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1 **Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae***  
2 **animal isolates**

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8

9 **Keywords:** Molecular typing, spoligotyping, bovine tuberculosis, *Mycobacterium bovis*,  
10 *Mycobacterium caprae*.

11

12 **Abstract**

13 The genetic diversity of 283 *Mycobacterium bovis* (*M. bovis*) and 10 *Mycobacterium*  
14 *caprae* (*M. caprae*) strains , isolated between 2002 and 2007 from cattle, goat, red  
15 deer and wild boar from six different geographical regions of Portugal was investigated  
16 by spoligotyping. The technique showed a good discriminatory power (Hunter-Gaston  
17 Index,  $h=0.9$ ) for the strains, revealing 29 different patterns. One pattern (SB0121)  
18 was clearly predominant, accounting for 26.3% of the isolates; ten patterns,  
19 representing 20.7% of the isolates, had never been reported previously. Multiple  
20 spoligotypes were detected in thirteen cattle and one goat herd, most of which were  
21 found in beef cattle and extensive management regions, suggesting different infection  
22 sources. With the exception of two spoligotypes, those in wildlife species were also  
23 found in domestic species.

24

25 **1. Introduction**

26 Bovine tuberculosis (bTB) is mainly caused by *Mycobacterium bovis* (*M. bovis*), but  
27 *Mycobacterium caprae* (*M. caprae*) has also been isolated from suspect tuberculous  
28 lesions. During the last decade, the incidence of bTB has been low in Portugal varying

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29 between 0.04% and 0.13% from 1995 to 2006 with a peak in 2003. In 2006 the  
30 estimated herd prevalence was 0.18% and animal incidence 0.05%. The most infected  
31 area was the Alentejo where 68.4% of animals were positive (Anonymous, 2006). A  
32 National Eradication Programme for bovine tuberculosis has been in operation since  
33 1987 and is presently based on systematic tuberculin testing and slaughter with  
34 bacteriological diagnosis carried out at the National Reference Laboratory (NRL-LNIV)  
35 and permanent abattoir surveillance. Despite this, bovine tuberculosis remains a major  
36 concern since no effective eradication has been achieved yet and sporadic outbreaks  
37 occur in some regions of Portugal with no evident epidemiological explanation. The  
38 possible role of wildlife reservoirs in the maintenance and reintroduction of bTB in  
39 certain herds has not been studied.

40 Spoligotyping has been considered a first line molecular typing method for *M. bovis*  
41 since it is PCR based, reproducible and less labour intensive than other fingerprinting  
42 techniques (Kamerbeek et al., 1997; Kremer et al., 1999). It also has the benefit of  
43 overcoming the limited discriminatory power of Restriction Fragment Length  
44 Polymorphism Analysis (RFLP) analysis of IS6110 (IS6110-RFLP) for microorganisms,  
45 such as *M. bovis* which have low IS6110 copies (Cousins et al., 1998). Furthermore,  
46 the results of spoligotyping can be promptly compared between laboratories as  
47 international databases (<http://www.mbovis.org> and [http://www.pasteur-](http://www.pasteur-guadeloupe.fr/tb)  
48 [guadeloupe.fr/tb](http://www.pasteur-guadeloupe.fr/tb)) are available.

49 In this study spoligotyping was used to produce genotyping data of *M. bovis* and *M.*  
50 *caprae* isolates from several animal species in different geographical regions of  
51 Portugal for the first time. The main aims were to examine the genetic diversity of these  
52 isolates, and to better understand the population structure of *M. bovis*, which will  
53 provide the basis for further studies on the transmission of bTB contributing for the  
54 improvement of the ongoing eradication program.

