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BOAR (*SUS SCROFA*) FROM PORTUGAL

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ABSTRACT: Tuberculosis has been diagnosed in wild boar (*Sus scrofa*) in several European countries during the last decade; however, almost no information has been reported to date for Portugal. This study aimed to investigate tuberculosis in wild boar in Portugal through characterization of *Mycobacterium bovis* infection and identification of disease risk factors. Tissue samples were obtained from hunted wild boar during the 2005 and 2006 hunting seasons. Samples were inspected for gross lesions and processed for culture. Acid-fast bacterial isolates were identified by polymerase chain reaction and spoligotyping. Associations between tuberculosis in wild boar and several variables linked to wild ungulate diversity and relative abundance, livestock density, and cattle tuberculosis incidence were investigated. *Mycobacterium bovis* isolates were identified in 18 of 162 wild boars from three of eight study areas. Infection rates ranged from 6% (95% confidence interval [$CI_{P95\%}$]=1–21%) to 46% ($CI_{P95\%}$=27–67%) in the three infected study areas; females in our sample were at greater risk of being infected than males (odds ratio=4.33; $CI_{P95\%}$=3.31–5.68). Spoligotyping grouped the *M. bovis* isolates in three clusters and one isolate was a novel spoligotype not previously reported in international databases. Detection of *M. bovis* was most consistently associated with variables linked to wild ungulate relative abundance, suggesting that these species, particularly the wild boar, might act as maintenance hosts in Portugal.

Key words: Descriptive epidemiology, *Mycobacterium bovis*, risk factors, spoligotypes, tuberculosis, wild boar.

INTRODUCTION

Mycobacterium bovis is the major etiologic agent of animal tuberculosis, infecting many species of wild and domestic mammals and also man (de Lisle et al., 2002). Tuberculosis occurs worldwide in livestock. Prevalence in livestock is unknown in several countries but it was estimated that over 50 million cattle could be infected, resulting in annual costs estimated at 3 billion US$ (Hewinson, 2001). Several countries have successfully eradicated bovine tuberculosis through test and removal programs reinforced by active surveillance of abattoirs. Nevertheless, in other countries (e.g., United Kingdom, Ireland, USA, New Zealand) similar strategies could not eradicate the disease, which is now re-emerging. Difficulties in eradicating bovine tuberculosis in cattle in some areas may relate to the occurrence of tuberculosis in wildlife reservoir species (Bengis, 1999; de Lisle et al., 2002).

Tuberculosis is presently considered an emerging infectious disease in wildlife (Dobson and Foufopoulods, 2001) and has been reported in a variety of wild species that may become maintenance hosts for *M. bovis*. Regarding free-ranging suids, *M. bovis* infection has been reported in feral pigs in New Zealand, Australia, and Hawaii; warthog (*Phacochoerus aethiopicus*) in Africa; and wild boar (*Sus scrofa*) in Europe (de Lisle et al., 2002; Machackova et al., 2003).

The detection of *M. bovis* in a wildlife species does not imply that the infection is

established in that particular species. Different epidemiologic situations may occur, from dead-end spillover hosts to maintenance hosts and reservoirs (Bengis, 1999). Feral pigs were considered spillover hosts in New Zealand and Australia (Corner, 2006). Moreover, wild suids were used in New Zealand as sentinels for *M. bovis* presence in the environment (Nugent et al., 2002). With respect to the wild boar, its contribution to the epidemiology of tuberculosis in Europe seems variable; it is considered a spillover host in Italy (Serraino et al., 1999) but a maintenance host in Spain (Naranjo et al., 2008).

Once established in wildlife, tuberculosis is extremely difficult to eradicate. The only successful example is in Australia, where *M. bovis* was eradicated by depopulation of its maintenance host, the water buffalo (*Bubalus bubalis*) (Bengis et al., 2004). Therefore, before any attempt to control tuberculosis is undertaken the precise role of each species in the epidemiologic cycle needs to be ascertained (Corner, 2006).

Tuberculosis incidence in cattle has been continuously decreasing since Portugal joined the European Community in 1986. In 2005, the cumulative incidence in cattle herds and in cattle was 0.22% and 0.06%, respectively, with some regional variation (Direcão Geral de Veterinária [DGV], 2006). Moreover, 70,267 cattle herds (99.3%) already were classified as "officially free of bovine tuberculosis" (DGV, 2006). This means that the national eradication program is approaching its final goal; however the existence of a wildlife reservoir may compromise progress toward eradication and waste financial investments made by Portugal and the European Union. The possibility of spillover to other domestic species such as free-ranging Iberian pigs and even to humans, particularly hunters and others that handle wild ungulate carcasses, also should be considered. Furthermore, tuberculosis affects the endangered Iberian lynx (*Lynx pardinus*) (Briones et al., 2000). Because Portugal is planning to reintroduce captive-bred lynx to their former range (Sarmento et al., 2005), the presence of infected prey and the persistence of *M. bovis* in environmental sources may represent a threat to this conservation program.

