Molecular epidemiology of bovine tuberculosis in the Czech Republic and Slovakia in the period 1965–2001 studied by spoligotyping

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ABSTRACT: Spoligotyping was used to examine IS*6110*-positive DNA of 26 *Mycobacterium bovis*, *M. bovis BCG* and *M. bovis* subsp. *caprae* non-viable isolates stored up to 10 years. All of these isolates were previously identified by biochemical tests and all 17/17 tested isolates were earlier found virulent for guinea pigs. In total seven spoligotypes, designated S1–S7, were detected and compared with the spoligotypes of 3 176 isolates in the database of the National Institute of Public Health and the Environment (RIVM) in Bilthoven, the Netherlands. A Neotype *M. bovis* strain, isolated in 1965 in the USA and thereafter stored in The Czechoslovak National Collection of Type Cultures (My 310/87) since 1987 was of an identical spoligotype S4 with the original reference *M. bovis* strain from the USA. The *M. bovis* isolates from capybara's (*Hydrochoerus hydrochaeris*) imported from Germany to the Czech Republic in 1989, as well as cattle isolates from 1966, 1991 and 1994, were of the most common type S1. Also a human isolate from 1981, a *M. bovis* BCG vaccine strain and clinical *M. bovis* BCG isolates from three children with post-vaccinal complications were of this most predominant spoligotype. The four unique spoligotypes S2, S3, S5 and S6 were identified in *M. bovis* isolates from cattle in the years 1965, 1996 and 1967 in the Czech Republic, respectively, but also in isolates from farmed red deer (*Cervus elaphus*) from 1991 and in cattle isolates from Slovakia from the year 1992. The scarcely occurring spoligotype S7, which is typical for *M. b. caprae* was detected in the Czech Republic from farmed red deer (1999), cattle isolates (1966, 1991, 1995) and in a strain isolated from an 80-year-old man (1999). Several strains isolated in each of three outbreaks in cattle herds were examined. Identical spoligotypes were detected in two outbreaks and different causal agents (*M. bovis* of spoligotype S1 and *M. b. caprae* of spoligotype S7) were identified in two cows from the third outbreak. The results confirm an effective control of bovine tuberculosis in the Czech Republic and Slovakia during 1959–1968, because previously circulating spoligotypes were successfully eradicated. The data also suggest other reservoirs of bovine tuberculosis may exist among free-living wild animals.

Keywords: IS6110; *Mycobacterium bovis* subsp. *caprae*; *Mycobacterium bovis* BCG, cattle; capybara; red deer; post-vaccinal complications in children

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Abbreviations:

ATCC = American Type Culture Collection; **CNCTC** = The Czechoslovak National Collection of Type Cultures; **DR** = Direct Repeat; **DVA** = District Veterinary Administration; **ETR** = Exact Tandem Repeats; **IS** = Insertion Sequence; **M.** = *Mycobacterium*; **MA** = *M. avium*; **MAA** = *M. avium* subsp. *avium;* **MAC** = *M. avium* complex; **MI** = *M. intracellulare;* **MPTR** = Major Polymorphic Tandem Repeat; **MTC** = *M. tuberculosis* complex; **OIE** = Office International des Epizooties; **PGRS** = Polymorphic GC-Rich Repeated Sequence; **RFLP** = Restriction Fragment Length Polymorphism; **RIVM** = National Institute of Public Health and the Environment, Bilthoven, The Netherlands; **SVDI** = State Veterinary Diagnostic Institute; **SVA** = State Veterinary Administration; **VNTR** = Variable Numbers of Tandem Repeats; **VRI** = Veterinary Research Institute

In the Czech Republic bovine tuberculosis in cattle was eliminated during a campaign of disease control between 1959–1968 (Polak, 1969; Pavlas, 1999; Kouba, 1999). New cattle outbreaks of bovine tuberculosis were recorded in 1969–1980, 1982–1986, 1991–1992 and 1994–1995 (Pavlik *et al.*, 1998, 2002a). Bovine tuberculosis was also confirmed in 120 animals of non-bovine animal species originating from farms, a zoological garden, a game park, a circus, the wild, and from 10 milk samples from infected cows in the period 1970–1996 (Pavlik *et al.*, 1998). In 1999 lung tuberculosis was detected in the Czech Republic for the first time in one farmed red deer (*Cervus elaphus*) (Machackova *et al.*, 2000; Pavlik *et al.*, 2002b).

In Slovakia the situation concerning bovine tuberculosis is also favourable. After the elimination of bovine tuberculosis during national control program 1959–1968, the number of new outbreaks decreased significantly, as was noted in the Czech Republic. Bovine tuberculosis was for the last time detected in three cattle herds in 1993 (Badalik *et al.*, 1997a, b, 1998, 1999). In the last decade, *Mycobacterium bovis* (*M. bovis*) was isolated from other animal species than cattle in 1992 from three wild boars (*Sus scrofa*) that came into contact with infected cattle in the pasture (Kalensky, 1992; Hanzlikova and Vilimek, 1992).

According to the definition of the International Animal Health Code of Office International des Epizooties (OIE) (prevalence of infected cattle herds up to 0.2%) the Czech Republic and Slovakia are free from bovine tuberculosis (Pavlik *et al.*, 2002a).