55

## 56 **2. Materials and methods**

### 57 2.1. Samples

58 Mycobacteria were isolated from tissue samples from slaughtered animals, that were  
59 either skin test reactors or showed tuberculous suspect lesions during meat inspection.  
60 The samples were submitted between July 2002 and June 2007 to the National  
61 Reference Laboratory (LNIV) by the National Veterinary Authority under the bTB  
62 Eradication Programme. A total of 293 *Mycobacterium tuberculosis* complex isolates  
63 from cattle (n=258), goat (n= 8), red deer (*Cervus elaphus*, n=21) and wild boar (*Sus*  
64 *scrofa*, n= 6) were randomly selected from all the six geographic regions of Portugal  
65 where bTB was reported: “Entre Douro e Minho” (EDM, n=47) “Trás-os-Montes” (TM,  
66 n=14) “Beira Litoral” (BL, n=6), “Beira Interior” (BI, n=38), “Ribatejo e Oeste” (RO, n=  
67 57) and Alentejo (AL, n=131). No samples were neither received from the Algarve, the  
68 Azores or Madeira islands as they are free of bovine TB. This represents 24% of the  
69 1228 *M. bovis* and *M. caprae* strains isolated during this period. Isolates were obtained  
70 from 132 cattle herds, 1 goat herd and deer and boar from game reserves. The data  
71 concerning cattle registration and movements for 221 of the 258 bovines studied were  
72 obtained from the National database (SNIRB database- *Sistema Nacional de*  
73 *Identificação e Registo Bovino*).

74

### 75 2.2 Bacterial isolation and identification

76 Samples were processed according to OIE standard procedures and bacterial isolation  
77 was performed by inoculation of BACTEC 9000 liquid medium, and of the following  
78 solid media: Stonebrink, Löwenstein-Jensen, Löwenstein-Jensen with thiophen-2-  
79 carboxylic acid hydrazide (TCH) and Löwenstein-Jensen with pyruvate. The isolates  
80 were identified by a PCR-REA system (Nieman et al., 2000) based on *gyrB* gene  
81 amplification and hydrolysis with *RsaI*, *SacII* and *TaqI* restriction enzymes.

82

### 83 2.3. DNA Extraction

84 DNA was extracted from isolates by a combined method of SDS/Proteinase K  
85 enzymatic lysis and bead-beating. Bacterial pellets from BACTEC liquid medium or  
86 colonies on Löwenstein-Jensen media were suspended in 500 µl TE (1 mM Tris-HCl-  
87 pH 7.6; 0.1 mM EDTA) and heated at 90°C for 20 min. After centrifugation the pellet  
88 was resuspended in 300 µl lysis buffer [40 mM NaCl; 4 mM Tris-HCl (pH 8); 0.6% SDS;  
89 2 mM EDTA; 0.034 mg/ml proteinase K], transferred to a tube with zirconium beads  
90 and incubated overnight at 37°C. Mechanic disruption was accomplished in a ribolyser  
91 FastPrep 120 (Savant), 6.5 m.sec<sup>-1</sup> for 45 sec. After cooling on ice, phenol/chloroform  
92 extraction was performed. The final pellet was resuspended in 50 µl TE.

93

#### 94 2.4. Spoligotyping

95 Spoligotyping (Spacer Oligonucleotide Typing; Kamerbeek et al.,1997) detects the  
96 polymorphism of the Direct Repeat (DR) chromosomal region that consists of unique  
97 spacer sequences (34-41 bp) interspersed with identical 36 bp direct repeats, termed  
98 together Direct Variant Repeat (DVR) (van Embden et al., 2000). The DR region is  
99 amplified by PCR with a biotin labelled primer and the presence or absence of 43  
100 spacer sequences is evaluated by hybridization to immobilized oligonucleotides on a  
101 membrane.

102 Forty three spacer oligonucleotides (Van Embden et al., 2000), synthesized with a 5'  
103 terminal amino group, were covalently linked to a negatively charged Biotodyne C  
104 membrane (Pall Corporation, USA) according to Kamerbeek et al. (1997). PCR  
105 targeting the DR region and hybridization of PCR products was performed according to  
106 the same authors with the following changes: the hybridization time was increased to  
107 90 minutes and membranes were reused after three washes at 80 °C in 5% SDS. *M.*  
108 *tuberculosis* H37Ra-ATCC 25177 and *M. bovis* ATCC 19015 reference strains were  
109 used as controls in each hybridization assay.