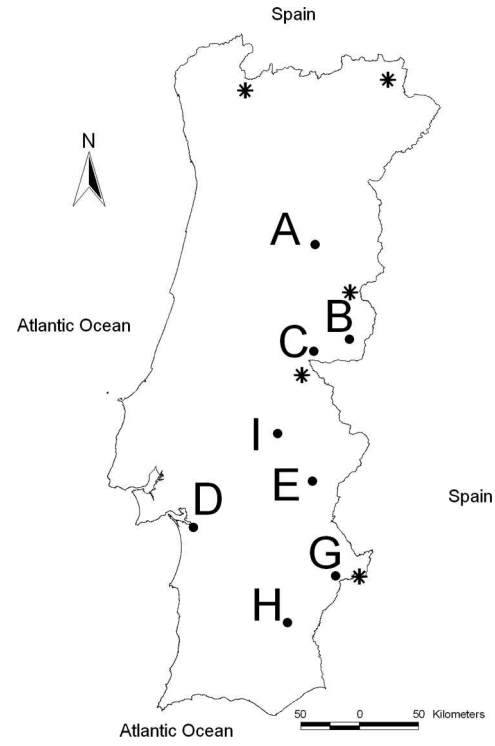

FIGURE 1. Historic refuges of wild boar in Portugal and study areas; * represents historic refuges of wild boar in Portugal. Study areas are represented by letters (A–E, G–I).

Wild boar populations declined in the Iberian Peninsula for centuries, probably because of direct persecution and increased human development of their habitats. Their distribution reached the lowest level during the first half of the 20th century (Nores et al., 1995; Lopes and Borges, 2004). By then the species was restricted, in Portugal, to a few historic refuges (Fig. 1) (Lopes and Borges, 2004; Ferreira et al., 2008). Thereafter, wild boar populations expanded during the second half of the 20th century (Sáez-Royuela and Tellería, 1986) and now the species occurs

throughout Portugal (Lopes and Borges, 2004; Ferreira et al., 2008).

The social organization of wild boar differs according to age and sex and comprises groups of one to several adult females—typically related to each other—along with offspring of the year, unstable groups of subadult males, and usually solitary adult males (Rosell et al., 2001). Home ranges are 40–60 km^2 for females, reaching 120–150 km^2 for adult males (Rosell et al., 2001). Densities reported in the Iberian Peninsula range from 1.7 wild boar/100 ha to 12.5 wild boar/100 ha (Rosell et al., 2001).

The aim of this study was to improve knowledge about tuberculosis in wild boar in Portugal. Specific objectives were to 1) investigate the infection by *M. bovis* in wild boar in major Portuguese hunting regions, 2) use spoligotyping to identify *M. bovis* strains involved, and 3) identify risk factors that may influence the occurrence of tuberculosis in wild boar populations.

MATERIALS AND METHODS

During the 2005–06 and the 2006–07 hunting seasons we collected submandibular, retropharyngeal, tracheobronchial, and mesenteric lymph nodes as well as lung samples from hunter-killed wild boars. Samples were taken in the field after evisceration of the carcasses, 2–10 hr after death. Tissues were stored in sterile 40-ml tubes and refrigerated. Gross pathology evaluation was conducted within 48 hr of sampling. Samples were then frozen at −20 C until used for bacterial culture. This sampling strategy, based on the targeted collection of samples from hunted animals, has been considered representative of the wild boar populations (Fernández-Llario et al., 2003).

Age and sex of the animals were recorded for most but not all animals because of constraints of field work. Age was determined by tooth eruption and wear patterns, according to Matschke (1967) and Buruaga et al. (2001). Animals were classified as juvenile (less than 1 yr old), subadult (1–2 yr old) or adult (>2 yr old). The epidemiologic unit was the area where sampled wild boars were potentially in direct contact. Considering the home ranges for the species, data from hunting areas within less than 25 km linear distance were grouped together in a study area.

Study areas

All study areas lie within the Mediterranean biogeographic region, with some Atlantic influence in study area A. The annual precipitation is 500–600 mm, the mean annual temperature is 14–17 C, and the number of sunlight hours is 3,000–3,100 hr/yr. The exception is study area A with 1,800 mm of annual precipitation, 9 C mean annual temperature, and 2,500 sunlight hr per yr. Altitude above sea level was 300–500 m for all study areas except A (800–1,000 m) and D (0–100 m) (Instituto do Ambiente, 2007).

All wild boars studied belonged to nonfenced, free-ranging populations.

Gross screening of tissues

Collected tissues were cut into slices approximately 3 mm thick. The presence of tuberculosis-like macroscopic lesions (TBL), identified as any granulomatous, caseous, purulent, necrotic, calcified, or proliferative lesion (Bollo et al., 2000; Gortazar et al., 2003), was recorded.