Within the *M. tuberculosis* complex (*MTC*), several genetically conserved sub-species can be distinguished; *M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG, *M. microti, M. bovis* subsp. *caprae* and *M. canetti* (Poulet and Cole, 1995; Van Soolingen *et al.*, 1997; Aranaz *et al.*, 1999; Niemann *et al.*, 2002). At present various molecular biological methods are available for detailed identification and typing of *MTC* isolates and the most frequently used is Restriction Fragment Length Polymorphism (RFLP) typing using different repetitive sequences as probe, like IS*6110* (Thierry *et al.*, 1990), and IS*1081* (Collins and Stephens, 1991).

For *MTC,* IS*6110* RFLP has become a widely used typing method for epidemiological studies. In *M. tuberculosis* isolates the large number of IS*6110* copies in the genome permits an excellent use of this element for strain typing facilitating molecular epidemiological analysis. *M. bovis* isolates often contain less than five IS*6110* copies. In *M. bovis* subsp. *caprae* (*M. b. caprae*) the usual copy number of IS*6110* appears to be higher than in *M. bovis*. In contrast to IS*6110*, the use of the remaining IS elements is limited by the small number of copies on the genome and the low degree of polymorphism (Van Soolingen *et al.*, 1998; Kremer *et al.*, 1999). However, six other types of short repetitive DNA with a varying degree of genetic diversity and potential usefulness were identified: DR (Direct Repeat) (Thierry *et al.*, 1990), PGRS (Polymorphic GC-Rich Repeated Sequence) (Ross *et al.*, 1992), GTG and MPTR (Major Polymorphic Tandem Repeat) (Hermans *et al.*, 1992), ETR (Exact Tandem Repeats) and VNTR (Variable Numbers of Tandem Repeats) (Frothingham and Meeker-O'Connell, 1998).

In the mid 1990s a PCR-based method for differentiation of *MTC* isolates designated spoligotyping (spacer oligo typing) was described (Hermans *et al.*, 1991; Mendiola *et al.*, 1992; Van Soolingen *et al.*, 1995; Kamerbeek *et al.*, 1997). It is an easy-to-perform, economical and rapid way for typing

of *MTC* isolates. Spoligotyping is based on DNA polymorphism at one chromosomal locus that is characterised by the presence of a high number of conserved direct repeats, and which was designated the Direct Repeat (DR) region (Thierry *et al*., 1990). The direct repeats are 36 bp in size and are interrupted by DNA spacers of 35 bp to 41 bp. When the DR regions of several isolates were compared, it was noted that the order of spacers is nearly the same in all isolates, but that many deletions or insertions occur in different strains. The presence or absence of 43 individual spacers can be detected using the spoligotyping method (Rastogi *et al*., 2001).

Spoligotyping is an excellent method for differentiation of *MTC* subtypes based on the presence and/or absence of certain combinations of spacers. The types, which can be differentiated, are given in Dvorska *et al*. (2001), which summarises unpublished results and data from the literature (Van Soolingen *et al*., 1997, 1998; Zumarraga *et al*., 1999; Aranaz *et al*., 1999; Niemann *et al*., 2000; Sola *et al*., 2000; Viana-Niero *et al*., 2001; Rastogi *et al*., 2001).

As spoligotyping is a PCR driven technique, only small amounts of DNA are required for analysis. Therefore, spoligotyping is particularly suitable for the analysis of slowly growing mycobacteria. It also permits the comparison of isolates, which are not re-culturable after prolonged storage. Typing can be of irreplaceable importance, for instance in the case of relapses, when it is necessary to compare new isolates from patients with isolates from former episodes of the disease.

The aim of this study was to investigate which spoligotypes of *M. bovis* isolates occurred in the Czech Republic and Slovakia in the period of elimination of bovine tuberculosis until 1968 and in the post-elimination period until 2001.

MATERIAL AND METHODS

The examined *M. bovis* isolates

A total of 27 *M. bovis* isolates were examined (Table 1): 25 isolates from seven District Veterinary Administrations (DVA) in the Czech Republic, one isolate from one DVA in Slovakia, and a Neotype strain *M. bovis* 19210 from the American Type Culture Collection (ATCC).

Anamnestic data on the origin of Neotype strain ATCC 19210

The ATCC 19210 strain was isolated in 1965 from a granulomatous lesion of a lymph node of a six-month-old heifer with positive skin test (Karlson and Lessel, 1970). The strain was obtained from The Czech National Collection of Type Cultures (CNCTC) from the USA in 1987 and designated as My 310/87. In 1995 the strain was revitalised and in 1999 examined by spoligotyping.

Anamnestic data on the origin of *M. bovis* isolates from cattle in the Czech Republic and Slovakia

Sixteen *M. bovis* isolates from cattle in the Czech Republic were included, which were isolated from eight independent outbreaks examined by three laboratories: State Veterinary Diagnostic Institute (SVDI) Prague, SVDI Brno and Veterinary Research Institute (VRI) Brno. One Slovak *M. bovis* isolate originated from SVDI Nitra.