110

#### 111 2.5. Data analysis

112 For each isolate, the presence or absence of each of the 43 oligonucleotides was  
113 recorded as 1 (presence) or 0 (absence) in an Excel file. The obtained patterns (a  
114 series of 43 digit binary numbers) were compared with the database patterns  
115 ([www.mbovis.org](http://www.mbovis.org)). As each herd rather than each animal is considered an  
116 epidemiological unit, and to avoid bias from the different number of isolates supplied  
117 per herd, spoligotypes frequencies and discriminatory index (Hunter et al., 1988) were  
118 calculated considering only one isolate for each particular spoligotype in an outbreak.

119

### 120 **3. Results**

#### 121 *3.1. Isolates identification*

122 From 293 *Mycobacterium* isolates [cattle (n=258), goats (n= 8), red deer (n=21) and  
123 wild boar (n= 6)], 283 were identified as *M. bovis* (256 from cattle, one from a goat, 21  
124 from deer, and 5 from a wild boar) and ten as *M. caprae* (7 from goats, 2 from cattle  
125 and one from a wild boar) by PCR-REA based on *gyr B* gene.

126

#### 127 *3.2. Global spoligotype patterns diversity*

128 In 293 *Mycobacteria* isolates, 29 different spoligotype patterns were identified (Table  
129 1), 28 for *M. bovis* strains and one for *M. caprae* strains, indicating a good  
130 discrimination power of this method (Hunter-Gaston index,  $h=0.9$ ). All the patterns  
131 presented the typical *M. bovis* (absence of spacers 3, 9, 16 and 39 to 43) and *M.*  
132 *caprae* (absence of spacers 1, 3-16, 28, 30-33 and 39-43) spoligotype signatures  
133 (Table 1). The most frequent deletion was of spacer 21, noticed in 19 spoligotypes.  
134 SB0121 was clearly the predominant spoligotype, representing 26.29 % of the isolates  
135 and was widely dispersed geographically.

136 Ten different spoligotype patterns were seen for the first time and a new SB number  
137 was allocated to the database [www.mbovis.org](http://www.mbovis.org). New spoligotypes were found in *M.*  
138 *bovis* isolates from different cattle breeds, both Portuguese autochthons and exotic  
139 (imported breeds). Three of these new patterns (SB1095, SB1090 and SB1093) differ

140 from the predominant SB0121 by deletion of a single spacer. Nineteen different  
141 patterns have been previously described in other countries (Table 1) having, like Spain,  
142 France, Belgium and Netherlands, trading ties with Portugal.

143

### 144 *3.3 Spoligotype diversity within herds*

145 Most (91.6%) of the outbreaks within the 133 herd studied presented a sole  
146 spoligotype, but for 14 outbreaks (8.4%) more than one spoligotype was found.  
147 Spoligotypes SB0140, SB1172, SB1174, SB0122, SB0130, SB0848, SB0833, SB1173,  
148 SB1175, SB1091, SB0867, SB0332, SB1093 and SB1267 (Table 1) were exclusively  
149 found in single-spoligotype outbreaks; while SB0121, SB0119, SB0886, SB1095,  
150 SB0295, SB0124, SB0120, SB0265, SB1090, SB1167, SB1230, SB1191, SB0849,  
151 SB0334 and SB0157 (Table 1) were also found in multi-genotype outbreaks. With the  
152 exception of TM and BL regions, these multi-genotype outbreaks were recorded in  
153 herds from every region, with a predominance in AL and RO.

154 From the available data, no cattle movements between herds were traced in 101 out of  
155 258 animals (45.7%). For the remaining animals, 31.7% of the inter-herd movements  
156 were made within the same geographical region, and 21.3% between neighbouring  
157 regions. Ten infected animals had been imported from France, Spain, Netherlands and  
158 Denmark but no inter-herd movement was recorded for those animals before slaughter.

