagnostic slaughter and collection of samples of other biological material from the shed from March 1, 1995. One week later other four animals (one heifer and three bulls) were slaughtered. Tuberculous lesions were found in the respiratory tract and *M. bovis* was subsequently identified in all animals (Table 2). All five pigs were also slaughtered, and tuberculous lesions were found in the liver, spleen and mesenteric lymph nodes in all of them. Cultures were produced only from the liver tissue of two large pigs and *M. bovis* was isolated from both of them. Of 33 samples of other biological material (Table 3), *M. bovis* was isolated only from three samples of scrapings collected in the shed from the wall and troughs (one tested strain was virulent for a guinea-pig in a biological trial). Two strains of *M. avium* subsp. *avium* of genotype IS*901*–, IS*1245*+ and serotype 8 were isolated from water (Table 3).

Second regional comparative skin testing on March 21, 1995. During this tuberculin skin test-

ing, only 19 remaining head of cattle (the youngest calf was not born until one day later on March 22, 1995) were examined. Skin reactions to Bovitubal and Avitubal were found in all animals (Table 2).

Announcement of the outbreak and implementation of all anti-infection measures. On the basis of all results of *intra-vitam* and *post-mortem* diagnostics, the outbreak of the infection was announced on March 28, 1995 with a proposal for its radical eradication that was carried out on April 3, 1995 (Table 2). Of 20 animals slaughtered, tuberculous lesions in the pulmonary tract (mainly in the pulmonary lymph nodes) were found in 16 animals. *M. bovis* was, however, isolated from all animals through culture examination.

Disinfection, disinsectisation and deratisation carried out in April 1995. After removing all animals (apart from the dog) from the shed, mechanical cleaning of all shed areas, disinfection, disinsectisation and deratisation were carried out by an authorised firm. The shed was newly whitewashed. New animals were bought and placed in the shed one year later.

DISCUSSION

Bovine tuberculosis in cattle in the Czech Republic was controlled in 1968 and the subsequent decrease in incidence as well as prevalence led to its complete eradication in 1995 in cattle and 1999 in other animals (Pavlik *et al.*, 2002b,c,d). This case, with its fast progress, i.e. the spread of *M. bovis* among all age categories of animals, was entirely unique in the Czech Republic. Since 1990, bovine tuberculosis has been diagnosed only in six other outbreaks in the Czech Republic (Table 1), but in none of them was such a fast spread recorded as in this herd (Pavlik *et al.*, 1998b, 2002b). Therefore it was necessary, from the epidemiological point of view, to find out what the source of infection was.

Presumed sources of the *M. bovis* infection for the first infected cow

Despite the negative tuberculin skin testing three months prior to slaughter, infected cow No. 24213 was the source of infection for cattle and pigs in the affected herd. Advanced patho-anatomical findings indicated that clinical symptoms must have occurred in this cow relatively soon after birth. The single-source-case theory is also supported by the identification of the same spoligotype in six isolates originating from six animals of all categories in this herd. From a pathogenic point of view it was most probably a case of endogenic reactivation of the infection in an old animal after 13 parturitions as a consequence of considerable stress. The persistence of infection in cattle and the same progress of disease were recorded in the Czech Republic (Liska, 1960; Zahor, 1964; Kacin *et al.*, 1966; Rossi *et al.*, 1969; Hejlicek *et al.*, 1970a,b; Chloupek, 1981; Hejlicek and Chloupek, 1982) as well as in other countries (Brown *et al.*, 1994; Körmendy, 1995).

The progress of infection was also indicated by the fact that a doubtful skin reaction to bovine tuberculin was recorded in three heifers in the herd at the time when this animal was still a heifer. No tuberculous lesions were found after slaughter in two heifers with doubtful skin reaction to bovine tuberculin, therefore the third one was repeatedly examined by comparative tuberculin skin testing. On the basis of the negative tuberculin skin testing result, the third heifer was left for further breeding at that time. It was impossible to determine on the basis of anamnestic data whether this animal was actually first infected cow No. 24213. However, these results may indicate that infection caused by *M. bovis* occurred in the herd in the past and could cause the persistent infection in this particular animal.