Cattle outbreaks Brno – 1965, 1966, 1967 (Czech Republic). *M. bovis* isolates originated from five different cattle outbreaks in 1965 (n = 1), 1966 (n = 3) and 1967 (n = 1). The available data did not allow determining the district in which the outbreak was recorded, nor the organ from the animal *M. bovis* was isolated from.

Cattle outbreak Prague-East – 1991 (Czech Republic). Three cows showed a dubious reaction following the yearly skin test with mammalian tuberculin. After slaughtering, calcified lesions in pulmonary lymph nodes were detected in all three animals, however, *M. bovis* was isolated only from two of them.

Cattle outbreak Znojmo – 1994 (Czech Republic). In two fattening bulls aged 12 and 16 months caseous lesions in pulmonary lymph nodes with caverns in pulmonary tissues were found and three *M. bovis* isolates were detected which were included in our study.

Cattle outbreak Zdar nad Sazavou – 1995 (Czech Republic). Bovine tuberculosis was detected in a 14-year old cow, which was subjected to emergency slaughter due to clinical signs (cough, emaciation, diarrhoea), The symptoms occurred one month after the last delivery. The whole herd was killed two months after the first detection of infection, including commonly reared pigs. Bovine

Table 1. Examined mycobacterial isolates

Spoligotype[1]	Country	Host			Year and place (DVA) of origin	Accu-Probe		Infection on guinea pig	Comparison with RIVM database
		species	born	tissue		*MTC*	*MA*		
S1	CR	capybara[2]	1988	l. lnn.	1989 – Zlin	+	–	+	
		bull No. 1	1993	l. lnn.		+	–	+	
		bull No. 1	1993	lungs	1994 – Znojmo	+	–	nt	
		bull No. 2	1993	lungs		+	–	+	
		cow No. 1	nk	l. lnn.	1966 – Brno	+	–	+	the most common spoligotype
		cow No. 1	1982	l. lnn.	1991 – Prague–East	+	–	+	
		patient No. 1	1986	bone		+	–	nt	
		patient No. 2	1995	bone	1997 – Prague–City	+	–	nt	
		patient No. 3	1996	bone		+	–	nt	
		patient No. 4	nk	sputum	1981 – Prague–City	+	–	+	
		BCG	nk	nk	1980 – nk	+	–	nt	
S2	CR	cow No. 1	nk	B	1966 – Brno	+	–	+	unique spoligotype
		cow No. 1	nk	nk	1967 – Brno	+	–	+	
S3	CR	wild deer[3]	nk	lungs	1991 – Chomutov	+	–	+	unique spoligotype
S4	USA	cow	nk	l. lnn.	1965[5] (1987[6]) – Neotype strain ATCC 19210	+	–	nt	original Neotype strain, two isolates from cows from South Africa, one isolate from England (host unknown)
S5	SK	cow	nk	l. lnn.	1992 – Levice	+	–	+	unique spoligotype
S6	CR	cow	nk	nk	1965 – Brno	+	–	+	unique spoligotype
S7	CR	cow No. 2	1981	l. lnn.	1991 – Prague–East	+	–	+	
		cow No. 1	1988	l. lnn.		+	–	nt	two deer isolates from Sweden
		cow No. 2	1988	l. lnn.		+	–	+	(deer originally imported from
		heifer No. 1	1994	l. lnn.	1995 – Zdar nad	+	–	+	Scotland),
		heifer No. 2	1994	l. lnn.	Sazavou	+	–	nt	one cattle isolate from Belgium
		bull No. 1	1993	lungs		+	–	+	and one isolate from Great Britain
		bull No. 2	1995	l. lnn.		+	–	nt	(host unknown)

cow	nk	l. lnn.	1966 – Brno	+	–	+	
farmed deer[4]	1993	l. lnn.	1999 – Prague–City	+	–	nt	
patient 5	1919	sputum	1999 – Prague–City	+	–	+	

Explanations:
RIVM (National Institute of Public Health and the Environment, Bilthoven, The Netherlands), [1]designation in our laboratory, [2]capybara (*Hydrochoerus hydrochaeris*), [3]isolate originated from dead free living red deer (*Cervus elaphus*), [4]isolate originated from farmed red deer (*Cervus elaphus*), [5]the Neotype strain was isolated in 1965 from a granulomatous lesion of lymph node from a six-month-old heifer with positive skin test (Karlson and Lessel, 1970), [6]the Neotype strain was acquired by CNCTC (The Czech National Collection of Type Cultures) from the USA in 1987 and designated as My 310/87 and in 1995 the strain was revitalised and submitted to CNCTC (The Czechoslovak National Collection of Type Cultures) in VRI Brno
nk – not known, nt – not tested, CR – the Czech Republic, SK – Slovakia, USA – United States of America, DVA – District Veterinary Administration, deer – red deer (*Cervus elaphus*), l. lnn. – lung's lymph nodes, ACCU-PROBE (Gen-Probe Incorporated, San Diego, California, USA), *MTC – Mycobacterium tuberculosis* complex, *MA* – examination with three probes for the detection of *M. avium* complex species: 1. probe for the identification of *M. avium* subsp. *avium* and *M. intracellulare* (serotypes 1 to 28), 2. probe for the identification of *M. avium* subsp. *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*- and IS*1245*+), 3. probe for the detection of *M. intracellulare* (serotypes 7, 12 to 20 and 22 to 28 of genotype IS*901*– and IS*1245*-)

tuberculosis was detected by pathological anatomic examination, direct microscopy or by culture in all 28 cattle of different age categories (9 cows, 7 bulls, 6 heifers and 6 calves) and in 5 commonly reared pigs (Pavlik *et al.*, 2001b, 2002c). Six *M. bovis* isolates were analysed in our study.