159

### 160 *3.4. Spoligotypes from different hosts*

161 Regarding pattern distribution in different animal species, with the exception of two *M.*  
162 *bovis* isolates from red deer (SB1091 and SB1267 patterns), wildlife and domestic  
163 species shared the same patterns (Figure 1). Shared patterns between wildlife and  
164 domestic animals within the same region were recorded in AL and BI regions for  
165 spoligotypes SB0121, SB0119, SB0120, SB0265 and SB0122, and in two  
166 neighbouring regions for spoligotype SB1174.

167



168 **4. Discussion**

169 The molecular typing results presented in this study are the first reported in Portuguese  
170 *M. bovis* and *M. caprae* animal isolates. The diversity of sub-types found (29) proves  
171 that spoligotyping is a valuable tool for epidemiological purposes ( $h= 0.9$ ). This high  
172 diversity could be a reflection of the low bTB prevalence in Portugal that leads to  
173 constricted ongoing transmission of the same or similar sub-types. Although it has  
174 been suggested that test and slaughter policies can reduce strain diversity favouring  
175 clonal expansion as a result of a bovine population bottleneck (Smith et al., 2006), this  
176 does not seem to be the case of in Portugal. A possible explanation could be the  
177 absence in Portugal of major cattle epidemics resulting in extensive animal slaughter.

178 The two most common patterns, SB0121 and SB0119, differ by a single spacer and  
179 together account for 35.7% of the spoligotypes found. These spoligotypes could  
180 represent strains with selective genetic advantages such as increased pathogenesis,  
181 transmissibility or poor immunogenicity that would escape detection by conventional  
182 serological or skin tests (Van Embden et al., 2000; Skuce et al., 2001).

183 The most common spoligotype, SB0121 (26.4%), which is also highly frequent in Spain  
184 (Aranaz et al., 1996; Javed et al., 2007), has a considerable geographic spread as it is  
185 present in all Portuguese regions where bTB exists. MIRU-VNTR typing results of  
186 Portuguese SB0121 strains showed that it represents a more heterogeneous group  
187 than other spoligotypes (unpublished data).

188 Interestingly pattern SB0140 (4.7%) is the predominant spoligotype in the UK and  
189 Ireland (Smith et al., 2006), but has not been reported in any other European country.

190 An explanation for this epidemiological link between Portugal and the British Isles is  
191 clearly the regular cattle trading, that occurred until the Bovine Spongiform  
192 Encephalopathy (BSE) embargo in 1996. Although uncommon, homoplasmy, *i.e.*  
193 convergence of different families of strains into the same spoligotype (Warren et al.,  
194 2002), can also be an explanation for this occurrence. Whether Portuguese and British  
195 SB0140 strains belong to the same lineage is not presently known.

196

197 Spoligotype SB0120, representing the BCG-like strains, is the most frequent pattern in  
198 France (Haddad et al., 2001) and the second most frequent from cattle in Spain  
199 (Aranaz et al., 1996) but is infrequent in Portugal (3.76%). A decreasing frequency of  
200 this pattern should be expected, since its few spacer deletions make it the probable  
201 ancestral spoligotype (Haddad et al., 2001; Smith et al., 2006).

202 Except for the very infrequent patterns (less than two isolates), newly described  
203 patterns were present in more than one region. This wide geographical distribution  
204 cannot be explained by cattle movements as most of the animals (73%) showed either  
205 no inter-herd movements or that occurred between herds from the same geographical  
206 region. These spoligotypes could have evolved by deletion events and became  
207 established in Portugal before herd classification and the establishment of strict  
208 movement control policies.

209 More than one spoligotype was found in 14 separate outbreaks, clearly indicating  
210 independent sources of infection. Outbreaks involving multiple genotypes have been  
211 found in both high and low prevalence settings (Costello et al., 1999; Michel et al.,  
212 2008). In the present study, herds with multiple spoligotypes were located in regions  
213 where beef cattle herds predominate (AL and RO regions). Beef cattle herds,  
214 comprising fattening animals from different origins, are large, and characterised by  
215 having high animal movements and replacement rates. These are known to be major  
216 risk factors for inter herd bTB transmission (Phillips et al., 2003) and statistically linked  
217 to a increased risk of bTB infection in Portuguese herds (Almeida et al., 2006.  
218 “Agregados geográficos de tuberculose bovina na DRAAL”, communication at the  
219 Portuguese Society of Veterinary Epidemiology Meeting). Furthermore, beef cattle  
220 herds have extensive management practices and grazing areas can overlap wildlife  
221 habitats, leading to possible inter-species transmission. Altogether, these features  
222 probably make multi-genotype infections more likely in these regions.