Bacterial culture and identification

All protocols were performed in a biosafety level 3 laboratory at Life and Health Sciences Research Institute, Braga, Portugal. Tissue samples were thawed and about 3 g of tissue was homogenized in 4 ml sterile water and then decontaminated with 0.75% hexa-decyl-pyridinium chloride, in a final volume of 35 ml. After a 2 hr-period for decontamination, a tube with Coletsos medium (BioMerieux, Marcy l'Étoile, France) and a petri dish with Middlebrook 7H11 medium (Becton Dickinson, Franklin Lakes, New Jersey, USA) were inoculated with the sediment/supernatant interface. Culture media were incubated at 37 C for 10 wk; they were checked after the first week to discard fast-growing bacteria, and then rechecked weekly from week 4 to week 10 to detect bacterial growth. Any bacterial growth was reinoculated into Coletsos and Middlebrook 7H11 media. A Ziehl-Neelsen stained smear was done from each culture and observed microscopically to determine the presence of acid-fast bacilli. A sample of each positive isolate was preserved at −80 C in sterile water. An aliquot of this suspension was heat-inactivated at 95 C for 45 min and DNA was extracted by standard phenol-chloroform method after 2×30 sec agitation with 0.1-mm zirconium beads in a Mini Bead-Beater

TABLE 1. Genes used for the identification of the mycobacterial isolates.

Target locus	Primer name	Nucleotidic sequence (5′–3′)	PCR fragment size (bp)[a]
16S RNA	16S RNA Fw	ACG GTG GGT ACT AGG TGT GGG TTT C	543
	16S RNA R	TCT GCG ATT ACT AGC GAC TCC GAC TTC A	
IS1081	IS1081 Fw	TCG CGT GAT CCT TCG AAA CG	238
	IS1081 R	GCC GTT GCG CTG ATT GGA CC	
Rv1510	Rv1510 Fw	GTG CGC TCC ACC CAA ATA GTT GC	1,033
	Rv1510 R	TGT CGA CCT GGG GCA CAA ATC AGT C	
Rv3120	Rv3120 Fw	GTC GGC GAT AGA CCA TGA GTC CGT CTC CAT	404
	Rv3120 R	GCG AAA AGT GGG CGG ATG CCA GAA TAG T	
IS1245	IS1245 Fw	GCC GCC GAA ACG ATC TAC	426
	IS1245 R	AGG TGG CGT CGA GGA AGA C	

[a] bp = base pairs.

(Biospec, Bartlesville, Oklahoma, USA). The DNA was stored at −20 C after quantification with an ultraviolet (UV) spectrophotometer (Beckman DU 650, Beckman Coulter, Fullerton, California, USA).

Bacterial isolates were identified by polymerase chain reaction (PCR) for a panel of selected genes: 16S RNA, IS1081, Rv3120, Rv1510, and IS1245 (Table 1). For the first four genes the PCR protocol described by Huard et al. (2003) was used. Five microliters of Taq buffer 10× (Fermentas, Burlington, Canada), 1.25 U Taq polymerase, 1 μl of each primer at 20 μM, and 1.25 μg of DNA were added to a solution containing 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 5% dimethyl sulfoxide (DMSO), in a final volume of 50 μl. This mix was submitted to the following PCR cycles: initial denaturation at 94 C for 5 min followed by 35 cycles of 94 C for 1 min, annealing at 60 C for 1 min, and extension at 72 C for 1 min with a final extension step of 72 C for 10 min. For IS1245 we followed protocols of Guerrero et al. (1995) and Bartos et al. (2006). Briefly, the same PCR mix was used, excluding the DMSO, with the following PCR cycles: 45 cycles of 94 C for 1 min, 65 C for 1 min and then 72 C for 1 min, with a final extension step of 72 C for 10 min. Polymerase chain reaction products were visualized by electrophoresis in 1% agarose gel with ethidium bromide and photographed under UV light (Alpha Imager, Alpha Innotech Corporation, San Leandro, California, USA). According to Huard et al. (2003) and Bartos et al. (2006), this set of genes allows for the identification of *M. bovis*, *Mycobacterium caprae*, other members of the *Mycobacterium tuberculosis* complex, members of the *Mycobacterium avium* complex, and other mycobacteria not belonging to these two complexes (Table 2).

Spolygotyping

The isolates were characterized by spoligotyping (Kamerbeek et al., 1997). The DNA from *M. bovis* BCG and *M. tuberculosis* H37Rv from the supplier's kit were used as positive controls and water as a negative control (Isogen Bioscience BV, Maarssen, The Netherlands). The spoligotyping patterns obtained were compared by visual examination and by analyzing the results in the BioNumerics software, version 3.5 (Applied Maths, Kortrijk, Belgium). A dendrogram was depicted to express similarities using Dice coefficient by unweighted pair group method using arithmetic average linkage. All the obtained spoligotype signatures were compared with those available in SpolDB4.0, the international spoligotyping database (Brudey et al., 2006). Additionally, spoligotype signatures were entered into the *M. bovis* spoligotype database (http://www.mbovis.org/spoligodatabase/singlepattern.php).

Risk factors

Because bacteriology is the gold standard for the diagnosis of tuberculosis, only culture-positive (confirmed by PCR) animals were considered tuberculosis-positive. Variables considered for potential association with *M. bovis* infection are listed in Table 3.

Relative abundance indices for wild ungulates were computed using the method described by Acevedo et al. (2007). One to three 4-km transects were walked in every study area, each divided into 50-m sections, determined with a pedometer. The presence of wild ungulate signs (feces for any species and wild boar roots) was recorded. Transects were standardized for season (summer), relative proportion of habitats covered (forest/shrub/river margins), and time since last rains (2–4 wk). The wild boar hunting bag for the county

TABLE 2. Polymerase chain reaction profile of mycobacterial species (*Mycobacterium* spp.) for the genes 16S RNA, IS1081, Rv1510, Rv3120, and IS1245 (Huard et al., 2003; Bartos et al., 2006).