Cattle outbreak Levice – 1992 (Slovakia). Infection was detected in the district Levice in Slovakia in an outbreak of bovine tuberculosis at a cattle farm owning 60 cows. *M. bovis* was detected only in one cow that was grazed on the pasture. After its slaughtering, bovine tuberculosis was not detected either in the district or in any other district in the following years (Badalik *et al.*, 1997a,b, 1998, 1999; Melicharek, 2000, 2001).

Anamnestic data on the origin of *M. bovis* isolates from other animals than cattle

Capybara isolate Zlin – 1989 (Czech Republic). Two young animals (male and female) of capybara (*Hydrochoerus hydrochaeris*) were imported from a zoological garden in Germany to the Czech Republic in May 1989. The female died in quarantine after few weeks, suffering from cough and emaciation. The male was dissected three weeks later for the same reasons. *Postmortem* examination revealed tuberculous lesions in lymph nodes and pulmonary tissue in both animals. *M. bovis* was isolated from the female in SVDI Prague have been examined in our study.

Red deer isolate Chomutov – 1991 (Czech Republic). Adult red deer living in the wild was found dead in a forest several hundred meters from a zoological garden in Chomutov. *Postmortem* examination revealed caverns and caseous lesions in pulmonary lymph nodes. Mixed infection of *M. bovis* and *M. avium* (serotype 2) was detected by culture in SVDI Prague. No bovine tuberculosis was found in this district in any domestic or wild animal for more than 20 years prior this finding.

Red deer isolate Prague-City – 1999 (Czech Republic). In May 1999 one farm reared red deer was slaughtered which was an offspring of a hind caught in the wild and a red deer originating from a zoological garden in Ostrava. It was sent to the slaughterhouse because of emaciation observed since the beginning of 1999 and a cough supposed to be caused by invasion of pulmonary worms. *Postmortem* examination showed severe emaciation, pearl-like lesions, caverns in pulmonary tissues and

caseous lesions in lymph nodes caused by *M. bovis* which was isolated in SVDI Prague.

Anamnestic data on the origin of human *M. bovis* isolates

***M. bovis* BCG isolates Prague-City – 1997 (Czech Republic)**. Three human isolates of *M. bovis* BCG originated from three girls aged 1, 2 and 11 years with post-vaccinal complications isolated in National Institute of Public Health in Prague in 1997 and one vaccine strain used in the Czech Republic during the same period.

Human isolates *M. bovis* Prague-City – 1999 and 1981 (Czech Republic). First *M. bovis* was isolated from sputum of one patient in 1981 (not more anamnestic data are available). In 1999 the second *M. bovis* was isolated from sputum of a 80-year-old man who previously worked in agriculture. The man had no signs of tuberculosis and laboratory examination was performed only within a preliminary examination.

Identification and storage of *M. bovis* isolates

Biochemical testing and isolate storage. *M. bovis* isolates were identified by biochemical methods (Wayne and Kubica, 1986), propagated on solid media by Stonebrink, Petragnani and Herrold (Kubin *et al.*, 1986, Whipple *et al.*, 1991), and since 1989 stored at room temperature. Isolates from the period 1965–1974 were, after isolation from biological material, lyophilised and in 1995 were revitalised, propagated and stored in tubes on solid media at room temperature.

Biological assay on guinea pig. Virulence testing in guinea pig was performed with 17 *M. bovis* isolates (Wayne and Kubica, 1986). Mycobacterial suspensions were inoculated into the pregenual skin fold. After 4 to 6 weeks the guinea pigs were killed, pathological anatomic findings were assessed and parenchymatous organs were cultured to detect the occurrence of mycobacteria. The finding of tuberculous nodes in parenchymatous organs with re-isolation of *M. bovis* was assessed as positive.

Accu-Probe method identification. The DNA of all 27 isolates had been examined by Accu-Probe according to the instructions of the producer (Gen-Probe Incorporated, San Diego, California, USA) using *MTC* probes (detection of *M. tuberculosis* complex strains: *M. tuberculosis, M. bovis, M. b. caprae, M. bovis* BCG, *M. microti, M. africanum* and *M. canetti*), *MAC* (detection of *M. avium* complex, serotypes 1 to 28), *MA* (detection of *M. avium* subsp. *avium* isolates of serotypes 1 to 6, 8 to 11 and 21), and *MI* (detection of *M. intracellulare of* serotypes 7, 12 to 20, 22 to 28).

IS*6110* PCR. The DNA from all isolates was examined with the primers for the detection of *MTC* specific insertion sequence IS*6110* (Kremer *et al.*, 1999).