223

224 Although the number of *M. caprae* isolates was low (10) they were remarkably  
225 homogeneous with only one spoligotype found in different regions. This spoligotype  
226 has already been reported in Spain (Aranaz et al., 1996). Further spoligotyping of  
227 Portuguese *M. caprae* isolates will confirm the need for an additional set of  
228 oligonucleotides spacers in order to enhance discrimination, as recently suggested  
229 (Javed et al., 2007). *M. caprae* was not exclusively isolated in goats, but also in cattle  
230 and boar. Although this species is adapted to goats, there have been several reports of  
231 transmission to other animals (Prodinger et al, 2002; Pate et al, 2006) and to humans  
232 (Gutierrez et al., 1997; Prodinger et al., 2002; Kubica et al., 2003).

233 Except for two newly described spoligotypes (SB1091 and SB1267) isolated in two  
234 deer, most wildlife spoligotypes were also found in domestic animals. Nevertheless, 8  
235 *loci* MIRU-VNTR typing (Supply et al., 2006) provided strong evidence of transmission  
236 on only two occasions: between cattle and wild boar in AL region and cattle and deer in  
237 BI region (unpublished data). This data constitute the first molecular evidence of inter-  
238 species transmission in Portugal. Although badgers (*Meles meles*) are recognised  
239 reservoirs of bTB in the British Isles (Costello et al., 1999; Reynolds, 2006), the  
240 importance of other animal species in the epidemiology of bTB elsewhere in Europe is  
241 largely unknown but is currently under investigation. Shared strain genotypes between  
242 feral animals and cattle have been reported in Italy and Spain (Serraino et al., 1999;  
243 Aranaz et al., 2004; Gortazar et al., 2005) and recent molecular, epidemiological and  
244 pathological evidence indicate that wild boar could act as a true reservoir  
245 (Martin.Hernando et al., 2007; Naranjo et al., 2008).

246 In this study we also noticed that *M. bovis* isolated from deer from the same herd  
247 presented different spoligotypes (data not shown), which would suggest the spillover of  
248 cattle strains into deer populations, rather than the maintenance and transmission of  
249 infection within it. More disease surveillance and strain typing would be expected to  
250 bring additional knowledge to support the later stages of the eradication process of bTB  
251 in Portugal, particularly in areas of known wildlife/livestock contact.

252

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 258 on herds and animal movements.