Green fodder the farmer obtained from his pastures near a forest could be considered as another possible source of *M. bovis*. The pastures could be entered by infected wild animals; but bovine tuberculosis was diagnosed in the wild in the red deer (*Cervus elaphus*) only in a different district (Chomutov) in 1991. *M. bovis* isolated from this deer was of different unique spoligotype detected in the Czech Republic during the period 1965–2001 (Pavlik *et al.*, 2002a). In one farmed red deer in the Czech Republic *M. bovis* subsp. *caprae* of the same spoligotype was detected. The source of infection of this 6 years old animal was not identified. Mother of this infected animal originated from the wild in the Czech Republic (Pavlik *et al.*, 2002a). These previously published results could demonstrate a possible existence of bovine tuberculosis reservoirs in free living wild animals in the Czech Republic. Despite of the fact that biomonitoring for mycobacterial infections in wild animals took place in the 1980s (Hejlicek *et al.*, 1994; Treml and Hejlicek,

1998), it is impossible to consider the screening of wild animals for bovine tuberculosis in the Czech Republic adequate.

A biological trial on a guinea-pig showed the virulence of all six strains that were selected for spoligotyping from animals of various age categories from variably affected tissue (three isolates from tissue with tuberculous lesions and three isolates from tissue without tuberculous lesions). A virulent strain for the guinea-pig was also isolated from the shed environment, which in the given herd of cattle confirms the importance of the external environment for the spread of the causal agent of bovine tuberculosis.

The first isolates of *M. bovis* subsp. *caprae* were characterised from samples of goats in Spain (Aranaz *et al.*, 1999). The association between occurrence of this subtype and animal species was not confirmed. In the Czech Republic were found ten strains of *M. bovis* subsp. *caprae* identified by spoligotyping (one spoligotype designated as spoligotype S7) in three cattle outbreaks in 1966, 1991 and 1995, in one farmed red deer in 1999 an in an 80-year-old patient in 1999 (Pavlik *et al.*, 2002a). Twelve cases of *M. bovis* subsp. *caprae* infection occurred in cattle, red deer and humans in western Austria since 1994 (Prodinger *et al.*, 2002). Based on the published results from Germany this *M. bovis* biovar seems much more widespread in Europe than previously assumed (Niemann *et al.*, 2002). From the epidemiological point of view it may be possible that *M. bovis* subsp. *caprae* present in the human population (Gutierrez *et al.*, 1997) could be transmitted from this source to the animal population and vice versa. Contact with cattle was found to be associated with three out of four human cases in Austria (Prodinger *et al.*, 2002).

Danger of infection in people

Of the family members on the farm only the farmer was exposed according to a positive skin test (Table 3). However, the outbreak caused a risk for the rest of the family members and other consumers of raw milk from milk cows of this herd. It is laid down by current legislation in the Czech Republic that according to **Veterinary Care** Act No. 166/1999 and § 49 of **Decree** No. 287/1999 **on Veterinary Requirements for Animal Products** it is possible to sell raw milk and products from such milk under certain conditions. The law sets down that raw milk and products made from it must not be sold for the purposes of public consumption. Nevertheless, raw milk can represent a risk factor in the transfer of the causal agent of bovine tuberculosis to the human population.

CONCLUSIONS

The described case has been the last recorded case of bovine tuberculosis in cattle in the Czech Republic so far. From its progress, it is possible to deduce the following information and conclusions:

1. The causal agent of bovine tuberculosis (*M. bovis* subsp. *caprae* was detected in the herd we monitored) can spread relatively fast to all animals in the shed including pigs, after penetrating into a herd free of the infection.
2. Older animals not reacting with bovine tuberculin in skin testing (so called anergents) can be the source of infection for the entire herd.
3. Even with high sensitivity and specificity of skin testing with tuberculin prepared from *M. bovis,* herds may still contain anergic infected animals that represent the greatest danger at this stage of epidemiological situation in the Czech Republic.
4. It need not be possible to restore the herd to health by the elimination method even if anti-infection measures are implemented in time.
5. All non-specific reactions that occur during tuberculin skin testing in cattle must be investigated further. In slaughterhouses, it is necessary to carefully conduct veterinary hygienic examinations and perform further laboratory investigations into all tuberculous lesions found in slaughtered domestic and wild animals (mainly in cattle, pigs, farmed cervids).
6. With regard to the detection of *M. bovis* subsp. *caprae* in 1999 in one farmed deer, in 1991 in a game-park kept European wild goat and in a wild deer in the open nature, so far unknown sources of the *M. bovis* subsp. *caprae* infection may exist in the Czech Republic.
7. To prevent the *M. bovis* infection from being introduced into a herd free of infection by a human being, workers in animal production must be required to have a preventative examination for tuberculosis. The risk is especially high in workers coming from countries with frequent occurrence of bovine tuberculosis.

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Corresponding Author

Assoc. Prof. MVDr. Ivo Pavlik, CSc., Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 541 321 241, fax +420 541 211 229, e-mail: pavlik@vri.cz
http://www.vri.cz/default.asp?page=people/pavlik.htm