Spoligotyping method. The spoligotyping method was applied according to Kamerbeek *et al.* (1997). Then the software Gel Compar (Applied Maths, Version 4.1, Kortrijk, Belgium) clustered isolates with the same spoligotyping patterns (Figure 1) and compared spoligotypes with the database (n = 3 176) existing in RIVM.

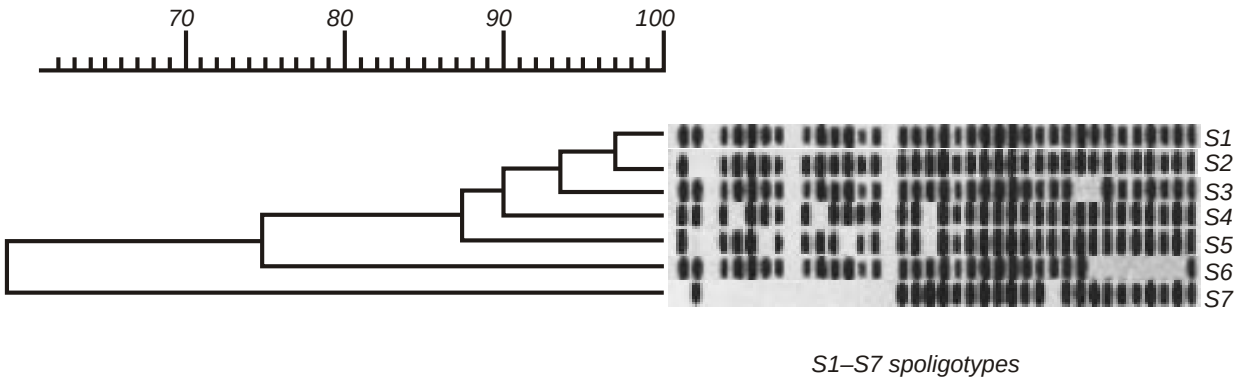

Figure 1. Dendrogram of spoligotypes

RESULTS

Identification of *M. bovis* isolates

All 27 isolates identified by biochemical methods as *M. bovis* reacted positively with *MTC* probe and negatively with *MAC*, *MA* and *MI* probes while using the Accu-Probe method, and contained IS*6110*. All 17 *M. bovis* isolates tested were fully virulent in biological assay on guinea pigs (Table 1).

Examination by spoligotyping

Examination of the isolates by spoligotyping yielded seven different spoligotypes designated as S1–S7 (Table 1, Figure 1).

Spoligotypes S1–S7 in individual years. The unique spoligotypes S2, S3, S5 and S6 were recorded in isolates from 1965, 1966, 1967 and 1992. The *M. bovis* strains of the common spoligotype S1 were found during the whole period from 1966 to 1997. The isolates of this spoligotype were isolated from both cattle and a capybara female from a zoological garden, as well as from three children with post-vaccinal complications and one patient with lung tuberculosis. Isolates of the same spoligotype were also isolated from one bull from pulmonary lymph node and from affected pulmonary tissue. This suggests of disseminated infection by the same *M. bovis* strain. The isolates of spoligotype S7 (specific for *M. b. caprae*), that have been only scarcely isolated in other European countries, were isolated during the period 1966–1999 from cattle, red deer in the farm and from an 80-year-old patient (Tables 1 and 2).

Spoligotype S1. A total of 11 *M. bovis* and *M. bovis* BCG isolates from; a female capybara imported from Germany, two cattle outbreaks in 1966 and 1994 (Brno – 1966 and Znojmo – 1994), three children (*M. bovis* BCG Prague-1997) with post-vaccinal complications, one human patient with lung tuberculosis, and a vaccine strain all shared the most common spoligotype S1 (Tables 1 and 2).

Spoligotypes S2, S3, S5 and S6. Each of the *M. bovis* spoligotypes S2, S3, S5 and S6 were represented in the database by a single isolate originating from four cattle outbreaks in the Czech Republic (Brno – 1965, 1966, 1967) and Slovakia (Levice – 1992) and from free living red deer in Chomutov district – 1991 (Tables 1 and 2).

Spoligotypes S4 and S7. No more than five isolates of each spoligotype S4 and S7 were present in the RIVM database, thus forming smaller clusters. Spoligotype cluster S4 included a Neotype strain *M. bovis* ATCC 19210 from the USA, which was identified in the original type strain, in two isolates from cows from South Africa and in one isolate from the UK (host unknown). Spoligotype S7 of *M. b. caprae* was identified in three cattle outbreaks in the Czech Republic in Brno – 1966, Prague-East – 1991 and Zdar nad Sazavou – 1995, in one farmed red deer (Prague-City-1991) and in an 80-year-old human patient (Prague-City – 1999). According to the data from the RIVM database, *M. b. caprae* of this spoligotype was further identified in two *M. bovis* isolates from red deer from Sweden (originally imported from Scotland), in one bovine isolate from Belgium and one animal isolate from unknown host from Great Britain (Table 1).