259

260 **References**

- 261 Anonymous 2006. Boletim Estatístico da DGV, 2006. Direcção Geral de Veterinária, Ministério  
 262 da Agricultura e Pescas, Lisboa, Portugal, pp 10-11.
- 263  
 264 Aranaz, A., Liebana, E., Mateos, A., Dominguez, L., Vidal, D., Domingo, M., Gonzolez, O.,  
 265 Rodriguez-Ferri, E.F., Bunschoten, A.E., van Embden, J.D., Cousins, D., 1996. Spacer  
 266 oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for  
 267 studying epidemiology of tuberculosis. J. Clin. Microbiol. 34, 2734-2740.
- 268  
 269 Aranaz, A., de Juan, L., Montero, N., Sanchez, C., Galka, M., Delso, C., Alvarez, J., Romero,  
 270 B., Bezos, J., Vela, A.I., Briones, V., Mateos, A., Dominguez, L., 2004. Bovine Tuberculosis  
 271 (*Mycobacterium bovis*) in Wildlife in Spain. J. Clin. Microbiol. 42, 2602-2608.
- 272  
 273 Costello, E., O' Grady, D., Flynn, O., O' Brien, R., Rogers, M., Quigley, F., Egan, J., Griffin, J.,  
 274 1999. Study of Restriction Fragment Length Polymorphism Analysis and Spoligotyping for  
 275 Epidemiological Investigation of *Mycobacterium bovis* Infection. J. Clin. Microbiol 37, 3217-  
 3222.
- 276  
 277 Cousins, D., Williams, S., Liebana, E., Aranaz, A., Bunschoten, A., Van Embden, J., Ellis, T.,  
 278 1998. Evaluation of Four DNA Typing Techniques in Epidemiological Investigations of Bovine  
 Tuberculosis. J. Clin. Microbiol 36, 168-178.
- 279  
 280 Gortazar, C., Vicente, J., Samper, S., Garrido, J., Fernández-De-Mera, I.G., Gavín, P., Juste,  
 281 R.A., Martín, C., Acevedo, P., De La Puente, M., Höfle, U., 2005. Molecular characterization of  
 282 *Mycobacterium tuberculosis* complex isolates from wild ungulates in South-Central Spain, Vet.  
 Res. 36, 43-52.
- 283  
 284 Gutierrez, M., Samper, S., Jimenez, M.S., van Embden, J.D., Marin, J.F., Martin, C., 1997  
 285 Identification by spoligotyping of a caprine genotype in *Mycobacterium bovis* strains causing  
 286 human tuberculosis. J. Clin. Microbiol 35, 3328-3330.
- 287  
 288 Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M.F., Hughes, S.L., Inwald, J.,  
 289 Hewinson, R.G., Durand, B., 2001. Spoligotype diversity of *Mycobacterium bovis* strains  
 290 isolated in France from 1979 to 2000. J. Clin. Microbiol. 39, 3623-3632.
- 291  
 292 Hunter, P.R. , Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing  
 systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465-2466.
- 293  
 294 Javed, M.T., Aranaz, A., de Juan, L., Bezos, J., Romero, B., Alvarez, J., Lozano, C., Mateos, A.,  
 295 Dominguez, L., 2007. Improvement of spoligotyping with additional spacer sequences for  
 296 characterization of *Mycobacterium bovis* and *M. caprae* isolates from Spain. Tuberculosis 87,  
 437-445.