Bacteria	16S RNA	IS1081	Rv1510	Rv3120	IS1245
Mycobacterium tuberculosis complex species other than *Mycobacterium bovis* and *Mycobacterium caprae*	+	+	+	+	−
Mycobacterium caprae	+	+	+	−	−
Mycobacterium bovis	+	+	−	−	−
Mycobacterium avium avium and *Mycobacterium avium hominissuis*	+	−	−	−	+
Mycobacterium avium paratuberculosis and *Mycobacterium intracellulare*	+	−	−	−	−
Other mycobacteria	+	−	−	−	−
Other bacteria	−	−	−	−	−

during the 2000–01 hunting season was obtained from Lopes and Borges (2004).

Wild ungulate species present in each study area were assessed by on-site interviews with hunters and hunting managers. The linear distance to the nearest historic refuge of wild boar was measured. Historic refuges of wild boar in Portugal correspond to their distribution during the first half of the 20th century (Fig. 1) according to Ferreira et al. (2008) and Lopes and Borges (2004).

Domestic ungulate densities were calculated using livestock data from the 1999 agricultural census of the Portuguese National Institute for Statistics (Instituto Nacional de Estatística, 2007). The number of cattle tuberculosis outbreaks and the number of tuberculin reactors were obtained from the animal health database run by the Portuguese Veterinary Directorate (DGV, 2006). Data from 2000 to 2005 were used to calculate annual cumulative incidences.

Statistical analysis

Statistical analysis was performed with Excel 2002 (Microsoft, Redmond, Washington, USA) and SPSS 14.0 (SPSS, Chicago, Illinois, USA). The 95% confidence intervals ($CI_{P95\%}$) surrounding estimated prevalence (p) and other proportions were calculated using a binomial distribution. Continuous variables were grouped into classes and a contingency analysis was applied to compare frequencies. To measure the strength of the association between risk factors and tuberculosis occurrence, we calculated prevalence odds ratio (OR_p) (Thrusfield, 2005).

TABLE 3. Variables investigated as possible risk factors for tuberculosis occurrence in wild boar (*Sus scrofa*) populations.

Class of variable	Variables investigated	Units	Geographical unit
Wild ungulate density	Wild boar relative abundance	n.a.[a]	Study area
	Cervid relative abundance	n.a.	Study area
	Wild ungulate relative abundance	n.a.	Study area
	Wild boar hunting bag (2000–01 season)	Wild boar/km^2	County
Wild ungulate species richness	Number of species of wild ungulates	Species	Study area
	Presence/absence of red deer	n.a.	Study area
Historic population dynamics	Distance to historic refuges of wild boar	km	Study area
Domestic ungulate density	Cattle density	Cattle/km^2	County
	Goat density	Goat/km^2	County
	Sheep density	Sheep/km^2	County
	Pig density	Pig/km^2	County
	Domestic ungulates density	Livestock/km^2	County
Tuberculosis prevalence in domestic ungulates (2000–05)	Tuberculosis incidence in cattle	%	County
	Tuberculosis incidence in cattle farms	%	County

[a] n.a. = not applicable.

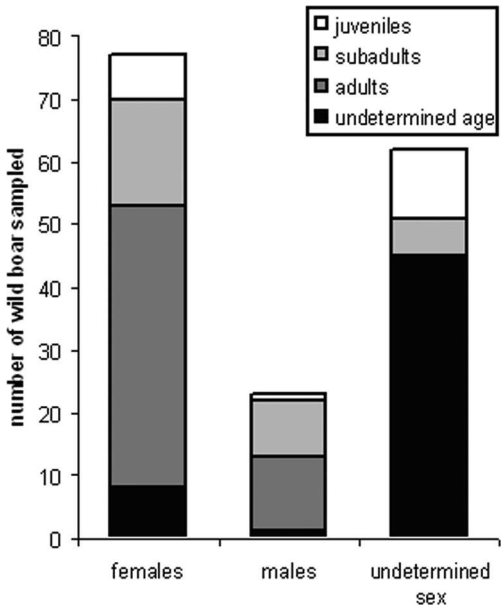

FIGURE 2. Age and sex distribution of animals sampled (n=162).

Multivariate exploratory analysis of data was performed by principal-components analysis (PCA) and logistic regression backwards stepwise. In the PCA, the dependent variable was the *M. bovis* infection status of the study area and the independent variables are listed in Table 3. In the logistic regression, all variables investigated were included in the initial model and sequentially removed, starting with those with a less significant effect. The dependent variable was the *M. bovis* infection status of individual wild boars and the independent variables were age, sex, and those listed in Table 3. Each wild boar was assigned the values of those variables obtained in the study area or county from which it came.

RESULTS

Tissue samples were collected from 162 hunter-killed wild boars in eight different study areas (Fig. 1). Sampled animals included 77 females, 23 males, and 62 of undetermined sex. Nineteen animals were juveniles, 32 were subadults, 57 were adults, and the remainder were of undetermined age (Fig. 2). The mean age of those animals for which we had age estimates was 2.4 yr; the oldest animal was 7–9 yr old.

