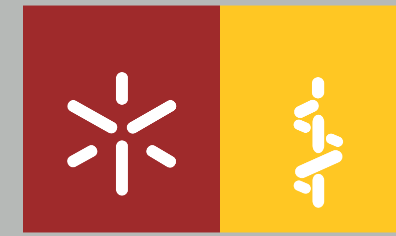


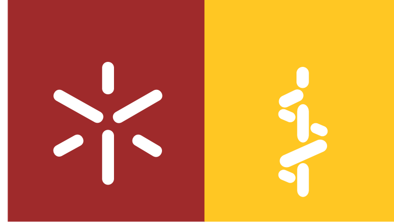
Universidade do Minho
Escola de Ciências da Saúde

Nuno Gonçalo Carvalho Caroço dos Santos

Tuberculosis in wild ungulates in the Iberian Peninsula: applying new methods for the epidemiological analysis of intra and interspecies transmission

janeiro de 2016

UMinho | 2016
Nuno Gonçalo Carvalho Caroço dos Santos
Tuberculosis in wild ungulates in the Iberian Peninsula: applying new methods for the epidemiological analysis of intra and interspeciestransmission



Universidade do Minho
Escola de Ciências da Saúde

Nuno Gonçalo Carvalho Caroço dos Santos

Tuberculosis in wild ungulates in the Iberian Peninsula: applying new methods for the epidemiological analysis of intra and interspecies transmission

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Prof. Doutora Margarida Correia-Neves
e do
Prof. Doutor Christian Gortázar Schmidt

janeiro de 2016

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 25 January 2016

Full name:

Nuno Gonçalo Carvalho Caroço dos Santos

Signature:

Nuno Gonçalo Carvalho Caroço dos Santos

Agradecimentos

À Escola de Ciências da Saúde agradeço a oportunidade de realizar o doutoramento num ambiente estimulante e de rigor científico.

À Prof. Margarida Correia Neves agradeço a confiança, a amizade, o apoio contínuo e a formação que me proporcionou.

Aos meus co-orientadores Prof. Christian Gortázar e Prof. Virgílio Almeida agradeço todo o apoio e ensinamentos transmitidos.

Aos colegas de laboratório agradeço as discussões proveitosas, apoio no laboratório e todos os bons momentos.

Aos meus amigos, por tudo.

À minha família por todo o apoio.

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

The work presented in this thesis was performed in the Microbiology and Infection Research Domain in the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal (ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal). The financial support was given by Fundação para a Ciência e Tecnologia (FCT) by means of a PhD grant (SFRH/BD/69390/2010).

Aos meus pais

Bovine tuberculosis (bTB) is a chronic slow-progressing zoonotic disease of livestock and wildlife caused by infection with *Mycobacterium bovis* or the closely related *Mycobacterium caprae*, both members of the *Mycobacterium tuberculosis* complex (MTC). In the Iberian Peninsula bTB is maintained in a multi-host pathogen system, with *M. bovis* and *M. caprae* circulating between sympatric wild ungulates and free-ranging domestic ungulates. This epidemiological model was investigated as part of the present PhD thesis in order to elucidate the mechanisms of intra- and inter-specific transmission and the spatial epidemiology of bTB in Iberian wildlife.

A systematic bibliographic review of the epidemiology of bTB in Iberian Peninsula suggests it is an endemic disease of autochthonous wild ungulates, with wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) acting as maintenance hosts. Bovine tuberculosis is an emergent disease in these species, with expansion from a core high-prevalence area in south-western Iberian Peninsula being fuelled by high host densities. Such high densities are due to intensive management for hunting purposes, including interventions such as removal of predators, fencing, translocation, artificial provision of food and water and even medication.

We investigated MTC excretion routes and concentration of MTC in biological samples from potential routes of excretion and reported for the first time the detection of MTC excretion from 83.0 % (CI_{95} 70.8–90.8 %) of naturally-infected wild boar and red deer. MTC DNA was amplified in all types of excretion routes (oronasal, bronchial-alveolar, fecal and urinary). MTC concentrations greater than the minimum infective doses for cattle, red deer or wild boar were estimated in excretion routes from wild boar and red deer. Also for the first time we provided evidence for the existence of a proportion of super-shedders within the naturally-infected populations of these host species (28.2 % of infected wild boar, CI_{95} 16.6–43.8 %; and 35.7 % of infected red deer, CI_{95} 16.3–61.2 %). These super-shedders are responsible for a disproportionately large amount of MTC excretion from infected wild ungulates.

Also we defined an improved protocol for the molecular detection and estimation of the concentration of MTC and *M. bovis/caprae* DNA in environmental samples and applied this protocol to assess MTC environmental contamination in areas with well-described distinct bTB

prevalence in wildlife. We reported for the first time the widespread occurrence of MTC DNA in the environment in areas where bTB has a high prevalence in wildlife. Seasonal rates of detection of MTC in environmental samples can be as high as 39.6 % (CI_{95} 27.6–53.6 %) in the spring. This contamination was detected in all types of *a priori* defined risk sites, where wild and domestic ungulates assemble, such as feeding and watering places.

We also assessed the spatial epidemiology of wildlife bTB in Portugal based on serological and bacteriological culture surveys. As a first step we confirmed that elutes from absorbent paper is a valuable new tool for bTB serological surveys in wild boar populations. Our data allowed for the confirmation of bTB as an emerging disease in wildlife in Portugal, documenting a 47 % increase in prevalence in one area from 2005-06 to 2009-14. Also we confirmed previous data suggesting a strong spatial structure of wildlife bTB, with 2 spatial clusters identified in south- and central-easternmost Portugal, in the periphery of the high-prevalence core area in central-southwestern Iberian Peninsula. Further we obtained 2 geographical risk models of bTB in wildlife at national and regional scales, both models generally agreeing with independent studies reporting MTC isolation from wild hosts.

These results have implications for the design of control programs in wildlife, including the selective targeting of super-shedder individuals in culling actions, the identification of high-risk transmission sites as targets for the implementation of biosecurity measures and risk-based surveillance and control based on spatial risk models.

A tuberculose bovina (bTB) é uma doença zoonótica crónica e de progressão lenta, que afeta animais domésticos e selvagens, sendo causada pela infeção por *Mycobacterium bovis* ou por *Mycobacterium caprae*, ambos pertencentes ao complexo *Mycobacterium tuberculosis* (MTC). Na Península Ibérica a bTB é mantida num sistema multi-hospedeiro, em que *M. bovis* e *M. caprae* circulam em populações simpátricas de ungulados selvagens e domésticos. Este modelo epidemiológico foi objeto de estudo na presente tese de doutoramento, tendo em vista contribuir para o conhecimento dos mecanismos de transmissão intra- e inter-específica e da epidemiologia espacial da bTB na fauna selvagem Ibérica.

Uma revisão sistemática da bibliografia sobre epidemiologia da bTB na Península Ibérica sugere que esta é uma doença endémica dos ungulados selvagens autóctones, sendo o javali (*Sus scrofa*) e o veado (*Cervus elaphus*) hospedeiros de manutenção. A bTB é uma doença emergente nestas espécies, sendo a expansão a partir do núcleo de alta prevalência no centro-sudoeste da Península Ibérica alimentado pelas altas densidades de hospedeiros selvagens. Essas densidades são mantidas artificialmente elevadas pelo maneio intensivo para fins cinegéticos, incluindo remoção de predadores, vedação, translocação, alimentação e abeberamento artificial e mesmo medicação.

Investigámos também as potenciais vias de excreção e respetivas concentrações de MTC e documentámos pela primeira vez a excreção de MTC em 83,0 % (IC_{95} 70,8–90,8 %) dos javalis e veados naturalmente infetados. Detetámos DNA de MTC em todos os tipos de vias de excreção estudadas (oronasal, bronquio-alveolar, fecal e urinária). Nestas vias de excreção estimámos concentrações de MTC superiores à dose mínima infetante para bovinos, veados e javali. Também pela primeira vez encontrámos evidência da existência de uma proporção de animais super-excretores na população infetada (28,2 % dos javalis infetados, IC_{95} 16,6–43,8 %; e 35,7 % dos veados infetados, IC_{95} 16,3–61,2 %), os quais são responsáveis por uma excreção de MTC desproporcionalmente elevada.

Também no âmbito desta tese descrevemos um protocolo melhorado para a deteção e estimativa da concentração de DNA de MTC e *M. bovis/caprae* em amostras ambientais, e

aplicámos esse protocolo para caracterizar a contaminação ambiental com estas micobactérias em zonas com diferentes prevalências de bTB na fauna selvagem. Pela primeira vez reportámos uma contaminação ambiental generalizada por MTC em zonas onde a bTB tem uma prevalência elevada em populações de ungulados selvagens. A proporção de amostras positivas alcançou os 39,6 % (IC_{95} 27,6–53,6 %) na primavera. Esta contaminação foi detetada em todos os tipos de zonas de risco previamente identificadas, onde ungulados domésticos e selvagens se concentram, como sejam zonas de alimentação e abeberamento.

Também avaliámos a epidemiologia espacial da bTB em Portugal com base em rastreios sorológicos e cultura bacteriológica. Inicialmente validámos as eluições de sangue embebido em papel absorvente como um novo método para rastreios sorológicos de bTB em javali. Os resultados confirmaram que a bTB é uma doença emergente da fauna selvagem em Portugal, documentando um aumento de 47 % da prevalência numa zona entre 2005-06 e 2009-14. Também confirmámos a ocorrência de uma marcada estruturação espacial da bTB na fauna selvagem, com 2 agregados espaciais no sul- e centro-leste de Portugal, localizados na periferia do núcleo da alta prevalência anteriormente descrito no centro-sudoeste da Península Ibérica. Produzimos 2 modelos geográficos de risco da bTB na fauna selvagem à escala nacional e regional, estando ambos em concordância com relatórios independentes do isolamento de MTC em animais selvagens.

O conjunto destes resultados tem implicações para o desenho de programas de controlo da bTB na fauna selvagem, nomeadamente através da remoção seletiva de super-excretores, da identificação de locais de alto risco de transmissão indireta como alvos de medidas de biossegurança e da vigilância e controlo da doença baseada nos modelos de risco espacial desenvolvidos no âmbito desta tese.

Acknowledgements v
Abstract ix
Abstract (Portuguese version) xi
Table of contents xiii

Chapter I – General introduction 1
1. Etiology of bovine tuberculosis 3
1.1. *Mycobacterium bovis* and *Mycobacterium caprae* 4
1.2. Other species of the *Mycobacterium tuberculosis* complex 5
2. Historical background and current epidemiological situation 6
2.1. Bovine tuberculosis in humans 6
2.2. Bovine tuberculosis in livestock 7
2.3. Bovine tuberculosis in wildlife 10
2.3.1. New Zealand 10
2.3.2. Australia 11
2.3.3. Southern Africa 12
2.3.4. North America 12
2.3.5. United Kingdom and Republic of Irelnd 14
2.3.6. Continental Europe (except Iberian Peninsula) 14
2.3.7. Iberian Peninsula 15
Santos N, Correia-Neves M, Almeida V, Gortázar C (2012) Wildlife tuberculosis: a systematic review of the epidemiology in Iberian Peninsula. In Cunha MLRS (ed.) Epidemiology Insights. Rijeka: InTech 17
3. The wildlife hosts of bovine tuberculosis in Iberian Peninsula 39
3.1. Ecology and population dynamics of wild boar 39
3.2. Ecology and population dynamics of red deer 40
4. Pathology of bovine tuberculosis 41
4.1. Pathology in cattle 41
4.2. Pathology in wild boar 43
4.3. Pathology in red deer 46
5. Transmission of bovine tuberculosis 47

6. Control of bovine tuberculosis in wildlife 50
6.1. Action upon the host population 51
6.2. Action upon the environment 53
6.3. Action upon human activities 53
7. Aims of the thesis 55
8. References 56

Chapter II– Shedding of *Mycobacterium tuberculosis* complex from naturally-infected wild boar and red deer 69
Santos N, Almeida V, Gortázar C, Correia-Neves M (2015) Patterns of *Mycobacterium tuberculosis*-complex excretion and characterization of super-shedders in naturally-infected wild boar and red deer. *Veterinary Research*, 46: 129 71

Chapter III – Environmental contamination by *Mycobacterium tuberculosis* complex 81
Santos N, Santos C, Valente T, Gortázar C, Almeida V, Correia-Neves M (2015) Widespread environmental contamination with *Mycobacterium tuberculosis* complex revealed by a molecular detection protocol. PLoS ONE, 10(11): e0142079 83

Chapter IV – Spatial analysis of bovine tuberculosis in wildlife in Portugal . 101
Santos et al. (in preparation) Spatial analysis and modeling of emerging wildlife tuberculosis in a multi-host pathogen system 103

Chapter V – General discussion and final conclusions 125
1. General discussion 126
1.1. Systematic review of the bibliography 126
1.2. *Mycobacterium tuberculosis* complex excretion 127
1.3. Environmental contamination with *Mycobacterium tuberculosis* complex 128
1.4. Spatial epidemiology of wildlife bovine tuberculosis 129
2. Future perspectives 131
3. References 133

Chapter I – General introduction

1. Etiology of bovine tuberculosis

Bovine tuberculosis (bTB) is a chronic slow-progressing disease caused by infection with *Mycobacterium bovis* or the closely related *Mycobacterium caprae*, both members of the *Mycobacterium tuberculosis* complex (MTC) (Pesciaroli et al., 2014; Rodriguez-Campos et al., 2014). These bacteria belong to the order *Actinomycetales*, family *Mycobacteriaceae* (Pfyffer, 2006).

Mycobacteria are defined as aerobic or microaerophilic, acid-alcohol fast, rod-shaped actinomycetes with occasional branching; the bacteria are non-motile, non-sporulating organisms that contain arabinose, galactose, and meso-diaminopimelic in the cell wall (Pfyffer, 2006). MTC species are aerobic and facultative intracellular parasites, showing slow growth in culture media (Pfyffer, 2006). Its virulence factors include the capacity to bind to manose receptors in macrophages, intracellular growth by inhibiting the phagosome-lisosome fusion and the production of free radicals during phagocytosis, mycolic acids inducing granuloma formation, allowing evading the immune response, inhibition of polimorphonuclear migration to tissues and modulation of cytokine secretion (Houben et al., 2006; Guenin-Macé et al., 2009).

The mycobacterial cellular envelope is characterized by high lipid content, including exceptionally long mycolic acids. Mycolic acids are covalently linked to peptidoglycan by an arabinogalactan polymer, forming the structural core of the cell wall (Brennan, 2003; Niederweiss et al., 2010). These features partially explain MTC resistance to many antibiotics and several host immune response mechanisms (Brennan, 2003; Niederweiss et al., 2010). They are also involved in the relative resistance to unfavorable environmental conditions (Brennan, 2003), such as the capacity to survive for extended periods in soil or water (Fine et al., 2011; Ghodbane et al., 2014) or to resist to mild disinfectants (Corner et al., 1995). They are also the cause of their almost unique staining properties, as the lipid cell wall takes up carbol-fuchsin but resists discoloration with acid-alcohol, thereby giving mycobacteria the name acid-fast bacilli (AFB) (Niederweiss et al., 2010).

1.1. *Mycobacterium bovis* and *Mycobacterium caprae*

M. bovis presents the widest host range of all MTC, naturally infecting many species of mammals, particularly ungulates of the families Bovidae, Cervidae, Suidae, Equidae and Camelidae, but also carnivores, rodents, lagomorphs, insectivores, marsupials and primates (including humans) (Thoen et al., 2006; Rodriguez-Campos et al., 2014). *M. caprae* was described as a separate species (Aranaz et al., 1999) and shown to naturally infect species of the families Bovidae, Cervidae, Suidae and occasionally humans (Prodinger et al., 2014; Rodriguez-Campos et al., 2014).

Egg-based culture media, such as Löwestein-Jensen supplemented with pyruvate or Stonebrink, are recommended for the isolation of *M. bovis* and *M. caprae*. Although requiring incubation periods of 12-15 weeks, egg-based media tend to yield more growth than agar-based media (de Lisle et al., 2002; Corner et al., 2011b; Gormley et al., 2014). It should be stressed that some media routinely used for the isolation of *M. tuberculosis*, such as Löwenstein-Jensen supplemented with glycerol, do not support growth of *M. bovis*, due to its inability to utilize glycerol as energy source (Thoen et al., 2006; Gormley et al., 2014).

Since the complete genome of *M. bovis* was published (Garnier et al., 2003), several clonal complexes have been described. Clonal complexes are groups of isolates with a common recent ancestor and can be identified based on stable molecular markers, such as chromosomal deletions or single nucleotide polymorphisms (Smith, 2012). African 1 clonal complex is found mostly in West-Central Africa, African 2 in East Africa, European 1 has a worldwide distribution although it seems to have originated in the British Isles while European 2 originated in the Iberian Peninsula although it presently displays a global distribution (Rodriguez-Campos et al., 2012; Smith, 2012). Although not defined as a clonal complex, a BCG-like spoligotyping group of isolates also shows worldwide distribution (Smith, 2012; Rodriguez-Campos et al., 2012). The global distribution of *M. bovis* clonal complexes of European origin is probably a result of being introduced along with domestic cattle during colonial times (Smith, 2012; Rodriguez-Campos et al., 2014).

M. caprae seems to be much less diverse than *M. bovis*, with only 2 clusters described to date: Iberian and Central-Eastern European (Rodriguez-Campos et al., 2014). Also its distribution is

more localized, described only in South-Central Europe and North Africa (Rodriguez-Campos et al., 2014).

1.2. Other species of the *Mycobacterium tuberculosis* complex

Other species traditionally recognized within the MTC include *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti* and *M. pinnipedii* (Brosch et al., 2002; Rodriguez-Campos et al., 2014). The first 3 species are almost exclusively human pathogens (de Jong et al., 2010; Fabre et al., 2010; Djelouadji et al., 2011; Rodriguez-Campos et al., 2014), *M. microti* natural hosts are rodents (Kipar et al., 2013) and *M. pinnipedii* infects primarily Southern Hemisphere pinnipeds (Cousins et al., 2003; Rodriguez-Campos et al., 2014). Recently 2 new members of this complex have been proposed: *M. orygis*, primarily isolated from African and Asian ungulates (van Ingen et al., 2012) and *M. mungi*, described in banded moongoses (*Mungos mungo*) from Southern Africa (Alexander et al., 2010). Other isolates whose status is uncertain include the “dassie bacillus” isolated from hyrax (*Procavia capensis*) in Southern Africa (Mostowy et al., 2004) and the “chimpanzee bacillus” from a chimpanzee (*Pan troglodytes*) in West Africa (Coscolla et al., 2013).

MTC species are highly homogeneous at genomic level, overall sharing >99 % of their genome (Rodriguez-Campos et al., 2014). Accordingly, it has been proposed that the complex consists of only one species, with several subspecies or host-adapted ecotypes (Djelouadji et al., 2011; Rodriguez-Campos et al., 2014). *M. bovis* and *M. caprae* seem to have recently diverged from other MTC species (Brosch et al., 2002; Rodriguez-Campos et al., 2014). In fact, mycobacterial DNA extracted from a 17,000 years old bison fossil was shown to be more closely related to *M. tuberculosis* than to *M. bovis*, although the former is not known to naturally infect ungulates (Rothschild *et al.*, 2001). The most plausible evolutionary scenario is that animal-adapted species like *M. bovis* and *M. caprae*, which all lack the region of difference 9 (RD9), evolved from a RD9-deleted species, such as the human-adapted *M. africanum* (Smith et al., 2006) (Figure 1). This probably happened in Africa, supported by the present geographical distribution of RD9-delected species and the fact that the most diverse assemblage of animal-adapted MTC is found in that continent (Rodriguez-Campos et al., 2014).

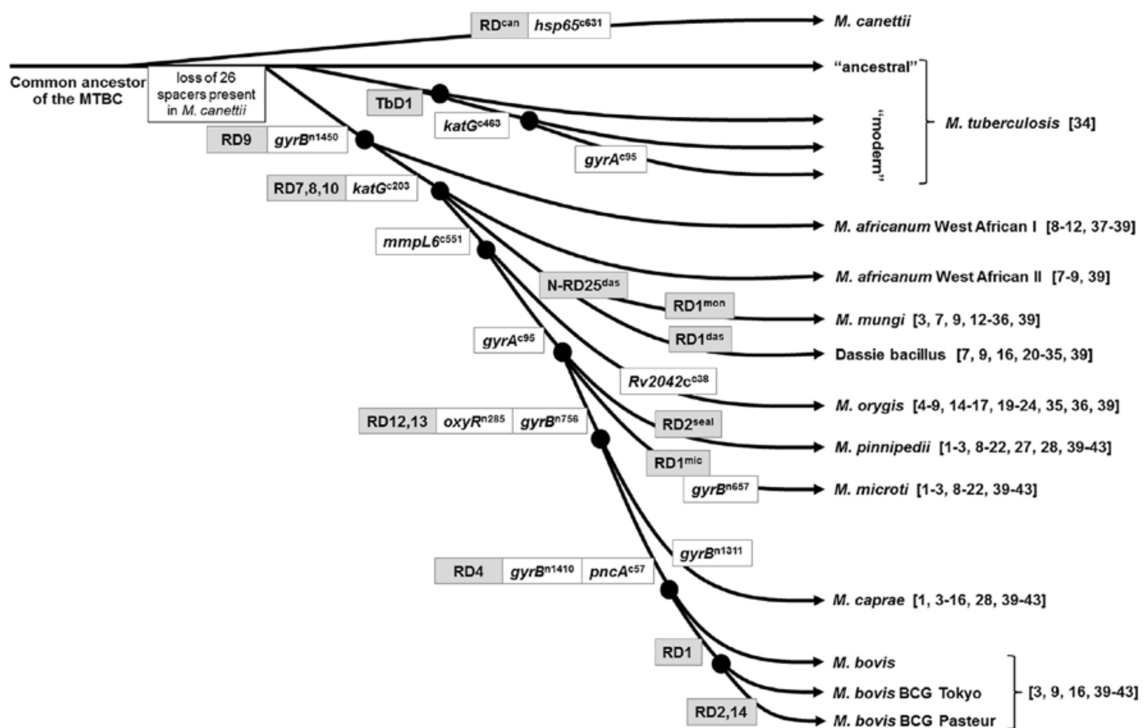


Figure 1 - Phylogeny of MTC based on regions of difference (RD) and single nucleotide polymorphisms (SNP). Grey boxes indicate the loss of a RD, white boxes SNP, black nodes represent common ancestors. From: Rodriguez-Campos et al. (2014), with permission from the publisher.

2. Historical background and current epidemiological situation of bovine tuberculosis

Bovine tuberculosis is known to affect humans and animals for millennia (Rothschild et al., 2001; Taylor et al., 2007). Nowadays vaccinations with BCG and improved sanitary conditions for humans as well as control programs in cattle have changed disease occurrence and distribution. In this section we briefly review the historical background of bTB and its current global epidemiological situation in humans, cattle and wildlife.

2.1. Bovine tuberculosis in humans

Bovine tuberculosis is a zoonosis known to affect humans for at least 2,000 years (Taylor et al., 2007), causing disease similar to tuberculosis by *M. tuberculosis* (de la Rua-Domenech, 2006). As most humans seem to become infected with *M. bovis* by ingestion of infected unpasteurized milk, lesions are usually extra-pulmonary, typically in the cervical lymph

nodes of children (de la Rua-Domenech, 2006; Thoen et al., 2006; Müller et al., 2013). Nevertheless pulmonary tuberculosis due to *M. bovis* can occur although human-to-human transmission seems to be a very rare event (Long et al., 1999).

There are no global data on the prevalence of human tuberculosis caused by *M. bovis*. This is due essentially to diagnostic issues, as diagnosis of human tuberculosis has been for decades based on methods that do not distinguish the mycobacteria species, like direct observation of acid-fast bacilli in human samples and molecular methods that target sequences common to all MTC; or that do not allow the detection of *M. bovis*, like bacteriological culture using media for *M. tuberculosis* isolation (e.g. Lowestein-Jensen with glycerol, among others) (Thoen et al., 2006). Müller et al. (2013) estimated the incidence of zoonotic tuberculosis to be approximately 1 case per 100,000 inhabitants/year in several countries outside Africa, while in that continent these figures were close to 7 cases per 100,000 inhabitants/year in those countries where surveys were available. Some surveys in Africa report up to 37.7 % of human tuberculosis cases caused by *M. bovis* (Thoen et al., 2006).

In developed countries human *M. bovis* infections are nowadays mostly an occupational disease of slaughterhouse or cattle farm workers; acquired abroad; or the reactivation of old infections when milk pasteurization was not widespread, consequently affecting elderly people (de la Rua-Domenech, 2006; Thoen et al., 2006; Rodriguez et al., 2009; Michel et al., 2010; Müller et al., 2013). In developing countries most infections seem to originate from the consumption of untreated milk from infected cows or herding of infected cattle (Michel et al., 2010). Most cases present the classical non-pulmonary lesions, although aerosol transmission can occur, albeit rarely (Michel et al., 2010; Müller et al., 2013).

M. caprae is also a zoonotic agent and was shown to be responsible for 13–67 % of the zoonotic tuberculosis cases in several European countries (Kubica et al., 2003; Rodriguez et al., 2009; Prodinger et al. 2014).

2.2. Bovine tuberculosis in livestock

M. bovis can infect all domestic mammal species, most notably cattle, goats, sheep and pigs (Humblet et al., 2009; Michel et al., 2010; Hardstaff et al., 2014). Bovine tuberculosis in cattle is

a chronic progressive disease primarily affecting the lungs and characterized by granulomatous lesions in organs or lymph nodes (Cassidy, 2006). Bovine tuberculosis is subject to control or eradication programs in cattle in many countries (Michel et al., 2010; Rivière et al., 2014).

M. bovis infection in sheep is relatively infrequent (Houlihan et al, 2008; Muñoz-Mendoza et al., 2011; Hardstaff et al., 2014) and pigs seem to be at risk only when free-ranging with infected wildlife populations (Parra et al., 2003; Humblet et al., 2009; Hardstaff et al., 2014; Pesciaroli et al., 2014). On the other hand goats are a competent maintenance host of *M. bovis* and *M. caprae*, although knowledge is scarce regarding bTB in this species and it is often not included in control programs (Crawshaw et al., 2008; Humblet et al., 2009; Hardstaff et al., 2014; Bezos et al., 2015).

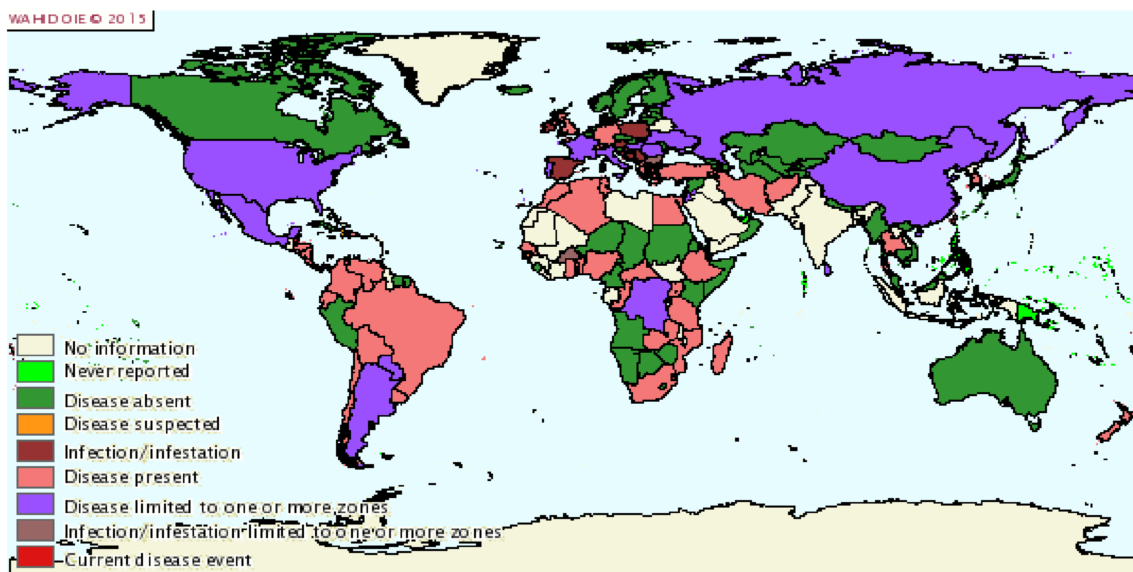


Figure 2 – Global situation of bovine tuberculosis in cattle. Worldwide report to the *Office International des Epizooties* of bovine tuberculosis in cattle by country, January to July 2014. Data from World Animal Health Information Database interface: www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

Bovine tuberculosis has been detected in almost every country in the world (Figure 2), although with extremely variable prevalence, depending on the existence and efficacy of control programs, management systems and geographical area (Humblet et al., 2009). Control programs in cattle are based on testing and removal of reactors and abattoir surveillance (Humblet et al., 2009). The test of choice is the intradermotuberculinization test (IDT) with the variants of single and

comparative tests, which differ on the injection of bovine purified protein derivative (bPPD) singly or alongside avian PPD (aPPD) (de la Rua-Domenech et al., 2006; Rivière et al., 2014). Although cumbersome, involving two manipulations of the animals 72 h apart, and with variable performance depending on the operator and bPPD source, it is still the favored screening test worldwide (de la Rua-Domenech et al., 2006; Downs et al., 2013). Where confirmatory tests are used, they usually involve measurement of γ-interferon levels in whole blood after stimulation with bPPD or specific antigens (de la Rua-Domenech et al., 2006; Bass et al., 2013; Rivière et al., 2014).

Although such control programs are the norm in developed nations they are rarely systematically applied in developing countries due to their high cost (Cosivi *et al.*, 1998; Humblet et al., 2009). Control programs have allowed several countries worldwide to eradicate bTB in cattle (e.g. Australia, USA, Canada and several European countries including France, Germany and Scandinavian countries) (Gortázar et al., 2012; Rivière et al., 2014). Nevertheless in several other countries eradication has not been achieved (e.g. New Zealand, United Kingdom, Republic of Ireland and southern European countries including Portugal and Spain) (Figure 3) and in many of those the existence of wildlife reservoirs of infection has been proposed as a contributing factor (Humblet et al., 2009; Hardstaff et al., 2014; Rivière et al., 2014).

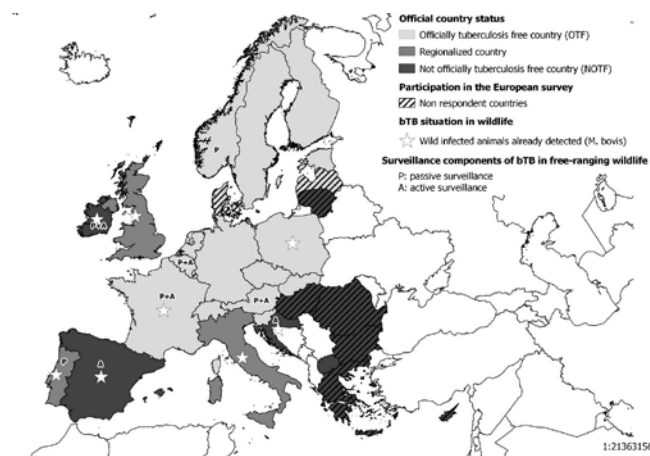


Figure 3 - Epidemiological situation regarding bovine tuberculosis in cattle and wildlife in Europe. European Union member states that have detected infection in wild animals and type of surveillance performed on free-ranging wildlife, in 2013, by official country status. From: Rivière et al. (2014), with permission from the publisher.

In Portugal, in 2014, the cattle herd prevalence was 0.34 %, the herd incidence was 0.25 % and the animal prevalence was 0.06 % (DGAV, 2015). In 2012, 21 % of the outbreaks in cattle were attributed to contact with wildlife, based on epidemiological enquiries. The region of Algarve is considered officially tuberculosis free (DGAV, 2013).

2.3. Bovine tuberculosis in wildlife

Wildlife species act as maintenance hosts for bTB in several regions throughout the world (Figure 4), in diverse epidemiological scenarios depending on specific environmental, ecological and management conditions. The best studied examples of such scenarios are briefly presented ahead.