- 297 Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., Van Soolingen, D., Kuijper, S.,  
298 Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., Van Embden, J., 1997. Simultaneous  
299 detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and  
300 epidemiology. J. Clin. Microbiol 35, 907-914.
- 301 Kremer, K., Van Soolingen, D., Frothingham, R., Haas, W.H., Hermans, P.W.M., Martin, C.,  
302 Palittapongarnpim, P., Plikaytis, B.B., Riley, L.W., Yakrus, M.A., Musser, J.M., van Embden,  
303 J.D.A., 1999. Comparison of methods based on different molecular epidemiological markers for  
304 typing of *Mycobacterium tuberculosis* complex strains: Interlaboratory study of discriminatory  
305 power and reproducibility. J. Clin. Microbiol. 37, 2607-2618.
- 306 Kubica, T., Rusch-Gerdes, S., Niemann, S., 2003. *Mycobacterium bovis* subsp. *caprae* caused  
307 one-third of human *M. bovis*-associated tuberculosis cases reported in Germany between 1999  
308 and 2001. J. Clin. Microbiol. 41, 3070-3077.
- 309 Martin-Hernando, M.P., Hofle, U., Vicente, J., Ruiz-Fons, F., Vidal, D., Barral, M., Garrido, J.M.,  
310 de la Fuente, J., Gortazar, C., 2007. Lesions associated with *Mycobacterium tuberculosis*  
311 complex infection in the European wild boar. Tuberculosis. 87, 360-367.
- 312 Michel, A.L., Hlokwé, T.M., Coetzee, M.L., Mare, L., Connoway, L., Rutten, V.P.M.G., Kremer,  
313 K., 2008. High *Mycobacterium bovis* genetic diversity in a low prevalence setting. Vet.  
314 Microbiol. 126, 151-159.
- 315 Naranjo, V., Gortazar, C., Vicente, J., de la Fuente, J., 2008. Evidence of the role of European  
316 wild boar as a reservoir of *Mycobacterium tuberculosis* complex. Vet. Microbiol. 127, 1-9.
- 317 Niemann, S., Harmsen, D., Rusch-Gerdes, S., Richter, E., 2000. Differentiation of Clinical  
318 *Mycobacterium tuberculosis* complex isolates by *gyr B* DNA sequence polymorphism analysis.  
319 J. Clin. Microbiol 38, 3231-3234.
- 320 Pate, M., Svára, T., Gombac, M., Paller, T., Zolnir-Dovc, M., Emersic, I., Prodingler, W.M.,  
321 Bartos, M., Zdovc, I., Krt, B., Pavlik, I., Cvetnic, Z., Pogacnik, M., Ocepek, M., 2006. Outbreak of  
322 Tuberculosis Caused by *Mycobacterium caprae* in a Zoological Garden. J Vet Med B 53, 387-  
323 392.
- 324 Phillips, C.J.C., Foster, C.R.W., Morris, P.A., Teverson, R., 2003. The transmission of  
325 *Mycobacterium bovis* infection to cattle. Res. Vet. Sci. 74, 1-15.
- 326 Prodingler, W.M., Eigentler, A., Allerberger, F., Schonbauer, M., Glawischnig, W., 2002.  
327 Infection of Red Deer, Cattle, and Humans with *Mycobacterium bovis* subsp. *caprae* in Western  
328 Austria. J. Clin. Microbiol 40, 2270-2272.
- 329 Reynolds, D., 2006. A review of tuberculosis science and policy in Great Britain, Vet. Microbiol.  
330 112, 119-126
- 331 Serraino A., Marchetti, G., Sanguinetti, V., Rossi, M.C., Zanoni, R.G., Catozzi, L., Bandera, A.,  
332 Dini, W., Mignone, W., Franzetti, F., Gori, A., 1999. Monitoring transmission of tuberculosis  
333 between wild boars and cattle: genotypical analysis of strains by molecular epidemiology  
334 techniques. J. Clin. Microbiol 37, 2766-2771.
- 335 Skuce, R.A., Neill, S.D., 2001. Molecular epidemiology of *Mycobacterium bovis*: exploiting  
336 molecular data. Tuberculosis 81, 169-175.
- 337 Smith, N.H., Gordon, S.V., de la Rua-Domenech, R., Clifton-Hadley, R.S., Hewinson, R.G.,  
338 2006. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat. Rev.  
339 Microbiol. 4, 670-681.
- 340 Supply, P., 2006. Protocol and Guidelines for Multilocus Variable Number Tandem Repeat  
341 Genotyping of *M. bovis* VENoMYC (Veterinary Network of Laboratories Researching into  
342 Improved Diagnosis and Epidemiology of Mycobacterial Diseases) WP7 Workshop. 2006.

343 Toledo, Spain, 19-22th October.

344 Van Embden, J.D.A., van Gorkom, T., Kremer, K., Jansen, R., Van der Zeijst, B.A.M., Schouls,  
345 L.M., 2000. genetic variation and evolutionary origin of the Direct Repeat locus of  
346 *Mycobacterium tuberculosis* complex bacteria. J. Bacteriol. 182, 2393-2401.

347 Warren, R.M., Streicher, E.M., Sampson, S.L., van der Spuy, G.D., Richardson, M., Nguyen, D.,  
348 Behr, M.A., Victor, T.C., van Helden, P.D., 2002. Microevolution of the Direct Repeat region of  
349 *Mycobacterium tuberculosis*: Implications for interpretation of Spoligotyping data. J. Clin.  
350 Microbiol. 40, 4457-4465.

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## CAPTIONS

377 Fig. 1. Sharing of *M. bovis* spoligotypes by different hosts. SB1091 and SB1267  
378 were exclusive to wildlife, while SB0265 and SB0157 were common to all hosts.  
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380 Table 1 – Diversity, distribution and frequency of *M. bovis* and *M. caprae* spoligotypes  
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