TABLE 4. Isolation rate of *Mycobacterium bovis* from wild boar (*Sus scrofa*) in each study area.

Study area	No. of animals sampled	*Mycobacterium bovis* isolation		
		n	%	CI_{P95} (%)
A	30	0	0	0–11.6
B	32	2	6.3	0.8–20.8
C	18	4	22.2	6.4–47.6
D	15	0	0	0–21.8
E	5	0	0	0–52.2
G	26	12	46.2	26.6–66.6
H	21	0	0	0–16.1
I	15	0	0	0–21.8

We collected 588 tissue samples, including 239 submandibular lymph nodes, 165 retropharyngeal lymph nodes, 75 tracheobronchial lymph nodes, 47 mesenteric lymph nodes, and 62 lung portions.

Mycobacterium bovis was isolated from 18 of 162 sampled animals (p=11.1%; $CI_{P95\%}$=6.3–16.0%). *Mycobacterium avium*–complex mycobacteria were isolated from eight animals and other mycobacteria from 13 animals. *Mycobacterium bovis* was isolated in wild boars from three out of eight study areas (Table 4); herein these are referred to as "tuberculosis-infected areas."

Mycobacterium bovis isolation rates were significantly different between study areas (χ^2=46.1; df=7; P<0.001). Considering only those areas where tuberculosis was detected, *M. bovis* was recovered from 6/16 juveniles, 3/23 subadults, 6/27 adults, and 3/10 whose age was not recorded (Fig. 3A). Isolation rates for the known age classes were 38% in juveniles ($CI_{P95\%}$=15–65%), 13% in subadults ($CI_{P95\%}$=3–34%), and 22% in adults ($CI_{P95\%}$=9–42%) (Fig. 3A). The risk of infection tended to be higher for juveniles compared to other age classes but the difference was not significant (OR_p=2.73; $CI_{P95\%}$=0.59–12.57). The oldest animal infected with *M. bovis* was 3–4 yr old; the youngest was 6–8 mo old.

Mycobacterium bovis was isolated from 13/46 females, 1/12 males and 2/18

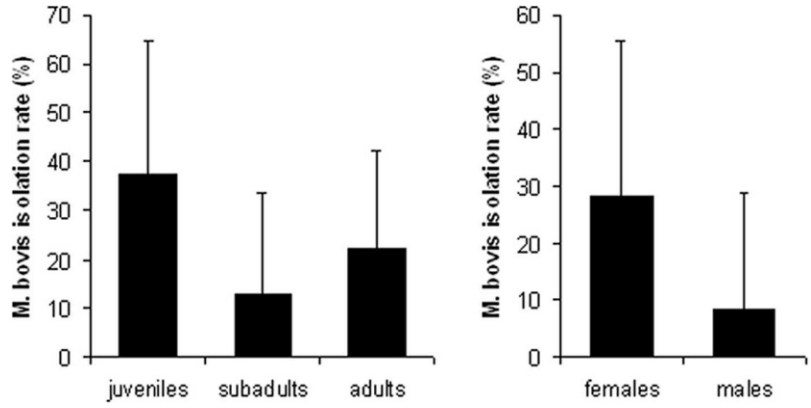

FIGURE 3. Isolation rates (%) of *Mycobacterium bovis* from sampled wild boar by age (A) and sex (B). The bars represent the upper bounds of binomial 95% confidence intervals ($CI_{P95\%}$).

animals whose sex was not recorded (Fig. 3B). Isolation rates were 28% ($CI_{P95\%}$=16–44%) for females and 8% ($CI_{P95\%}$=0.2–39%) for males (Fig. 3B). The risk of infection was significantly higher for females in comparison to males (OR_p=4.33; $CI_{P95\%}$=3.31–5.68).

The spoligotypes of 14 *M. bovis* isolates obtained from study areas B and G (Table 5) grouped into three clusters containing two, three, or eight isolates each, with one unique isolate (Fig. 4). Clusters 1 and 2 were only found in study area G, whereas cluster 3 was obtained from study areas B and G. The unique spoligotype originated from study area B. Isolates from the study area C were not spoligotyped.

The spoligotype patterns were compared to those available in the international databases SpolDB4 and www.mbovis.org. The spoligopattern of isolates in Cluster 1, the largest cluster in our study (n=8) was registered in the *M. bovis* database as spoligotype international type (SIT) SB1190 but was not found in SpolDB4. Isolates with this spoligotype were identified in wild boar from Cáceres, Spain (www.mbovis.org), which is located 200 km north from study area G. The spoligopattern of isolates in Cluster 2 (n=2) was reported previously as SIT1819, from

TABLE 5. Spoligotype signatures of the wild boar (*Sus scrofa*) *Mycobacterium bovis* isolates as defined in the SpolDB4 spoligotype database and the database at www.mbovis.org.