Table 2. Occurrence of spoligotypes of *M. bovis* strains isolated during 1965 and 1999

Spoligotype	1965	1966	1967	1980	1981	1989	1991	1992	1994	1995	1997	1999	Total
S1	0	1	0	1	1	1	1	0	3	0	3	0	11
S2	0	1	1	0	0	0	0	0	0	0	0	0	2
S3	0	0	0	0	0	0	1	0	0	0	0	0	1
S4	1	0	0	0	0	0	0	0	0	0	0	0	1
S5	0	0	0	0	0	0	0	1	0	0	0	0	1
S6	1	0	0	0	0	0	0	0	0	0	0	0	1
S7	0	1	0	0	0	0	1	0	0	6	0	2	10
No. of strains	2	3	1	1	1	1	3	1	3	6	3	2	27

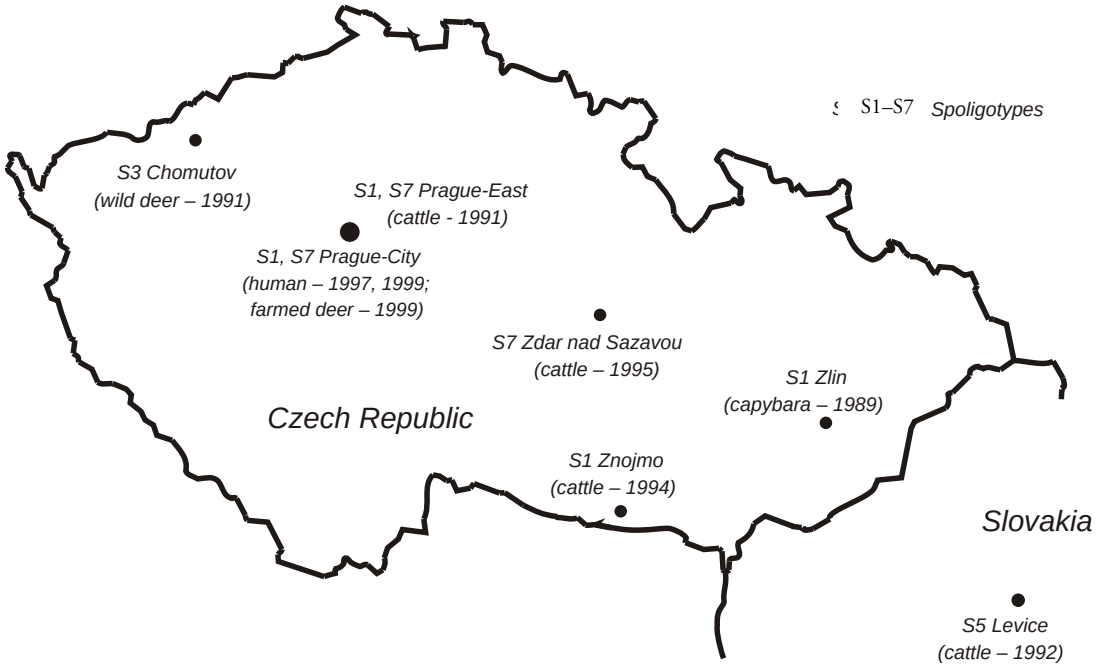

Figure 2. Distribution of spoligotypes of *M. bovis* and *M. b. caprae* isolates from the Czech Republic and Slovakia during the 1989–1999

Geographic distribution of spoligotypes in the Czech Republic and Slovakia. A total of 19 examined *M. bovis* isolates from animals and humans were isolated at different places of the Czech Republic and Slovakia in the period 1989–2001 (Figure 2). In no region or in any district isolates of a certain spoligotype occurred predominantly. All five human isolates were isolated in Prague. The three *M. bovis* isolates from children were determined as vaccine strain (of the most common spoligotype) and two isolates were isolated from infected patients with lung's tuberculosis.

Distribution of spoligotypes within individual infected cattle herds. Several isolates from three cattle herds were examined by spoligotyping. Six isolates from two cows, two heifers, one bull and one calf from an outbreak in Zdar nad Sazavou in 1995 were of the same spoligotype S7 (*M. b. caprae*). Three isolates from two bulls from an outbreak Znojmo – 1994 were of the spoligotype S1 (*M. bovis*), and two isolates from an outbreak Prague-East – 1991 were of spoligotypes S1 (*M. bovis*) and S7 (*M. b. caprae*).

DISCUSSION

All 27 isolates of *M. bovis*, *M. bovis* BCG and *M. b. caprae* were classified as *MTC* following the Accu-Probe examination. At present it is the most frequently used routine method for identification of mycobacterial isolates from humans and animals affected by tuberculosis. The distinction of *M. bovis* and *M. tuberculosis* isolates can be done by biological experiments in animals, biochemical methods or by spoligotyping (Wayne and Kubica, 1986; Kremer *et al.*, 1999; Dvorska *et al.*, 2001). The spoligotyping method based on chromosomal DNA amplification enables not only isolate classification but also further typing of individual isolates which can be used in epidemiological investigations (Aranaz *et al.*, 1996; de la Salmoniere *et al.*, 1997; Cousins *et al.*, 1998; Roring *et al.*, 1998; Aranaz *et al.*, 1999). We used the later method as it allowed identification of isolates that were in the form of dead mycobacterial mass, non-growing in repeated subcultures.