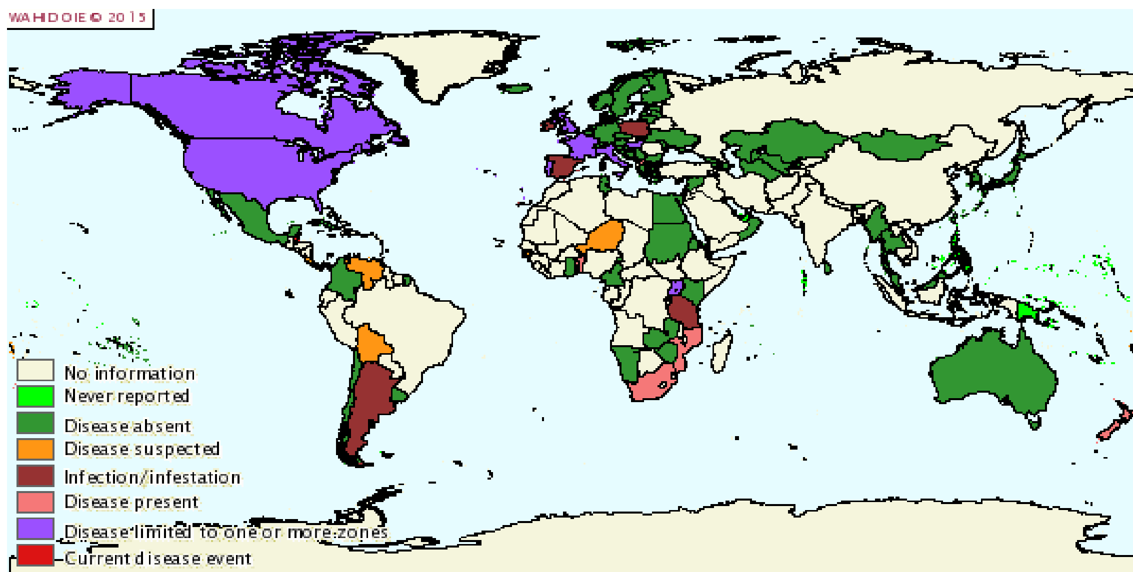


Figure 4 – Global situation of bovine tuberculosis in wildlife. Worldwide report to the *Office International des Epizooties* of bovine tuberculosis in wildlife by country, January to July 2014. Data from World Animal Health Information Database interface: www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

2.3.1. New Zealand

In New Zealand bTB is maintained in brushtail possum (*Trichosurus vulpecula*) populations from several regions in North and South islands (Livingstone et al., 2015). Possums are extremely susceptible to bTB with survival times after infection ranging 3-14 months (Ryan et al., 2006).

They develop generalized disease with lesions located in organs such as lungs and in lymph nodes, often involving draining tracts (Coleman & Cooke, 2001). Almost half the infected animals show no visible lesions (deLisle et al, 2009). Prevalence typically ranges 1-10 % (Ryan et al., 2006).

As possums are an introduced pest species in New Zealand, bTB control programs are based on massive culling campaigns using trapping and poisoning (Fitzgerald et al., 2013). This strategy as led to a decline of 95 % in bTB cattle herd incidence and to the eradication of the disease in wildlife from over 800,000 hectares (Livingstone et al., 2015). Nowadays the goal of the New Zealand bTB control program is to eradicate the disease by 2026 (Livingstone et al., 2015).

Other wildlife species in New Zealand are known to be infected by *M. bovis*, such as feral pigs, red deer (*Cervus elaphus*), ferrets (*Mustela putorius*) and stoats (*Mustela erminea*), nevertheless they have been considered spillover hosts (Fitzgerald et al., 2013; Livingstone et al., 2015). Feral pigs have been used as indicator species to locate bTB-infected regions due to their high susceptibility to infection (Nugent *et al.*, 2002).

2.3.2. Australia

In Australia bTB was maintained in populations of introduced water buffalo (*Bubalus arnee*), which by the 1980's numbered over 300,000 animals (Tweddle & Livingstone, 1994; Bradshaw et al., 2012). Prevalence was estimated at 16.4 % in the 1960´s (Clifton-Hadley *et al.*, 2001). In this species bTB is similar to that in cattle, with lesions located in lungs and associated lymph nodes (Clifton-Hadley *et al.*, 2001).

bTB eradication in water buffalo was achieved by massive depopulation through culling, which almost led to the elimination of this species in Australia (Radunz, 2006; Bradshaw et al., 2012). This strategy was possible because water buffalo is an exotic species in Australia with scarce socio-economic relevance. The Australian case remains the only example of a successful large-scale eradication program for bTB in a wildlife species (Palmer et al., 2012).

2.3.3. Southern Africa

In Southern Africa many wildlife species are known to be infected with *M. bovis* nevertheless the main maintenance hosts are African buffalo (*Syncerus caffer*) and lechwe antelopes (*Kobus leche*) (Fitzgerald et al, 2013; de Garine-Wichatitsky et al., 2013).

The best known bTB host is the African buffalo where the disease was diagnosed in 1990 in Kruger National Park, South Africa and prevalence ranged 1.5–38.2 % depending on the herd (Rodwell et al., 2001; de Garine-Wichatitsky et al., 2013). Disease has spread to buffalo herds in the Greater Limpopo Transfrontier Park (Tanner et al., 2015). In this species bTB is very similar to the disease in cattle, with lesions mainly located in lungs and associated lymph nodes (Renwick *et al.*, 2006; Laisse et al., 2011).

The IDT shows a good sensitivity in this species (84-91 %, Clifton-Hadley *et al.*, 2001) and so control programs involve capturing, testing and maintaining in captivity for 72h whole herds of African buffalo, with subsequent culling of reactors. A captive bTB-free herd was established to allow for the future reintroduction into the Kruger National Park, in case depopulation is considered viable (de Lisle *et al.*, 2002). Nevertheless, presently bTB seems to be out of control in South Africa, having increased its host and geographical range (Hlokwe et al., 2014).

2.3.4. North America

In mainland United States of America the only known maintenance host for bTB is the white-tailed deer (*Odocoileus virginianus*). After being declared eradicated in cattle, bTB was discovered in this species in Michigan in 1975 and again in 1995, with a prevalence of 3.5 % and an additional 2 % culture-negative deer showing bTB-like lesions (Schmitt *et al.*, 1997; O'Brien et al., 2004). Lesions predominate in the retropharyngeal lymph nodes, usually as purulent lymphadenopathies or less frequently as caseogranulomas (O'Brien *et al.*, 2001).

High deer densities maintained for hunting purposes by means of winter feeding were identified as the main risk factor for bTB persistence (Miller et al., 2003). Spillover to livestock, to other wildlife species and even to humans has been reported (Payeur et al., 2002; Wilkins et al., 2008). Control is based on intensified hunting and prohibition of supplemental feeding of deer,

improved biosecurity of cattle farms and surveillance of hunted deer (O'Brien et al., 2011a). This strategy led to a decrease in apparent prevalence to <2 %, nevertheless eradication cannot be foreseen (Okafor et al., 2011; Miller and Sweeney, 2013).

The efficacy and safety of BCG as vaccine for bTB in white–tailed deer has been investigated. While vaccination was shown to significantly decrease disease severity in white-tailed deer, long-term infection with BCG and transmission to in-contact deer were described, precluding further field studies on the efficacy of vaccination as a control action in free-ranging populations (Palmer et al., 2009, 2010).

Another outbreak of bTB in white-tailed deer occurred in Minnesota in 2005 in the vicinity of an infected cattle herd. Aggressive control measures were taken, including liberalized hunting and massive culling of deer and prohibition of supplemental feeding (Carstensen and Doncarlos, 2011). This aggressive strategy with an estimated cost of 86 million USD, together with the fact that bTB prevalence and host density were lower than in Michigan, led to the eradication of the disease in Minnesota deer by 2011 (Carstensen and Doncarlos, 2011).

Although Canada has eradicated bTB in livestock, the disease is maintained in wildlife by white-tailed deer, wapiti (*Cervus elaphus*) and bison (*Bison bison*) (Nishi et al., 2006; Wobeser, 2009). In Riding Mountain National Park, Manitoba, bTB is maintained by wapiti (3.6 % prevalence in live-captured, 0.4 % in hunted wapiti) and white-tailed deer (0.2 % prevalence in hunted deer) (Lees et al., 2003; 2004). Spillover to adjacent cattle herds occurs through shared feeding grounds outside the National Park (Nishi et al., 2006). Management with the goal of containing bTB in and around the National Park consists of reducing host density, improving biosecurity of cattle farms, habitat management inside protected areas and surveillance of hunted and live-captured hosts (Nishi et al., 2006).

In Wood Buffalo National Park, Alberta, bTB is maintained by endangered bison populations at a prevalence of 49 % (Joly and Messier, 2004). No spillover to livestock has occurred and management consists only of surveillance and improved biosecurity trough enforcement of bison exclusion zones (Nishi et al., 2006; Shury et al., 2015). A captive breeding herd of bison was established for reintroduction in the wild in case depopulation was considered as a management tool, nevertheless in 2005 an outbreak of bTB was detected in this herd, which was then depopulated (Himsworth et al., 2010; Shury et al., 2015).

2.3.5. United Kingdom and Republic of Ireland

In the United Kingdom (UK) and Republic of Ireland (RoI) bTB is maintained in populations of Eurasian badger (*Meles meles*), which locally reach very high densities, up to >25/km^2. After being detected in this species in the 1970's, large-scale surveys of dead badgers yielded 4.3 % prevalence in >20,000 badgers in the UK and 14.3 % in >7,000 badgers in the RoI (Clifton-Hadley *et al.*, 2001). In this species bTB leads to no significant increase in mortality rates of infected animals (Gallagher & Clifton-Hadley, 2000). Lesions predominate in lymph nodes throughout the body and also in organs such as lungs, kidneys, spleen, liver and intestines (Gavier-Widén at al., 2001, Jenkins et al., 2008). Although lesions tend to be well encapsulated, hematogenous spread can occur in late stages of the disease or when transmission occurs through bite wounds (Gallagher et al., 1998; Gallagher & Clifton-Hadley, 2000; Gavier-Widén et al., 2001). Besides bite wounds, aerosol transmission seems to be the most important transmission route among badgers (Jenkins et al., 2008).

Culling was initially the cornerstone of control programs in the UK and RoI (Clifton-Hadley *et al.*, 2001). Starting in 1998, an experimental large-scale study took place in the UK, in which 9 triplicates of proactive culling (widespread culling of badgers), reactive culling (culling in response to bTB outbreaks in cattle) and control areas (no management) were compared. The scientific group that analyzed the study recommended not using culling as a management action due to increased incidence in the vicinity of culling areas (Donnely et al., 2006). By contrast a similar smaller scale study performed in the RoI concluded that culling badgers is an effective method to control bTB in cattle and so this remains the main control action for wildlife bTB in that country (Griffin *et al.*, 2005).

2.3.6. Continental Europe (except Iberian Peninsula)

Across Continental Europe sporadic cases of bTB in wildlife have been described in several countries (Table 1). In France and Austria, red deer (*Cervus elaphus*) are considered a maintenance host for bTB caused by *M. bovis* and *M. caprae*, respectively (Zanella et al., 2008a; Fink et al., 2015). In France also wild boar and badger could be maintenance hosts for bTB (Payne et al., 2012; Richomme et al., 2013; Rivière et al., 2013).

Table 1 – Host species for bovine tuberculosis in continental Europe (except Iberian Peninsula). Continental European countries and wildlife species where bovine tuberculosis was identified and epidemiological status attributed.

Country	Wildlife species	Status	Reference
	Red deer	Maintenance	Zanella et al. (2008a)
	Wild boar	Maintenance	Hars et al. (2010)
France	Badger	Maintenance	Payne et al. (2012)
	Roe deer	Spillover	Richomme et al. (2013)
	Red fox	Spillover	Rivière et al. (2013)
Austria	Red deer	Maintenance	Fink et al. (2015)
Poland	Wild boar	Spillover	Krajewska et al. (2014)
	European bison	Spillover	
Germany	Wild boar	Spillover	Gortázar et al. (2012)
Croatia	Wild boar	Spillover	Machackova et al. (2003)
Italy	Wild boar	Spillover?	Serraino *et al.* (1999)
Hungary	Wild boar	Spillover	Machackova et al. (2003)
Slovakia	Wild boar	Spillover	Machackova et al. (2003)
Bulgaria	Wild boar	Spillover	Machackova et al. (2003)

It is of notice that most central European countries are classified as officially bTB-free in cattle (Gortázar et al., 2012; Rivière et al., 2014) (Figure 3). A notable example is France, which was declared officially bTB-free in 2000 but in early 2001, *M. bovis* infection was confirmed in red deer in an 80 km^2 isolated forest in Normandy. An epidemiological survey during the following hunting season confirmed the occurrence of bTB in both free-ranging red deer and wild boar (*Sus scrofa*). Management actions consisting of depopulation of red deer, reduction of density in wild boar and proper disposal of hunting offal led to the eradication of disease in this population after 10 years (Hars et al., 2010; Hars et al., 2012).

2.3.7. Iberian Peninsula

In the Iberian Peninsula bTB is maintained in a multi-host pathogen system (Renwick et al., 2007), with *M. bovis* and *M. caprae* circulating between sympatric wild ungulates (mostly wild boar and red deer) and free-ranging domestic ungulates (cattle, goats, sheep and pigs) (Gortázar et al., 2012). Several other ungulates (Balseiro et al., 2011) and carnivores (Briones et al., 2000; Sobrino et al., 2008; Matos et al., 2014) are considered spillover hosts for bTB. This

epidemiological model was investigated as part of the present PhD thesis by means of a systematic review of the literature in Portugal and Spain.

Santos N, Correia-Neves M, Almeida V, Gortázar V (2012) Wildlife tuberculosis: A systematic review of the epidemiology in Iberian Peninsula. In Maria de Lourdes Ribeiro de Souza da Cunha (Ed.) Epidemiology Insights, InTech, ISBN: 978-953-51-0565-7

14

Wildlife Tuberculosis: A Systematic Review of the Epidemiology in Iberian Peninsula

Nuno Santos[1], Margarida Correia-Neves[1],
Virgílio Almeida[2] and Christian Gortázar[3]
[1]*Life and Health Sciences Research Institute (ICVS)*
School of Health Sciences, University of Minho, Campus de Gualtar, Braga
[2]*Centro de Investigação Interdisciplinar em Sanidade Animal – Faculdade de Medicina Veterinária (CIISA/FMV), TULisbon, Pólo Universitário do Alto da Ajuda, Lisbon*
[3]*IREC National Wildlife Research Institute (CSIC-UCLM-JCCM), Ciudad Real*
[1,2]*Portugal*
[3]*Spain*

1. Introduction

Mycobacterium bovis is the main etiological agent of bovine tuberculosis, infecting many species of wild and domestic mammals and also man. Bovine tuberculosis is a chronic and contagious infectious disease that has been reported to infect wild ungulates, carnivores, marsupials and primates (de Lisle *et al*., 2002). Bovine tuberculosis (bTB) also occurs worldwide in livestock (Humblet *et al*., 2009), causing annual economic losses estimated at 3 billion USD in 1995 (Steele, 1995). It remains a serious risk for animal health, and a threat for human health in many developing countries (Etter *et al*., 2006). Several countries successfully eradicated bovine tuberculosis in livestock through test-and-slaughter and/or abattoir surveillance programs. Yet other countries, using similar strategies, did not achieve eradication and some even face the re-emergence of the disease (Schiller *et al*., 2010). In Europe for instance, the prevalence of bTB in cattle is increasing in several countries (Gordejo & Vermeersch, 2006). Moreover current eradication and control programs in livestock in Europe are facing a range of challenges as stamping out is becoming a less attractive option for economic and environmental reasons and due to animal welfare concerns (Whiting, 2003).

Some of the abovementioned difficulties in eradicating bTB in cattle may relate with the occurrence of the disease in wildlife (Schiller *et al*., 2010). In fact it has been demonstrated that the complete elimination of bTB can be extremely complicated by persistent infection of wild hosts, such as badgers in the United Kingdom, white tailed deer in the United States and brushtail possum in New Zealand (Corner, 2006). The single successful example of bTB eradication in a wildlife host is the Australian case, where it was accomplished through stamping out, which eliminated introduced water buffalo *Bubalus arnee*, the only maintenance host in that ecosystem, (Corner, 2006). This is not an option when autochthonous, protected or economic and socially valuable species are involved (Artois *et al*., 2001). In most cases, an integrated control program is needed (Horan *et al*.,

2008), but this is often hampered by the lack of epidemiological data (Artois *et al.*, 2001; Corner, 2006).

Bovine tuberculosis control programs in cattle are in place for several decades in Iberian Peninsula and consequently incidence has been decreasing (Allepuz *et al.*, 2011; Cunha *et al.*, 2011). However in the last few years incidence has stabilized, or even slightly increased in both Portugal and Spain (Allepuz *et al.*, 2011; Cunha *et al.*, 2011). The role of wildlife hosts in this scenario remains speculative; nevertheless the existence of wildlife reservoirs may compromise the goal of eradication in cattle. Besides livestock, attention should be given to spill-over from wildlife to other domestic animals (e.g. goats and free-ranging pigs) and even to humans, namely hunters and others that handle wild ungulate carcasses (Gortazar *et al.*, 2011b, in press). Wildlife-to-human transmission of *M. bovis* is hard to prove and no single case has been documented in Iberian Peninsula, but it is known to occur elsewhere (e.g. USA – Wilkins *et al.*, 2008). Bovine tuberculosis is also one of the main infectious diseases affecting the critically endangered Iberian lynx *Lynx pardinus*, with several free-ranging and captive lynx killed by this infection (Millán *et al.*, 2009). Iberian lynx is subject to an intensive multinational conservation program in Iberian Peninsula, which includes releasing captive-bred animals to former range. The persistence of *M. bovis* on the environment and in prey species poses a threat to this conservation action (Millán *et al.*, 2009).

Iberian Peninsula ecosystems display a high degree of human intervention and have experienced some profound changes in the last decades. The most important alterations were a shift from domestic ungulate to wild ungulate production for hunting purposes (Miguel *et al.* 1999) and an increasing intensification of the later (Vargas *et al.* 1995). This management of wild ungulate populations aims to increase profits by increasing harvest, translating into increased densities of hunted species. This has been accomplished through introduction/restocking, provision of food and water (mostly during the summer shortage), fencing and sometimes even medication (Miguel *et al.* 1999, Gortázar *et al.*, 2006). All these changes have potential implications on bTB epidemiology (Gortázar *et al.*, 2006).

In the Iberian Peninsula, ungulates such as the wild boar *Sus scrofa* and the red deer *Cervus elaphus* have been recognized as the most important maintenance hosts for wildlife tuberculosis (Gortázar *et al.*, 2011b). Nevertheless other species have also been identified as locally non-negligible hosts, such as the fallow deer *Dama dama* and the badger *Meles meles* (Gortázar *et al.*, 2011b; Balseiro *et al.*, 2011). Several other species of ungulates and carnivores were also found infected (Rodriguez *et al.*, 2010). This situation fits the definition of a multi-host pathogen within a multi-species ecosystem (Renwick *et al.*, 2007; Gortázar *et al.*, in press), in which pathogen persistence and spread is dependent on the density of each maintenance host species and also on the effective interspecies contact rate (dependent on the ecology of each species).

Research on host-pathogen interaction usually deals with single-host single-pathogen systems, where disease persistence depends solely on the intra-species transmission rate (Tompkins *et al.*, 2001). If transmission is density-dependent, then population thresholds for disease invasion and persistence are expected and have been described (Swinton *et al.*, 2001). By contrast, in multi-host pathogens systems, disease persistence is dependent on both intra and inter-species transmission rates and densities of several host species (Renwick *et al.*, 2007). Moreover, these rates depend on pathological, epidemiological, ecological and behavioural factors (Corner, 2006).

In such a complex epidemiological setting, it is imperative to determine the precise role of each host species in pathogen maintenance before comprehensive control measures are undertaken. Much has been investigated in the last decade regarding wildlife tuberculosis epidemiology in Iberian Peninsula. In order to contribute to understanding the mechanisms underlying wildlife tuberculosis persistence in the multi-host ecosystems of this region, under widely different ecological and management pressures, we report a systematic bibliographic review on this subject. The aim of this review was to survey the peer-reviewed literature for evidence of the: *i*) epidemiological status of each host species; *ii*) determinants of wildlife tuberculosis occurrence; *iii*) geographical structuring of wildlife tuberculosis in the Iberian Peninsula; *iv*) time trends in wildlife tuberculosis occurrence.

2. Methods

We conducted a systematic bibliographic review for epidemiological studies on tuberculosis in wildlife in Iberian Peninsula by searching MEDLINE/PubMed, up to the 31st of August 2011, using MeSH and keywords: "*Mycobacterium bovis*", "*Mycobacterium caprae*", "wild boar", "deer", "epidemiology", "Iberian Peninsula", "Portugal" and "Spain". Combinations used were: ("Portugal" OR "Spain") AND ("*Mycobacterium bovis*" OR "*Mycobacterium caprae*"), ("*Mycobacterium bovis*" OR "*Mycobacterium caprae*") AND "wild boar" AND "epidemiology" and ("*Mycobacterium bovis*" OR "*Mycobacterium caprae*") AND "deer" AND "epidemiology". Abstracts were selected according to their relevancy and excluded if dealing exclusively with laboratory or pathology investigations, domestic species or humans or other geographical regions. Articles were reviewed in full text.

For each article, information about the type of epidemiological study and study design, sample size and sampling methodology, screening and diagnostic tests used, prevalence rate, time frame of the study, study areas, characteristics of the populations studied, risk factors identified and host epidemiological status was summarized and presented in table format for easy comparison. Due to their idiosyncrasies, molecular epidemiology articles were characterized differently according to the number of isolates studied, genotyping technique, mycobacterial species reported, number of genotypes found, host and geographical clustering of genotypes and study areas. Due to differing methodologies and sometimes incomplete reporting of results, meta-analysis was not applicable except for a small number of studies.

For the purpose of this review, wildlife tuberculosis was defined according to the OIE definition of bovine tuberculosis, but *Mycobacterium caprae* was also considered etiological agent, besides *M. bovis*.

3. Results

The bibliographic search yielded 286 articles. Initially, title and abstracts were reviewed and 247 articles excluded because they deal only with laboratory/pathology investigations (n=74), domestic animals (n=41), humans (n=50), other geographical regions (n=79), or were review/model articles (n=3). Full text papers were then reviewed and further 6 papers were excluded because they focused exclusively on laboratory/pathology investigations. Therefore 33 articles were selected as of interest to the present review.

Reference	Type	Sampling strategy	Sample n	Screening test	Diagnostic test	Time frame & tendency	Prevalence (rate)	Fencing	Study areas
Aranaz *et al.* (2004)	SU	Targeted (hunted)	96		BC		51 (53,1%)	MX	7 area SW Spain
Acevedo-Whitehouse *et al.* (2005)	CS	Targeted (hunted)	175		BC	2000-2003	82 (47%)	MX	7 areas SW Spain
Parra *et al.* (2005)	CS	Scanning (hunted)	112	MI	BC		112	FE	1 region W Spain
de Mendoza *et al.* (2006)	CS	Scanning (hunted)	8.478	MI	BC	1992-2004 increasing	333 (3,92%)	MX	1 area W Spain
Parra *et al.* (2006)	CS	Scanning (hunted)	34.582	MI	BC	1997-2002 increasing	625 (1,81%)	MX	1 region W Spain
Vicente *et al.* (2006a)	CS	Targeted (hunted)	1.060		GP BC (not all)	1999-2004	(42,51%, mean estate rate)	MX	57 areas SW Spain
Vicente *et al.* (2006b)	CS	Targeted (hunted)	412		GP BC (not all)	1999-2004	(18,2%-100%)	FE	19 area SW Spain
Gortázar *et al.* (2008)	CS	Targeted (culled)	124		BC	2006-2007	65 (52,4%)	FR	1 area SW Spain
Romero *et al.* (2008)	SU	Targeted (culled)	214		BC	1998-2003	60 (28,0%)	FR	1 area SW Spain
Santos *et al.* (2009)	CS	Targeted (hunted)	162		BC	2005-2007	18 (11,1%)	FR	8 areas South-central Portugal
Cunha *et al.* (2011)	SU	Scanning (hunted)	343	MI	BC	2002-2010	(63%)	MX	Several areas across Portugal
Gortázar *et al.* (2011a)	CS	Targeted (culled)	124		BC	2006-2007	62 (50%)	FR	1 area SW Spain
Pinto *et al.* (2011)	CS	Targeted (hunted)	132	GP	BC	2008-2009	21 (15,9%)	MX	1 area Central Portugal

Table 1. Studies dealing with wild boar included in the analysis. Classification: SU – survey; CS - cross sectional study; CC – case-control study; Screening/diagnostic test: MI – official meat inspection scheme; GP – gross pathology; BC – bacteriological culture; SE – serology; Fencing: FR – free-ranging populations; FE – fenced populations; MX – mixed free-ranging and fenced populations.

3.1 Characterization of published articles

Investigation of bTB epidemiology in wild boar and red deer (most often studied hosts) are mostly cross-sectional (11/14), the rest being surveys (Tables 1-2). Most studies opt for

targeted surveillance on hunted (6/14) or culled (3/14) animals, the rest relying on scanning surveillance in routine meat inspection schemes for detection of macroscopic lesions-like lesions (Table 1-2). The mean number of animals studied in targeted-design studies is 278 for wild boar (n=9, range 96-1.060) and 401 for red deer (n=6, range 95-1.368). Thirteen out of fourteen studies use bacteriological culture as the diagnostic test. Nevertheless most of them (9/14) also include a previous screening test (usually gross pathology or routine meat inspection schemes), followed by bacteriological culture when macroscopic lesions were observed (Table 1-2).

As regards studies on other host species (ungulates and carnivores), 5/14 are case reports, 6/14 surveys while 3/14 are cross sectional studies (Table 3). Five out of twelve studies rely on passive surveillance of haphazardly found carcasses and 3/12 on targeted surveillance of purposefully trapped animals. Most of these studies deal with carnivore species. As expected regarding novel host species, 3/12 studies are case reports (Table 3). Mean number of animals studied in survey studies is 105 for fallow deer (n=4, range 89-134), 63 for badger (n=3, range 2-157) and 15 for Iberian lynx (n=5, range 1-39). Most other species (Table 5) are dealt in single studies, usually as case reports. Serologic tests were used in 3/9 studies investigating other host species, such as Barbary sheep and carnivores (Table 3).

3.2 Prevalence rates

For the wild boar populations surveyed by targeted-design studies using bacteriological culture as diagnostic test on all animals (n=6), prevalence rates ranged 0,11-0,53, with a meta prevalence rate of 0,36 (Table 5). Including all studies, regardless of design, prevalence rates ranged 0,18-1 (Table 1). For the red deer populations surveyed by targeted-design studies using bacteriological culture as diagnostic test on all animals samples (n=3), prevalence rates ranged 0,02-0,27, with a meta prevalence rate of 0,21 (Table 5). Including all studies, regardless of design, prevalence rates ranged 0,01-0,44 (Table 2). For the fallow deer populations surveyed by targeted-design studies using bacteriological culture as diagnostic test on all animals samples (n=4), prevalence rates ranged 0,13-0,67, with a meta prevalence rate of 0,28 (Table 5). For other host species, the sample size and/or the study design do not allow meta analysis.

3.3 Trends

Few studies address or allow addressing the time trend of bTB prevalence rates. In Doñana, bTB was not detected in targeted wildlife health surveillance until 1990's, when the population of cattle greatly increased, while in 2000's high prevalence rates were found in all ungulate species (Gortázar *et al*., 2008). In fact, prevalence rates in this area increased from 1998-2003 to 2006-2007 by 100% in wild boar and 50% in red deer (Gortázar *et al*., 2011b). In Extremadura region, West-central Spain, prevalence rates detected in routine meat inspection schemes steadily raised from 1994-2004, while not detected in 1992-1993 (de Mendonza *et al*., 2006). One study area in South-eastern Portugal showed an increase in *M. bovis* infection rates in wild boar from 0,46 in 2005/06 (Santos *et al*., 2009) to 0,78 in 2009/11 (Santos *et al*., unpublished data).

Reference	Type	Sampling strategy	Sample n	Screening test	Diagnostic test	Time frame & tendency	Prevalence (rate)	Fencing	Study areas
Aranaz *et al.* (2004)	SU	Targeted (hunted)	108		BC		26 (24,1%)	MX	5 areas SW Spain
Parra *et al.* (2005)	CS	Scanning (hunted)	59	MI	BC		59	FE	1 region W Spain
de Mendoza *et al.* (2006)	CS	Scanning (hunted)	36.144	MI	BC	1992-2004 increasing	394 (1,09%)	MX	1 area W Spain
Parra *et al.* (2006)	CS	Scanning (hunted)	50.009	MI	BC	1997-2002 increasing	591 (1,18%)	MX	1 region W Spain
Vicente *et al.* (2006a)	CS	Targeted (hunted)	1.368		GP BC (not all)	1999-2004	(13,71% mean rate)	MX	21 areas SW Spain
Vicente *et al.* (2006b)	CS	Targeted (hunted)	574		GP BC (not all)	1999-2004	(0-44,0%)	FE	19 areas SW Spain
Gortázar *et al.* (2008)	CS	Targeted (culled)	95		BC	2006-2007	26 (27,4%)	FR	1 area SW Spain
Romero *et al.* (2008)	SU	Targeted (culled)	168		BC	1998-2003	26 (15,5%)	FR	1 area SW Spain
Castillo *et al.* (2010)	CS	Scanning (hunted)	551	MI	BC	2007-2009	28 (5,1%)	MX	2 areas SW Spain
Cunha *et al.* (2011)	SU	Scanning (hunted)	544 samples with lesion	MI	BC	2002-2010	(51%)	MX	Several areas across Portugal
Gortázar *et al.* (2011a)	CS	Targeted (culled)	95		BC	2006-2007	24 (25,3%)	FR	1 study area SW Spain
Pinto *et al.* (2011)	CS	Targeted (hunted)	339	GP	BC	2008-2009	35 (10,3%)	MX	1 area Central Portugal

Table 2. Studies dealing with red deer included in the analysis. Classification: SU – survey; CS - cross sectional study; CC – case-control study; Screening/diagnostic test: MI – official meat inspection scheme; GP – gross pathology; BC – bacteriological culture; SE – serology; Fencing: FR – free-ranging populations; FE – fenced populations; MX – mixed free-ranging and fenced populations.

Reference	Type	Sampling strategy	Sample n	Diagnostic test	Time frame	Prevalence (rate)	Fencing	Study areas
Briones *et al.* (2000)	CR		1 Iberian lynx	BC		1	FR	1 - SW Spain
Pérez *et al.* (2001)	CR		1 Iberian lynx	BC		1	FR	1 - SW Spain
Aranaz *et al.* (2004)	SU	Targeted (hunted)	89 fallow deer	BC		60 fallow deer (67,4%)	MX	2 area SW Spain
		Scanning (carcasses)	4 Iberian lynx			3 Iberian lynx		
Atance *et al.* (2005)	SU	Scanning (carcasses)	7 red fox 2 mongoose 2 genets 1 Iberian lynx 4 mustelids	BC		1 red fox	FR	1 area SW Spain
Atance *et al.* (2006)	SU	Targeted (trapped)	118 red fox 5 mongoose 4 genets 39 Iberian lynx 32 mustelids	SE (ELISA MPB70)		5 red fox (4%) 1 Iberian lynx (3%) 7 badger (23%)	FR	1 area SW Spain
Gortázar *et al.* (2008)	CS	Targeted (culled)	97 fallow deer	BC	2006-2007	18 (18,5%)	FR	1 area SW Spain
Millán *et al.* (2008)	CR		1 red fox	BC		1	FR	1 area SW Spain
Romero *et al.* (2008)	SU	Targeted (culled)	134 fallow deer	BC	1998-2003	17 (12,7%)	FR	1 area SW Spain
		Scanning (carcasses)	10 Iberian lynx			4 (40%)		
			5 red fox			2 (40%)		
Sobrino *et al.* (2008)	CR		1 badger	BC		1	FR	1 area SW Spain
Candela *et al.* (2009)	CS	Targeted (hunted)	61 Barbary sheep	SE (icELISA MPB70)	1999	(50%)	FR	1 area SE Spain
Millán *et al.* (2009)	SU	Targeted (trapped) Scanning (carcasses)	26 Iberian lynx 33 red fox 24 mongoose 10 gennet 2 badger	BC PCR SE (cELISA MPB70)	2004-2006	SE: 1 red fox 1 mongoose 2 badger BC: 2 red fox 2 Iberian lynx	FR	2 area SW Spain
Balseiro *et al.* (2009)	CR		1 roe deer	PCR IHC		1	FR	1 area N Spain
Gortázar *et al.* (2011a)	CS	Targeted (culled)	100 fallow deer	BC	2006-2007	21 (21%)	FR	1 area SW Spain
Balseiro *et al.* (2011)	SU	Targeted (trapped) Passive (carcasses)	157 badger (121 found dead, 36 trapped)	BC	2006-2010	8 found dead (6,6%) 0 trapped	FR	Several areas across Spain

Table 3. Studies dealing with other host species included in the analysis. Classification: SU – survey; CS - cross sectional study; CC – case-control study; Screening/diagnostic test: MI – official meat inspection scheme; GP – gross pathology; BC – bacteriological culture; SE – serology; IHC – immunohistochemistry; ELISA - enzyme-linked immune serum assay; Fencing: FR – free-ranging populations; FE – fenced populations; MX – mixed free-ranging and fenced populations.