Sample	Study area of origin	Cluster	SpolDB4 signature	www.mbovis.org signature
ID-47	G	1	No	SB1190
ID-49	G	1	No	SB1190
ID-54	G	1	No	SB1190
ID-90	G	1	No	SB1190
ID-92	G	1	No	SB1190
ID-171	G	1	No	SB1190
ID-173	G	1	No	SB1190
ID-91	G	1	No	SB1190
ID-18	G	2	SIT1819	SB1053
ID-52	G	2	SIT1819	SB1053
ID-80	B	Unique	No	No
ID-12	G	3	SIT684	SB0265
ID-13	G	3	SIT684	SB0265
ID-97	B	3	SIT684	SB0265

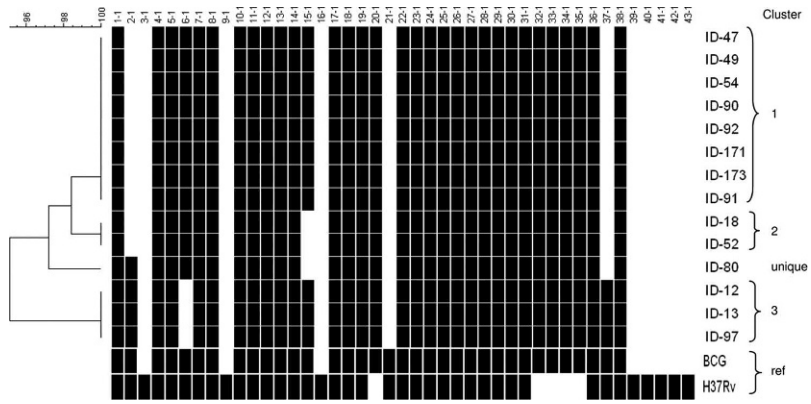

FIGURE 4. Spoligotypes of *Mycobacterium bovis* isolates obtained from wild boar and their clustering.

Mexico, in the SpolDB4 database. The same spoligo signature was registered as SB1053 in the www.mbovis.org database from Argentina, Brazil, Paraguay, Mexico, Uruguay, and Costa Rica. The pattern of Cluster 3 isolates ($n=3$) was found as SIT 684 in SpolDB4 and was previously reported from Germany, Belgium, France, South Africa, Taiwan, and the USA. It was also found in the www.mbovis.org database as SB0265. This pattern was reported in isolates from wild boar, red deer, and cattle from Extremadura, western Spain (Parra et al., 2005). This region is contiguous to the south and west with study areas B and C. The unique isolate (our designation ID-80) was not found in any database.

By gross pathology, we detected TBL in 37 tissue samples collected from 18 animals (13 of these were culture-positive). Lesions were seen in submandibular, tracheobronchial, retropharyngeal, and mesenteric lymph nodes and in lung. Most of the TBL detected in culture-positive animals were caseous granulomas with variable sizes and degrees of calcification. The TBL were usually well encapsulated by a fibrous layer. One lesion was characterized by fibrotic hypertrophy of the affected lymph node and two lesions were purulent abscesses. One abscess was invading the contiguous parotid salivary gland. All 18 wild boars in which TBL were detected originated from tuberculosis-infected areas, from which a total of 76 animals were examined. No TBL were detected in any of the 86 wild boars from the other study areas in which no *M. bovis* isolates were found.

In the univariate analysis, variables significantly associated with tuberculosis detection in wild boar were "cervid relative abundance," "wild ungulate relative abundance," "presence of red deer," and "distance to historic refuges" (Table 6). The multivariate exploratory analysis by PCA identified four components with eigenvalues >1, which explain 89.5% of the variation on tuberculosis detection at the study areas. All variables significantly correlated with component 1 (37.3% of the variation explained) were linked to wild ungulate abundance (i.e., "cervid relative abundance," "wild ungulate relative abundance," and "wild boar hunting bag"). Variables significantly correlated with other components were "sheep density" and "tuberculosis incidence in cattle."

The logistic regression model incorporating the variables "wild boar relative abundance" and "sex" classified correctly 89.0% of the animals regarding their infection status (93.7% of the negative and 58.3% of the positive). However, the only variable significantly associated with the presence of *M. bovis* infection in individual wild boars was "wild boar

TABLE 6. Association between tuberculosis detection at study areas ($n=8$) and variables investigated in the univariate analysis.

Variables investigated	Infected mean (SD)	Noninfected mean (SD)	Cut-off	χ^2	df	P
Wild boar relative abundance	1.69 (0.63)	1.43 (0.68)	1.5	1.07	1	0.30
Cervid relative abundance	1.12 (1.30)	0.71 (1.13)	1	4.71	1	0.03
Wild ungulate relative abundance	2.51 (1.31)	1.96 (1.29)	2	5.33	1	0.02
Wild boar hunting bag	n.a.[a]	n.a.	1.5 wild boar/km^2	2.55	1	0.11
Number of species of wild ungulates	2.50 (1.05)	2.11 (1.05)	2 species	3.61	1	0.06
Presence of red deer	n.a.	n.a.	n.a.	5.33	1	0.02
Distance to historic refuges	18.3 (20.2)	51.9 (44.9)	40 km	5.33	1	0.02
Cattle density	9.88 (8.36)	8.11 (7.51)	9 cattle/km^2	1.78	1	0.18
Goat density	6.75 (5.92)	5.78 (5.15)	6 goat/km^2	1.07	1	0.30
Sheep density	45.41 (16.95)	49.91 (17.96)	48 sheep/km^2	0.62	1	0.43
Pig density	8.27 (6.72)	6.64 (5.85)	8 pig/km^2	1.07	1	0.30
Domestic ungulate density	62.73 (34.17)	64.90 (29.10)	64 ungulate/km^2	2.58	1	0.11
Tuberculosis incidence in cattle	0.84 (1.07)	0.96 (1.34)	0.9 %	2.19	1	0.14
Tuberculosis incidence in cattle farms	3.26 (3.58)	7.17 (12.98)	6.0 %	0.62	1	0.43

[a] n.a. = not applicable.

relative abundance" (Wald $\chi^2=12.82$; df=1; $P<0.001$).