Examination of the Neotype strain *M. bovis* ATCC 19210, stored in CNCTC in Prague under the number My 310/87 since 1987, showed consistency of spoligotype with the original type strain *M. bovis* isolated in 1965 (Table 1). The spoligotype remained unchanged even after repeated subculture that proceeded at its revitalisation and repeated lyophilisation as is commonly done in the collections of microorganisms.

The isolates of *M. bovis* from cattle outbreaks in the 1960s' and 1990s' (outbreaks: Brno – 1966,

Prague-East – 1991 and Znojmo – 1994) were of the same spoligotype S1 as the isolates of the most common spoligotype detected in European countries. This knowledge corresponds with spreading of bovine tuberculosis in Europe during the last century. This spreading was partly due to cattle transfer during the World Wars as important domestic animals (Pavlas, 1999). After World War II and social changes in 1948 the transfer of cattle between the previous Czechoslovakia (today's Czech Republic and Slovakia) and other West European countries ceased. It was renewed after 1989, especially in the years 1992–1996, when more than 30 000 cattle of more than 20 breeds from about 15 countries were imported. In the imported animals no infection with bovine tuberculosis was found (Holejsovsky, 1995) but of other mycobacterial infections primarily paratuberculosis was detected in those animals (Pavlik *et al.*, 1999, 2000, 2001a).

It is interesting from the epizootiological point of view that *M. bovis* isolates of quite unique spoligotypes S2, S3, S5 and S6 in the Czech Republic and Slovakia, not found in RIVM database, were detected in cattle in 1965, 1966, 1967, and 1992 (Table 2). It is tempting to conclude that the isolates of these spoligotypes were not spread throughout the Czech Republic and Slovakia due to a successful control of bovine tuberculosis in farm animals and minimal cattle trade. The origin of spoligotype S7, which corresponds to the spoligotype of *M. b. caprae* in the Czech Republic is unclear (Aranaz *et al.*, 1999, Niemann *et al.*, 2002). However, this *M. bovis* biovar with some characteristics of *M. tuberculosis* seems much more widespread in Europe than previously assumed (Niemann *et al.*, 2002).

M. bovis isolates from more animals involved in three cattle outbreaks were identified by spoligotyping. Two isolates from the first outbreak (Prague-East – 1991) originated from two slaughtered old cows, that reacted dubiously at preventive skin test with bovine tuberculin. In each cow, *M. bovis* (spoligotype S1) and *M. b. caprae* (spoligotype S7) has been identified. Heterogeneity of these isolates was likely due to other sources of infection, which could not be detected with regard to cow transfer within a farm and because of the cows age (Plhal, 1992).

In the second outbreak (Znojmo – 1994), the most common spoligotype S1 has been detected in all three *M. bovis* isolates from two fattening bulls aged 12 and 16 months. One isolate from tuberculous pulmonary tissue was examined from one animal, and two isolates from a pulmonary lymph node and pulmonary tissue from another bull. These results suggest spreading of only one *M. bovis* strain in the outbreak. It was also confirmed by the disease progression when rapid spreading of the infection in pulmonary tissue and lymph nodes was recorded in other bulls. Neither the infection sources nor animal origin were revealed in this case, as the herd was formed by purchase of animals from several districts of the Czech Republic, from Slovakia and some animals probably imported from Poland and/or from the Ukraine. At downfall of the firm the owner destroyed individual animal identification, so that searching for their origin was impossible (Docekal *et al.*, 1995). The following control measures prevented further spreading of bovine tuberculosis to other cattle herds in the Czech Republic (Pavlik *et al.*, 1998).

From the third outbreak Zdar nad Sazavou – 1995 isolates from animals of all categories (cows, bulls and heifers) were randomly selected for identification. All 6 animals were infected with *M. b. caprae* of the same spoligotype S7 (Figure 1). This result is suggestive of a unique infection source for cattle herd of 28 animals at the time of the elimination of the infection. Infection source in this herd was a 14-year-old cow, which was in the herd since her birth. She had 12 calves in total (Pavlik *et al.*, 2001b). The source of *M. b. caprae* infection was not detected. Bovine tuberculosis of cattle was eradicated in this district as early as in 1964 (Juranek, 1965). Since finding of this case and consequently till the end of 2001 no bovine tuberculosis was diagnosed in any animal of this district (Pavlik *et al.*, 1998; Machackova *et al.*, 2000; Statistical data of the State Veterinary Administrations – SVA, Prague, Czech Republic, 2001).