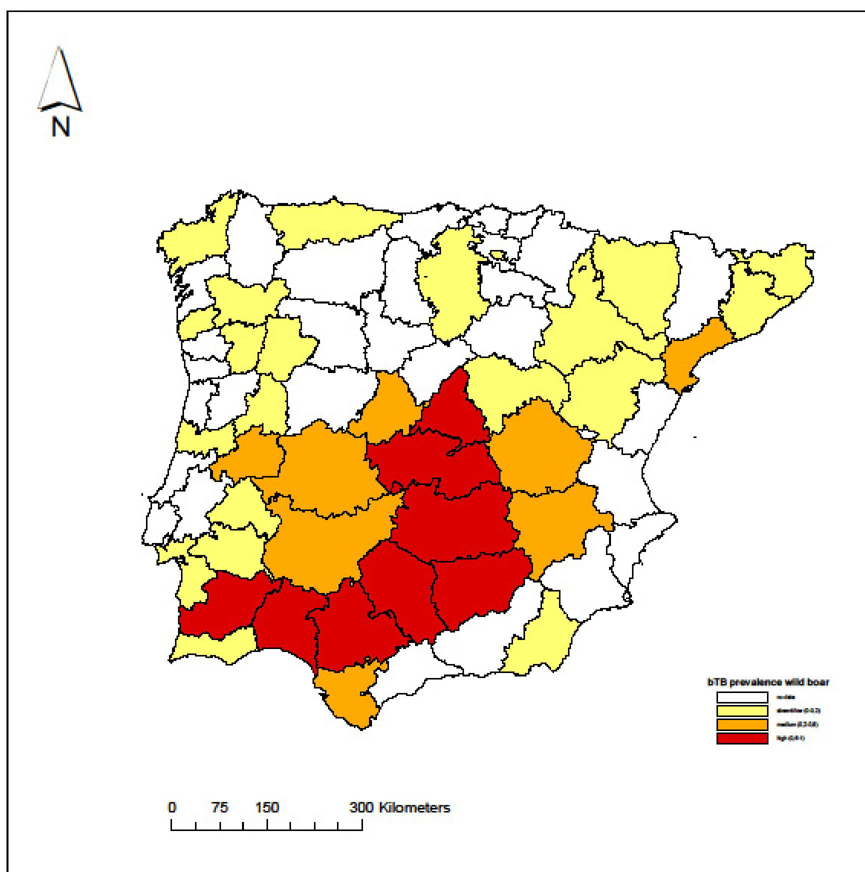


Fig. 1. Map displaying reported prevalence rates for bTB in the wild boar by administrative divisions of Iberian Peninsula (provinces in Spain, districts in Portugal). Bacteriological culture data (Aranaz *et al*., 2004; de Mendoza *et al*., 2006; Vicente *et al*., 2006a; Gortázar *et al*., 2008; Santos *et al*., 2009; Pinto *et al*., 2011) and serology data (Boadella *et al*., 2011; Santos *et al*., unpublished data) combined. The highest recorded prevalence for each administrative division is shown.

Again, few published articles address or allow addressing the geographical trend in bTB prevalence rates. In South-central Spain, an area roughly corresponding to Sierra Morena and Montes de Toledo was shown to have high prevalence rates, which declined towards the periphery of the area (Vicente *et al*., 2006a). In Doñana, wild boar and red deer show an increasing South-North gradient in prevalence rates (Gortázar *et al*., 2008). In Portugal, bTB was not detected in western regions, while present in the eastern portion of the country (Santos *et al*., 2009). Also in Eastern-central Portugal, wild boar and red deer populations show an increasing North-South gradient in prevalence rates (Pinto *et al*., 2011). In South-central Spain, lack of geographical autocorrelation in prevalence rates was reported (Vicente *et al*., 2006b).

3.4 Determinant factors of disease

Several risk and protective factors for bTB in both wild boar and red deer have been identified (Table 4). Most of the identified risk factors relate to host and other sympatric host's population factors, but also to environmental, management and historical factors. On the other hand, protective factors are mainly associated with environmental variables (Table 4). Notably, only one study has identified fencing, feeding and watering of wild ungulate populations as risk factors.

Determinants of disease		Wild boar	Red deer
	Type of risk factor		
Risk factors	Host population	Reproductive season Age Sex bTB prevalence rate in sympatric wild boar Wild boar abundance	Reproductive season Age Sex bTB prevalence rate in sympatric red deer
	Other hosts	Red deer presence Red deer abundance bTb prevalence rate in sympatric red deer	bTb prevalence rate in sympatric wild boar
	Environmental	Agro forestry land cover	
	Management	Aggregation at watering sites	Aggregation of wild boar at watering and feeding sites Fencing Supplementary feeding Presence water ponds Presence of livestock
	Historical	Past cattle density Distance to historical refuges	Past cattle density
Protective factors	All	Shrub land cover Distance to freshwater Sparse forestry land cover Genetic variability	Distance to freshwater

Table 4. Determinant factors of bTB occurrence identified in wild boar and red deer epidemiological studies in the Iberian Peninsula.

3.5 Host epidemiological status

Wild boar and red deer are usually considered maintenance hosts in Iberian Peninsula and epidemiological evidence has been gathered to support this view (Table 5) based on the

characterization of populations maintaining high bTB prevalence rates despite long-term lack of contacts with cattle. Fallow deer, Barbary sheep and badger are also discussed as possible maintenance hosts, while all other reported hosts are considered spillover. Wild boar, red and fallow deer have been suggested as possible reservoirs of infection for livestock.

3.6 Molecular epidemiology

The most commonly identified causative agent of bTB in Iberian Peninsula has been *M. bovis*, although a small proportion (0,05, n=829) of *Mycobacterium caprae* was reported in 6/15 studies. *M. caprae* is much more frequent among isolates from wild boar (0,08, n=502) than from red deer (0,01, n=327). *Mycobacterium avium*-complex mycobacteria and other mycobacteria have also been isolated from wild hosts, but they fall out of the scope of the present review. Molecular epidemiology studies rely mostly on spoligotyping (14/15), usually coupled with MIRU-VNTR typing (9/15) (Table 6).

4. Discussion

4.1 Characterization of published articles

Most epidemiological studies on wild boar or red deer are cross-sectional, allowing for the estimation of prevalence rates and simultaneously the identification of risk or protective factors. A few of the earliest studies were surveys; also classified as such were some molecular epidemiology articles that allow calculating prevalence rates. As knowledge of bTB on other species is more recent, a larger proportion of these studies are case reports and surveys. A comparatively large number of studies address molecular epidemiology.

Notably absent from the literature are case-control studies, which could shed light on the importance of specific determinants of disease, such as fencing and provision of feed and water. The same should be mentioned for experimental studies, were exposure to a certain determinant of disease is manipulated and the effect on disease occurrence is then measured. This design could be of great help to ascertain the role of each species in the persistence of bTB, trough manipulation of host density. The same can be said for epidemiological modelling, which could provide a theoretical framework for understanding bTB persistence in Iberian Peninsula and test the effect of different control measures (Thrushfield *et al*., 1995) and also to identify key data on host populations and wildlife tuberculosis that is missing or that is not feasible or up to date.

Most articles resort to targeted surveillance of hunted or culled animals, which allows prevalence estimation. Culling is expected to be less sex and age-biased than recreational hunting, which focuses on specific age (adults) and sex (males) classes. The hunting method used for harvesting the animals (drive hunts) is less selective than trophy hunting, allowing access also to females and juvenile/subadult animals (Fernández-Llario & Mateos-Quesada, 2003; Martínez *et al*., 2005). Hunted animals are usually considered a representative sample of the population for health monitoring, at least for non neurological or debilitating diseases (Conner *et al*., 2000). Nevertheless it should be kept in mind that some sampling biases can be present (Wilson *et al*., 2001).

Taxonomic Order	Species	Diagnostic technique	Mycobacterial species	Meta prevalence	Epidemiological status	References
Artiodactyla	Wild boar *Sus scrofa*	Bacteriology culture other	*M. bovis* *M. caprae*	276/771 (35,8%)	Maintenance host Reservoir?	Aranaz *et al*. (2004) Acevedo-Whitehouse *et al*. (2005) Gortázar *et al*. (2008) Romero *et al*. (2008) Santos *et al*. (2009) Pinto *et al*. (2011) others
	Red deer *Cervus elaphus*	Bacteriology culture other	*M. bovis* *M. caprae*	78/371 (21,0%)	Maintenance host Reservoir?	Aranaz *et al*. (2004) Gortázar *et al*. (2008) Romero *et al*. (2008) Pinto *et al*. (2011) others
	Fallow deer *Dama dama*	Bacteriology culture other	*M. bovis*	116/420 (27,6%)	Maintenance host? Spillover host? Reservoir host?	Aranaz *et al*. (2004) Gortázar *et al*. (2008, 2011) Romero *et al*. (2008) others
	Chamois *Rupicapra pyrenaica*	Bacteriology culture	*M. bovis*		Spillover host	Rodríguez *et al*. (2010)
	Mouflon *Ovis orientalis*	Bacteriology culture	*M. bovis*		Spillover host	Rodríguez *et al*. (2010)
	Barbary sheep *Ammotragus lervia*	Bacteriology culture other	*M. bovis*		Spillover host? Maintenance host?	Candela *et al*. (2009) Rodríguez *et al*. (2010)
	Roe deer *Capreolus capreolus*	IHC PCR	*M. bovis*		Spillover host	Balseiro *et al*. (2009)
Carnivora	Iberian lynx *Lynx pardina*	Bacteriology culture other	*M. bovis*	9/40	Spillover host	Briones *et al*. (2000) Pérez *et al*. (2001) Aranaz *et al*. (2004) Atance *et al*. (2006) Romero *et al*. (2008) Millán *et al*. (2009) Rodríguez *et al*. (2010)
	Red fox *Vulpes vulpes*	Bacteriology culture other	*M. bovis* *M. caprae*	5/45	Spillover host	Atance *et al*. (2005, 2006) Millán *et al*. (2008, 2009) Romero *et al*. (2008) Rodríguez *et al*. (2010)
	Badger *Meles meles*	Bacteriology culture other	*M. bovis*	8/121	Maintenance host	Atance *et al*. (2006) Sobrino *et al*. (2008) Rodríguez *et al*. (2010) Balseiro *et al*. (2011)

Table 5. Bovine tuberculosis host species described in the Iberian Peninsula. For references and meta prevalence rate calculations for wild boar, red and fallow deer only targeted-design studies using bacteriological culture as diagnostic test on all animals samples are used. Meta prevalence in carnivores is exclusively based on passive-design studies.

Reference	Sample n (Isolates)	Technique	Time frame	Genotypes n	Host clustering	Study areas
Aranaz *et al*. (1996)	4 wild boar 2 red deer (129 cattle, 44 goat, 1 sheep, 2 cat)	SP		24 spoligotypes (2 clusters)	Sheep/goat isolates clustered apart from other species	
Parra *et al*. (2003)	37 wild boar (25 Iberian pig)	SP MV	1998-2001	8 spoligotypes 43 combined (14 clusters, 21 unique profiles)	4 Iberian pig-only clusters 7 wild boar-only clusters 2 common clusters (14 genotypes)	1 area W Spain
Aranaz *et al*. (2004)	33 red deer 62 fallow deer 58 wild boar 3 Iberian lynx (50 cattle)	SP	1996-2002	21 spoligotypes	17 genotypes in wild boar (4 exclusive) 8 genotypes red deer (none exclusive) 6 fallow deer (1 exclusive) 10 cattle (3 exclusive)	7 areas SW Spain
Gortázar *et al*. (2005)	58 wild boar 19 red deer	SP MV	1999-2002	11 spoligotypes 19 combined	10 spoligotypes wild boar (5 exclusive) 6 spoligotypes red deer (1 exclusive)	24 areas SW Spain
Parra *et al*. (2005)	112 wild boar 59 red deer (6 cattle, 28 Iberian pig, 2 goat)	SP MV	1998-2003	14 spoligotypes 131 combined (28 clusters, 76 unique profiles)	22 clusters wild boar (8 exclusive) 13 clusters red deer (3 exclusive) 7 clusters pig (2 exclusive) 3 clusters cattle (1 exclusive) 1 cluster goat	1 area W Spain
de Mendoza *et al*. (2006)	11 wild boar 8 red deer (5 cattle)	SP MV	1992-2004	(4 clusters, 10 unique profiles)		1 area W Spain
Duarte *et al*. (2008)	21 red deer 6 wild boar (258 cattle, 8 goat)	SP	2002-2007	29 spoligotypes	11 spoligotypes red deer (2 exclusive) 5 spoligotypes wild boar (none exclusive) 27 spoligotypes cattle (15 exclusive)	Portugal
Romero *et al*. (2008)	60 wild boar 26 red deer 17 fallow deer 4 Iberian lynx 2 red fox (54 cattle)	SP MV	1998-2003	9 spoligotypes	3 spoligotypes wild boar (none exclusive) 2 spoligotypes red & fallow deer & red fox (none exclusive) 2 spoligotypes Iberian lynx (1 exclusive) 11 spoligotypes cattle (8 exclusive)	1 area SW Spain
Duarte *et al*. (2009)	13 red deer 4 wild boar (157 cattle, 7 goat)	MV	2002-2007	87 genotypes	12 genotypes red deer (8 exclusive) 4 genotypes wild boar (1 exclusive) 78 genotypes cattle (71 exclusive)	Portugal
Santos *et al*. (2009)	14 wild boar	SP	2005-2006	4 spoligotypes		3 areas Portugal
Rodríguez *et al*. (2010)	204 wild boar 141 red deer 229 fallow deer 2 chamois 1 mouflon 6 Iberian lynx 2 red fox 1 badger	SP	1992-2007	252 spoligotypes	26 spoligotypes wild boar (6 exclusive) 22 spoligotypes red deer (2 exclusive) 13 spoligotypes fallow deer (1 exclusive) 1 spoligotype chamois (none exclusive) 1 spoligotype mouflon (1 exclusive) 3 spoligotypes lynx (none exclusive)	Spain

	(5585 cattle, 33 goat, 7 pig, 3 cat, 1 dog)				2 spoligotypes red fox (none exclusive) 1 spoligotype badger (none exclusive) 239 spoligotypes cattle (207 exclusive) 3 spoligotypes goat (1 exclusive) 2 spoligotypes pig (none exclusive) 3 spoligotypes cat (1 exclusive) 1 spoligotype dog (none exclusive)	
Cunha *et al*. (2012)	74 red deer 36 wild boar	SP MV	2008-2009	27 spoligotypes	21 spoligotypes red deer (11 exclusive) 15 spoligotypes wild boar (5 exclusive) 6 spoligotypes exclusive of wildlife vs 23 spoligotypes exclusive of domestic species	4 regions South-Central Portugal
Gortázar *et al*. (2011)	24 red deer 21 fallow deer 62 wild boar	SP MV	2006-2007	9 spoligotypes 13 genotypes combined	8 genotypes red deer (2 exclusive) 6 genotypes fallow deer (none exclusive) 5 genotypes wild boar (none exclusive)	1 area SW Spain
Pinto *et al*. (2011)	27 red deer 21 wild boar	SP	2008-2009	8 spoligotypes	8 spoligotypes red deer (4 exclusive) 4 spoligotypes wild boar (none exclusive)	1 area Central Portugal
Rodriguez *et al*. (2011)	14 wild boar 1 red deer 1 red fox (542 goat, 229 cattle, 2 sheep, 2 pig)	SP MV	1992-2009	15 spoligotypes	4 spoligotypes wild boar (none exclusive) 1 spoligotype red (none exclusive) 1 spoligotype red fox (none exclusive) 12 spoligotypes goat (6 exclusive) 9 spoligotypes cattle (2 exclusive) 2 spoligotypes sheep, pig (none)	Spain

Table 6. Molecular biology studies included in the analysis. SP: spoligotyping, MV: MIRU-VNTR mycobacterial interspersed repetitive units-variable number of tandem repeats.

On the other hand, studies of wild ungulates relying on routine meat inspection for detection of macroscopic tuberculosis-like lesions, do not allow for a reliable estimation of prevalence, which is underestimated in this situation (de Mendonza *et al*., 2006). Nevertheless this type of design allows increasing sample size, which makes them suited for long-term surveillance rather than detailed epidemiological studies (de Mendonza *et al*., 2006) and were mostly used in the first surveys and cross-sectional studies after bTB was detected in wildlife in Iberian Peninsula. The investigations on carnivore species, most of which are not hunted, tend to rely on passive surveillance schemes based on haphazardly found carcasses. This sampling design does not allow to estimate prevalence rates due to extensive sampling bias (e.g. Taylor *et al*., 2002). Targeted sampling in these species has been attempted using serological tests but results should be interpreted with caution since these techniques have not yet been validated in these species.

The number of animals studied is usually adequate to determine prevalence rates with relatively small confidence intervals, at least in the easily available hunted species. The same cannot be said for most studies on protected carnivore species, where the collection of biological samples from a large number of animals is inherently difficult.

Bacteriological culture is the reference test for diagnosing bTB although it is expensive and time-consuming (de Lisle *et al*., 2002). As the financial resources needed to perform bacteriological culture on a large number of samples are scarcely available, most surveys use

other methods (usually gross pathology) as screening tests and only perform bacteriological culture for lesion-positive animals, sometimes as pooled samples. This introduces a bias and it was shown that the sensitivity of gross pathology was 72,2% of that obtained from bacteriology in the wild boar (Santos *et al.*, 2010). The same trend has been reported elsewhere for deer (Rohonczy *et al.*, 1996; O'Brien *et al.*, 2004).

4.2 Prevalence rates

Overall prevalence rates reported for bTB in wild boar, red deer and fallow deer in Iberian Peninsula are among the highest recorded for these species worldwide (Corner, 2006; Nishi *et al.*; 2006, Wilson *et al.*, 2008). Interestingly, prevalence rates in wild boar are invariably higher than in sympatric red or fallow deer (Gortázar *et al.*, in press).

Most studies report no sex differences in infection rates, but Santos *et al.* (2009) reported a significantly higher infection rate in female wild boar, presumably linked to more frequent social behaviour of females compared to males. Several studies report age differences in infection rates in wild boar, but data is conflicting since some authors reported increasing prevalence rates with age (e.g. Vicente *et al.*, 2006a,b), while others found higher prevalence rate in juveniles (e.g. Gortázar *et al.*, 2008; Santos *et al.*, 2009). Age and sex differences in prevalence rates were also reported in red deer (Vicente *et al.*, 2006a), which were higher for males and increased with age. This gender difference was already reported for cervids in North America (O'Brien *et al.*, 2006).

4.3 Trends

The few published data about the temporal dynamics of bTB prevalence rates are unanimous in showing an increasing trend across Iberian Peninsula in both wild boar and red deer (de Mendonza *et al.*, 2006; Gortázar *et al.*, 2008, 2011b, in press; Santos *et al.*, 2009, unpublished data). Gortázar *et al.*, (2011b) recently reported that 11/14 wild ungulate populations from central Spain show increasing bTB prevalence rates as assessed by gross pathology. This strongly supports previous interpretations that bTB is an emerging disease in wildlife in Iberian Peninsula.

The highest prevalence rates for bTB reported in wild ungulates in Iberian Peninsula lie in the central-south-western mountain chains of Montes de Toledo-Sierra Morena-Contenda (e.g. Vicente *et al.*, 2006a; Santos *et al.*, 2009) and Doñana (Gortázar *et al.*, 2008). Prevalence rates decline to the periphery of this region; the detected limits of this bTB core area are the provinces of Cáceres/Ávila to the north, eastern Portugal to the West, the Mediterranean coast to the South and Teruel to the East. bTB has not been detected or only sporadically in the northern, western and eastern periphery of Iberian Peninsula, despite locally intense surveillance (Gortázar *et al.*, 2011b). This pattern, coupled with the abovementioned increase in prevalence over time, strongly suggests that the disease is expanding from the central core area.

Interestingly, this core region of high bTB prevalence rates coincides with the main historical refuge of the wild boar in Spain (Tellería & Saez-Royuela, 1985) and, to some extent, in Portugal (Lopes & Borges, 2004). In the beginning of the XXth century, Iberian populations of wild ungulates were at their lowest level due to intense direct persecution and were largely restricted to a few mountain regions. Starting in 1960's, wild boar populations expanded from these refuges (Tellería & Saez-Royuela, 1985; Acevedo *et al.*, 2011) to a point they

nowadays occupy almost all Iberian Peninsula (Rosell, 2001). Natural expansion of red deer also occurred but not to such a great extent as in the wild boar case and was much dependent upon translocations (Soriguer, 1998; Acevedo *et al*., 2011).

As suggested by Santos *et al*. (2009) for Portugal, wildlife bTB could be similarly expanding from the historical refuges with a lag comparative to its host's expansion. This lag could be explained by the threshold theory for disease persistence, as reported for other bTB hosts such as the possum *Trichosurus vulpecula* in New Zealand – Lloyd-Smith *et al*., 2005). As wild ungulate populations expanded, densities at the front of the expansion wave were too low (Holland *et al*., 2007) to allow for the persistence of bTB, even if presumably some infected hosts were involved in that expansion event. As a consequence, wildlife bTB initially remained confined to the historical refuges, despite dispersion of infected hosts. As ungulate distribution continued to expand, densities increased in a gradient centred at the historical refuges and eventually reached the threshold level. At that point, bTB, introduced by infected immigrants from the historical refuges, could persist and spread its distribution, a process seemingly still taking place.

This hypothesis could be tested by comprehensive geographical spatial analysis of the distribution of bTB in Iberian Peninsula, but the proposed natural expansion pattern has probably been much obscured by translocation and intensive management of ungulates for hunting purposes (Vargas *et al*. 1995; Miguel *et al*. 1999; Castillo *et al*., 2010). In fact, in South-central Spain lack of geographical autocorrelation in prevalence rates was suggested to be due to extensive fencing of intensively-managed big game hunting estates, which impair animal movements (Vicente *et al*., 2006b). On the other hand, wild ungulate translocations for hunting purposes occur frequently and may spread *M. bovis* to areas where it is absent today. Interestingly, *M. bovis* was isolated from wild boar in Portugal in two areas widely out of the known distribution of the disease (Santos *et al*., 2009; Cunha *et al*., 2012), one of which coincides with the release site of red deer originating from a population harbouring the same genotype of *M. bovis*. This provides circumstantial evidence for the role of translocations on bTB geographical spread.

More spatial data of bTb occurrence in Iberian Peninsula is urgently needed. The advent of sensitive, specific, reproducible and cheap serologic tests allows such large-scale research to be conducted, at least for wild boar (Boadella *et al*., 2011). This should improve the understanding of bTB occurrence across Iberian Peninsula.

4.4 Disease determinant factors

Most risk factors for bTB in wild boar and red deer identified in Iberian Peninsula are host population factors, most of them abundance-related. It is interesting to note that in the wild boar-red deer system, the abundance of each species influences bTB occurrence in the other species, further supporting the multi-host pathogen status of bTB in Iberian Peninsula ecosystems.

The number of risk factors related to management is greater for the red deer (n=5) than for the wild boar (n=1). This suggests that bTB occurrence in red deer populations is more dependent on management practices, while wild boar is competent to act as maintenance host under low-intensity management. This hypothesis could be tested by a case-control study of bTB occurrence in both species across a gradient of intensity of management.

Interestingly, among the protective risk factors described for bTB in Doñana, distance to freshwater sources is highlighted. Much remains to be known on the conditions necessary for the survival of mycobacteria in the environment, but humidity seems to favour it (Humblet *et al*., 2009), particularly in the arid summer conditions of southern Iberian Peninsula. This suggests that environmental contamination with mycobacteria, particularly at watering sites, and indirect routes could play a role in disease transmission among wild ungulate species.

4.5 Host status

Wild boar and red deer are usually referred as maintenance hosts in Iberian Peninsula and evidence is available as populations maintaining high prevalence rates for several years, even decades, in the absence of domestic cattle which could theoretically serve as reservoirs for wildlife (e.g. Vicente *et al*., 2006a; Gortázar *et al*., 2008). It seems consensual that high-density sympatric populations of wild boar and red deer can maintain bTB at a high prevalence independent of the existence of other hosts (e.g. de Mendonza *et al*., 2006; Vicente *et al*., 2006a; Gortázar *et al*., in press). This seems also to be independent of intensity of management for hunting purposes, favouring high density of animals through habitat management, feeding and watering (Miguel *et al*., 1999), as even non-intensively managed but high-density populations of wild boar show high bTB prevalence rates (Santos *et al*., 2009).

It should be noted that in most of Iberian Peninsula densities far above the natural carrying capacity of wild boar and red deer occur, even in the absence of intensive management, because natural predators of these species (essentially wolf *Canis lupus*) have been eliminated during the last 50 years (Rico & Torrente, 2000). Packer *et al*. (2003) have shown through modelling that removal of predators can lead to an increase on pathogens' prevalence. Furthermore, Barber-Meyer *et al*. (2007) have shown that wolf restoration in Yellowstone had significant impacts on the seroprevalence of several pathogens of deer, even though those populations were previously subject to predation by other species.

It could be hypothesized that the current bTB high prevalence rates in wildlife in Iberian Peninsula derives from severe changes on the ecosystems caused by intensive management for hunting purposes (Gortázar *et al*., 2006) and eventually also predator eradication (Rico & Torrente, 2000). Experimental studies where host density is manipulated through large-scale culling are absent from the literature and could help to understand the role of artificialization of the ecosystems in the persistence and expansion of bTB. The picture is further complicated by the difficulty in separating the effect of each host species, as they usually occur in sympatry in the core area. Nevertheless, wild boar populations have been reported to show high bTB prevalence rates even in the absence of sympatric deer (Vicente *et al*., 2006a).

Fallow deer and badger are most likely local maintenance hosts where they occur at high density, notably in scattered populations of fallow deer and in Atlantic Iberian Peninsula for the badger. On the other hand, other carnivore and ungulate species infected in Iberian Peninsula are most likely spillover hosts, with the possible exception of exotic Barbary sheep.

4.6 Molecular epidemiology

Studies reviewed are rather concordant in concluding that genotypes seem to be geographically clustered as each location has a few predominant genotypes, responsible for

the majority of the infections. Concurrently, there is also a wide variety of locally rare genotypes. Local genotypes tend to be the same in different sympatric species, both domestic and wild, supporting the local interspecies transmission of *M. bovis*.

5. Conclusion

In summary, published evidence suggests that bTB is a natural pathogen of autochthonous wild ungulates in Iberian Peninsula, where wild boar and red deer act as maintenance hosts. Bovine tuberculosis is an emergent disease in these hosts, the expansion from the core high prevalence area in south-western Iberian Peninsula being fuelled by high densities of these species due to intensive management for hunting purposes. Several other species of ungulates and carnivores are affected by bTB, most probably as spillover hosts, but fallow deer and badger could serve as maintenance host in some locations. Although shown to be an important emerging infection, large gaps remain in the knowledge of the epidemiology of bTB in wildlife, such as intra and inter-species transmission routes, geographical distribution and effectiveness of control methods. Applying different epidemiological study designs, such as case-control and experimental studies, spatial analysis and modelling could shed light on this subject.

6. References

Acevedo, P., Farfán, M., Márquez, A., Delibes-Mateos, M., Real, R. & Vargas, J. (2011) Past, present and future of wild ungulates in relation to changes in land use. *Landscape Ecology*, 26:19–31, ISSN 0921-2973

Acevedo-Whitehouse, K., Vicente, J., Gortázar, C., Höfle, U., Fernández-de-Mera, I. & Amos, W. (2005) Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Molecular Ecology*, 14: 3209–3217, ISSN 0962-1083

Allepuz, A., Casals, J., Nappa, S., Saez, M., Alba, A., Vilar, M., Domingo, M., González, M., DuranFerrer, M., Vicente, J., Álvarez, J., Muñoz, M. & Saez, J. (2011) Analysis of the spatial variation of bovine tuberculosis disease risk in Spain (2006–2009). *Preventive Veterinary Medicine*, 100(1): 44-52, ISSN 0167-5877

Aranaz, A., Liébana, E., Mateos, A., Domínguez, L., Vidal, D., Domingo, M., González, O., Rodriguez-Ferri, E., bunschoten, A., van Embden, J. & Cousins, D. (1996) Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for studying epidemiology of tuberculosis. *Journal of Clinical Microbiology*, 34(11): 2734–2740

Aranaz, A., de Juan, L., Montero, N., Sánchez, C., Galka, M., Delso, C., Álvarez, J., Romero, B., Bezos, J., Vela, A., Briones, V., Mateos, A. & Domínguez, L. (2004) Bovine tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. *Journal of Clinical Microbiology*, 42(6): 2602–2608

Artois, M., Delahay, R., Guberti, V. & Cheeseman, C. (2001) Control of infectious diseases of wildlife in Europe. *Veterinary Journal*, 162: 141–152, ISSN 1090-0233

Atance, P., Palomares, F., Candela, M., Revilla, E., Cubero, M., Calzada, J. & Vizcaíno, L. (2005) Bovine tuberculosis in a free ranging red fox (*Vulpes vulpes*) from Doñana National Park (Spain). *Journal of Wildlife Diseases*, 41(2): 435–436, ISSN 0090-3558

Atance, P., Vizcaíno, L., Palomares, F., Revilla, E., Candela, M., Calzada, Cubero-Pablo, J. & Delibes, M. (2006) Antibodies to *Mycobacterium bovis* in Wild Carnivores from

Doñana National Park (Spain). *Journal of Wildlife Diseases*, 42(3): 704–708, ISSN 0090-3558

Balseiro, A., Oleaga, A., Orusa, R., Robetto, S., Domenis, L., Zoppi, S., Dondo, A., Goria, M., Gortázar, C. & Marín, J. (2009) Tuberculosis in roe deer from Spain and Italy. *Veterinary Record,* 164: 468-470, ISSN 0042-4900

Balseiro, A., Rodríguez, O., González-Quirós, P., Merediz, I., Sevilla, I., Davé, D., Dalley, D., Lesellier, D., Chambers, M., Bezos, J., Muñoz, M., Delahay, R., Gortázar, C. & Prieto, J. (2011) Infection of Eurasian badgers (*Meles meles*) with *Mycobacterium bovis* and *Mycobacterium avium* complex in Spain. *Veterinary Journal,* 190(2): 21-25, ISSN 1090-0233

Barber-Meyer, S., Whit, P. & Mech, L. (2007) Survey of Selected Pathogens and Blood Parameters of Northern Yellowstone Elk: Wolf Sanitation Effect Implications. *American Midland Naturalist*, 158: 369–381, ISSN 0003-0031

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J., Vicente, J., de la Fuente, J. & Gortázar, C. (2011) Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium* subspecies *paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). *Journal Veterinary Diagnostic Investigations*, 23: 77-83, ISSN 1040-6387

Boadella, M., Acevedo, P., Vicente, J., Mentaberre, G., Balseiro, A., Arnal, M., Martínez, D., García-Bocanegra, I., Casal, C., Álvarez, J., Oleaga, A., Lavín, S., Muñoz, M., Sáez-Llorente, J., de la Fuente, J. & Gortázar, C. (2011) Spatio-temporal trends of Iberian wild boar contact with *Mycobacterium tuberculosis* complex detected by ELISA. *EcoHealth*, doi: 10.1007/s10393-011-0713-y, ISSN 1612-9210

Briones, V., de Juan, L., Sánchez, C., Vela, A., Galka, M., Montero, N., Goyache, J., Aranaz, A., Mateos, A. & Domínguez, L. (2000) Bovine tuberculosis and the endangered Iberian Lynx. *Emerging Infectious Diseases*, 6(2): 189-191, ISSN 1080-6040

Candela, M., Serrano, E., Martinez-Carrasco, C., Martín-Atance, C., Cubero, M., Alonso, F. & Leon, L. (2009) Coinfection is an important factor in epidemiological studies: the first serosurvey of the aoudad (*Ammotragus lervia*). *European Journal of Clinical Microbiology Infectious Diseases*, 28:481–489, ISSN 0934-9723

Castillo, L., Fernández-Llario, p., Mateos, C., Carranza, J., Benítez-Medina, J., García-Jiménez, W., Bermejo-Martín, F. & de Mendoza, J. (2010) Management practices and their association with *Mycobacterium tuberculosis* complex prevalence in red deer populations in Southwestern Spain. *Preventive Veterinary Medicine*, 98(1): 58-63, ISSN 0167-5877

Clifton-Hadley, R., Sauter-Louis, C., Lugton, I., Jackson, R., Durr, P. & Wilesmith, J. (2001) *Mycobacterium bovis* infections, In: *Infectious diseases of wild mammals*. E. Williams & I. Barker, pp. 340-361, 3rd edition, Manson Publishing/The Veterinary Press, ISBN 9781840760057, London

Conner, M., McCarty, C. & Miller, M. (2000) Detection of bias in harvest-based estimates of Chronic Wasting Disease prevalence in Mule deer. *Journal of Wildlife Diseases*, 36(4): 691-699, ISSN 0090-3558

Corner, L. (2006) The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. *Veterinary Microbiology*, 112: 303–312, ISSN 0378-1135

Cunha, M., Monteiro, M., Carvalho, P., Mendonça, P., Albuquerque, T. & Botelho, A. (2011) Multihost tuberculosis: insights from the Portuguese control program. *Veterinary Medicine International*, 2011: doi:10.4061/2011/795165, ISSN 2042-0048