DISCUSSION

The epidemiology of *M. bovis* infection in wild boar populations in Portugal was not previously described. Because infection rate varied between study areas, results are discussed by study area and not as a whole. Isolation rates for *M. bovis* were 6, 22, and 46% in tuberculosis-infected areas. In this study, as well as in the study by Aranaz et al. (2004), *M. bovis* infection rates in wild boar were estimated by systematically employing bacteriology and not relying on gross pathology as the screening test. This is of relevance because gross pathology alone can substantially underestimate disease prevalence, as shown in free-ranging cervids (O'Brien et al., 2004).

Vicente et al. (2006) estimated a TBL prevalence rate of 43% in wild boar in Sierra Morena, southwestern Spain. At study area G, located in the foothills of Sierra Morena, our estimated *M. bovis* infection rate was similar (46%). In contrast, Parra et al. (2006) reported a 2.3% TBL prevalence rate in wild boar in the Spanish region of Extremadura. This value is lower than the *M. bovis* infection rate we estimated in nearby study areas B and C (12% for both areas combined; $CI_{P95\%}=5$–24%). Although this difference in prevalence may be real, it may as well be the result of 1) the use of different diagnostic approaches (bacterial culture vs. gross pathology), 2) the use of different study designs (targeted surveys vs. official veterinary inspection of hunted animals), or 3) the lapse in time between the two studies; the study by Parra et al. (2006) was performed in 2002 and the authors suggested that the disease incidence was increasing.

Most previous studies on wildlife tuberculosis have not reported any association with gender, but some studies reported a higher prevalence in males (Bengis, 1999). This is usually explained by sexual differences in behavior. For example, *M. bovis* may be transmitted during fights in breeding season in white-tailed deer (*Odoicoleus virginianus*) (O'Brien et al., 2006). Vicente et al. (2006) reported that TBL prevalence tended to be higher in male wild boar on intensively managed populations with apparently high overall prevalence. From our results, female wild boar were 4.3 times more likely to be infected with *M. bovis* than were males.

One possible explanation for this pattern is the social behavior of females, which tend to live in groups and are therefore expected to have higher intraspecific contact rates compared to males.

Because tuberculosis is usually a chronic disease, prevalence tends to increase with age for most species. However, a few studies have reported higher infection rates in juveniles compared to older age classes (Bengis, 1999). The infection rate in our sample tended to be higher in juveniles compared to other age classes. This may be the result of increased mortality rates in infected animals (Naranjo et al., 2008). Another possible explanation is that wild boar are mainly infected during the first year of life, either through maternal milk (Martín-Hernando et al., 2007) or by horizontal transmission associated with living in large familiar groups (Vicente et al., 2006). If this is the case, then the infection rate in each age cohort could reflect the transmission rate during the first year of life of that age cohort. Therefore, our results may be explained by an increased transmission of *M. bovis* during the last few years. In that case, tuberculosis should be considered an emerging disease in wild boar in Portugal. This hypothesis could be tested by following the dynamics of the infection rates in each age cohort during the coming years. If tuberculosis is emerging, then infection rates should increase in older age cohorts (subadults and adults) over time. On the other hand, our results may reflect the small sample size of juveniles, as differences in infection rates were not significant.

Juvenile wild boars with tuberculosis have been previously reported by Serraino et al. (1999) and Vicente et al. (2006). Possible infection routes are the ingestion of infected milk (Martín-Hernando et al., 2007), direct contact with infected members of the family group (Vicente et al., 2006), or feeding on carrion from infected mammals (Naranjo et al., 2008). *Mycobacterium bovis* may remain viable several weeks in carcasses and juveniles are the main age class consuming carrion (Dardaillon, 1989).

Besides *M. bovis* (43.9% of the mycobacterial isolates), we identified *M. avium* (19.5%) and other mycobacteria (36.6%). Whether the presence of these other mycobacterial species is due to contamination during the collection of the samples or to infection in vivo is not known, because they might be present in the environment (e.g., water, soil) and in animal hosts (e.g., rodents) (Skoric et al., 2007).

Characterization of the *M. bovis* spoligotypes obtained from Portuguese wild boars adds to knowledge on regional strain variation in wild and domestic ungulates. The Cluster 3 spoligopattern has recently been reported from cattle, red deer, and wild boar from the same regions of Portugal (Duarte et al., 2008). The Cluster 1 and Cluster 3 spoligopatterns were previously identified in wild boar isolates from Spain. Additionally, isolates with the Cluster 3 spoligotype were also reported in Spanish cattle and red deer. Isolates with the Cluster 2 spoligotype only have been reported from cattle in Latin America and could have been introduced there with cattle brought from the Iberian Peninsula by colonists. The unique spoligotype of an isolate from area B was not previously reported in any international database. These findings seem to reflect host and/or geographic clustering of *M. bovis* as reported by Gortazar et al. (2005); however, none of the spoligotypes found by these authors in Sierra Morena, Spain, approximately 250 km from Portugal, were identical to those reported in the present study.