The isolate capybara Zlin – 1989 obtained from female capybara (isolate from the imported male was not available) was of the most common spoligotype S1 as compared with RIVM database. Both the infected one-year-old animals (male and female) were kept in a quarantine after their importation from Germany in 1989, where the female died after few weeks due to progressed tuberculosis. After dissection tuberculosis was also detected in previously ill male. Thus it is evident that both animals were infected prior to importation to the Czech Republic. However, the source of infection was not found. In 1994 and 1995 a similar case was recorded in three tapirs in the quarantine of the zoological garden in Jihlava (Statistical data of

SVA, Prague, Czech Republic, 1994, 1995; Pavlik *et al.*, 1998, 2002b). The animals were imported from Poznan zoological garden in Poland in 1994. Isolates from those animals were not available for our study. The consequent collection of anamnestic data revealed that bovine tuberculosis had already been detected in that zoological garden in a tapir in 1997 (Pavlik *et al.*, 2002b)! Therefore, it is evident that this group of animals was infected with *M. bovis* and that importation of animals to Jihlava zoological garden only forwarded the course of clinical infection. Animal keeping in zoological gardens is to some extent dependent on exchanges or purchases of animals. Furthermore, it is evident from the above data that animal business among different zoological gardens has to be considered as highly hazardous with regards to a possible spreading of bovine tuberculosis (Pavlik *et. al.*, 1998).

The isolate red deer Chomutov – 1991 of *M. bovis* originating from red deer in the wild in the district Chomutov was of unique spoligotype S3 compared with the isolate of *M. b. caprae* from red deer Prague-City – 1999 isolated from a farmed red deer, which was of spoligotype S7. This result shows different infection sources on one hand, but on the other hand suggests that dangerous reservoirs of bovine tuberculosis are present in the wild. At game farms, introduction of causal agent of bovine tuberculosis can results in rapid spreading and consequently a very difficult control of the infection (Robinson *et al.*, 1989; Schmitt *et al.*, 1997). Therefore, it would be advisable to perform laboratory examinations of pulmonary lymph nodes with tuberculous lesions for mycobacteria in wild ruminant reared at farms in the Czech Republic. Transmission of the infection from farm-reared red deer to wild animals has been described in the USA (Whipple *et al.*, 1997), which is another risk factor that has to be taken into consideration. Detection of the same spoligotype of an isolate of *M. b. caprae* from farmed red deer Prague-City – 1999 and isolates from cattle in 1966, 1991 and 1995 (Table 1 and Figure 2) are suggestive of the same infection source for both domestic and wild ruminants.

Analysis of the course of death of wild ruminants in the period 1945–1958, of red deer (n = 10), roe deer (n = 348), fallow deer (*Dama dama*) (n = 5), moufflon (*Ovis musimon*) (n = 6), did not reveal any pathological anatomic lesions suggestive of tuberculosis. On the other hand, caseous lesions were found at *postmortem* examinations in 10 of 584 dead hares (*Lepus europaeus*) (Vyvlecka, 1960). Bovine tuberculosis was in the past detected in free-living animals in the Czech Republic and Slovakia, like red deer (Herkner, 1913), roe deer (Krul, 1962), in the above mentioned red deer and goat (*Capra aegagrus*) (Pavlik *et al.*, 1998), in wild boar (Kalensky, 1992), and in the liver of a dead badger (*Meles meles*) (Bukovjan – personal communication, 1998). The risk of *M. bovis* transmission from wild animals to domestic ruminants is increasing due to the higher number of herds in the Czech Republic and Slovakia that are grazed either in summer (May to September) only or during the whole year. Domestic pigs started to be exposed to the same risk as they are also quite often kept under "natural conditions", for example in forest pens (Vanicek and Prasek, 1995).

Sources of *M. b. caprae* infections in red deer (Prague – 1999) and a 6-year-old red deer have not been elucidated (Table 1). The anamnestic data show that the red deer was an offspring of a hind caught in the wild and a red deer kept in a zoological garden where bovine tuberculosis has never been detected. Therefore, the red deer most presumably was infected by his mother. However, no bovine tuberculosis was ever detected in her, nor in other red deer (about 90 animals) originating from the wild of the whole Czech Republic. Another possible source could be the contact of red deer with workers coming from the Ukraine who worked nearby the red deer farm. Local veterinarian confirmed that they fed the animals with leftovers of food and occasionally spat at them (Zaoralek – personal communication, 1999). In case they came from villages with cattle rearing, the risk of their infection and transmission of the infection to red deer was relatively high. According to the statistical data of OIE, bovine tuberculosis was diagnosed in the Ukraine in 1998 in 67 cattle outbreaks with 14 425 animals, and in 1999 even in 100 cattle outbreaks with 21 395 animals (OIE, 1999, 2000).

From the epidemiological point of view it may be possible that *M. b. caprae* present in human population (Gutierrez *et al.*, 1997) could be transmitted from this source to animal population.

CONCLUSIONS

The spoligotyping method can be used in epidemiological studies of bovine tuberculosis in the countries with low incidence and prevalence of the infection. Due to the fact that spoligotyp-

ing is based on PCR this method could also be used for identification of samples which contain low amounts of DNA (for example dead isolates stored in liquid or on solid media for several years). Different spoligotypes of our isolates and in some cases time lapses from the incidence of the infection with exclusion of possible contacts among the herds are suggestive of different sources of causal agent in individual herds. As a risk factor has to be considered nowadays the farm rearing of red deer, animals purchase in zoological gardens and reservoirs in the wild. Therefore it appears necessary from the epidemiological point of view to examine in the laboratory all tuberculous lesions detected at necropsy in shot dead or dissected wild animals originating from zoological gardens, game parks and farms with wild ruminants and from wild ruminants from the nature.

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