Cunha, M., Matos, F., Canto, A., Albuquerque, T., Alberto, J., Aranha, J., Pinto, M. & Botelho, A. (2012) Implications and challenges of tuberculosis in wildlife ungulates in Portugal: A molecular epidemiology perspective. *Research in Veterinary Science*, 92(2): 225-235, ISSN 0034-5288

Etter, E., Donado, P., Jori, F., Caron, A., Goutard, F. & Roger, F. (2006) Risk analysis and bovine tuberculosis, a re-emerging zoonosis, *Annals New York Academy Sciences*, 1081:61–73, ISSN 0077-8923

Fernández-Llario, P. & Mateos-Quesada, P. (2003) Population structure of the wild boar (*Sus scrofa*) in two Mediterranean habitats in the western Iberian Peninsula. *Folia Zoologica*, 52(2): 143–148, ISSN 1573-1189

Gordejo, F. & Vermeersch, J. (2006) Towards eradication of bovine tuberculosis in the European Union. *Veterinary Microbiology*, 112: 101–109, ISSN 0378-1135

Gortázar, C., Acevedo, P., Ruiz-Fons, F. & Vicente, J. (2006) Disease risks and overabundance of game species. *European Journal Wildlife Research*, 52: 81–87, ISSN 1439-0574

Gortázar, C., Torres, M., Vicente, J., Acevedo, P., Reglero, M., de la Fuente, J., Negro, J. & Aznar-Martín, J. (2008) Bovine tuberculosis in Doñana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian Lynx strongholds. *PLoS ONE*, 3(7): e2776. doi:10.1371/journal.pone.0002776, ISSN 1932-6203

Gortázar, C., Torres, M., Acevedo, P., Aznar, J., Negro, J., de la Fuente, J. & Vicente, J. (2011a) Fine-tuning the space, time, and host distribution of mycobacteria in wildlife. *BMC Microbiology*, 2011, 11:27, ISSN 1471-2180

Gortázar, C., Vicente, J., Boadella, M., Ballesteros, C., Galindo, R., Garrido, J., Aranaz, A. & de la Fuente, J. (2011b) Progress in the control of bovine tuberculosis in Spanish wildlife. *Veterinary Microbiology*, 151(1-2): 170-178, ISSN 0378-1135

Gortázar, C., Delahay, R., McDonal, R., Boadella, M., Gavier-Widen, D., Acevedo, P. (in press) The status of tuberculosis in European wild mammals. *Mammal Review*, ISSN 1365-2907

Holland, E., Aegerter, J. & Smith, G. (2007) Spatial sensitivity of a generic population model, using wild boar (*Sus scrofa*) as a test case. *Ecological Modelling*, 205: 146–158, ISSN 0304-3800

Horan, R., Wolf, C., Fenichel, E. & Mathews, K. (2008) Joint management of wildlife and livestock disease. *Environmental & Resources Economics*, 41:47–70 doi: 10.1007/s10640-007-9180-x, ISSN 1573-1502

Humblet, M., Boschiroli, M. & Saegerman, C. (2009) Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach. *Veterinary Research*, 40:50 doi: 10.1051/vetres/20090 , ISSN 0928-4249

de Lisle, G., Bengis, R., Schmitt, S. & O'Brien, D. (2002) Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue Scientific et Technique Office International des Epizooties*, 21: 317–334, ISSN 0253-1933

Lloyd-smith, J., Cross, P., Briggs, C., Daugherty, M., Getz, W., Latto, J., Sanchez, M., Smith, A. & Swei, A. (2005) Should we expect population thresholds for wildlife disease? *Trends in Ecology and Evolution*, 20: 511–520, ISSN 0169-5347

Lopes, F. & Borges, J. (2004) Wild boar in Portugal. *Galemys*, 16: 243–251, ISSN 1137-8700

Lyashchenko, K., Greenwald, R., Esfandiari, J., Chambers, A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B., Jackson, R., O'Brien, D., Schmitt, S., Palmer, M., Delahay, R. & Waters, R. (2008) Animal-side serologic assay for rapid

detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Veterinary Microbiology*, 132: 283-292, ISSN 0378-1135

Martínez, M., Rodríguez-Vigal, C., Jones, O., Coulson, T. & San Miguel, A. (2005) Different hunting strategies select for different weights in red deer. *Biology Letters*, 1: 353–356, ISSN 1744-9561

Matos, F., Cunha, M., Canto, A., Albuquerque, T., Amado, A. & Botelho, A. (2010) Snapshot of *Mycobacterium bovis* and *Mycobacterium caprae* infections in livestock in a bovine tuberculosis low incidence scenario. *Journal of Clinical Microbiology*, 48: 4337–4339, ISSN 0095-1137

de Mendoza, J., Parra, A., Tato, A., Alonso, J., Rey, J., Peña, J., García-Sánchez, A., Larrasa, J., Teixidó, J., Manzano, G., Cerrato, R., Pereira, G., Fernández-Llario, P. & de Mendoza, M. (2006) Bovine tuberculosis in wild boar (*Sus scrofa*), red deer (*Cervus elaphus*) and cattle (*Bos taurus*) in a Mediterranean ecosystem (1992–2004). *Preventive Veterinary Medicine*, 74: 239–247, ISSN 0167-5877

Miguel, A., Pérez-Carral, C. & Roig, S. (1999) Deer and traditional agrosilvopastoral systems of Mediterranean Spain. A new problem of sustainability for a new concept of land use. *Options Méditerranéennes*, 39: 261-264, ISSN 1016-1228.

Millán, J., Jiménez, M., Viota, M., Candela, M., Peña, L. & Vizcaíno, L. (2008) Disseminated bovine tuberculosis in a wild red fox (*Vulpes vulpes*) in Southern Spain. *Journal of Wildlife Diseases*, 44(3): 701–706, ISSN 0090-3558

Millán, J., Candela, M., Palomares, F., Cubero, M., Rodríguez, A., Barral, M., de la Fuente, J., Almería, S. & Vizcaíno, L. (2009) Disease threats to the endangered Iberian lynx (*Lynx pardinus*). *Veterinary Journal*, 182: 114–124, ISSN 1090-0233

Nishi, J., Shury, T. &, Elkin, B. (2006) Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: strategies for management and research. *Veterinary Microbiology*, 112: 325–338, ISSN 0378-1135

O'Brien, D., Schmitt, S., Berry, D., Fitzgerald, S., Vanneste, J., Lyon, T., Magsig, D., Fierke, J., Cooley, T., Zwick, L. & Thomsen, B. (2004) Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan. *Journal of Wildlife Diseases*, 40(1): 42–52, ISSN 0090-3558

O'Brien, D., Fitzgerald, S., Berry, D. & Hickling, G. (2006) Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience. *Veterinary Microbiology*, 112: 313– 323, ISSN 0378-1135

Packer, C., Holt, R., Hudson, P., Lafferty, K. & Dobson, A. (2003) Keeping the herds healthy and alert: implications of predator control for infectious disease. *Ecology Letters*, 6: 797–802, ISSN 1461-023X

Parra, A., Larrasa, J., García, A., Alonso, J. & de Mendoza, J. (2005) Molecular epidemiology of bovine tuberculosis in wild animals in Spain: A first approach to risk factor analysis. *Veterinary Microbiology*, 110 (3-4): 293-300, ISSN 0378-1135

Parra, A., Inglis, N., Tato, A., Alonso, J., de Mendoza, M., de Mendoza, J. & Larrasa, J. (2006) An epidemiological evaluation of *Mycobacterium bovis* infections in wild game animals of the Spanish Mediterranean ecosystem. *Research in Veterinary Science*, 80: 140–146, ISSN 0034-5288

Pérez, J., Calzada, J., Vizcaíno, L., Cubero, M., Velarde, J. & Mozos, E. (2001) Tuberculosis in an Iberian lynx (*Lynx pardina*). *Veterinary Record*, 148: 414-415, ISSN 0042-4900

Pinto, M., Alberto, J., Aranha, J., Serejo, J., Canto, A., Cunha, M. & Botelho, A. (2011) Combined evaluation of bovine tuberculosis in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) from Central-East Portugal. *European Journal of Wildlife Research*, doi 10.1007/s10344-011-0532-z, ISSN 1612-4642

Renwick, A., White, P. & Bengis, R. (2007) Bovine tuberculosis in southern African wildlife : a multi-species host–pathogen system. *Epidemiology and Infection*, 135: 529-540, ISSN 0950-2688

Rico, M. & Torrente, J. (2000) Caza y rarificación del lobo en españa: investigación histórica y conclusiones biológicas. *Galemys*, 12:163-179, ISSN 1137-8700

Rodríguez, S., Romero, B., Bezos, J., de Juan, L., Álvarez, J., Castellanos, E., Moya, N., Lozano, F., González, S., Sáez-Llorente, J., Mateos, A., Domínguez, L., Aranaz, A. (2010) High spoligotype diversity within a *Mycobacterium bovis* population: clues to understanding the demography of the pathogen in Europe. *Veterinary Microbiology*, 141: 89-95, ISSN 0378-1135

Rodríguez, S., Bezos, J., Romero, B., de Juan, L., Álvarez, J., Castellanos, E., Moya, N., Lozano, F., Javed, M., Sáez-Llorente, J., Liébana, E., Mateos, A., Domínguez, L., Aranaz, A. (2011) *Mycobacterium caprae* infection in livestock and wildlife, Spain. Emerging Infectious Diseases, 17(3): 532-535, ISSN 1080-6059

Rohonczy, E., Balachandran, A., Dukes, T., Payeur, J., Rhyan, J., Saari, D., Whiting, T., Wilson, S. & Jarnagin, J. (1996) A comparison of gross pathology, histopathology, and mycobacterial culture for the diagnosis of tuberculosis in elk (*Cervus elaphus*). *Canadian Journal of Veterinary Research*, 60: 108–114, ISSN 0830-9000

Romero, B., Aranaz, A., Sandoval, A., Álvarez, J., de Juan, L., Bezos, J., Sánchez, C., Galka, M., Fernández, P., Mateos, A. & Domínguez, L. (2008) Persistence and molecular evolution of *Mycobacterium bovis* population from cattle and wildlife in Doñana National Park revealed by genotype variation. *Veterinary Microbiology*, 132: 87–95, ISSN 0378-1135

Rosell, C., Fernández-Llario, P. & Herrero, J. (2001) El jabalí (*Sus scrofa* Linnaeus, 1758). *Galemys*, 13(2): 1-25, ISSN 1137-8700

Santos, N., Correia-Neves, M., Ghebremichael, S., Källenius, G., Svenson, S. & Almeida, V. (2009) Epidemiology of *Mycobacterium bovis* infection in wild boar *Sus scrofa* from Portugal. *Journal of Wildlife Diseases*, 45(4): 1048-1061, ISSN 0090-3558

Santos, N., Geraldes, M., Afonso, A., Almeida, V. & Correia-Neves, M. (2010) Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): a comparison of methods applicable to hunter-harvested animals. *PLoS-ONE*, 5(9): e12663, ISSN 1932-6203

Schiller, I., Oesch, B., Vordermeier, M., Palmer, M., Harris, B., Orloski, K., Buddle, B., Thacker, T., Lyashchenko, K. & Waters, W. (2010) Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and Emerging Diseases* doi:10.1111/j.1865-1682.2010.01148.x, ISSN 1865-1674

Sobrino, R., Martín-Hernando, M., Vicente, J., Aurtenetxe, O., Garrido, J. & Gortázar, C. (2008) Bovine tuberculosis in a badger (*Meles meles*) from Spain. *Veterinary Record*, 163(5): 159-160, ISSN 0042-4900

Soriguer, R., Márquez, F. & Pérez, J. (1998) Las translocaciones (introducciones y reintroducciones) de espécies cinegéticas y sus effectos medioambientales. *Galemys*, 10(2): 19-35, ISSN 1137-8700

Steele, J. (1995) Regional and Country Status Report, In: *Mycobacterium bovis infection in animals and humans*, Thoen, C.O. & J.H. Steele, pp. 169–172, Iowa Press, ISBN 9780470344538, Ames

Swinton, J., Woolhouse, M., Begon, M., Dobson, A., Ferroglio, E., Grenfell, B., Guberti, V., Hails, R., Heesterbeek, J., Lavazza, A., Roberts, M., White, P. & Wilson, K. (2001) Microparasite transmission and persistence, In: *The Ecology of Wildlife Diseases*, P. Hudson, A. Rizzoli, B. Grenfell, H. Heesterbeek, A. Dobson, pp. 83-101, Oxford University Press, ISBN 0198506198, Oxford

Taylor, S., Buergelt, C., Roelke-Parker, M., Homer, B. & Rotstein, D. (2002) Causes of mortality of free-ranging Florida panthers. *Journal of Wildlife Diseases*, 38(1): 107-114, ISSN 0090-3558

Tellería, J. & Saez-Royuela, Y. (1985) L'évolution démographique du sanglier (*Sus scrofa*) en Espagne. *Mammalia* 49: 195–202, ISSN 0025-1461

Thrushfield, M. (1995) *Veterinary epidemiology*. 2nd edition, Blackwell Science, ISBN 0632048514, Oxford

Tompkins, D., Dobson, A., Arneberg, P., Begon, M., Cattadori, I., Greenman, J., Heesterbeek, J., Hudson, P., Newborn, D., Pugliese, A., Rizzoli, A., Rosa, R., Rosso, F. & Wilson, K. (2001) Parasites and host population dynamics, In: *The Ecology of Wildlife Disease,*. P. Hudson, A. Rizzoli, B. Grenfell, H. Heesterbeek, A. Dobson, pp. 45-62, Oxford University Press, ISBN 0198506198, Oxford

Vargas, J. Calvo, J. & Aparicio, M, (1995) Red deer *(Cervus elaphus hispanicus)* management in the dehesa system in Central Extremadura, Spain. *Agroforestry Systems*, 29: 77-89, ISSN 0167-4366.

Vicente, J., Höfle, U., Garrido, J., Fernández-de-Mera, I., Juste, R., Barral, M. & Gortazar, C. (2006a) Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. *Veterinary Research*, 37: 107–119, ISSN 0928-4249

Vicente, J., Höfle, U., Garrido, J., Fernández-de-Mera, I., acevedo, P., Juste, R., Barral, M. & Gortazar, C. (2006b) Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain. *Veterinary Research*, 38: 1-15, ISSN 0928-4249

Whiting, T. (2003) Foreign animal disease outbreaks, the animal welfare implications for Canada: risks apparent from international experience. *Canadian Veterinary Journal*: 44: 805–815, ISSN 0008-5286

Wilkins, M., Meyerson, J., Bartlett, P., Spieldenner, S., Berry, D., Mosher, L., Kaneene, J., Robinson-Dunn, B., Stobierski, M. & Boulton, M. (2008) Human *Mycobacterium bovis* infection and bovine tuberculosis outbreak, Michigan, 1994–2007. *Emerging Infectious Diseases*, 14(4):657-660, ISSN 1080-6040

Wilson, K., Bjornstad, O., Dobson, A., Merler, S., Poglayen, G., Randolph, S., Read, A. & Skorping, A. (2001) Heterogenities in macroparasite infections: patterns and processes, In *The Ecology of Wildlife Diseases,* P. Hudson, A. Rizzoli, B. Grenfell, H. Heesterbeek, A. Dobson, pp. 6-44, Oxford University Press, ISBN 0198506198, Oxford

Wilson, G., Broughan, J., Chambers, M., Clifton-Hadley, R., Crawshaw, T., de la Fuente, J., Delahay, R., Gavier-Widen, D., Gortazar, C., Hewinson, G., Jackson, V., Martín-Hernando, M., Neimanis, A., Salguero, F., Vicente, J., Ward, A. & McDonald, R. (2008) Scientific review on Tuberculosis in wildlife in the European Union. *Technical Report submitted to EFSA*, 117 pp.

3. The wildlife hosts of bovine tuberculosis in Iberian Peninsula

Relevant aspects of the ecology and population dynamics of wild boar and red deer will be briefly outlined next, as these are the main wildlife maintenance hosts for bTB in the multi-host pathogen system of Iberian Peninsula (Gortázar et al., 2012; Santos et al., 2012).

3.1. Ecology and population dynamics of the wild boar

The wild boar belongs to the taxonomic family Suidae, order Artiodactyla (Figure 5). Originally distributed through much of Europe, Asia and northern Africa it was introduced in historical times to the Americas, Australia, New Zealand and numerous islands throughout the world, usually in its domesticated form (Rosell, 2001). Hunting and habitat change reduced wild boar populations in Europe (Nores et al., 1995) to the point that in mid-XXth century the species was almost extinct in Portugal, surviving only in a few isolated populations (Lopes and Borges, 2004; Ferreira et al., 2008).

Figure 5 – Image of wild boar. Group of free-ranging wild boar, including adults and juveniles. Photographed at *Tapada de Mafra*, Portugal.

Since the mid-XXth century wild boar populations in Europe increased markedly (Acevedo et al., 2006) to the point where the species is currently present all over the Portuguese continental territory, except the most urbanized areas (Lopes and Borges, 2004; Bosh et al., 2012). Wild boar density in the Iberian Peninsula varies with habitat and management and is in the range of 2-12 individuals/km^2. Hunting usually removes 11-40 % of the populations each year (Rosell,

2001; Nores et al., Nores et al., 2008). The prolificacy of the wild boar is explained by the early age at sexual maturity (8-12 months for females), the short length of pregnancy (average 120 days) and the high number of piglets born per litter (average 4.1) (Rosell, 2001; Fonseca et al., 2011). Mating can occur throughout the year but tends to concentrate in the autumn with births occurring mostly in the following spring (Fonseca et al., 2011).

As a generalist and opportunistic species, diet is composed mainly of vegetable matter, although a small proportion of animal matter is present, usually invertebrates, amphibians, small mammals and even carrion. Being markedly fossatorial, the wild boar is an important seed-dispersing agent and an ecological keystone species (Rosell, 2001). Females tend to live in social groups composed of one to several females with their offspring of the year (Figure 5). Young males live in social groups while mature males tend to be solitary (Rosell, 2001; Poteaux et al., 2009).

3.2. Ecology and population dynamics of the red deer

The red deer belongs to the taxonomical family Cervidae, order Artiodactyla (Figure 6). Originally distributed through much of Europe, Asia, northern Africa and northern America, it was introduced in Australia, New Zealand and several islands throughout the world (Ludt et al., 2004).

Figure 6 – Images of red deer. (A) Group of females with offspring of different ages; (B) group of males, highlighting their ability to jump over cattle fences. Photographed by the author at *Herdade da Contenda*, Portugal.

Similarly to the wild boar, red deer populations went almost extinct in Portugal but rebounded since the mid-XXth century, nowadays presenting a fragmented distribution throughout continental Portugal. The main populations are located in Algarve, Alentejo, Beira Baixa, Serra da Lousã and Bragança (Salazar, 2009). In the Iberian Peninsula red deer density can be as high as 67 individuals/km^2 (Acevedo et al., 2008).

Females reach sexual maturity at the age of 2 years and males at 5-6 years. Being strictly seasonal, mating occurs in the autumn and females give birth in the following spring (García et al., 2002). Females tend to live in family groups consisting of a mature female, its offspring of the year and eventually the one from 2 years before, if it is female. These family groups can temporarily coalesce into larger groups. Males outside the breeding season also live in social male-only groups (Bocci et al., 2012) (Figure 6). The red deer has a mixed browser and grazer diet with large variation between seasons and wet/dry years (Bugalho and Milne, 2003).

4. Pathology of bovine tuberculosis

Bovine tuberculosis is a chronic disease giving rise to lesions known as tuberculous granulomas (Pesciaroli et al., 2014). Granulomas can be defined as a chronic inflammation characterized by organized collections of mature mononuclear phagocytes (macrophages, epithelioid and multinucleated giant cells) (Williams and Williams, 1983). Cells such as lymphocytes, plasma cells and fibroblasts and features such as necrosis and calcification can be present. The characteristics and anatomical distribution of tuberculous granulomas vary depending on the host species. In the following sections we briefly outline the current knowledge on the pathology of bTB in wild boar and red deer and also in cattle as this latter species has been more thoroughly studied.

4.1. Pathology in cattle

In naturally infected cattle, bTB lesions predominate in the upper and lower respiratory tract and associated lymph nodes, giving rise to a “primary complex” (Pollock and Neill, 2002; Cassidy, 2006; Liebana et al., 2008). Cephalic lymph nodes are also often affected and lesions in the retropharyngeal and submaxillary lymph nodes can occur in the absence of detectable lung

lesions, possibly due to the difficulty of detecting small lesions in the large pulmonary parenchyma (Cassidy 2006). Lesions are also reported in tonsils from natural infections (Neill et al., 2001; Liebana et al., 2008).

Based on the anatomical distribution of bTB lesions, inhalation of *M. bovis* is considered the most important route of bovine infection. Experimental infections through nasal and tracheal inoculations and in-contact secondary infections all support the effectiveness of the aerogenous route for bovine infection (Pollock and Neill, 2002). Infection with *M. bovis* can be established in cattle by the nasal instillation of as few as 500 CFU (Neill et al., 1988; Dean et al., 2005).

In respect to route of infection, ingestion of *M. bovis* is considered secondary to respiratory infections (Pollock and Neill, 2002). Nevertheless tuberculous lesions are common in the mesenteric lymph nodes and may result from ingestion or dissemination from primary lesions in other sites (Neill et al., 2001). Generalized bTB is nowadays rarely found in countries with active eradication programs. It is characterized by lesions in organs such as liver, kidneys and udder and serous cavities. Dissemination is also considered to arise from primary lesions elsewhere (Neill et al., 2001).

In experimentally-infected cattle by the nasal route, *M. bovis* was isolated 3 days post-infection (dpi) from upper respiratory tract lymph nodes, tonsil and the caudal lobe of the lungs; 7 dpi from bronchial-mediastinal and mesenteric lymph nodes, and subsequently thymus and spleen. At this stage, microscopic lesions were seen on the mucosa of the upper respiratory tract, lungs and their associated lymph nodes and at 11 dpi in upper respiratory tract lymph nodes (retropharyngeal, submandibular and cervical), trachea and palatine tonsil (Cassidy et al., 1998).

Once in tissues, mycobacteria are phagocytosed by macrophages which then interact with other cells of the innate immune system and activate cells of the acquired immune responses, within tissue but mostly in the draining lymph nodes. Macrophages are the preferred host cell for intracellular mycobacteria, but they are also the key cells for their control (Pollock & Neill, 2002; Álvarez et al., 2009). In many of the exposures, mycobacteria could be eliminated by the action of the innate immune response, involving mechanisms such as lysosome pH, lysosomal hydrolyses, bactericidal peptides and superoxide (Rastogi et al, 2001). Phagocytic vacuoles containing viable mycobacteria may have impaired fusion with lysozymes (Rastogi et al, 2001).

This interplay between macrophages and mycobacteria largely decides the consequences of the exposure to *M. bovis*.

Bovine tuberculous granulomas usually have the macroscopic appearance of white to yellowish nodules. Within these lesions, macrophages generally have an epithelioid cell appearance and often are observed as multinucleated giant cells (Langhan's cells) formed by macrophage fusion (Neill et al., 2001). These epithelioid and giant cells constitute the central area of young granulomas, and where shown to correlate negatively with bacterial counts in tissues (Menin et al., 2013). Tuberculous granulomas are subsequently surrounded by a zone of lymphocytes, plasma cells and monocytes. Later in the infection, granulomas eventually develop a central caseous necrosis that might be followed by mineralization and enveloping fibrosis (Cassidy 2006, Liebana et al., 2008). The presence of fibrosis was shown to negatively correlate with bacterial counts and lesion severity (Menin et al., 2013). Neutrophils tend to be present in the early stages of lesion formation and correlate positively with *M. bovis* proliferation (Menin et al., 2003; Cassidy 2006).

Tuberculous granulomas are the focal expression of granulomatous inflammation aimed at restricting mycobacterial growth by allowing infected macrophages and T cells to interact. In this context, T cells can both upregulate macrophage microbicidal activity or lyse heavily infected macrophages (Cassidy, 2006; Álvarez et al., 2009). These complex interactions within granulomas reflect macrophage and helper T cell function, cytokine production and mycobacterial activity, which influence its features. Necrosis, liquefaction and mineralization are outcomes of these interactions that determine lesion size and morphology and ultimately the evolution of disease (Cassidy, 2006; Álvarez et al., 2009). Thacker and colleagues (2007) suggested that cattle experimentally infected with *M. bovis* develop distinct histopathological features depending on the predominance of Th1 or Th2 cytokine profile.

4.2. Pathology in wild boar

Macroscopic lesions compatible with bTB are present in 53-83 % of naturally-infected wild boar, with microscopic-only lesions present in an additional 9-10 %. Lesions can be located in only one anatomical region (42-55 %) or generalized (45-58 %) (Martín-Hernando et al., 2007; Zanella et

al., 2008b; Santos et al., 2010; García-Jiménez et al., 2013b). Generalized bTB is more common on juvenile wild boar (Martín-Hernando et al., 2007).

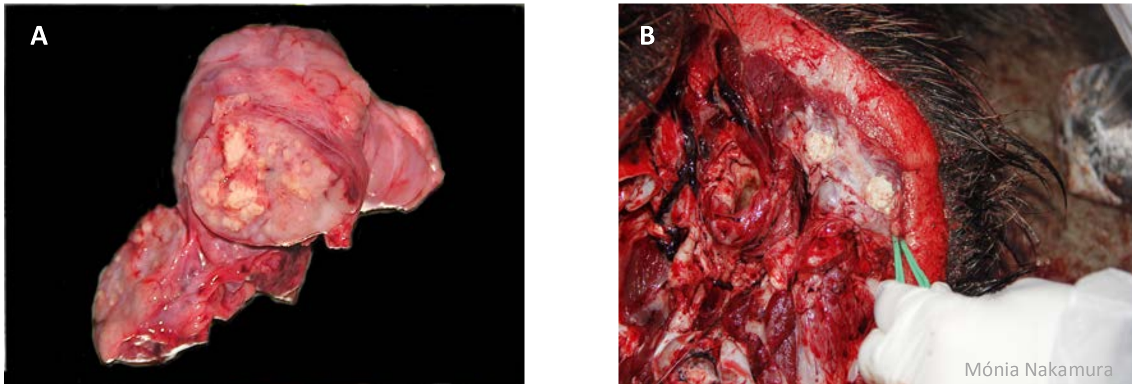


Figure 7 – Macroscopic aspects of bovine tuberculosis lesions in mandibular lymph nodes of naturally-infected wild boar. (A) Typical necrotic-calcified granulomas; (B) Sometimes lesions are immediately visible after cutting the head of hunted animals. Photographs of lesions detected by the author at sanitary inspection of hunted wild boar.

Cephalic lymph nodes are the most common location of bTB lesions (up to >90 %), particularly the mandibular lymph nodes (Figure 7), being less frequent in thoracic and mesenteric lymph nodes, tonsils and ileocecal valve (Martín-Hernando et al., 2007; Santos et al., 2010; Vieira-Pinto et al., 2011). Lesions in organs are most commonly found in the lungs (38 %), liver (23 %) and spleen (13 %), while they are rare in mammary glands and meninges and have not been reported in kidneys (García-Sánchez et al., 2007; Martín-Hernando et al., 2007).

Tuberculous granulomas in the wild boar can reach up to 15 cm in diameter and typically consist of epithelioid macrophages and low numbers of multinucleated giant cells surrounded by lymphocytes, plasma cells and macrophages (Martín-Hernando et al., 2007; García-Jiménez et al., 2013b). Early-stage granulomas are composed of inflammatory cells (mostly macrophages) eventually surrounding an area of central necrosis and constitute the majority of the granulomas found in wild boar (63 % - García-Jiménez et al., 2013b) (Figure 8). More advanced granulomas show peripheral fibroplasia and central necrosis, with different degrees of calcification and were found to be 38 % of all granulomas in this species (Martin-Hernando et al., 2007; Santos et al., 2010; Vieira-Pinto et al., 2011; García-Jiménez et al., 2013b). Early-stage granulomas are more frequent in localized lesions, while advanced-stage granulomas are more common in generalized

bTB (Martin-Hernando et al., 2007). Acid-fast bacilli are detected in 5-71 % of granulomas, usually in low numbers (Martín-Hernando et al., 2007; Santos et al., 2010; García-Jiménez et al., 2013b).

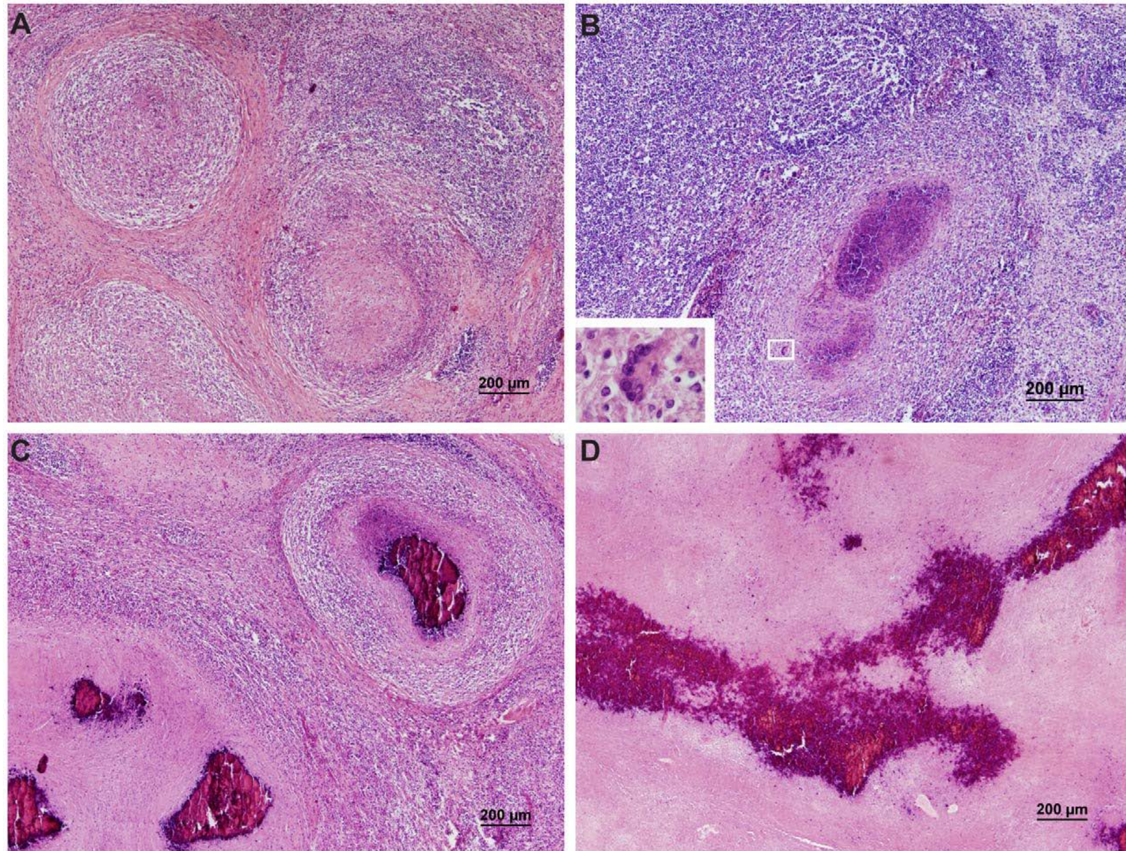


Figure 8 – Microscopic images of bovine tuberculosis lesions in wild boar. Light micrograph of lymph node lesions in naturally-infected wild boar stained with hematoxylin-eosin. (A) Granulomatous lesions with necrotic core; (B) Granulomatous lesions with a central caseous necrosis with light mineralization, surrounded by macrophage-like cells with abundant granular eosinophilic cytoplasm and multinucleate giant cells – Langhans' giant cells (inset); (C) Two adjacent granulomatous lesions with central mineralized caseous necrosis, bound by macrophage-like cells and fibrosis; (D) Advanced lesion showing extensive caseous necrotic areas with strong mineralization and fibrosis. From: Santos et al. (2010).

Infection with distinct mycobacteria species or strains may give rise to different presentations of disease. García-Jiménez and collegues (2013c) reported that granulomas caused by *M. caprae* tend to be of more advanced stages, show more multinucleated giant cells and higher counts of mycobacteria than those caused by *M. bovis*. Also differences in granuloma stage and bacterial counts were found for spoligotypes of *M. bovis* and *M. caprae* (García-Jiménez et al., 2013c).

By immuno-histochemical analysis it was shown that macrophages are more predominant and scattered in early stage granulomas compared to more advanced ones, where they tend to form a rim surrounding the central necrotic area. Lymphocyte numbers show no difference according to granuloma stages but while they are scattered throughout early stage granulomas, they tend to concentrate in the external layers, just within the fibrous capsule, in more advanced lesions. IFNγ and iNOS were present at high levels in all types of granulomas (García-Jiménez et al., 2013b).