We detected TBL in head lymph nodes that drain the nasal and oral cavities, larynx, pharynx, and tonsils (Barone, 1996), as well as in tracheobronchial and mesenteric lymph nodes, suggesting both the respiratory and the alimentary routes of natural infection, although their relative importance is not known. Bollo et al.

(2000), Gortazar et al. (2003), and Martín-Hernando et al. (2007) all found that TBL usually were well encapsulated and contained few mycobacteria, which should reduce the likelihood of excretion. However, these authors also reported some animals with lesions not contained by a fibrous layer, potentially allowing excretion by several routes. The same patterns were observed in our study.

Infection by *M. bovis* in wild boar in Portugal was most consistently associated with several indices of wild ungulate abundance. Cervid, wild ungulate, and wild boar relative abundance indices all were higher in the areas where tuberculosis was detected. This is not surprising because tuberculosis prevalence rates and persistence have been associated with density in other maintenance hosts such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Lloyd-Smith et al., 2005). This suggests that wild ungulates may be maintenance hosts of *M. bovis* in Portugal, because if they were spillover hosts and the infection was maintained primarily by cattle, we would not expect the occurrence of *M. bovis* to be linked with their density, but rather with cattle variables. Others already have suggested that the wild boar is a maintenance host of *M. bovis* in Iberian Peninsula Mediterranean habitats (Aranaz et al., 2004; Vicente et al., 2006), and our data support their hypothesis.

The presence of red deer also was significantly and positively associated with tuberculosis occurrence in wild boar populations. This provides additional evidence of interaction between several hosts in a multihost-pathogen system. Vicente et al. (2006) suggested this interaction when they reported that TBL prevalence among red deer was significantly associated with TBL prevalence among sympatric wild boar.

We found that tuberculosis infection in wild boar varied with the distance to historic refuges of this species, which were $\leq$40 km away in infected areas and farther in noninfected areas. It is not known how long *M. bovis* has been present in wild boar populations in Portugal. One possible explanation of the pattern we observed is that five decades ago wild boar tuberculosis was restricted to historic refuges (Ferreira et al., 2008). From these refugia, wild boar populations expanded quickly throughout Portugal. Tuberculosis seems to have spread similarly, although at a much slower pace. One possible mechanism for such a lag between wild boar and *M. bovis* expansion is a population threshold for disease persistence in the tuberculosis-wild boar system. This was reported for the possum in New Zealand (Lloyd-Smith et al., 2005). Under this scenario, as wild boar populations expanded, densities at the front of the expansion wave were low (Holland et al., 2007), so tuberculosis could not be maintained even with the recruitment of infected animals from the high-density historic refuges. As expansion continued and wild boar densities increased to approximate densities in historic refuges (Holland et al., 2007), the range of *M. bovis* expanded. Considering that wild boar populations started expanding throughout Portugal some four decades ago but tuberculosis presently appears restricted to areas within 40 km of historic refuges of that species, we hypothesize that the disease is expanding at a rate of approximately 1 km/yr. The seemingly slow pace of tuberculosis expansion may be explained by the predominantly sedentary nature of the wild boar (Truvé et al., 2004). Taken together, these observations further support the notion that tuberculosis should be considered an emerging disease of wild boar in Portugal.

Tuberculosis incidence in cattle was significantly but negatively associated with wild boar infection in the multivariate analysis by PCA. Although we found no evidence of spillover to cattle, we cannot rule out that this may have occurred in the past and still may occur sporadically.

Delahay et al. (2001) and Corner (2006) stated that the potential for a

wildlife species to be a reservoir of tuberculosis is influenced by the prevalence (quantity of disease) and the pathology (which influences the capacity to produce and excrete viable bacilli) of the disease and also by the ecology of that species (which influences the potential for interspecies transmission). Our data show that *M. bovis* infection rates in wild boar in Portugal can be quite high and, although most lesions contained few mycobacteria and were well encapsulated, some were not and were located in organs with excretion potential, such as lungs and salivary glands. The potential for wild boar to excrete enough viable bacilli to infect other species has not been demonstrated and seems to be a research priority. More studies also are needed to improve knowledge about the ecologic and behavioral determinants that facilitate interspecies transmission between wild and domestic ungulates.

One relevant issue to consider regarding the epidemiology of wildlife tuberculosis in Portugal is the increasing frequency of wild ungulate translocations for hunting purposes. The movement of animals of unchecked health status has the potential to introduce *M. bovis* to wildlife in tuberculosis-free areas.

Our study reports *M. bovis* infection in wild boar in Portugal and provides evidence for its role as maintenance host in the tuberculosis epidemiologic cycle. Further studies are needed to improve understanding of the epidemiology of tuberculosis in wild ungulates and to assess the odds of interspecies transmission involving wild and domestic ungulates. Research priorities should be the investigations of excretion frequency and exit routes, contact rates among susceptible species, and detailed molecular epidemiology.

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