4.3. Pathology in red deer

Macroscopic lesions compatible with bTB are present in 70-100 % of naturally-infected red deer, with microscopic-only lesions present in up to 11 % (Martín-Hernando et al., 2010). Lesions can be either located in only one anatomical region (54-66 %) or generalized (34-46 %) (Zanella et al., 2008b; Martín-Hernando et al., 2010).

The most common location of bTB lesions in the red deer are the retropharyngeal lymph nodes (12-64 %), mesenteric (75-80 %) and ileocecal lymph nodes (Figure 9) (Lugton et al., 1998; Zanella et al., 2008b; Martín-Hernando et al., 2010; Vieira-Pinto et al., 2011). Tonsilar lesions have been reported as absent by some authors (Martín-Hernando et al., 2010) but as common by others (Lugton et al., 1998; Shurry and Bergeson, 2011). Lesions in organs are most commonly found in the ileocecal valve and lungs but also on the spleen, mammary glands, rumen, diaphragm, aorta, joints and subcutaneous tissues (Zanella et al., 2008b; Martín-Hernando et al., 2010). Pleuritis can be present (Lugton et al., 1998; Zanella et al., 2008b).

Tuberculous granulomas in the red deer can have more than 30 cm in diameter and typically consist of a large central caseous-necrotic area surrounded by mononuclear cells and macrophages, including multinucleated giant cells (Lugton et al., 1998; Zanella et al., 2008b; Martín-Hernando et al., 2010). They are similar to abscess containing creamy yellowish pus and calcification is absent (Zanella et al., 2008b; Palmer et al., 2015) (Figure 9).

Figure 9 – Macroscopic aspects of bovine tuberculosis lesions in lymph nodes of naturally-infected red deer. (A) Caseo-purulent lesion in retropharyngeal lymph node; (B) Large abscess-like lesion in mesenteric lymph node draining caseo-purulent material after being incised. Photographs of lesions detected by the author at sanitary inspection of hunted red deer.

A thick external connective tissue capsule has been described by some authors as usually present (Zanella et al., 2008b; Martín-Hernando et al., 2010), or by others as usually absent (Shurry and Bergeson, 2011; Palmer et al., 2015). These granulomas are frequently large and eventually affected the entire organ (Martín-Hernando et al., 2010). Mycobacteria are present in 44 % of the granulomas, but their numbers were reported either as low (Martín-Hernando et al., 2010) or high (Lugton et al., 1998; Palmer et al., 2015).

5. Transmission of bovine tuberculosis

In order to control bTB in wildlife it is essential to gather deep knowledge on factors that affect the intra- and inter-specific transmission of infection, both individually and at population level (Corner, 2006; Riviére et al., 2015). Transmission of *M. bovis* from an infected excretor to a susceptible host can occur by direct or indirect routes (Phillips et al., 2003; Humblet et al., 2009). Direct transmission requires close contact between infected excretors and susceptible hosts (Corner, 2006). Therefore, it is expected to play a major role in intraspecific transmission of infection, as close contact is common among individuals of the same species (Cowie et al., 2015). However, close contact between individuals of different species seems to be rare (Phillips et al., 2003; Corner, 2006; Courtenay et al., 2006; Kukielka et al., 2013, Cowie et al., 2015) and so indirect routes are expected to play a crucial role in interspecific transmission. Indirect routes of transmission require the contamination of the environment with mycobacteria which

maintain their viability for a sufficient amount of time, allowing infection of other hosts (Phillips et al., 2003; Cowie et al., 2015).

Several studies showed that indirect transmission of *M. bovis* occurs scarcely in cattle grazing in either naturally or artificially infected pasture (reviewed by Morris et al., 1994; Humblet et al., 2009). Nevertheless it is strongly suspected to play a major role in the white-tailed deer-cattle system of North America, where it has been experimentally shown to occur through contaminated feed (Palmer et al., 2001; 2004a; 2004b). It is also suspected to occur in other wildlife-cattle systems, such as badger-cattle in the United Kingdom and Ireland (Phillips et al., 2003; Courtenay et al., 2006) and wild ungulates-cattle in the Iberian Peninsula (Kukielka et al., 2013; Cowie et al., 2015). In this later situation, environmental contamination of watering and feeding areas was proposed to be of epidemiological relevance (Kukielka et al., 2013; Cowie et al., 2015).

Detection of *M. bovis* in soil samples has been reported to endure several weeks or months after inoculation, depending on the initial concentration used (Morris et al., 1994; Tanner and Michel, 1999; Phillips et al., 2003; Fine et al., 2011; Ghodbane et al., 2014). However, Young and colleagues (2005) reported that mycobacterial DNA does not persist in the environment for more than 10 days outside a viable cell. Although it was experimentally shown that *M. bovis* DNA can persist in the environment for several months after no longer being recoverable by culture, this may reflect the lower sensitivity of bacteriological culture applied to environmental samples, when compared to molecular biology methods (Young et al., 2005; Adams et al., 2013).

The lack of clear data on environmental contamination with MTC might be due mainly to the lack of sensitive and mass-scalable techniques to detect MTC in the environment (Young et al., 2005; Courtenay et al., 2006; Fine et al., 2011; Adams et al., 2013). Molecular techniques show a greater promise over bacteriological techniques to detect MTC in environmental samples (Adams et al., 2013). Nevertheless, available protocols have exceedingly high detection limits, rendering them of limited usefulness as screening techniques, possibly due to the uneven distribution of mycobacteria in soil samples and the co-extraction of PCR inhibitors (Young et al., 2014). Young and colleagues (2005) reported a protocol with detection limits of 10^2-10^3 cells/g soil, however, this protocol was not replicated by other research groups. Pontirolli and colleagues (2011) optimized a protocol with a detection limit of 4.25×10^5 cells/g soil, which is too high for the

mycobacterial loads expected to occur in nature (Adams et al., 2013). These two studies used direct extraction techniques, where DNA was extracted from an environmental sample, typically of 0.1-0.5 g. On the other hand, Sweeney and colleagues (2006) described an immunomagnetic capture technique allowing the isolation and molecular detection of *M. bovis* from naturally contaminated soil samples. Despite this technical breakthrough in the study of the environmental contamination with pathogenic mycobacteria, this technique is difficult to scale up to test large numbers of samples and has not been replicated by other research groups.

Key to understanding transmission of MTC is information on routes of infection, pathology (structure and anatomical location of lesions), routes and levels of excretion and minimum infective doses (Corner et al., 2006). While pathology of bTB has been thoroughly documented in many wildlife species (e.g. Martín-Hernando et al., 2007; 2010; Gavier-Widén et al., 2009), routes of infection have not been demonstrated but only presumed based upon the location of the lesions (e.g. Martín-Hernando et al., 2007 for wild boar; Griffin and Buchan, 1994 for red deer; Corner et al., 2011a for badgers). In addition, information on the minimum infective doses have been estimated by experimental infections (e.g. Griffin et al., 2006 for red deer; Ballesteros et al., 2009 for wild boar) while data on MTC excretion is notably scarce. The only study addressing *M. bovis* routes of excretion from naturally infected wild ungulates was performed by Lugton and colleagues (1998), who detected excretion by several routes from red deer: oral (4/53 oropharyngeal swabs), nasal (1/53 nasal swabs), tracheal (1/53 tracheal swabs) and rectal (1/53 fecal samples). Urinary excretion was also investigated but not detected by these authors.

In experimentally infected white-tailed deer excretion was shown to occur sporadically by the oral route for up to 90 dpi, by the nasal route for up to 85 dpi and it was not detected by the fecal route (Palmer et al., 1999; 2004b). Using experimental infection with high doses of *M. bovis,* Palmer and colleagues (2001) detected excretion for up to 113 dpi by oral, nasal and fecal routes. In this study, naïve deer in contact with experimentally infected animals showed excretion by the oral and nasal routes for up to 90 days post-contact. In some studies shedding has been inferred based on the location and structure of the lesions (e.g. Martín-Hernando et al., 2007; Gavier-Widén et al., 2009), but *M. bovis* has been cultured from the feces of calves that only presented lesions in the lungs and cephalic-thoracic lymphoid tissues (Cassidy et al., 1999). This

has been attributed to swallowing of infected pulmonary secretions (Gallagher and Clifton-Hadley, 2000; Phillips et al., 2003).

The shedding of *M. bovis* has been extensively assessed only in Eurasian badgers, both in natural and experimental infections. Excretion was shown by bacteriological culture to occur by the fecal, urinary, pulmonary and oronasal routes and wound discharges in 25-50 % of infected animals (Clifton-Hadley et al., 1993; Delahay et al., 2000; Gallagher and Clifton-Hadley, 2000; Corner et al., 2008; 2011a). In this species a “super-shedder” state has been described encompassing those badgers where MTC shedding is detected by culture persistently or by more than one route, in contrast to the standard, intermittent shedders (Delahay et al., 2000). “Super-shedders” have been hypothesized to occur also in the wild boar (Gortázar et al., 2013) but evidence to support this supposition was lacking.

6. Control of bovine tuberculosis in wildlife

The concept of disease control in wildlife applies to actions aimed at reducing the prevalence of infection or to limit its effects to an acceptable level (Artois et al., 2001; Carter et al., 2009). It differs from control of the same infection in domestic species in several aspects. Wild animals are by definition free-living, less accessible and therefore signs of disease can be hard to detect. Also, population size usually cannot be estimated with confidence and so morbidity and mortality rates remain poorly understood (Wobeser, 2007). Therefore controlling disease in wildlife usually implies dealing with a great deal of uncertainty (Artois et al., 2001; Nugent, 2011; O’Brien et al., 2011b; Riviére et al., 2015). Such uncertainty, together with the complexity of host-pathogen systems, results that frequently the best option is no intervention (Wobeser, 2007; Bolzoni et al., 2013; Gortázar et al., 2015). Nevertheless, even in the event of no control actions being undertaken, it is essential to ensure surveillance to allow identifying future situations or locations where control should be undertaken (Gortázar et al., 2015; Riviére et al., 2015).

Wild animals are often the subject of much affection from the general public, which is usually not aware that infections can be transmitted from wildlife to humans or domestic species, so the rationale for control is difficult to understand (Artois et al., 2001; Gortázar et al., 2005; O’Brien et al., 2011b). Also as wildlife is usually neither private nor public ownership, no one is responsible

for them, so it is often hunters or wildlife conservationists who report disease cases and carry out control actions, often in the absence of wildlife management professionals (Artois et al., 2001).

Control actions may be grouped into the following strategy types (Wobeser, 2007):

i. Action upon the pathogen or vector (e.g. treatment, biological control of vectors);

ii. Action upon the host population (e.g. reducing density through culling or contraception, vaccination);

iii. Action upon the environment (e.g. eliminating high-risk transmission sites);

iv. Action upon human activity (e.g. biosecurity of farms, eliminating high-risk transmission practices).

Regarding wildlife bTB, tentative control programs have focused on the host population, environment and human activity (Phillips et al., 2003; Gortázar et al., 2011).

6. 1. Action upon the host population

Reducing host density through culling (lethal control) has been the mainstay of most attempts to control bTB in wildlife throughout the world (O'Brien et al., 2011b). The rationale is that by reducing both the infected and susceptible subsets of the population the density will eventually fall below a certain threshold level (usually not quantified) where disease will disappear due to a low transmission rate (Artois et al., 2001; Carter et al., 2009). This type of actions allowed for the successful eradication of bTB in water buffalo in Australia and is showing promising results in New Zealand (Radunz, 2006; Bradshaw et al., 2012; Livingstone et al., 2015). Both situations have in common that a single non-autochthonous and economically and socially not-valued wildlife species acted as maintenance host (Gortázar et al., 2015). Host population reduction, combined with action upon human activities, has also prevented the establishment of localized bTB-endemic areas in Minesotta and France (Carstensen and Doncarlos, 2011; Palmer et al., 2012; Hars et al., 2012). In Spain, culling of wild boar as been tested as management tool for bTB control, with positive effects on prevalence in other host species (Boadella et al., 2012; García-Jiménez et al., 2013a).

The example of attempts to control bTB in the UK by culling badgers, together with other examples such as rabies in foxes (*Vulpes vulpes*) and classical swine fever in wild boars in Europe underscores that lethal control is hard to implement over large areas (Blancou et al., 2009; Artois et al., 2011; Gortázar et al., 2015). In situations where rates of birth, death and disease transmission are high, mathematical models predict that culling is the most efficient control action (Barlow, 1996). Elsewhere, vaccination would be the preferred method to control disease, but depends on the availability of efficient vaccines and workable delivery systems (Blancou et al., 2009; Buddle et al., 2013).

Selective reduction of the susceptible subset of the host population through vaccination has been the subject of much research (Waters et al., 2012; Buddle et al., 2013). Vaccine candidates being studied include BCG and inactivated field strains of *M. bovis*. Host species where vaccination is being tested include the possum, African buffalo, white-tailed deer, badger and wild boar (Buddle et al., 2013). A major challenge for vaccination of wildlife is its delivery to the host. Oral baits are the most convenient delivery system, but it is difficult to control the dose of vaccine consumed, number of animals vaccinated, age of vaccination, uptake by non-target species and vaccine viability in the field (Waters et al., 2012; Buddle et al., 2013).

Regarding the host species involved on the epidemiology of bTB in Iberian Peninsula, oral vaccination with BCG or inactivated *M. bovis* field strain have shown promising results in the laboratory (Ballesteros et al., 2009; Garrido et al., 2011; Béltran-Beck et al., 2012; 2014; Gortázar et al., 2014). Although they do not fully protect from infection, lesion severity was considerably reduced (Béltran-Beck et al., 2014; Gortázar et al., 2014). Field trials are now under way to determine vaccine efficacy in free-ranging populations of wild boar (Béltran-Beck et al., 2012).

Reducing host population through fertility control shows promise but has not been attempted for bTB control and has only been used to control pest species (Carter et al., 2009; Artois et al., 2011; O'Brien et al., 2011b).

6.2. Action upon the environment

Determinants of disease include not only host and pathogen but also the environment (Ward et al., 2009; Artois et al., 2011). Soil, climate, vegetation and the spatial distribution of resources influence the dynamics of host-parasite systems (Ward et al., 2009). As a consequence, modifying environmental conditions may contribute to control the transmission of disease. The goal of environmental management may be either to render local conditions unfavorable for the host or the pathogen or to limit contacts between the infected and the susceptible populations (Ward et al., 2009; Artois et al., 2011). For the environmental management to be effective it is essential that our knowledge of the system is enough to anticipate all the consequences of the actions undertaken. Environmental management may help in reducing disease risk but, alone, is rarely sufficient (Artois et al., 2011).

Such actions have been experimentally used in the context of bTB control in Spain, where clustering around waterholes was identified as a risk factor (Vicente et al, 2006; Gortázar et al., 2011; Barasona et al., 2013; 2014). As a consequence, wildlife- or cattle-specific fences have been setup around such sites, aiming at separating the infected wildlife populations from livestock subjected to an eradication program (Barasona et al., 2013). Also in Riding Mountain National Park environmental management through controlled burns is used to improve habitat inside the protected area in an attempt to avoid infected red deer herds to feed on agricultural areas where they may contact livestock (Nishi et al., 2006).

6.3. Action upon human activities

Throughout the world, human activities influence ecosystems in a more or less profound way (Delahay et al., 2009; Mateos-Delibes et al., 2009). Such influence is perceptible on the epidemiology of many wildlife diseases, including bTB, but in turn it might also provide means for control (Gortázar et al., 2005; 2011). An example is white-tailed deer in Michigan, where clustering around winter feeders provided by local hunters was identified as a risk factor (Miller et al., 2003; O'Brien et al., 2011b; Palmer et al., 2012). Such practice was since prohibited which, in the context of an integrated bTB control program, lead to a decline in prevalence (Palmer et al., 2012). In many other instances clustering of hosts around resources artificially provided by

humans was identified as a risk factor, including in the multi-host pathogen system of Iberian Peninsula (Gortázar et al., 2005; 2011; Barasona at al., 2013; 2014).

When the issue is inter-specific transmission between wildlife and domestic species, biosecurity measures fall in this scope. Improved fencing and livestock-guarding dogs have been used to strengthen the biosecurity of livestock farms (Gehring et al., 2010; Judge et al., 2011; O'Brien et al., 2011b). Biosecurity translates into the concept of "compartimentalization" adopted by the *Office International des Epizzoties* where populations with different health status can coexist in a country if they are proved to be compartimentalized, e.g. no potentially disease-transmitting contacts occur between different compartments (Artois et al., 2011). A related concept is that of "zoning", in which such a separation is achieved on a geographical basis (Artois et al., 2011).

Preventive actions such as sanitary checks of translocated wildlife and proper disposal of hunting offal have also been used for bTB control in wildlife populations, including in the Iberian Peninsula (Gortázar et al., 2010; 2015; Zanella et al., 2012). In instances where bTB control in wildlife is not feasible, farmers have been advised to change from cattle to sheep farming, as the latter are more disease-resistant (Gortázar et al., 2015).

7. Aims of the thesis

Analyzing the state of the knowledge on the epidemiology of bTB in wildlife, particularly in the multi-host pathogen scenario of Iberian Peninsula, several gaps were identified. Particularly relevant are the ones related to the intra- and inter-specific routes of transmission and the spatial analysis of bTB distribution in the Iberian Peninsula. This PhD thesis aimed to contribute to fulfill these gaps in knowledge, and so its main goals were:

i) To identify MTC excretion routes and concentration of MTC in naturally-infected wild boar and red deer;

ii) To assess environmental contamination with MTC in areas with well-described distinct bTB prevalence in wildlife;

iii) To perform a spatial analysis of bTB in Portugal, aiming to identify the spatial structure, factors related to the presence of disease and produce risk models.

Each main goal corresponds to several secondary aims which are highlighted in the following sections of the thesis. To accomplish these goals several novel protocols were implemented and applied, such as high-sensitivity MTC molecular detection protocols, efficient DNA extraction protocols from environmental samples and blood collection methods for serological surveys, previously not used in the context of bTB research.

8. References

Acevedo P et al. (2006) Factors affecting wild boar abundance across an environmental gradient in Spain. *Acta Theriol*, 51:327–336.

Acevedo P et al. (2008) Estimating red deer abundance in a wide range of management situations in Mediterranean habitats. *J Zool*, 276 (1):37-47.

Adams AP et al. (2013) Comparison of PCR versus culture for detection of *Mycobacterium bovis* after experimental inoculation of various matrices held under environmental conditions for extended periods*. Appl Environ Microbiol*, 79(20):6501-6506.

Alexander KA et al. (2010) Novel *Mycobacterium tuberculosis* complex pathogen, *M. mungi*. *Emerg Infect Dis*, 16(8):1296.

Álvarez AH et al. (2008) Molecular findings and approaches spotlighting *Mycobacterium bovis* persistence in cattle. *Vet Res*, 40:22. doi: 10.1051/vetres/2009005

Aranaz A et al. (1999) *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. *Int J Syst Bact*, 49(3):1263-1273.

Artois M et al. (2001) Control of infectious diseases of wildlife in Europe. *Vet J*, 162:141–152.

Artois M et al. (2011) Sustainable control of zoonotic pathogens in wildlife: how to be fair to wild animals? *Rev Sci Tech Off Int Epiz*, 30(3):733-743.

Ballesteros C et al. (2009) First data on Eurasian wild boar response to oral immunization with BCG and challenge with a *Mycobacterium bovis* field strain. *Vaccine*, 27:6662-6668.

Balseiro A et al. (2009) Tuberculosis in roe deer from Spain and Italy. *Vet Rec*, 164:0.

Barasona JA et al. (2013) Effectiveness of cattle operated bump gates and exclusion fences in preventing ungulate multi-host sanitary interaction. *Prev Vet Med*, 111(1–2):42–50.

Barasona JA et al. (2014) Spatiotemporal interactions between wild boar and cattle: implications for cross-species disease transmission. *Vet Res*, 45:122.

Barlow ND (1996) The ecology of wildlife disease control: simple models revisited*. J Appl Ecol*, 33(2):303–314.

Bass KE et al. (2013) Clinical and diagnostic developments of a gamma interferon release assay for use in bovine tuberculosis control programs. *Clin Vaccine Immunol*, 20(12):1827-1835.

Beltrán-Beck B et al. (2012) Progress in oral vaccination against tuberculosis in its main wildlife reservoir in Iberia, the Eurasian wild boar. *Vet Med Int*, (2012):978501, doi: 10.1155/2012/978501

Beltrán-Beck B et al. (2014) Oral vaccination with heat inactivated *Mycobacterium bovis* activates the complement system to protect against tuberculosis. *PLoS ONE*, 9(5):e98048. doi: 10.1371/journal.pone.0098048

Bengis R et al. (1999) Tuberculosis in free-ranging mammals. *in* M. Fowler e R. Miller (eds.) Zoo and wild animal medicine, current therapy 4. W. B. Saunders Company, Philadelphia, p. 101-114.

Bezos J et al. (2015) Goats challenged with different members of the *Mycobacterium tuberculosis* complex display different clinical pictures. *Vet Immunol Immunopathol*, 16783):185-189.

Blancou J et al. (2009) Options for the control of disease 1: targeting the infectious or parasitic agent. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 97–120.

Boadella M et al. (2012) Effects of culling Eurasian wild boar on the prevalence of *Mycobacterium bovis* and Aujeszky's disease virus. *Prev Vet Med*, 107(3):214-221.

Bocci A, et al. (2012) Shifter and resident red deer: intrapopulation and intersexual behavioural diversities in a predator-free area. *Wildl Res*, 39(7):573-582.

Bolzoni L et al. (2013) React or wait: which optimal culling strategy to control infectious diseases in wildlife. *J Math Biol*, 69(4): 1001-1025. doi 10.1007/s00285-013-0726-y

Bosch J et al. (2012) Distribution, abundance and density of the wild boar on the Iberian Peninsula, based on the CORINE program and hunting statistics. *Folia Zool*, 61(2):138–151.

Brennan PJ (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis* 83(1):91–97.

Briones V et al. (2000) Bovine tuberculosis and the endangered Iberian lynx. *Emerg Infect Dis*, 6(2):189.

Brosch R et al. (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *P Natl Acad Sci USA*, 99(6):3684–3689.

Buddle BM et al. (2013) Overview of vaccination trials for control of tuberculosis in cattle, wildlife and humans. *Transbound Emerg Dis*, 60(1):136–146.

Bugalho MN and Milne JA (2003) The composition of the diet of red deer (*Cervus elaphus*) in a Mediterranean environment: a case of summer nutritional constraint? *Forest Ecol Manag*, 181:23–29.

Carstensen M and Doncarlos MW (2011) Preventing the establishment of a wildlife disease reservoir: a case study of bovine tuberculosis in wild deer in Minnesota, USA. *Vet Med Int*, 2011:1–10.

Carter SP et al. (2009) Options for the control of disease 2: targeting hosts. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 121–146.

Cassidy JP et al. (1998) Early lesion formation in cattle experimentally infected with *Mycobacterium bovis*. *J Comp Pathol*, 119:27-44.

Cassidy JP et al. (1999) Lesions in cattle exposed to *Mycobacterium bovis*-inoculated calves. *J Comp Pathol*, 121:321-337

Cassidy JP (2006) The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models. *Vet Microbiol*, 112:151–161.

Clifton-Hadley RS et al. (2001) *Mycobacterium bovis* infections. *In* E Williams & I Barker (eds.) Infectious diseases of wild mammals. 3rd edition, Manson Publishing/The Veterinary Press, London, p. 340-361.

Clifton-Hadley RS et al. (1993) *Mycobacterium bovis* in the European badger (*Meles meles*): epidemiological findings in tuberculous badgers from a naturally infected population. *Epidemiol Infect*, 111:9-19.

Coleman J and Cooke M (2001) *Mycobacterium bovis* infection in wildlife in New Zealand. *Tuberculosis*, 81(3): 191-202.

Corner LAL et al. (1995) Determination of the optimum concentration of decontaminants for the primary isolation of *Mycobacterium bovis*. *New Zeal Vet J*, 43:129-133.

Corner LAL (2006) The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. *Vet Microbiol*, 112:303–312.

Corner LAL et al. (2008) Experimental tuberculosis in the European badger (*Meles meles*) after endobronchial inoculation with *Mycobacterium bovis*: II. Progression of infection. *Res Vet Sci*, 85:481–490.

Corner LAL et al. (2011a) *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*): the disease, pathogenesis, epidemiology and control. *J Comp Pathol*, 144:1-24.

Corner LAL et al. (2011b) Primary isolation of *Mycobacterium bovis* from bovine tissues: conditions for maximizing the number of positive cultures. *Vet Microbiol*, 156(1):162-171.

Coscolla M et al. (2013). Novel *Mycobacterium tuberculosis* complex isolate from a wild chimpanzee. *Emerg Infect Dis*, 19(6):969.

Cosivi O et al. (1998) Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerg Infect Dis*, 4(1):59-70.

Courtenay O et al. (2006) Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis? *Biol Lett*, 2(3):460-462.

Cowie CE et al. (2015) Interactions between four species in a complex wildlife: livestock disease community: implications for Mycobacterium bovis maintenance and transmission. *Eur J Wildl Res*, in press. doi: 10.1007/s10344-015-0973-x

Crawshaw T et al. (2008) TB in goats caused by *Mycobacterium bovis*. *Vet Rec*, 163:127.

Dean GS et al. (2005) Minimum infective dose of *Mycobacterium bovis* in cattle. *Infect Immun*, 73(10):6467–6471.

Delahay RJ et al. (2000) The spatio-temporal distribution of *Mycobacterium bovis* (bovine tuberculosis) infection in a high-density badger population. *J Anim Ecol*, 69:428-441.

Delahay RJ et al. (2009) The science of wildlife disease management. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 1–8.

de Garine-Wichatitsky et al. (2013) A review of bovine tuberculosis at the wildlife–livestock–human interface in sub-Saharan Africa. *Epidemiol Infect*, 141(7):1342-1356.

de Jong BC et al. (2010) *Mycobacterium africanum*—review of an important cause of human tuberculosis in West Africa. *PLoS Neg Trop Dis*, 4(9):e744. doi: 10.1371/journal.pntd.0000744

de la Rua-Domenech R (2006) Human *Mycobacterium bovis* infection in the United Kingdom: incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis*, 86:77–109.

de la Rua-Domenech R et al. (2006) Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci*, 1:190–210.

de Lisle G et al. (2002) Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Rev Sci Tech Office Int Epiz*, 21(2):317-334.

de Lisle GW et al. (2009) Isolation of *Mycobacterium bovis* from brushtail possums with non-visible lesions. *New Zeal Vet J*, 57:221–224.

Donnelly CA et al. (2006) Positive and negative effects of widespread badger culling on tuberculosis in cattle. *Nature*, 439:843-845.

DGAV (2013) Implementation of bovine tuberculosis eradication program in Portugal 2012. Brussels, SCOFCAH, 2 July 2013.

DGAV (2015). *Relatório técnico de sanidade animal: tuberculose bovina, 2014*. Lisboa, Accessed December 2015. http://www.dgv.min-agricultura.pt/portal/page/portal/DGV/genericos?actualmenu= 8961117&generico=166927&cboui=166927.

Djelouadji Z et al. (2011) Palaeogenomics of *Mycobacterium tuberculosis*: epidemic bursts with a degrading genome. *Lancet Infect Dis*, 11:641–50

Downs SH et al. (2013) Tuberculin manufacturing source and breakdown incidence rate of bovine tuberculosis in British cattle, 2005–2009. *Vet Rec*, 172(4):98. doi: 10.1136/vr.100679

Fabre M et al. (2010) Molecular characteristics of "*Mycobacterium canettii*" the smooth *Mycobacterium tuberculosis* bacilli. *Infect Genetics Evol*, 10(8):1165-1173.

Ferreira E et al. (2008) Genetic structure of the wild boar population in Portugal: evidence of a recent bottleneck. *Mamm Biol*, 74(4):274-285. doi: 10.1016/j.mambio.2008.05.009.

Fine AE et al. (2011) A study of the persistence of *Mycobacterium bovis* in the environment under natural weather conditions in Michigan, USA. *Vet Med Int*, 2011:765430.

Fink M et al. (2015) Red deer as maintenance host for bovine tuberculosis, Alpine Region. *Emerg Inf Dis*, 21(3):464.

Fitzgerald SD and Kaneene JB (2012) Wildlife reservoirs of bovine tuberculosis worldwide: hosts, pathology, surveillance, and control. *Vet Pathol*, 50:488. doi: 10.1177/0300985812467472

Fonseca C et al. (2011) Reproductive performance of wild boar females in Portugal. *Eur J Wildl Res*, 57:363–371.

Gallagher J et al. (1989) Role of infected, non-diseased badgers in the pathogenesis of tuberculosis in the badger. *Vet Rec*, 142(26):710–714.

Gallagher J and Clifton-Hadley RS (2000) Tuberculosis in badgers; a review of the disease and its significance for other animals. *Res Vet Sci*, 69:203–217.

García AJ, et al. (2002) Reproductive seasonality in female Iberian red deer (*Cervus elaphus hispanicus*). *Theriogen*, 58(8):1553-1562.

García-Jiménez WL et al. (2013a) Reducing Eurasian wild boar (*Sus scrofa*) population density as a measure for bovine tuberculosis control: effects in wild boar and a sympatric fallow deer (*Dama dama*) population in Central Spain. *Prev Vet Med*, 110(3):435-446.

García-Jiménez WL et al. (2013b) Immunopathology of granulomas produced by M*ycobacterium bovis* in naturally infected wild boar. *Vet Immunol Immunopathol*, 156:54–63.

García-Jiménez WL et al. (2013c) Comparative pathology of the natural infections by *Mycobacterium bovis* and by *Mycobacterium caprae* in wild boar (*Sus scrofa*). *Transbound Emerg Dis*, 60:102–109.

García-Sánchez A et al. (2007) Tuberculous meningoencephalitis in a wild boar. *J Wildl Dis*, 43(4):780–783.

Garnier T et al. (2003) The complete genome sequence of *Mycobacterium bovis*. *P Natl Acad Sci*, 100(13):7877-7882.

Garrido JM et al. (2011) Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated *Mycobacterium bovis*. *PLoS ONE*, 6(9): e24905. doi: 10.1371/journal.pone.0024905

Gavier-Widén D et al. (2001) Pathology of natural *Mycobacterium bovis* infection in European badgers (*Meles meles*) and its relationship with bacterial excretion. *Vet Rec*, 148:299-304.

Gavier-Widén D et al. (2009) A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the 'no visible lesion' presentation. *New Zeal Vet J*, 57:122-131.

Gehring TM et al. (2010) Utility of livestock-protection dogs for deterring wildlife from cattle farms. *Wildl Res*, 37:715–721.

Ghodbane R et al. (2014) Long-term survival of tuberculosis complex mycobacteria in soil. *Microbiol*, 160:496–501.

Gormley E et al. (2014) Bacteriological diagnosis and molecular strain typing of *Mycobacterium bovis* and *Mycobacterium caprae*. *Res Vet Sci*, 97:S30–S43.

Gortázar C et al. (2005) Disease risks and overabundance of game species. *Eur J Wildl Res*, 52(2):81-87. doi: 10.1007/s10344-005-0022-2

Gortázar C et al. (2010) Disease-related conflicts in mammal conservation. *Wildl Res*, 37(8):668–675.

Gortázar C et al. (2011) Progress in the control of bovine tuberculosis in Spanish wildlife. *Vet Microbiol*, 151:170-178.

Gortázar C et al. (2012) The status of tuberculosis in European wild mammals. *Mamm Rev*, 42(3):193-206.

Gortázar C et al. (2013) Which factors drive tuberculosis transmission? Identifying super-shedders in wild boar populations. Proceedings WDA International Conference, Knoxville, Tennesse, USA, p124.

Gortázar C et al. (2014) Oral re-vaccination of Eurasian wild boar with *Mycobacterium bovis* BCG yields a strong protective response against challenge with a field strain. *BMC Vet Res*, 10(1):96.

Gortázar C et al. (2015) The wild side of disease control at the wildlife-livestock-human interface: a review. *Front Vet Sci*, 1(27):1-12.

Grange J (2001) *Mycobacterium bovis* infection in human beings. *Tuberculosis*, 81(1/2):71-77.

Griffin JFT and Buchan GS (1994) Aetiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. *Vet Microbiol*, 40:193-205.

Griffin J et al. (2005) The impact of badger removal on the control of tuberculosis in cattle herds in Ireland. *Prev Vet Med*, 67:237-266.

Griffin JFT et al. (2006) Tuberculosis in ruminants: characteristics of intra-tonsilar *Mycobacterium bovis* infection models in cattle and deer. *Tuberculosis*, 86:404-418.

Griffiths RI et al. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Appl Environ Microbiol*, 66(12):5488–5491.

Guenin-Macé L et al. (2009) Lipids of pathogenic Mycobacteria: contributions to virulence and host immune suppression. *Transbound Emerg Dis*, 56(6-7):255-268.

Hardstaff JL et al. (2014) Evaluating the tuberculosis hazard posed to cattle from wildlife across Europe. *Res Vet Sci*, 97:S86-S93.

Hars J et al. (2010) *La tuberculose bovine dans la faune sauvage en France*. *Bull Epid Santé Anim Alim*, 38:25-27.

Hars J et al. (2012) Culling red deer to eradicate *Mycobacterium bovis* in wildlife in the Brotonne forest (Normandy, France). Proceedings WDA-EWDA Joint Conference, Lyon, France, p. 380.

Himsworth CG et al. (2010) An outbreak of bovine tuberculosis in an intensively managed conservation herd of wild bison in the Northwest Territories. *Can Vet J*, 51:593–597.

Hlokwe TM et al. (2014) Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: are we losing the battle? *Prev Vet Med*, 115(1):10-17.

Houben EN et al. (2006) Interaction of pathogenic mycobacteria with the host immune system. *Curr Opin Microbiol*, 9(1):76-85.

Houlihan MG et al. (2008) *Mycobacterium bovis* isolated from a sheep during routine surveillance. *Vet Rec*, 163(3):94–95.

Humblet MF et al. (2009) Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach. *Vet Res*, 40(5):1-24.

Jenkins HE et al. (2008) The prevalence, distribution and severity of detectable pathological lesions in badgers naturally infected with *Mycobacterium bovis*. *Epidemiol Infect*, 30:1–12.

Joly DO and Messier F (2004) Factors affecting apparent prevalence of tuberculosis and brucellosis in wood bison. *J Anim Ecol*, 73:623–631.

Judge J et al. (2011) Effectiveness of biosecurity measures in preventing badger visits to farm buildings. *PLoS ONE*, 6(12):e28941. doi: 10.1371/journal.pone.0028941

Kaneene JB et al. (2010) Tuberculosis in wild animals. *Int J Tuberc Lung Dis*, 14(12):1508-1512.

Kipar A et al. (2013) *Mycobacterium microti* tuberculosis in its maintenance host, the field vole (*Microtus agrestis*): characterization of the disease and possible routes of transmission. *Vet Pathol Onl*, 2013:0300985813513040.

Krajewska M et al. (2014) Bovine tuberculosis in a wild boar (*Sus scrofa*) in Poland. *J Wildl Dis*, 50(4):1001-1002.

Kubica T et al. (2003) *Mycobacterium bovis* subsp. *caprae* caused one-third of human *M. bovis*-associated tuberculosis cases reported in Germany between 1999 and 2001. *J Clin Microbiol*, 41:3070–3077.

Kukielka E et al. (2013) Spatial and temporal interactions between livestock and wildlife in South Central Spain assessed by camera traps. *Prev Vet Med*, 112(3):213-221.

Laisse CJM et al. (2011) Characterization of tuberculous lesions in naturally infected African buffalo (*Syncerus caffer*). *J Vet Diagn Invest*, 23(5):1022–1027.

Lees V (2003) Bovine tuberculosis in elk (*Cervus elaphus manitobensis*) near Riding Mountain National Park, Manitoba, from 1992 to 2002. *Can Vet J*, 44:830-831.

Lees V (2004) Learning from outbreaks of bovine tuberculosis near Riding Mountain National Park: applications to a foreign animal disease outbreak. *Can Vet J*, 45:28-34.

Liebana E et al. (2008) Pathology of naturally occurring bovine tuberculosis in England and Wales. *Vet J*, 176:354–360.

Livingstone PG et al. (2015) Development of the New Zealand strategy for local eradication of tuberculosis from wildlife and livestock. *New Zeal Vet J*, 2015:1-10.

Long R et al. (1999) Transcontinental spread of multidrugresistant *Mycobacterium bovis*. *Am J Resp Crit Care Med*, 159:2014–2017.

Lopes F and Borges J (2004) Wild boar in Portugal. *Galemys*, 16(special number):243–251.

Ludt CJ, et al. (2004) Mitochondrial DNA phylogeography of red deer (*Cervus elaphus*). *Mol Phylogen Evol*, 31(3):1064-1083.

Lugton IW et al. (1998) Epidemiology and pathogenesis of *Mycobacterium bovis* infection of red deer (*Cervus elaphus*) in New Zealand. *New Zeal Vet J*, 46:147-156.

Machackova M et al. (2003) Wild boar (*Sus scrofa*) as a possible vector of mycobacterial infections: review of literature and critical analysis of data from Central Europe between 1983 to 2001. *Vet Med – Czech*, 48(3):51–65.

Martín-Hernando MP et al. (2007) Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. *Tuberculosis*, 87:360-367.

Martín-Hernando MP et al. (2010) Distribution of lesions in red and fallow deer naturally infected with *Mycobacterium bovis*. *J Comp Pathol*, 142:43-50.

Delibes-Mateos M et al. (2009) Long-term changes in game species over a long period of transformation in the Iberian Mediterranean landscape. *Environ Manage*, 43(6):1256-1268.

Matos AC et al. (2014) New insights into *Mycobacterium bovis* prevalence in wild mammals in Portugal. *Transbound Emerg Dis*, 2014:1-10. doi: 10.1111/tbed.12306

Menin Á et al. (2013) Asymptomatic cattle naturally infected with *Mycobacterium bovis* present exacerbated tissue pathology and bacterial dissemination. *PLoS ONE*, 8(1):e53884. doi: 10.1371/journal.pone.0053884

Michel AL et al. (2010) *Mycobacterium bovis* at the animal–human interface: a problem, or not? *Vet Microbiol*, 140:371–381.

Miller R et al. (2003) Evaluation of the influence of supplemental feeding of white-tailed deer (*Odocoileus virginianus*) on the prevalence of bovine tuberculosis in the Michigan wild deer population. *J Wildl Dis*, 39(1):84-95.

Miller RS and Sweeney SJ (2013) *Mycobacterium bovis* (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. *Epidemiol Infect*, 141:1357–1370.

Morris RS et al. (1994) The epidemiology of *Mycobacterium bovis* infections. *Vet Microbiol*, 40:153-177.

Mostowy S et al. (2004) Genomic interrogation of the dassie bacillus reveals it as a unique RD1 mutant within the *Mycobacterium tuberculosis* complex. *J Bacteriol*, 186(1):104-109.

Müller B et al. (2013) Zoonotic *Mycobacterium bovis*–induced tuberculosis in humans. *Emerg Infect Dis*, 19(6):899-908.

Muñoz-Mendoza MM et al. (2011) Tuberculosis due to *Mycobacterium bovis* and *Mycobacterium caprae* in sheep. *Vet J*, 19182):267-269.

Neill SD et al. (1988) Excretion of *Mycobacterium bovis* by experimentally infected cattle. *Vet Rec*, 123(13):340–343.

Neill SD et al. (2001) Pathogenesis of tuberculosis in cattle. *Tuberculosis*, 81(1/2):79-86.

Niederweis M et al. (2010) Mycobacterial outer membranes: in search of proteins. *Trends Microbiol*, 18(3):109-116.

Nishi JS et al. (2006) Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: strategies for management and research. *Vet Microbiol*, 112:325–338.

Nores C et al. (1995) Wild boar distribution trends in the last two centuries: an example in northern Spain. *Ibex*, 3:137–140.

Nores C et al. (2008) Wild boar *Sus scrofa* mortality by hunting and wolf *Canis lupus* predation: an example in northern Spain. *Wildl Biol*, 14:44-51.

Nugent G et al. (2002) Use of released pigs as sentinels for *Mycobacterium bovis*. *J Wildl Dis*, 38(4):665-677.

Nugent G (2011) Maintenance, spillover and spillback transmission of bovine tuberculosis in multi-host wildlife complexes: A New Zealand case study. *Vet Microbiol*, 151: 34-42.

Okafor CC et al. (2011) Descriptive epidemiology of bovine tuberculosis in Michigan (1975–2010): lessons learned. *Vet Med Int*, 2011:874924. doi: 10.4061/2011/874924

O'Brien DJ et al. (2001) Tuberculous lesions in free-ranging whit-tailed deer in Michigan. *J Wildl Dis*, 37(3):608-613.

O'Brien DJ et al. (2004) Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan. *J Wildl Dis*, 40(1):42-52.

O'Brien DJ, Schmitt SM, Fitzgerald SD, Berry DE (2011a) Management of bovine tuberculosis in Michigan wildlife: current status and near term prospects. *Vet Microbiol*, 151(1):179-187.

O'Brien DJ et al. (2011b) Recent advances in the management of bovine tuberculosis in free-ranging wildlife. *Vet Microbiol*, 151(1–2):23–33.

Palmer MV et al. (1999) Development of a model of natural infection with *Mycobacterium bovis* in white-tailed deer. *J Wildl Dis*, 35:450–457.

Palmer MV et al. (2001) Experimental deer-to-deer transmission of *Mycobacterium bovis*. *Am J Vet Res*, 62:692-696.

Palmer MV et al. (2004a) Shared feed as a means of deer-to-deer transmission of *Mycobacterium bovis*. *J Wildl Dis*, 40(1):87–91.

Palmer MV et al. (2004b) Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. *Am J Vet Res*, 65:1483–1489.

Palmer MV et al. (2009) Vaccination with *Mycobacterium bovis* BCG strains Danish and Pasteur in white-tailed deer (*Odocoileus virginianus*) experimentally challenged with *Mycobacterium bovis*. *Zoon Pub Health*, 56:243–251.

Palmer MV et al. (2010) Persistence of *Mycobacterium bovis* Bacillus Calmette-Guérin in white-Tailed Deer (*Odocoileus virginianus*) after oral or parenteral Vaccination. *Zoon Pub Health*, 57:206–212.

Palmer MV et al. (2015) Tuberculosis in wild and captive deer. *In* Mukundan H et al. (eds.) Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria. CAB International, Oxfordshire, UK, pp. 334-364.

Parra A et al. (2003) Epidemiology of *Mycobacterium bovis* infections of pigs and wild boars using a molecular approach. *Vet Microbiol*, 97:123-133.

Payeur J et al. (2002) Bovine tuberculosis in Michigan wildlife. *Ann N Y Acad Sci*, 969:259-261.

Payne A et al. (2012) Bovine tuberculosis in "Eurasian" badgers (*Meles meles*) in France. *Eur J Wildl Res*, 59(3):331-339. doi: 10.1007/s10344-012-0678-3

Pesciaroli M, Álvarez J, Boniotti MB, Cagiola M, Di Marco V, Marianelli C, Pacciarini M, Pasquali P (2014) Tuberculosis in domestic animal species. *Res Vet Sci*, 97:S78–S85.

Pfyffer GE (2006) *Mycobacterium*: general characteristics, laboratory detection, and staining procedures. *In* Murray PR et al. (eds.) Manual of clinical microbiology, Volume 1. ASM Press, Washington DC, USA, pp. 543-572.

Phillips CJC et al. (2003) The transmission of *Mycobacterium bovis* infection to cattle. *Res Vet Sci*, 74:1–15.

Pollock JM and Neill SD (2002) *Mycobacterium bovis* Infection and tuberculosis in cattle. *Vet J*, 163:115-127.

Pontiroli A et al. (2011) Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. *PLoS ONE*, 6(3):e17916. doi: 10.1371/journal.pone.0017916

Poteaux C et al. (2009) Socio-genetic structure and mating system of a wild boar population. *J Zool*, 2009:1–10.

Prodinger et al. (2014) *Mycobacterium caprae* infection in humans. *Exp Rev Anti-Infect Ther*, 12(12):1501-1513.

Radunz B (2006) Surveillance and risk management during the latter stages of eradication: experiences from Australia. *Vet Microbiol*, 112:283–290.

Rastogi N et al. (2001) The mycobacteria: an introduction to nomenclature and pathogenesis. *Rev Sci Tech Off Int Epiz*, 20(1):21-54.

Renwick AR e al. (2006) Bovine tuberculosis in southern African wildlife: a multi-species host–pathogen system. *Epidemiol Infect*, 135(4):529-540.

Richomme C et al. (2013) Exposure of wild boar to *Mycobacterium tuberculosis c*omplex in France since 2000 is consistent with the distribution of bovine tuberculosis outbreaks in cattle. *PLoS ONE*, 8(10):e77842. doi:10.1371/journal.pone.0077842

Rivière J et al. (2013) *Sylvatub: bilan d'une première année de surveillance de la tuberculose bovine dans la faune sauvage en France*. *Bull Epid Santé Anim Alim*, 57:10-15.

Rivière J et al. (2014) Bovine tuberculosis surveillance in cattle and free-ranging wildlife in EU Member States in 2013: A survey-based review. *Vet Microbiol*, 173(3):323-331.

Rivière J et al. (2015) Sensitivity of bovine tuberculosis surveillance in wildlife in France: a scenario tree approach. *PLoS ONE*, 10(10):e0141884. doi: 10.1371/journal.pone.0141884

Rodríguez E et al. (2009) Human tuberculosis due to *Mycobacterium bovis* and *M. caprae* in Spain, 2004–2007. *Int J Tuberc Lung Dis*, 13(12):1536–1541.

Rodriguez-Campos S et al. (2012) European 2–A clonal complex of *Mycobacterium bovis* dominant in the Iberian Peninsula. *Infect Gen Evol*, 12(4):866-872.

Rodriguez-Campos S et al. (2014) Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: implications for diagnostics and legislation of bovine tuberculosis. *Res Vet Sci*, 97:S5-S19.

Rosell C et al. (2001) El jabalí (*Sus scrofa* Linnaeus, 1758). *Galemys*, 13(2):1-25.

Rothschild B et al. (2001) *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present. *Clin Infect Dis*, 33(3):305-311.

Rodwell TC et al. (2001) Prevalence of bovine tuberculosis in African buffalo at Kruger national park. *J Wildl Dis,* 37(2):258–264.

Ryan TJ et al. (2006) Advances in understanding disease epidemiology and implications for control and eradication of tuberculosis in livestock: the experience from New Zealand. *Vet Microbiol*, 112:211–219.

Salazar D (2009) Distribuição e estatuto do veado e corço em Portugal. MSc Thesis, Universidade de Aveiro, Portugal, 101 pp.

Santos N et al. (2010) Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): a comparison of methods applicable to hunter-harvested animals. *PLoS ONE*, 5(9):e12663. doi: 10.1371/journal.pone.0012663

Schmitt S et al. (1997) Bovine tuberculosis in free-ranging white-tailed deer from Michigan*. J Wildl Dis*, 33(4):749-758.

Serraino A et al. (1999) Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. *J Clin Microbiol*, 37(9):2766-2771.

Shury TK and Bergeson D (2011) Lesion distribution and epidemiology of *Mycobacterium bovis* in elk and white-tailed deer in South-Western Manitoba, Canada. *Vet Med Int*, 2011:591980. doi: 10.4061/2011/591980

Shury TK et al. (2015) Tuberculosis and brucellosis in wood bison (*Bison bison athabascae*) in Northern Canada: a renewed need to develop options for future management. *J Wildl Dis*, 51(3):543-554.

Smith NH et al. (2006) Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. *Nat Rev Microbiol*, 4(9):670-681.

Smith NH (2012) The global distribution and phylogeography of *Mycobacterium bovis* clonal complexes. *Infect Gen Evol*, 12(4):857-865.

Sobrino R et al. (2008) Bovine tuberculosis in a badger (*Meles meles*) in Spain. *Vet Rec,* 163(5):159.

Sweeney FP et al. (2006) Immunomagnetic recovery of *Mycobacterium bovis* from naturally infected environmental samples. *Lett Appl Microbiol*,43(4):364-369.

Tanner M et al. (1999) Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park. *Onderstepoort J Vet Res*, 66:185-190.

Tanner M et al. (2014) Bovine tuberculosis and brucellosis in cattle and African buffalo in the Limpopo National Park, Mozambique. *Transbound Emerg Dis*, 62:632–638.

Taylor GM et al. (2007) First report of *Mycobacterium bovis* DNA in human remains from the Iron Age. *Microbiol*, 153:1243–1249.

Thacker TC et al. (2007) Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. *Vet Immunol Immunopathol*, 119:204–213.

Thoen C et al. (2006) The importance of *Mycobacterium bovis* as a zoonosis. *Vet Microbiol*,112(2):339-345.

Tweddle N and Livingstone P (1994) Bovine tuberculosis control and eradication programs in Australia and New Zealand. *Vet Microbiol*, 40:23-39.

Vicente J et al. (2006) Risk factors associated with prevalence of tuberculosis-like lesions in wild boar and red deer in South Central Spain. *Vet Res*, 38:451–464.

Vieira-Pinto M et al. (2011) Combined evaluation of bovine tuberculosis in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) from Central-East Portugal. *Eur J Wildl Res*, 57:1189–1201.

Ward AI et al. (2009) Options for the control of disease 3: targeting the environment. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 147–168.

Waters WR et al. (2012) Bovine tuberculosis vaccine research: historical perspectives and recent advances. *Vaccine*, 30(16):2611-2622. doi: 10.1016/j.vaccine.2012.02.018

Wilkins MJ et al. (2008) Human *Mycobacterium bovis* infection and bovine tuberculosis outbreak, Michigan, 1994–2007. *Emerg Infect Dis*, 14(4):657.

Wobeser GA (2007) Investigation and management of disease in wild animals. 2nd edition, Springer-Verlag, Berlin, Heidelberg, 393 pp.

Wobeser GA (2009) Bovine tuberculosis in Canadian wildlife: an updated history. *Can Vet J*, 50:1169–1176

Young JS et al. (2005) Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. *Appl Environ Microbiol*, 71:1946–1952.

Young JM et al. (2014) Limitations and recommendations for successful DNA extraction from forensic soil samples: a review. *Sci Just*, 54(3):238-244.

Zanella G et al. (2008a) *Mycobacterium bovis* in wildlife in France. *J Wildl Dis*, 44(1):99–108.

Zanella G et al. (2008) Patterns of lesions of bovine tuberculosis in wild red deer and wild boar. *Vet Rec,* 163(2):43-47.

Zanella G et al. (2012) Modelling transmission of bovine tuberculosis in red deer and wild boar in Normandy, France. *Zoon Pub Health*, 59(2):170–178.

Chapter II – Shedding of *Mycobacterium tuberculosis* complex from naturally-infected wild boar and red deer

Santos N, Almeida V, Gortázar C, Correia-Neves M (2015) Patterns of *Mycobacterium tuberculosis*-complex excretion and characterization of super-shedders in naturally-infected wild boar and red deer. Veterinary Research, 46: 129. doi 10.1186/s13567-015-0270-4

Santos *et al. Vet Res (2015) 46:129*
DOI 10.1186/s13567-015-0270-4

VETERINARY RESEARCH

RESEARCH ARTICLE

Open Access

Patterns of *Mycobacterium tuberculosis*-complex excretion and characterization of super-shedders in naturally-infected wild boar and red deer

Nuno Santos[1,2*], Virgílio Almeida[3], Christian Gortázar[4] and Margarida Correia-Neves[1,2]

Abstract

Wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) are the main maintenance hosts for bovine tuberculosis (bTB) in continental Europe. Understanding *Mycobacterium tuberculosis* complex (MTC) excretion routes is crucial to define strategies to control bTB in free-ranging populations, nevertheless available information is scarce. Aiming at filling this gap, four different MTC excretion routes (oronasal, bronchial-alveolar, fecal and urinary) were investigated by molecular methods in naturally infected hunter-harvested wild boar and red deer. In addition MTC concentrations were estimated by the Most Probable Number method. MTC DNA was amplified in all types of excretion routes. MTC DNA was amplified in at least one excretion route from 83.0% (CI_{95} 70.8–90.8) of wild ungulates with bTB-like lesions. Oronasal or bronchial-alveolar shedding were detected with higher frequency than fecal shedding ($p < 0.001$). The majority of shedders yielded MTC concentrations $<10^3$ CFU/g or mL. However, from those ungulates from which oronasal, bronchial-alveolar and fecal samples were available, 28.2% of wild boar (CI_{95} 16.6–43.8) and 35.7% of red deer (CI_{95} 16.3–61.2) yielded MTC concentrations $>10^3$ CFU/g or mL (referred here as super-shedders). Red deer have a significantly higher risk of being super-shedders compared to wild boar (OR = 11.8, CI_{95} 2.3–60.2). The existence of super-shedders among the naturally infected population of wild boar and red deer is thus reported here for the first time and MTC DNA concentrations greater than the minimum infective doses were estimated in excretion samples from both species.

Introduction

Bovine tuberculosis (bTB) is a zoonotic disease whose natural hosts are wild and domestic mammals. Bovine tuberculosis is a disease of economic and public health relevance subjected to eradication programs on livestock in many countries, usually based on test and slaughter and abattoir surveillance strategies [1]. The existence of wildlife reservoirs has been shown to hinder such eradication programs in cattle, as reported to occur with possums (*Trichosurus vulpecula*) in New Zealand, Eurasian badgers (*Meles meles*) in the United Kingdom and Ireland and cervids in North America [2]. In several regions throughout continental Europe bTB is maintained in a multi-host-pathogen system, with *Mycobacterium bovis* and *Mycobacterium caprae* circulating between sympatric wild ungulates (mostly wild boar *Sus scrofa* and red deer *Cervus elaphus*) and free-ranging domestic ungulates (cattle, goats, sheep and pigs) [3]. The wild boar has been shown to act as a maintenance host for bTB in Iberian Peninsula [4]. The red deer is also considered as part of the bTB maintenance community in France [5], Spain [6] and Austria [7]. Wildlife bTB is increasing its host range, geographical distribution and/or frequency of occurrence in several countries and so is considered an emerging disease in Europe [3].

In order to control bTB in wildlife it is essential to gather deep knowledge on factors that affect the

*Correspondence: nunosantos@ecsaude.uminho.pt
[1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
Full list of author information is available at the end of the article

BioMed Central

© 2015 Santos et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

intra- and inter-specific transmission of infection, both individually and at a population level. As part of this essential information are the routes of infection, pathology (structure and anatomical location of lesions), routes and levels of excretion and minimum infective doses [2]. While pathology of bTB has been thoroughly documented in many wildlife host species [e.g. 8–10], routes of infection have not been demonstrated but only presumed based upon the location of the lesions [e.g. [8] for wild boar, [11] for red deer, [12] for badgers]. In addition, information on the minimum infective doses have been determined by experimental infections [e.g. [13] for red deer and [14] for wild boar] while data on *Mycobacterium tuberculosis* complex (MTC) excretion is notably scarce. The only study addressing *M. bovis* routes of excretion from naturally infected wild ungulates was performed by Lugton et al. [15], who detected excretion by several routes from red deer: oral (4/53 oropharyngeal swabs), nasal (1/53 nasal swabs), tracheal (1/53 tracheal swabs) and rectal (1/53 fecal samples). Urinary excretion was also investigated but not detected by these authors.

In experimentally infected white-tailed deer (*Odocoileus virginianus*) excretion was shown to occur sporadically by the oral route for up to 90 days post infection (dpi), by the nasal route for up to 85 dpi and it was not detected by the fecal route [16, 17]. Using experimental infection with high doses of *M. bovis,* Palmer and collaborators [18] detected excretion for up to 113 dpi by oral, nasal and fecal routes. In this same study, naïve deer in contact with the experimentally infected animals showed excretion by the oral and nasal routes for up to 90 days post-contact. In some studies shedding has been inferred based on the location and structure of the lesions [8, 10], but *M. bovis* has been cultured from the feces of calves that only presented lesions in the lungs and cephalic-thoracic lymphoid tissues [19]. This has been attributed to swallowing of infected pulmonary secretions [20, 21].

Shedding has been extensively assessed only in Eurasian badgers (*Meles meles*) in natural and experimental infections. It was shown by bacteriological culture to occur by the fecal, urinary, pulmonary and oronasal routes and wound discharges in 25–50% of infected animals [12, 20, 22–24]. In this species a "super-shedder" state has been described encompassing those badgers where MTC shedding is detected by culture persistently or by more than one route, in contrast to the standard, intermittent shedders [23]. "Super-shedders" have been hypothesized to occur also in the wild boar [25] but evidence to support this supposition was lacking.

Knowledge of the excretion routes is crucial to improve the control strategies to reduce bTB in wildlife. Determining which excretion route(s) is(are) more prevalent is fundamental to define the likelihood of interspecific transmission as fecal shedding tends to promote indirect transmission through contamination of the environment, while oronasal shedding, in addition to environmental contamination, allows also an easier direct transmission by aerosols, usually involving conspecifics [2, 26]. Although the anatomical location of lesions *per se* provides some information on the potential routes of transmission, this indirect association needs to be interpreted carefully as abdominal lesions can be caused by swallowing of infected pulmonary secretions or hematogenous spread of infection [2, 20, 26].

As the understanding of the bTB excretion routes and MTC excretion doses is critical for defining the best control strategies for wild reservoirs, it is surprising that so little solid data is available on this subject [1]. The aim of this study was thus to determine the MTC excretion routes and concentration of MTC in the biological samples from the potential transmission routes. This was performed by molecular biology methods using samples from naturally infected hunter-harvested wild boar and red deer, for which the bTB status was defined.

Among several protocols tested, a nested PCR was selected as it revealed the highest sensitivity for the MTC molecular detection. Besides detection of MTC shedding it is of utmost importance to quantify excretion. Since DNA present in samples is not quantifiable by nested PCR protocols, we combined this with the Most Probable Number (MPN) method [27]. The MPN is an established and well documented technique to obtain estimates of microbial concentrations from binomial data [27].

Materials and methods

Study design

In order to investigate the MTC excretion routes from naturally infected wild ungulates we collected, from hunter-harvested wild boar (n = 116) and red deer (n = 62), the head and distal third of the neck (66 wild boar and 33 deer), lungs and proximal third of the trachea (66 wild boar and 54 deer), feces from the rectum (93 wild boar and 41 deer) and urine samples from the urinary bladder (3 wild boar and 1 red deer). We obtained bronchial-alveolar lavages (BAL) by aseptically pouring 100 mL of sterile water into the trachea, inverting the lungs and collecting the washes from the trachea, and also oronasal lavages (ONL) by pouring 100 mL of sterile water into the pharynx and collecting the washes from the nose and mouth. All samples were stored at −20 °C until processing, up to 12 weeks post-collection.

Infection status of hunter-harvested wild boar and red deer was assessed by gross pathology, PCR in the lymph nodes with macroscopic lesions and bacteriological culture following protocols previously described [28]. Taking into account these results, the animals were categorized

into the following groups: (1) bTB-confirmed—macroscopic lesions detected and MTC demonstrated in tissues by culture or molecular methods (56 wild boar and 43 red deer); (2) bTB-suspected—macroscopic lesions detected, bacteriological culture and PCR-negative in tissues (21 wild boar and 15 red deer); (3) bTB-free—negative for gross pathology, PCR and culture in tissues and collected in regions where bTB has not been detected in wildlife despite surveillance (31 wild boar and 4 red deer).

Regions with known bTB infection status in wild ungulate populations were identified from published results [29] and consisted of the following Portuguese counties: Idanha a Nova (centroid coordinates, utm wgs84: 661408, 4418202), Castelo de Vide (629710, 4369235), Moura (650202, 4221830) and Mértola (614207, 4167236).

DNA extraction protocol

40 mL of lavages (BAL or ONL) or urine were centrifuged at 2566 *g* for 30 min (Heraeus Multifuge 3SR Plus, ThermoFisher Scientific, Waltham, MA, USA), after which most of the supernatant was discarded and 0.5 mL aliquots of the sediment/supernatant interface were collected for DNA extraction. 15 g of fecal material were agitated overnight at 150 rpm at 8 °C in an incubation shaker (Multitron II, Infors AG, Bottmingen, Switzerland) in order to homogenize the sample. After resting for 2 h at room temperature, 14 mL of the supernatant/sediment interface were collected and processed as previously described for lavages and urine samples.

For lavages, DNA extraction was performed by a standard phenol–chloroform protocol. Briefly, 55 μL of 10 × TEN buffer and 0.25 mL phenol were added to 0.5 mL of sample in a 2 mL screw-cap conical tube containing 100 μL of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK, USA). The mixture was subjected to 2 cycles of 30 s agitation at 5 m/s in a FastPrep 24 (MP Biomedicals, Santa Ana, CA, USA), after which 0.25 mL chloroform were added and gently agitated for 60 s, followed by 5 min centrifugation at 16 627 *g* at 4 °C. 500 μL of the aqueous phase was then transferred to a new tube and an equal volume of chloroform added, mixed by gentle agitation for 60 s and again centrifuged for 5 min at 16 627 *g* at 4 °C. 300 μL of the aqueous phase were then transferred to a new tube and 40 μL of sodium acetate and 800 μL absolute EtHO were added and this mix was left to rest for 2 h at room temperature, followed by 10 min centrifugation at 19 283 *g* at 4 °C. The supernatant was discarded and the pellet washed with 70% EtHO, centrifuged for 5 min at 16 627 *g* at 4 °C, the supernatant again discarded and the pellet suspended in 50 μL of TE buffer.

For fecal and urine samples, DNA extraction was performed using a slight modification of the protocol by Griffiths et al. [30]. The differences to the abovementioned phenol–chloroform extraction protocol were: 0.5 mL of 5% hexadecyltrimethylammonium bromide buffer were used instead of 10× TEN buffer and DNA was precipitated by the addition of 400 μL of 30% PEG 6000 solution in 1.6 M $NaCl_2$ and kept at room temperature for 2 h.

Quantification and purity assessment of DNA was performed using NanoDrop (ThermoScientific, Wilmington, DE, USA). DNA extraction negative controls were included at a rate of 1 for every 6 samples.

Molecular detection

As screening test for MTC DNA, a modification of the nested PCR protocol targeting IS6110 described by Soo et al. [31] was used, including the same set of internal and external primers (external forward: 5′ CGTGAGGGCATCGAGGTGGC 3′, external reverse: 5′ GCGTAGGCGTCGGTGACAAA 3′, internal forward: 5′ CTCGTCCAGCGCCGCTTCGG 3′, internal reverse: 5′ GCGTCGGTGACAAAGGCCAC 3′). Briefly, 250 ng DNA were added to a solution of 7.5 μL of NZYTech Green Master Mix (NZYTech, Lisbon, Portugal), containing 1.5 U Taq polymerase, 1.5 mM $MgCl_2$, 1 μL of each primer at 20 mM and 5% DMSO, in a final volume of 25 μL. For the internal PCR 1 μL of the products of the external PCR was used as template. External PCR mix were submitted to the following PCR protocol: initial denaturation at 94 °C for 5 min, followed by 26 cycles of 94 °C for 30 s, annealing at 64 °C for 15 s and extension at 72 °C for 30 s, with a final extension step of 72 °C for 3 min. Internal PCR mix were submitted to the same protocol, except that 30 cycles were used. Negative controls were included in all PCR at a rate of 1 for every 3 samples.

As an external control for PCR inhibition, every sample negative for MTC DNA was inoculated with 7×10^4 copies of a PCN1 construct inserted in a pGEM plasmid and subjected to a standard PCR using the primers forward: 5′ ATACGACTCACTATAGGGCG 3′, reverse: 5′ GGTGACACTATAGAATACTC 3′. Briefly, 0.25 pg of pGEM PCN1 DNA and 250 ng of DNA extracted from the biological samples were added to a solution of 12.5 μL of NZYTech Green Master Mix, containing 2.5 U Taq polymerase, 3.0 mM $MgCl_2$, 1 μL of each primer at 20 mM and 5% DMSO, in a final volume of 25 μL. This mix was submitted to the following PCR protocol: initial denaturation at 94 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 30 s, with a final extension step of 72 °C for 3 min. Inhibition was detected in 44/209 samples, which were then diluted 1:2 or 1:4 until inhibition disappeared. In all but 18 samples PCR inhibition was avoided

using these method; these 18 samples from fecal extracts ($n = 16$), ONL and BAL ($n = 1$ each) were removed from the analysis.

PCR products were visualized by electrophoresis in 2% agarose gel with GreenSafe Premium (NZYTech, Lisbon, Portugal) and photographed under UV light with Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The preparation of the nested PCR master mixes took place in a room not used for other work with MTC and physically separate from the rooms where the addition of the DNA templates was performed. Negative controls were included at a rate of 1 for every 3 samples.

Bacteriological culture

In a restricted set of samples (18 ONL, 13 BAL and 12 fecal samples from 5 wild boar and 7 red deer) bacteriological culture for *M. bovis* detection was performed. Briefly, 15 mL of lavages or 15 g of feces were decontaminated for 2 h with 30 mL of 0.75% hexa-decyl-pyridinium chloride solution, after which they were centrifuged at 2566*g* for 30 min; most supernatant was discarded and 0.25 mL aliquots of the sediment-supernatant inoculated in Coletsos medium (2 tubes for each sample) (BioMerieux, Marcy l'Étoile, France). The inoculated tubes were incubated at 37 °C for 15 weeks, checked weekly for any growth suspected to be MTC, which was then re-inoculated again in Coletsos medium. Isolates were identified by PCR for a panel of selected genes: 16S RNA, IS1081, Rv3120 and Rv1510 following the protocol by Huard et al. [32]. Briefly, 250 ng DNA were added to a solution of 6.5 μL of NZYTech Green Master Mix (NZYTech, Lisbon, Portugal), containing 1.3 U Taq polymerase, 1.5 mM $MgCl_2$, 1 μL of each primer at 20 mM and 5% DMSO, in a final volume of 25 μL. This mix was submitted to the following PCR protocol: denaturation at 94 °C for 5 min, 35 cycles at 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.

Most probable number

MTC concentration was estimated using the method Most Probable Number (MPN) [27] based on positive/negative nested PCR data on serial dilutions of DNA. Briefly, serial tenfold dilutions of MTC-positive DNA samples were submitted to the previously described nested PCR protocol targeting IS6110 [31]. Undiluted DNA was assayed in triplicate, 1:10, 1:10^2, 1:10^3 and 1:10^4 DNA were assayed 1–2 times. The dilution at which no detection begins to occur indicates that the DNA has been diluted so much as to be absent and is used to estimate the original concentration. The software MPN Calculator Build 23 [33] was used to compute the MTC DNA concentration.

Samples of excretion routes obtained from animals negative for bTB and where no MTC DNA was detected were inoculated with twofold decreasing concentrations of *M. bovis* bacillus Calmette-Guérin (BCG) strain Pasteur, determined by colony-forming units (CFU). Negative controls were included in each assay, consisting of the same substrate inoculated with the same volume of sterile water. After seeding, the samples were manually agitated to homogenize the mycobacterial distribution and subjected to the molecular detection techniques previously described. The 100% limit of detection (LD100) was determined after repeating 7 times the molecular detection protocols in the inoculated samples.

Calibration lines were calculated by applying the MPN technique to inoculated biological samples. BCG concentrations and MPN estimates were log transformed and their least squares linear relation was calculated and used to convert MPN estimates of MTC DNA concentration to MTC concentration in CFU/g or mL. For ONL and BAL the relation between inoculated BCG concentration and MPN estimates was linear over 5 log, for wild boar and red deer feces over 4 log (Figure 1).

Statistical analysis

Fisher's exact test, Mann–Whitney U test and binary logistic regression were performed in IBM SPSS Statistics (SPSS, Chicago, ILL, USA); graphics were produced in Excel 2007 (Microsoft, Redmond, WA, USA); and confidence intervals for the positivity rates were calculated using VassarStats [34].

Results

MTC excretion was detected in most bTB-confirmed or -suspected ungulates

In order to characterize MTC excretion the first step was to determine the limits of detection of the techniques used. For ONL and BAL the LD100 was 5×10^2 CFU/mL. For wild boar feces the LD100 was 5×10^4 CFU/g, while for red deer feces the LD100 was 4×10^6 CFU/g.

Overall, MTC DNA was detected in 82/173 wild boar samples and 61/118 red deer samples from bTB-confirmed or suspected animals (Table 1). MTC DNA was not detected in any of 43 samples from negative controls, i.e. animals from bTB-negative regions and lesion- and culture-negative: red deer BAL ($n = 4$) and ONL ($n = 2$) and wild boar BAL ($n = 10$) and feces ($n = 27$). Considering only those bTB-confirmed or suspected ungulates for which all three types of biological samples (ONL, BAL and feces) were available, MTC DNA was detected in at least one biological sample in 31/39 wild boar (79.5%, CI_{95} 64.5–89.2) and 13/14 red deer (92.9%, CI_{95} 68.5–98.7). Moreover, MTC DNA was amplified in all three types of biological samples in 5/39 wild boar (12.8%,

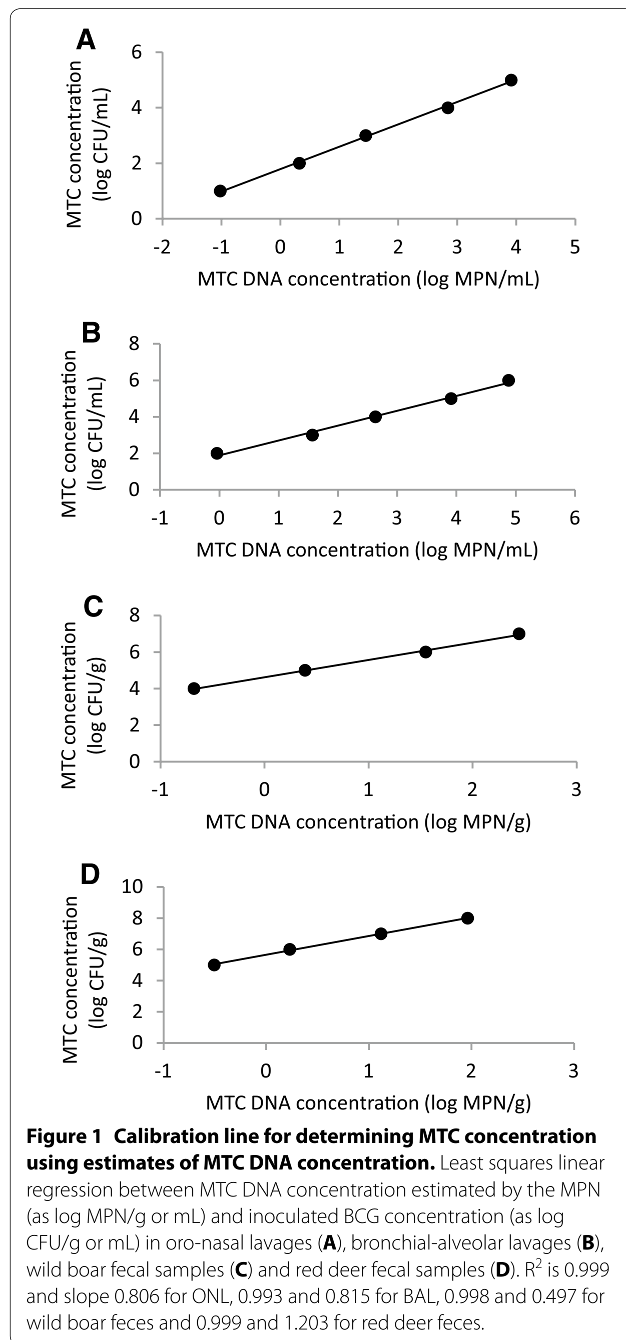


Figure 1 Calibration line for determining MTC concentration using estimates of MTC DNA concentration. Least squares linear regression between MTC DNA concentration estimated by the MPN (as log MPN/g or mL) and inoculated BCG concentration (as log CFU/g or mL) in oro-nasal lavages (**A**), bronchial-alveolar lavages (**B**), wild boar fecal samples (**C**) and red deer fecal samples (**D**). R^2 is 0.999 and slope 0.806 for ONL, 0.993 and 0.815 for BAL, 0.998 and 0.497 for wild boar feces and 0.999 and 1.203 for red deer feces.

CI_{95} 5.6–26.7) and 2/14 red deer (14.3%, CI_{95} 4.0–40.0). No statistically significant differences in MTC detection rate were found between species. MTC excretion was detected at approximately the same rates in bTB-confirmed or suspected groups, with the exception of red deer BAL, where MTC DNA was amplified significantly more often in bTB-confirmed than in bTB-suspected deer ($p = 0.004$, Fisher's exact test) (Table 1). *M. bovis* was isolated by culture from the feces of one infected wild boar out of 7 samples that were not overgrown by other microorganisms (3 ONL, 2 BAL and 2 feces).

No seasonal, age or gender differences were found for the other types of biological samples or species. In a binary logistic regression analysis with MTC detection as dependent variable and species, gender, age, season, bTB status and type of biological sample as independent variables, the only factor affecting the proportion of MTC DNA positive samples was the type of biological sample, with fecal shedding being detected less often than oronasal or bronchial-alveolar shedding in both species ($p < 0.001$).

A proportion of the infected ungulates excrete large concentrations of MTC DNA by several routes

MTC DNA concentration in positive samples revealed a bimodal pattern separated at the concentration 10^3 CFU/g or mL (Figure 2). The ungulates with $>10^3$ CFU/g or mL in at least 1 sample were 14 wild boar and 22 red deer. Considering only those ungulates for which all three biological samples (ONL, BAL and feces) were available for this study, 28.2% of wild boar (CI_{95} 16.6–43.8) and 35.7% of red deer (CI_{95} 16.3–61.2) had at least one excretion route with $>10^3$ CFU/g or mL.

The proportion of male wild boar with MTC DNA concentrations $>10^3$ CFU/g or mL was higher than that of females (42.9 vs 21.4%), while in red deer the opposite was true (0% for males vs 36.4% for females), although these differences were not statistically significant (Table 2).

In a binary logistic regression analysis for those samples where MTC DNA was amplified (80 wild boar and 60 red deer samples), with MTC DNA concentration classified as lower or higher than 10^3 CFU/g or mL as dependent variable and species, age, gender, season, bTB status and type of biological sample as independent variables, host species was significantly related with concentration ($p < 0.01$). Red deer showed a tendency for MTC DNA concentrations $>10^3$ CFU/g or mL (OR = 11.8, CI_{95} 2.3–60.2).

Red deer with at least one sample with $>10^3$ CFU/g or mL showed significantly higher MTC DNA concentrations in BAL compared to wild boar ($p = 0.05$, Mann–Whitney U). When considering only ungulates with $<10^3$ CFU/g or mL in all samples tested no differences were found between species. Super-shedder ungulates excreted significantly higher concentrations of MTC than standard shedders as detected in lavages ($p < 0.01$ for both species, Mann–Whitney U) (Table 3).

Discussion

We provide here evidence and quantify, for the first time, MTC excretion by several routes from naturally infected

Table 1 Proportion of excretion samples positive for MTC DNA.

Host species	Excretion route	bTB-confirmed status				bTB-suspected status			
		No. tested	MTC-positive samples			No. tested	MTC-positive samples		
			no.	%	CI_{95} (%)		no.	%	CI_{95} (%)
Wild boar	Oronasal	47	26	55.3	41.3–68.6	17	7	41.2	21.6–64.0
	Bronchial-alveolar	39	25	64.1	4.48–77.3	17	10	58.8	36.0–78.4
	Fecal	34	9	26.5	14.6–43.1	17	3	17.7	6.2–41.0
	Urinary	3	2	66.7	20.8–93.9	0			
	Total	123	62	50.4	41.7–59.1	51	20	39.2	27.0–52.9
Red deer	Oronasal	22	10	45.5	26.9–65.3	8	5	62.5	30.6–86.3
	Bronchial-alveolar	36	29	80.6	65.0–90.3	12	4	33.3**	13.8–60.9
	Fecal	30	9	30.0	16.7–47.9	9	3	33.3	12.1–64.6
	Urinary	0				1	1	100	20.7–100
	Total	88	48	54.6	44.2–64.5	30	13	43.3	27.4–60.8

Proportion of MTC positive biological samples by species, excretion route and infection status, with confidence intervals and statistically significant differences between infection status highlighted (Fisher's exact test).

** $p < 0.01$.

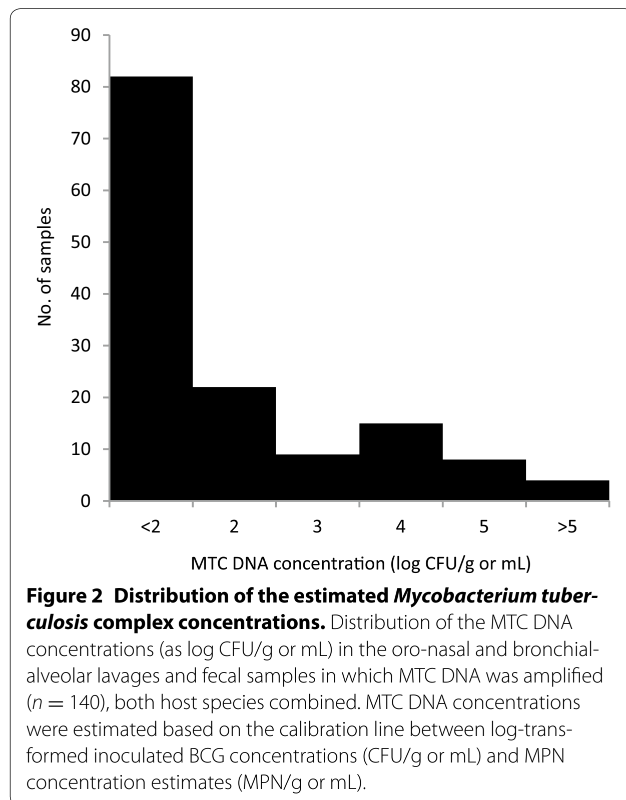


Figure 2 Distribution of the estimated *Mycobacterium tuberculosis* complex concentrations. Distribution of the MTC DNA concentrations (as log CFU/g or mL) in the oro-nasal and bronchial-alveolar lavages and fecal samples in which MTC DNA was amplified (*n* = 140), both host species combined. MTC DNA concentrations were estimated based on the calibration line between log-transformed inoculated BCG concentrations (CFU/g or mL) and MPN concentration estimates (MPN/g or mL).

wild boar and red deer. MTC DNA was detected in all types of biological samples investigated (oronasal and bronchial-alveolar lavages, feces and urine). In 80% of all naturally-infected wild ungulates for which ONL, BAL and feces were available we amplified MTC DNA in at least one sample. This proportion of shedders is higher than reported previously for red deer [15]. This discrepancy might be due to a lower sensitivity of bacteriological culture from swabs performed in the previous study, compared to the molecular detection in lavages and fecal samples used here [24, 35]. This proportion of shedders is also much higher than the one reported for badgers [23] and the difference could be due to the same methodological factors or to differences in bTB pathology between species [10]. In fact, there is evidence that most excretor badgers could be those in advanced, terminal stages of bTB [20, 36] whereas in wild ungulates excretion seems to occur intermittently from early stages of disease [18].

Our results may have been influenced by the fact that bTB lesions could perforate either during hunting or during evisceration of the carcasses, releasing previously encapsulated MTC into the bronchial-alveolar compartment or into the oronasal cavity. This could have led to distorted rates of excretion and estimates of MTC DNA concentration in some samples. Nevertheless the differences found on excretion in our sample associated with known host disease determinants such as species and gender cannot be explained by these methodological issues and should reveal true biological processes. Also our molecular detection protocol targets all MTC species, which could lead to the detection of other mycobacteria not responsible for bTB. Among these, *M. microti* has been reported to infect wild boar [37], although this has never been reported in Iberian Peninsula. Nevertheless all our negative control samples yielded no amplification of MTC DNA, which leads to the assumption that *M. microti* excretion does not occur, at least to a significant

Table 2 Characterization of super-shedders.

Host species	Gender	Age	bTB status	MTC DNA concentration (CFU/g or mL)		
				Feces	Bronchial-alveolar lavages	Oro-nasal lavages
Red deer	Female	Adult	Suspected	Neg	70	4.3×10^3
	Female	Adult	Confirmed	9.9×10^4	248	4.3×10^3
	Female	Adult	Confirmed	Neg	<10	4.3×10^3
	Female	Adult	Confirmed	9.9×10^4	1.9×10^4	99
Wild boar	n.a.	n.a.	Confirmed	Neg	93	4.3×10^3
	n.a.	Adult	Confirmed	1.3×10^4	<10	557
	Female	Subadult	Confirmed	3.2×10^4	14	557
	Male	Adult	Confirmed	6.0×10^6	<10	178
	Female	Adult	Confirmed	1.3×10^4	<10	557
	n.a.	n.a.	Confirmed	Neg	<10	9.9×10^3
	Female	Adult	Suspected	1.0×10^5	Neg	178
	n.a.	Subadult	Confirmed	1.0×10^5	Neg	Neg
	n.a.	Adult	Confirmed	1.9×10^5	Neg	679
	Male	Adult	Suspected	3.2×10^4	<10	Neg
	Male	n.a.	Confirmed	1.3×10^4	Neg	Neg

Species, age, gender, infection status and MTC DNA concentration in samples from the ungulates with at least one sample with MTC DNA concentration $>10^3$ CFU/g or mL (highlighted in bold) out of 3 tested samples. MTC DNA concentrations were estimated based on the calibration line between log-transformed inoculated BCG concentrations (CFU/g or mL) and MPN concentration estimates (MPN/g or mL).

n.a not available.

Table 3 Average MTC DNA concentration in excretion samples from wild ungulates with ONL, BAL and fecal samples tested.

Host species	Excretion route	Average MTC DNA concentration (CFU/g or mL)			
		No.	Super-shedders	No.	Standard shedders
Wild boar	Oronasal	11	1.5×10^3	20	182.2
	Bronchial-alveolar	11	15.2	20	22.8
	Fecal	11	5.9×10^5	20	0
Red deer	Oronasal	5	2.6×10^3	7	83.7
	Bronchial-alveolar	5	3.8×10^3*	7	12.9
	Fecal	5	9.8×10^4	7	0

Average MTC DNA concentration by host species and excretion route, including ungulates with $>10^3$ CFU/g or mL in at least one sample (super shedders) or only those with $<10^3$ CFU/g or mL in all samples (standard shedders). Statistically significant differences between species are highlighted (Mann–Whitney U). MTC DNA concentrations were estimated based on the calibration line between log-transformed inoculated BCG concentrations (CFU/g or mL) and MPN concentration estimates (MPN/g or mL).

* $p = 0.05$.

extent, in our sample. Also freezing of the biological samples at −20 °C could have affected the viability of mycobacteria and so contributed to the low success of the bacteriological culture. Nevertheless Tessema et al. [38] found no effect of freezing sputum samples at −20 °C on the success of *M. tuberculosis* bacteriological culture.

For the first time we report evidence of the occurrence in wild ungulates of a class of infected hosts that fit into the definition of super-shedders. Super-shedders have been described in the Eurasian badger as those in which MTC excretion is detected consistently trough time or by several routes [23]. Although our study design is cross-sectional, so we do not assess the temporal dimension, we found that a proportion of infected ungulates (28.2% of wild boar and 35.7% of red deer) excrete MTC at large concentrations by at least one route. Also shedding was detected to occur by all three routes analyzed in a proportion of the infected ungulates (12.8–14.3% of wild boar and red deer, respectively). Moreover in 6 out of 15 super-shedders for which all 3 routes were available, MTC DNA was amplified in all routes. Furthermore, 13 out of 15 super-shedders had at least another MTC DNA-positive excretion route (in two cases also with MTC concentrations $>10^3$ CFU/g or mL) (Table 2). This means that a large proportion of the ungulates excreting large concentrations of MTC by one route, are in fact excreting by several routes, further supporting their classification as super-shedders.

Although MTC DNA concentration was not directly measured on the excretion samples, our estimates suggest that a super-shedder ungulate sheds on average $>10^5$ CFU/g or mL through all routes combined (Table 3). Given the known infectious doses for cattle (10^2–10^3 CFU by inhalatory route and 5×10^3 CFU by

oral route), red deer (10–5 × 10^2 CFU orally) and wild boar (10^4 CFU oropharingeal route) [13, 14, 17, 39], the estimated quantity of MTC excreted by a super-shedder wild boar or red deer would be sufficient to infect these hosts. Also MTC DNA concentrations excreted by super-shedders are at least one order of magnitude higher than those excreted by standard shedders. This supports the super-shedder subset of the infected population of wild ungulates has having a disproportionally large role in the transmission and maintenance of bTB in multi-host pathogen systems.

The existence of super-shedders in bTB-infected wild ungulates has implications for the design of control programs in these species. In fact, the removal of super-shedders from the population could reduce drastically the horizontal transmission and environmental contamination with MTC, which should lead to a decline on bTB incidence. The elimination of super-shedders could be accomplished by selective culling, but requires the previous identification of correlates of super-excretion, allowing targeting these animals in culling actions. Red deer females and wild boar males tend to be overrepresented in the super-shedder subset, although the differences are not statistically significant. Further studies are needed to characterize the super-shedder subset of the infected population of both species.

A future approach to reduce super-shedders would be to vaccinate against bTB with live or inactivated oral vaccines that are presently under development and validation for use in free-ranging wild ungulate populations, namely the wild boar [14, 40–42] and the white-tailed deer [43, 44]. Although these vaccines do not protect from infection or disease they diminish the severity of lesions and mycobacterial load in tissues [e.g. 40, 42] and so could potentially hamper the build-up to a super-shedder status.

Excretion was detected in a significantly lower proportion of fecal samples compared to oronasal or bronchial-alveolar samples in both host species. Abdominal lesions are detected less often compared to thoracic and cephalic lesions in the wild boar [8] but not in the red deer [9]. Nevertheless, cephalic and thoracic lesions can also give rise to fecal excretion by swallowing oral and pulmonary secretions [21]. Another possible explanation is the higher detection limit of our protocol when applied to fecal samples compared to lavages, which could give rise to a greater proportion of false negative results in fecal samples. In fact while the LD100 was equal in both lavages, it was 100–10 000× greater in feces, which explains why MTC DNA was amplified in fecal extracts only from super-shedders, as the standard shedders by this route would not be detected with the protocol we describe. The DNA extraction protocol by Griffiths et al. [30] was adopted for fecal samples because it allows controlling co-extracted PCR inhibitors in the fecal material [45], which were found to hamper PCR reactions in preliminary assays when the standard phenol–chloroform method was used.

Although we could only collect a limited number of urine samples due to the processing of hunted ungulates carcasses, which usually leads to rupture of the urinary bladder, it was surprising to find such a high proportion of shedders by this route (3 out of 4). In fact, the reported prevalence of kidney lesions is low in both wild boar and red deer [8, 9], which may be explained by the difficulty in detecting bTB lesions in organs with a large parenchyma or to the presence of microscopic lesions often missed by gross pathology [10, 20]. These results highlight that further studies on the urinary excretion of MTC and prevalence of kidney bTB lesions in wild ungulates are needed.

In super-shedder ungulates in our sample, MTC DNA concentrations in bronchial-alveolar lavages were significantly higher in red deer than in wild boar. This is expected given the structure of the lesions in each species, with red deer usually showing abscesses often located in the lungs and moderate numbers of acid-fact bacilli, while wild boar tend to show caseocalcareous lesions predominantly located in lymph nodes with a small number of acid-fast bacilli [5, 8, 9]. In wild boar the biological samples with higher average MTC DNA concentration (oronasal lavages) coincide with the most frequent anatomical location of bTB lesions (cephalic lymph nodes) [8].

On this article we report the detection of MTC excretion in 80% of bTB-naturally-infected wild boar and red deer. For the first time we provide evidence for the existence of a proportion of super-shedders within the naturally infected population of these host species. These super-shedders are responsible for a disproportionately large amount of MTC excretion from infected wild ungulates. MTC DNA concentrations greater than the minimum infective doses for cattle, red deer or wild boar are present in excretion routes from both species. These results have implications for the design of control programs in multi-host pathogen systems where these species are maintenance hosts for bTB.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal. [2] ICVS/3B's, PT Government Associate Laboratory, Braga, Portugal. [3] Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-ULisboa), Lisbon, Portugal. [4] SaBio (Health and Biotechnology), IREC, National Wildlife Research Institute (CSIC-UCLM-JCCM), Ciudad Real, Spain.

Authors' contributions

NS participated in the design of the study, performed field and laboratory work, analyzed data and drafted the manuscript. VA participated in the design of the study, analyzed data and participated in writing the final version of the manuscript. CG participated in the design of the study, analyzed data and participated in writing the final version of the manuscript. MCN conceived the study, participated in the design of the study and in writing the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to all owners, hunters and hunting organizations that helped us to collect samples. Thanks are also due to Mónica Cunha (INIAV, Portugal) for her advice during the setup of the laboratorial diagnosis techniques. Nuno Santos was supported by the *Fundação para a Ciência e a Tecnologia* (Ph.D. Grant SFRH/BD/69390/2010). This is also a contribution to MINECO Plan Nacional grant AGL2014-56305 and FEDER, and to the EU FP7 grant ANTIGONE #278976.

Received: 9 April 2015 Accepted: 22 September 2015
Published online: 30 October 2015

References

1. Humblet MF, Boschiroli ML, Saegerman C (2009) Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach. Vet Res 40:50
2. Corner LAL (2006) The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Vet Microbiol 112:303–312
3. Gortázar C, Delahay RJ, Mcdonald RA, Boadella M, Wilson GJ, Gavier-Widen D, Acevedo P (2012) The status of tuberculosis in European wild mammals. Mammal Rev 42:193–206
4. Naranjo V, Gortázar C, Vicente J, de la Fuente J (2008) Evidence of the role of European wild boar as a reservoir of Mycobacterium tuberculosis complex. Vet Microbiol 127:1–9
5. Zanella G, Duvauchelle A, Hars J, Moutou F, Boschiroli ML, Durand B (2008) Patterns of lesions of bovine tuberculosis in wild red deer and wild boar. Vet Rec 163:43–47
6. Vicente J, Höfle U, Garrido JM, Acevedo P, Juste R, Barral M, Gortázar C (2007) Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain. Vet Res 38:451–464
7. Fink M, Schleicher C, Gonano M, Prodinger WM, Pacciarini M, Glawischnig W, Ryser-Degiorgis MP, Walzer C, Stalder GL, Lombardo D, Schobesberger H, Winter P, Büttner M (2015) Red deer as maintenance host for bovine tuberculosis. Alpine Region. Emerg Infect Dis 21:464
8. Martín-Hernando MP, Höfle U, Vicente J, Ruiz-Fons F, Vidal D, Barral M, Garrido JM, de la Fuente J, Gortázar C (2007) Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. Tuberculosis 87:360–367
9. Martín-Hernando MP, Torres MJ, Aznar J, Negro JJ, Gandía A, Gortázar C (2010) Distribution of lesions in red and fallow deer naturally infected with *Mycobacterium bovis*. J Comp Pathol 142:43–50
10. Gavier-Widén D, Cooke MM, Gallagher J, Chambers MA, Gortázar C (2009) A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the 'no visible lesion' presentation. N Z Vet J 57:122–131
11. Griffin JFT, Buchan GS (1994) Aetiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. Vet Microbiol 40:193–205
12. Corner LAL, Murphy D, Gormley E (2011) *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*): the disease, pathogenesis, epidemiology and control. J Comp Pathol 144:1–24
13. Griffin JFT, Rodgers CR, Liggett S, Mackintosh CG (2006) Tuberculosis in ruminants: characteristics of intra-tonsilar *Mycobacterium bovis* infection models in cattle and deer. Tuberculosis 86:404–418
14. Ballesteros C, Garrido JM, Vicente J, Romero B, Galindo RC, Minguijón E, Villar M, Martín-Hernando MP, Sevilla I, Juste R, Aranaz A, de la Fuente J, Gortázar C (2009) First data on Eurasian wild boar response to oral immunization with BCG and challenge with a *Mycobacterium bovis* field strain. Vaccine 27:6662–6668
15. Lugton IW, Wilson PR, Morris RS, Nugent G (1998) Epidemiology and pathogenesis of *Mycobacterium bovis* infection of red deer (*Cervus elaphus*) in New Zealand. N Z Vet J 46:147–156
16. Palmer MV, Whipple DL, Olsen SC (1999) Development of a model of natural infection with *Mycobacterium bovis* in white-tailed deer. J Wildl Dis 35:450–457
17. Palmer MV, Waters WR, Whipple DL (2004) Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. Am J Vet Res 65:1483–1489
18. Palmer MV, Whipple DL, Waters WR (2001) Experimental deer-to-deer transmission of *Mycobacterium bovis*. Am J Vet Res 62:692–696
19. Cassidy JP, Bryson DG, Pollock JM, Evans RT, Forster F, Neill SD (1999) Lesions in cattle exposed to *Mycobacterium bovis*-inoculated calves. J Comp Pathol 121:321–337
20. Gallagher J, Clifton-Hadley RS (2000) Tuberculosis in badgers; a review of the disease and its significance for other animals. Res Vet Sci 69:203–217
21. Phillips CJC, Foster CRW, Morris PA, Teverson R (2003) The transmission of *Mycobacterium bovis* infection to cattle. Res Vet Sci 74:1–15
22. Clifton-Hadley RS, Wilesmith JW, Stuart FA (1993) *Mycobacterium bovis* in the European badger (Meles meles): epidemiological findings in tuberculous badgers from a naturally infected population. Epidemiol Infect 111:9–19
23. Delahay RJ, Langton S, Smith GC, Clifton-Hadley RS, Cheeseman CL (2000) The spatio-temporal distribution of Mycobacterium bovis (bovine tuberculosis) infection in a high-density badger population. J Anim Ecol 69:428–441
24. Corner LAL, Costello E, Lesellier S, O'Meara D, Gormley E (2008) Experimental tuberculosis in the European badger (*Meles meles*) after endobronchial inoculation with *Mycobacterium bovis*: II. Progression of infection. Res Vet Sci 85:481–490
25. Gortázar C, Díez-Delgado I, Barasona JA, Boadella M, Garrido JM, Vicente J (2013) Which factors drive tuberculosis transmission? Identifying super-shedders in wild boar populations. Wildlife Disease Association International Conference, Knoxville, Tennesse, USA, p124
26. Morris R, Pfeiffer D, Jackson R (1994) The epidemiology of *Mycobacterium bovis* infections. Vet Microbiol 40:153–177
27. Garthright WE, Blodgett RJ (2003) FDA's preferred MPN methods for standard, large or unusual tests, with a spreadsheet. Food Microbiol 20:439–445
28. Santos N, Geraldes M, Afonso A, Almeida V, Correia-Neves M (2010) Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): a comparison of methods applicable to hunter-harvested animals. PLoS One 5:e12663
29. Santos N, Correia-Neves M, Ghebremichael S, Källenius G, Svenson S, Almeida V (2009) Epidemiology of *Mycobacterium bovis* infection in wild boar *Sus scrofa* from Portugal. J Wildl Dis 45:1048–1061
30. Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl Environ Microbiol 66:5488–5491
31. Soo P, Horng Y, Hsueh P, Shen B, Wang J, Tu H, Wei J, Hsieh S, Huang C, Lai H (2006) Direct and simultaneous identification of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium tuberculosis* (MTB) by rapid multiplex nested PCR-ICT assay. J Microbiol Methods 66:440–448
32. Huard R, Lazzarini L, Butler W, van Soolingen D, Ho J (2003) PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J Clin Microbiol 41:1637–1650
33. MPN Calculator. http://www.i2workout.com/mcuriale/mpn/. Accessed 10 Mar 2015
34. VassarStats: Website for Statistical Computation. http://vassarstats.net/. Accessed 10 Mar 2015
35. Chambers MA, Pressling WA, Cheeseman CL, Clifton-Hadley RS, Hewinson RG (2002) Value of existing serological tests for identifying badgers that shed *Mycobacterium bovis*. Vet Microbiol 86:183–189
36. Wilkinson D, Smith GC, Delahay RJ, Rogers LM, Cheeseman CL, Clifton-Hadley RS (2000) The effects of bovine tuberculosis (*Mycobacterium bovis*) on mortality in a badger (*Meles meles*) population in England. J Zool 250:389–395
37. Chiari M, Ferrari N, Giardiello D, Avisani D, Pacciarini ML, Alborali L, Zanoni M, Boniotti MB: Spatiotemporal and ecological patterns of *Mycobacterium microti* infection in wild boar (*Sus scrofa*). Transbound Emerg Dis (in press)

38. Tessema B, Beer J, Emmrich F, Sack U, Rodloff AC (2011) Rate of recovery of *Mycobacterium tuberculosis* from frozen acid-fast-bacillus smear-positive sputum samples subjected to long-term storage in Northwest Ethiopia. J Clin Microbiol 49:2557–2561
39. Neill SD, Bryson DG, Pollock JM (2001) Pathogenesis of tuberculosis in cattle. Tuberculosis 81:79–86
40. Garrido JM, Sevilla IA, Beltrán-Beck B, Minguijón E, Ballesteros C, Galindo RC, Boadella M, Lyashchenko K, Romero B, Geijo MV, Ruiz-Fons F, Aranaz A, Juste RA, Vicente J, de la Fuente J, Gortázar C (2011) Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated *Mycobacterium bovis*. PLoS One 6:e24905
41. Beltrán-Beck B, de la Fuente J, Garrido JM, Aranaz A, Sevilla I, Villar M, Boadella M, Galindo RC, de la Lastra JMP, Moreno-Cid JA, de Mera IGF, Alberdi P, Santos G, Ballesteros C, Lyashchenko KP, Minguijón E, Romero B, de Juan L, Domínguez L, Juste R, Gortázar C (2014) Oral vaccination with heat inactivated *Mycobacterium bovis* activates the complement system to protect against tuberculosis. PLoS One 9:e98048
42. Gortázar C, Beltrán-Beck B, Garrido JM, Aranaz A, Sevilla IA, Boadella M, Lyashchenko K, Galindo R, Montoro V, Domínguez L, Juste R, de la Fuente J (2014) Oral re-vaccination of Eurasian wild boar with *Mycobacterium bovis* BCG yields a strong protective response against challenge with a field strain. BMC Vet Res 10:96
43. Nol P, Palmer MV, Waters WR, Aldwell FE, Buddle BM, Triantis JM, Linke LM, Phillips GE, Thacker TC, Rhyan JC, Dunbar MR, Salman MD (2008) Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (*Odocoileus virginianus*): a feasibility study. J Wildl Dis 44:247–259
44. Palmer MV, Thacker TC, Waters WR (2009) Vaccination with Mycobacterium bovis BCG strains Danish and Pasteur in white-tailed deer (Odocoileus virginianus) experimentally challenged with Mycobacterium bovis. Zoonoses Public Health 56:243–251
45. Kreader CA (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol 62:1102–1106

Submit your next manuscript to BioMed Central and take full advantage of:

- **Convenient online submission**
- **Thorough peer review**
- **No space constraints or color figure charges**
- **Immediate publication on acceptance**
- **Inclusion in PubMed, CAS, Scopus and Google Scholar**
- **Research which is freely available for redistribution**

Submit your manuscript at
www.biomedcentral.com/submit

Chapter III – Environmental contamination by *Mycobacterium tuberculosis* complex

Santos N, Santos C, Valente T, Gortázar C, Almeida V, Correia-Neves M (2015) Widespread environmental contamination with *Mycobacterium tuberculosis* complex revealed by a molecular detection protocol. PLoS ONE, 10(11): e0142079. doi: 10.1371/journal.pone.0142079

RESEARCH ARTICLE

Widespread Environmental Contamination with *Mycobacterium tuberculosis* Complex Revealed by a Molecular Detection Protocol

Nuno Santos[1,2]*, Catarina Santos[1,2], Teresa Valente[3], Christian Gortázar[4], Virgílio Almeida[5], Margarida Correia-Neves[1,2]

1 Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, **2** ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal, **3** Earth Sciences Institute (ICT), Pole of the University of Minho, Earth Sciences Department, University of Minho, Braga, Portugal, **4** SaBio (Health and Biotechnology), IREC, National Wildlife Research Institute (CSIC-UCLM-JCCM), Ciudad Real, Spain, **5** Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-ULisboa), Lisboa, Portugal

* nunosantos@ecsaude.uminho.pt

CrossMark
click for updates

OPEN ACCESS

Citation: Santos N, Santos C, Valente T, Gortázar C, Almeida V, Correia-Neves M (2015) Widespread Environmental Contamination with *Mycobacterium tuberculosis* Complex Revealed by a Molecular Detection Protocol. PLoS ONE 10(11): e0142079. doi:10.1371/journal.pone.0142079

Editor: Srinand Sreevatsan, University of Minnesota, UNITED STATES

Received: August 3, 2015

Accepted: October 16, 2015

Published: November 11, 2015

Copyright: © 2015 Santos et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Environmental contamination with *Mycobacterium tuberculosis* complex (MTC) has been considered crucial for bovine tuberculosis persistence in multi-host-pathogen systems. However, MTC contamination has been difficult to detect due to methodological issues. In an attempt to overcome this limitation we developed an improved protocol for the detection of MTC DNA. MTC DNA concentration was estimated by the Most Probable Number (MPN) method. Making use of this protocol we showed that MTC contamination is widespread in different types of environmental samples from the Iberian Peninsula, which supports indirect transmission as a contributing mechanism for the maintenance of bovine tuberculosis in this multi-host-pathogen system. The proportion of MTC DNA positive samples was higher in the bovine tuberculosis-infected than in presumed negative area (0.32 and 0.18, respectively). Detection varied with the type of environmental sample and was more frequent in sediment from dams and less frequent in water also from dams (0.22 and 0.05, respectively). The proportion of MTC-positive samples was significantly higher in spring ($p<0.001$), but MTC DNA concentration per sample was higher in autumn and lower in summer. The average MTC DNA concentration in positive samples was 0.82 MPN/g (CI_{95} 0.70–0.98 MPN/g). We were further able to amplify a DNA sequence specific of *Mycobacterium bovis/ caprae* in 4 environmental samples from the bTB-infected area.

Introduction

Bovine tuberculosis (bTB) is a zoonosis caused by *Mycobacterium bovis* or *Mycobacterium caprae*, both members of the *Mycobacterium tuberculosis* complex (MTC), whose natural hosts are wild and domestic mammals [1,2]. Bovine tuberculosis is a disease of economic and public health relevance subjected to eradication programs in livestock in many countries. As a

consequence, bTB has been eradicated in a few countries but in others the disease persists despite massive investment in prevention, control and surveillance. This scenario has been attributed to the existence of wildlife reservoirs, such as possums (*Trichosurus vulpecula*) in New Zealand, Eurasian badgers (*Meles meles*) in the United Kingdom and Ireland and cervids in North America [3]. In several regions of Continental Europe, notably the Iberian Peninsula, bTB is maintained in a multi-host-pathogen system, with *M. bovis* and *M. caprae* circulating between sympatric wild ungulates (mostly wild boar *Sus scrofa* and red deer *Cervus elaphus*) and free-ranging domestic ungulates [2,4,5].

Transmission of *M. bovis* from an excretor to a susceptible host can occur by direct or indirect routes [6,7]. Direct transmission requires close contact between infected excretors and susceptible hosts [3]. Therefore, it is expected to play a major role in intraspecific transmission of infection, as close contact is common among individuals of the same species. However, close contact between individuals of different species seems to be rare [3,6,8,9] and so indirect routes are expected to play a crucial role in interspecific transmission. Indirect routes of transmission require the contamination of the environment with viable mycobacteria [6].

Indirect transmission of *M. bovis* was shown to occur scarcely in cattle grazing in either naturally or artificially infected pasture [7,10]. Nevertheless it is strongly suspected to play a major role in the white-tailed deer-cattle system of North America, where it has been experimentally shown to occur through contaminated feed [11,12,13]. It is also suspected to occur in other wildlife-cattle systems, such as badger-cattle in the United Kingdom and Ireland [6,8] and wild ungulates-cattle in the Iberian Peninsula [9]. In this later situation, environmental contamination of watering and feeding areas was proposed to be of epidemiological relevance [9].

Environmental contamination with MTC remains controversial and has not been thoroughly addressed in recent studies. Detection of *M. bovis* in soil samples has been reported to endure several weeks or months after inoculation, depending on the initial concentration used [6,10,14–16]. However, Young *et al.* [17] reported that mycobacterial DNA does not persist in the environment for more than 10 days outside a viable cell. Although it was experimentally shown that *M. bovis* DNA can persist in the environment for several months after no longer being recoverable by culture, this may reflect the lower sensitivity of bacteriological culture applied to environmental samples, when compared to molecular biology methods [17,18].

The lack of clear data on environmental contamination with MTC might be due mainly to the lack of sensitive and mass-scalable techniques to detect MTC in the environment [8,15,17,18]. Molecular techniques show a greater promise over bacteriological techniques to detect MTC in environmental samples [18]. Nevertheless, available protocols have exceedingly high detection limits, rendering them of limited usefulness as screening techniques, possibly due to the uneven distribution of mycobacteria in soil samples and the co-extraction of PCR inhibitors [19]. Young et al. [17] reported a protocol with detection limits of 10^2–10^3 cells/g soil, however, this protocol was not replicated by other research groups. Pontirolli et al. [20] optimized a protocol with a detection limit of 4.25 x 10^5 cells/g soil, which is too high for the mycobacterial loads expected to occur in nature [18]. These two studies used direct extraction techniques, where DNA was extracted from an environmental sample, typically of 0.1–0.5 g. On the other hand, Sweeney et al. [21] described an immunomagnetic capture technique allowing the isolation and molecular detection of *M. bovis* from naturally contaminated soil samples. Despite this technical breakthrough in the study of the environmental contamination with pathogenic mycobacteria, this technique is difficult to scale up to test large numbers of samples and has not been replicated by other research groups.

In the present study we explore the real-life model of the multi-host pathogen system of Iberian Peninsula to assess the occurrence of environmental contamination with MTC at the interface between wild and domestic ungulates. The two central aims were: i) to define an

improved protocol for the molecular detection and estimation of the concentration of MTC and *M*. *bovis* DNA in environmental samples, easy to scale-up and with higher sensitivity than previously published methods; ii) to apply this protocol to assess MTC environmental contamination in areas with well-described distinct bTB prevalence in wildlife.

Material and Methods

Study areas

Environmental samples (soil, sediment and water) were collected from two regions 70 km apart in southern Portugal, one known to be bTB-infected (geographical coordinates 4217747/673542 utm wgs84), where *M*. *bovis* or *M*. *caprae* have been isolated from the tissues of 42/60 hunted wild boar and 13/78 hunted red deer from 2009–2014; and another presumably bTB-free (geographical coordinates 4184462/615257 utm wgs84), where MTC have not been isolated from tissues of 84 wild boar and 3 red deer from 2009–2014. Wildlife bTB prevalence in these two areas was based on previously published data [22] and subsequent unpublished results. Both areas belong to the Mesomediterranean biogeographical region of the Iberian Peninsula [23], characterized by hot, dry summers and temperate humid winters, with a strongly seasonal pattern of precipitation. Landowners allowed the collection of the environmental samples from their properties. No other permissions were needed to collect soil, sediment and water samples. The study did not involve any endangered or protected species.

Study design

Three types of *a priori* risk sites for the occurrence of environmental contamination with MTC were defined: i) small dams; ii) rivers (many seasonal) and iii) feeding areas (where hay or feed is provided, on the ground or in troughs, for cattle but also used by wild ungulates). Relevant aspects of the collection sites used for environmental samples are represented in Fig 1.

We collected a total of 319 environmental samples in the following subsets (Table 1): i) 71 sediment/water samples collected from dams in May and July 2013 and April 2014 in parallel at bTB-infected and presumed bTB-free study areas to compare the MTC DNA detection rates; ii) 204 samples from the bTB-infected study area, stratified by season (spring/summer/autumn/winter 2012) with the aim of describing the patterns of environmental contamination with MTC; iii) 44 samples opportunistically collected from soil rooted by wild boar, soil from vulture feeding stations and vulture feces.

DNA extraction protocol

Environmental samples were collected in hermetic 1000 mlL polyethylene containers and kept refrigerated until analysis, which was performed 1–3 days post-collection. In order to homogenize the distribution of mycobacteria eventually present, on average 1,087±262 g (wet weight) of soil or sediment samples were soaked with a slight excess of distilled water in a 1,000 ml cylindrical container and agitated overnight at 150 rpm at 8°C in an incubator shaker (Multitron II, Infors AG, Bottmingen, Switzerland). After resting for 2 h at room temperature, 14 ml of the supernatant/sediment interface were collected and centrifuged at 2,566 g for 30 min, after which most of the supernatant was discarded and 0.5 ml aliquots of the sediment/supernatant interface collected for DNA extraction. 50 ml water samples were centrifuged at 2,566 g for 30 min, after which the extraction protocol was equal to soil and sediment samples.

DNA extraction was performed in triplicate for each environmental sample, using a slight modification of the protocol by Griffiths et al. [19]. Briefly, 0.5 ml of sample, 0.5 ml of 5% hexadecyltrimethylammonium bromide buffer and 0.25 ml phenol were added to a 2 ml screw-cap

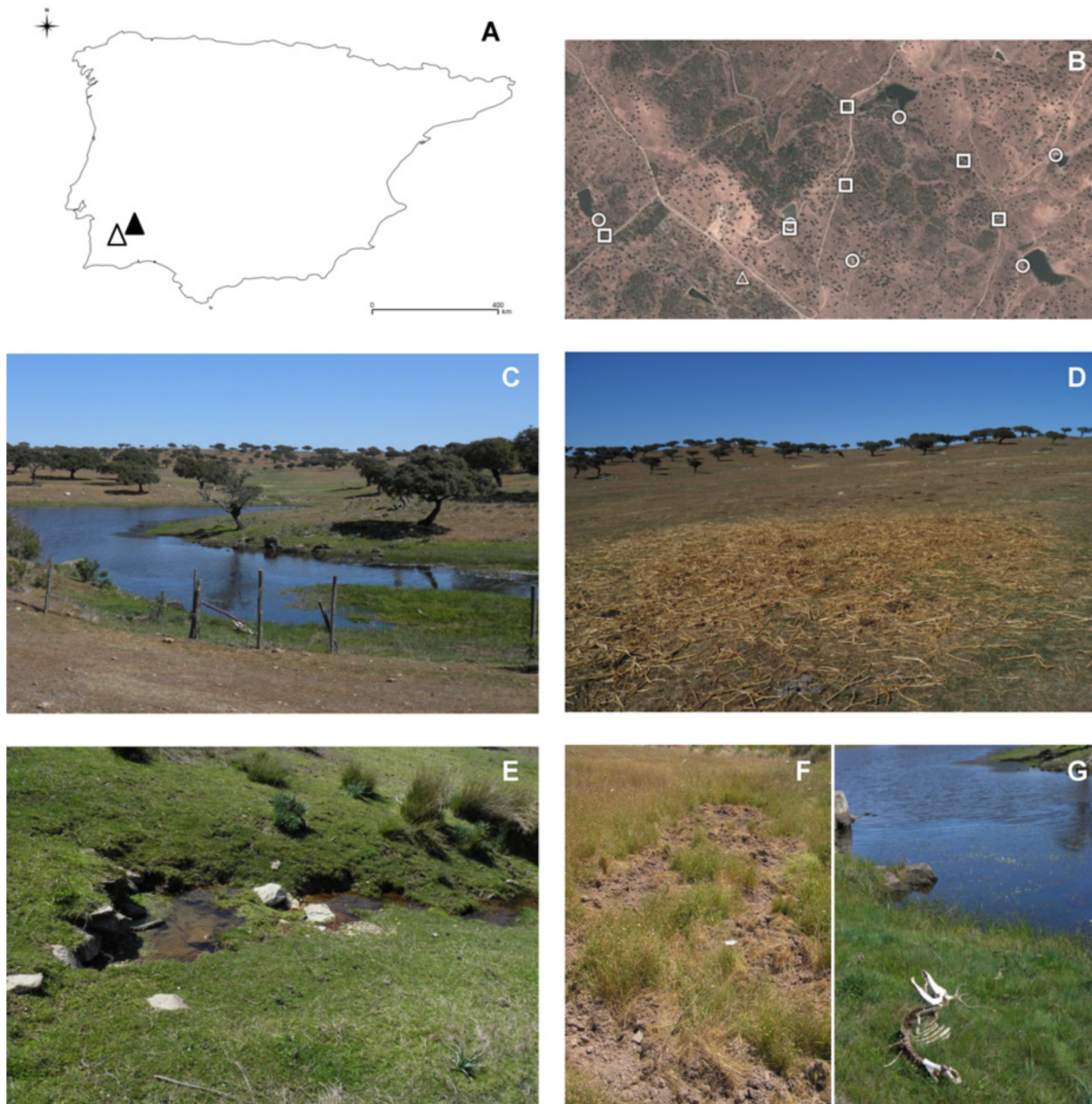


Fig 1. Aspects of the collection sites of environmental samples. (A) Map of the Iberian Peninsula highlighting the location of the bTB-positive (black triangle) and presumed bTB-negative (white triangle) study areas; (B) detail of sites in the bTB-infected area where samples were collected (squares: rivers, circles: dams; triangles: feeding sites); images of sample collection sites: (C) small dam, (D) feeding site, (E) seasonal river, (F) wild boar roots; (G) red deer skeleton besides a small dam.

doi:10.1371/journal.pone.0142079.g001

conical tube containing 100 μl of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, USA). The mixture was subjected to 2 cycles of 30 s agitation at 5 m/s in a FastPrep 24 (MP Biomedicals, Santa Ana, USA), after which 0.25 ml chlorophorm were added and gently agitated for 60 s, followed by 5 min centrifugation at 16,627 g at 4°C. 500 μl of the aqueous phase was then extracted to a new tube and an equal volume of chlorophorm added, mixed by gentle agitation for 60 s and again centrifuged for 5 min at 16,627 g at 4°C. 300 μl of the aqueous phase were then extracted to a new tube and 400 μl of 30% polyethyleneglycol 6,000 solution in 1.6 M $NaCl_2$ were added. The phase containing the precipitated DNA was collected and left to rest for 2 h at room temperature, followed by 10 min centrifugation at 19,283 g at 4°C. The

Table 1. Samples collected and positive for MTC DNA by study area and sample type.

Environmental samples		Subsets of environmental samples		
		Patterns of MTC environmental contamination (no. positive/total tested)	Comparison between study areas (no. positive /total tested)	
		bTB-infected	bTB-infected	Presumed bTB-free
Standard samples	Dams (sediment)	13/58	12/37	6/34
	Dams (water)	3/57	n.a.	n.a.
	Rivers (sediment/water)	11/61	n.a.	n.a.
	Feeding areas (soil)	5/28	n.a.	n.a.
Opportunistic samples	Wild boar roots (soil)	4/16	n.a.	n.a.
	Vulture feeding stations (soil)	1/19	n.a.	n.a.
	Vulture feces (feces)	1/9	n.a.	n.a.
Total		38/248	12/37	6/34

Number of samples collected and number of samples positive for MTC DNA by study area and sample type. n.a.—not applicable.

doi:10.1371/journal.pone.0142079.t001

supernatant was discarded and the pellet washed with 70% EtHO, centrifuged for 5 min at 16,627 g at 4°C, the supernatant again discarded and the pellet suspended in 50 μl of Tris-EDTA buffer. DNA was quantified and purity assessed using NanoDrop (ThermoScientific, Wilmington, USA). Negative controls for DNA extraction, consisting of 0.5 ml of water submitted to the same extraction protocol and interspersed with the environmental samples, were included at a rate of one for every 6 samples.

Molecular detection

Every sample was subjected to a PCR targeting a 16SRNA sequence (1218–1432 bp sequence, depending on the microorganism [24]), common to all bacteria, as an inhibition external control. A modification of the protocol described by Hiraishi [25] was used, including the same set of primers described by this author (forward: 5' AGAGTTTGATCCTGGCTCAG 3', reverse: 5' ACGGGCGGTGTGTACAAG 3'). Briefly, 250 ng DNA were added to a solution of 6.5 μl of NZYTech Green Master Mix (NZYTech, Lisbon, Portugal), containing 1.3 U Taq polymerase, 1.5 mM $MgCl_2$, 1 μl of each primer at 10 mM and 5% dimethylsulfoxide, in a final volume of 25 μl. This mix was submitted to the following PCR cycles: initial denaturation at 93°C for 5 min, followed by 35 cycles of 93°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s, with a final extension step of 72°C for 10 min. Inhibition was detected in 60/319 samples, which were then diluted 1:2 or 1:4 until inhibition disappeared. In all but 4 samples, PCR inhibition was avoided using this method; these 4 samples (1 water and 2 sediments from dams and 1 vulture feces) were removed from the analysis.

Previously to testing environmental samples we evaluated several conventional, nested and real-time PCR protocols, either previously published or developed in-house. The one showing the best performance was selected as screening protocol to detect MTC DNA in excretion routes from wild ungulates. As screening test for MTC DNA we selected a modification of the nested PCR protocol targeting a 110 bp sequence in IS6110 as described by Soo et al. [26], including the same set of primers described by those authors (external forward: 5' CGTGAGGGCATCGAGGTGGC 3', external reverse: 5' GCGTAGGCGTCGGTGACAAA 3', internal forward: 5' CTCGTCCAGCGCCGCTTCGG 3', internal reverse: 5' GCGTCGGTGA CAAAGGCCAC 3'). Briefly, 250 ng DNA were added to a solution of 7.5 μl of NZYTech

Green Master Mix, containing 1.5 U Taq polymerase, 1.5 mM $MgCl_2$, 1 μl of each primer at 10 mM and 5% dimethylsulfoxide, in a final volume of 25 μl. For the internal PCR 1 μl of the products of the external PCR was used as template. External PCR mix was submitted to the following PCR cycles: initial denaturation at 94°C for 5 min, followed by 26 cycles of 94°C for 30 s, annealing at 64°C for 15 s and extension at 72°C for 30 s, with a final extension step of 72°C for 3 min. Internal PCR mix was submitted to the same protocol, except that 30 cycles were used.

MTC-positive samples were submitted in triplicate to a hemi-nested PCR protocol specific for *M. bovis/caprae*, targeting a 306 bp sequence of RD12 (external and internal forward: 5' AGCAGGAGCGGTTGGATATTC 3', external reverse: 5' CGCCTACGCGTACTGGTATT 3', internal reverse: 5' GTGTTGCGGGAATTACTCGG 3'). The internal and external forward primers were previously described [27], while the external reverse primer was designed *in silico* using the software Primer-Blast [24]. Briefly, 250 ng DNA were added to a solution of 7.5 μl of NZYTech Green Master Mix, containing 1.5 U Taq polymerase, 2.5 mM $MgCl_2$, 1 μl of each primer at 10 mM and 5% dimethylsulfoxide, in a final volume of 25 μl. In the internal PCR, 1 μl of the products of the external PCR was used as template. External PCR mix was submitted to the following PCR cycles: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, with a final extension step of 72°C for 5 min. Internal PCR mix was submitted to the same protocol, except that 45 cycles were used.

MTC-positive samples were also submitted in triplicate to another hemi-nested PCR that allows for the differentiation of *M. microti*, *M. tuberculosis*, *M. africanum* and *M. pinnipedii* from other members of the MTC, targeting a 369 bp sequence of RD12 (forward: 5' AGCA GGAGCGGTTGGATATTC 3', external reverse: 5' CGATCGCCGTGATCACAAAC 3', internal reverse: 5' GGGAGCCCAGCATTTACCTC 3'). The internal and external forward primers were previously described [27], while the external reverse primer was designed *in silico* using the software Primer-Blast [24]. Briefly, 250 ng DNA were added to a solution of 7.5 μl of NZYTech Green Master Mix, containing 1.5 U Taq polymerase, 2.5 mM $MgCl_2$, 1 μl of each primer at 10 mM and 5% dimethylsulfoxide, in a final volume of 25 μl. In the second (internal) PCR, 1 μl of the products of the first (external) PCR was used as template. External PCR mix was submitted to the following PCR cycles: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, with a final extension step of 72°C for 3 min. Internal PCR mix was submitted to the same protocol, except that we used 45 cycles, 65°C annealing temperature and 2 mM $MgCl_2$ were used.

PCR products were visualized by electrophoresis in 2% agarose gel with GreenSafe Premium (NZYTech, Lisbon, Portugal) and GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Walthman, Massachusetts, USA) and photographed under UV light with Alpha Imager (ProteinSimple, San Jose, California, USA) (Fig 2). The preparation of the nested PCR master mixes took place in a room where no other work with MTC took place and physically separated from the rooms where the addition of the DNA templates was performed. Negative controls for the PCR protocol, consisting of 1 μl sterile water instead of extracted DNA, were included in all PCR runs at a rate of one for every 6 samples. This means that every PCR run included 1 negative control (either PCR or DNA extraction control) interspersed with every 3 samples (S1 Dataset). Negative controls were handled as the samples to be tested. The specificity of the PCR assays is supported in the literature [26,27] and was confirmed by *in silico* analysis and tested in an assay including *M. tuberculosis*, *M. bovis* BCG, *M. bovis* and *M. caprae* field isolates, *M. avium*, *M. smegmatis* and *E. coli*.

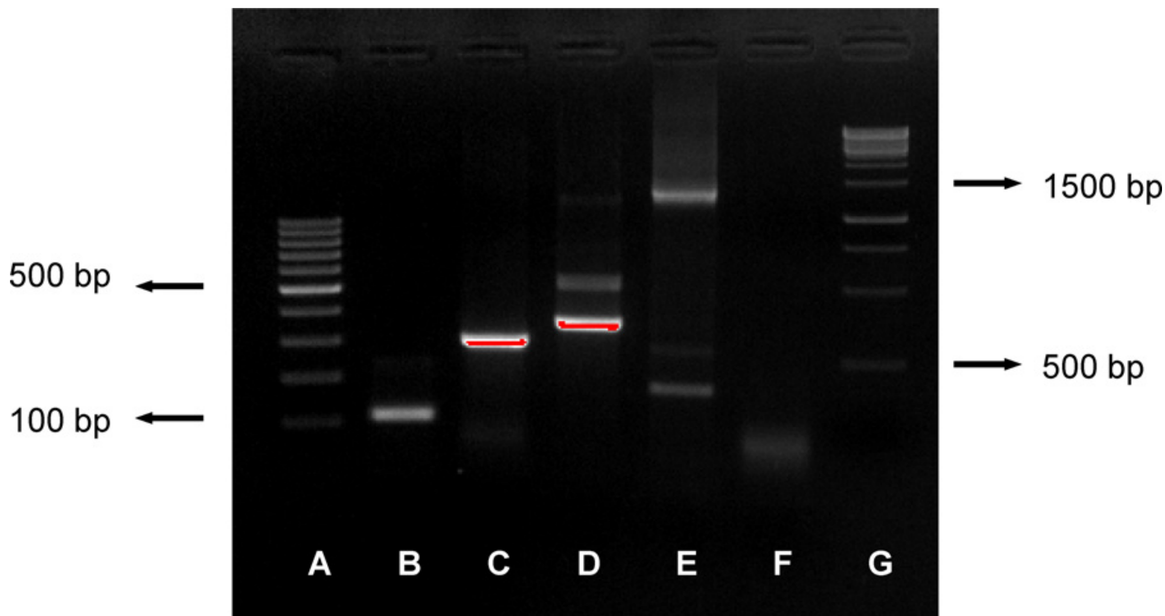


Fig 2. Image of gel showing all bands obtained with the present protocol. Image of gel showing amplification of the PCR protocols described: (A) GeneRuler 100bp DNA ladder; (B) MTC DNA IS6110 (110 bp); (C) *M. bovis/caprae* DNA RD12 (306 bp); (D) *M. microti/tuberculosis/ africanum/pinnipedii* DNA RD12 (369 bp); (E) 16SRNA (1218–1432 bp); (F) negative control; (G) GeneRuler 1kbp.

doi:10.1371/journal.pone.0142079.g002

Limits of detection

Soil samples were collected from peri-urban soils in Braga, Portugal where no MTC contamination was expected to occur, while water from ponds from the same region was used for bacterial suspensions. Ten replicates of whole-community DNA extraction and nested PCR assays were performed to assure these substrates were free from detectable levels of MTC contamination. Soil and water samples were seeded with 2-fold decreasing concentrations of *M. bovis* Bacillus Calmette-Guérin (BCG) strain Pasteur, determined by colony-forming units (CFU). Negative controls were included in each assay, consisting of the same substrate (soil or water) inoculated with the same volume of sterile water. After seeding, the samples were manually agitated to homogenize the mycobacterial distribution and subjected to the molecular detection techniques previously described. The 100% limit of detection (LD100) and 50% limit of detection (LD50) were determined based on the results of 7 molecular detection assays in the seeded samples. In order to assess the effect of increasing sample volume in the efficiency of the MTC detection in environmental samples, 0.5, 5, 50 and 500 g of soil were inoculated with 10^6 CFU/g of BCG and subjected to the previously described DNA extraction and PCR amplification protocols.

Most Probable Number

MTC DNA concentration was estimated by the Most Probable Number (MPN) [28] based on positive/negative nested PCR data on serial dilutions of DNA. Briefly, serial 10 fold dilutions of MTC-positive DNA samples were submitted to multiple nested PCR protocols as previously described. Undiluted DNA was assayed 3–8 times, 1:10 DNA 3–6 times and $1{:}10^2$ DNA 1–2 times until one dilution yielded at least two negative results. The dilution at which no amplification begins to occur indicates that the DNA has been diluted so much as to be absent and is used to estimate the original concentration. The software MPN Calculator Build 23

(http://www.i2workout.com/mcuriale/mpn/) was used to calculate the MPN MTC DNA concentration in environmental samples.

Meteorological data

Meteorological data were obtained from IPMA [29] concerning the weather station located at Beja (geographical coordinates 593635/4215076 utm wgs84), 33 and 75 km from the study areas. Data consisted of air temperature and humidity (average, minimum, maximum), wind speed, soil temperatures (grass, 5 cm, 10 cm), soil water content, evapotranspiration (ET0, Penman-Monteith, model Aladin, FAO method), global solar radiation, precipitation and number of days with fog or rain. Overall the second half of 2012 was characterized by heavy rainfall, after 2011 and the first half of 2012 being very dry, with extreme drought in both study areas and over much of Iberian Peninsula [29]. A Principal Components Analysis was performed in order to highlight which meteorological variables are more strongly related to the probability of detecting MTC DNA in the environment.

Physical-chemical characterization of the samples

After collection, samples were immediately refrigerated, transported in polyethylene bottles and stored in the dark at 4°C until analysis. A subset of sediments or soil samples (n = 7) were dried at 40°C for 72 h and the organic matter content was estimated by loss on ignition method. Quantitative assessment of percentage for different grain sizes in the coarser fractions was performed by screening, using a standard series of sieves between 0.062 and 2 mm. Silt- and clay-sized material classification was obtained using automated SediGraph 5100 (Micromeritics, Norcross, USA). The texture classification was based on the United States Department of Agriculture soil texture diagram [30].

In a subset of water samples (n = 12) pH and electric conductivity were measured with multiparameter Crison MM40+ (Crison Instruments, Barcelona, Spain). Before use, electrodes were calibrated and/or tested for accuracy, according to the manufacturer's instructions. Laboratory analyses were performed for anions by ion chromatography with suppressed conductivity detection (761 Compact IC, Metrohm AG, Herisau, Switzerland) and for alkalinity by volumetric determination [31].

Statistical analysis

Principal Components Analysis and Pearson's χ^2 were performed using IBM SPSS Statistics (SPSS, Chicago, Illinois, USA); graphics were produced in Excel 2007 (Microsoft, Redmond, Washington, USA); and confidence intervals for the positivity rates were calculated using VassarStats (http://vassarstats.net/).

Results

Limits of detection depend on the type and amount of substrate

As PCR results have been shown to be influenced by characteristics of the substrate we considered of relevance to perform physical and chemical characterization of the soil and water used in this study. Overall the soil and sediment samples analyzed were of sandy loam texture and with low clay content, while the water showed neutral pH and low total dissolved solids (Table 2).

The determination of the MTC DNA detection limit, using BCG inoculation, revealed that it varies between soil and water. We observed that in soil both LD100 and LD50 were 4 x 10^4 CFU/g while in water the LD100 was 5 x 10^5 CFU/ml and the LD50 was 10^5 CFU/ml.

Table 2. Physical-chemical parameters of the environmental samples.

Type of sample	Analytical parameter	Avg ± SD
	Texture	**Sandy loam**
Soil/sediment	Sand (%)	56.3 ± 26.5
(n = 7)	Silt (%)	36.4 ± 22.0
	Clay (%)	7.3 ± 4.7
	Organic matter (%)	5.8 ± 4.3
	pH	6.9 ± 0.16
	Electric conductivity (μS/cm)	108.1 ± 38.7
	Total Dissolved Solids (mg/l)	69.2 ± 24.9
	Total alkalinity (mg/l $CaCO_3$)	37.2 ± 11.9
Water	Fluoride (mg/l F^-)	0.077 ± 0.044
(n = 12)	Chloride (mg/l Cl^-)	17.717 ± 7.220
	Nitrite (mg/l NO_2^-)	0.064 ± 0.070
	Nitrate (mg/l NO_3^-)	13.839 ± 14.144
	Phosphate (mg/l PO_4^{3-})	0.548 ± 0.816
	Sulphate (mg/l SO_4^{2-})	6.655 ± 3.274

Physical-chemical characteristics of the environmental samples analyzed.

doi:10.1371/journal.pone.0142079.t002

Regarding the *M*. *bovis*/*caprae* molecular detection protocol in water the LD100 was 5 x 10^5 CFU/ml and the LD50 was 10^5 CFU/ml, while in soil the LD100 was 10^6 CFU/g and the LD50 was 4 x 10^4 CFU/g.

Interestingly we observed that the initial volume of the sample had an impact on the detectability of MTC DNA, as 500 g of soil inoculated with 10^6 CFU/g BCG yielded 100% positive results with an estimated concentration of 42 MPN/g (CI_{95} 13–130 MPN/g), while 50 g of soil inoculated with the same concentration of BCG yielded 80% positive results with an estimated concentration of 1.9 MPN/g (CI_{95} 0.7–5.2 MPN/g). No positive results were obtained for 5 g and 0.5 g of soil inoculated with the same concentration of BCG. Furthermore, samples of 500 g of soil inoculated with 10^5 CFU/g BCG yielded a concentration of 1.9 MPN/g (CI_{95} 1.1–3.5 MPN/g) in the sediment, 0.8 MPN/g (CI_{95} 0.4–1.1 MPN/g) in the sediment/supernatant interface and no detection in the supernatant.

Environmental MTCcontamination was detected in all types of samples

The proportion of MTC-positive samples in the bTB-infected area was higher (0.32, CI_{95} 0.20–0.49) than in the bTB presumed negative area (0.18, CI_{95} 0.08–0.34), although this difference did not reach statistical significance (p = 0.15, Pearson's χ^2) (Table 1).

From the bTB-infected area, 38/248 (0.15, CI_{95} 0.11–0.20) environmental samples were positive for MTC DNA (Table 1). MTC DNA was detected more often in sediment from dams (0.22, CI_{95} 0.14–0.35), in mixed sediment/water from rivers (0.18, CI_{95} 0.10–0.29) and soil from feeding points (0.18, CI_{95} 0.08–0.36) and significantly less in water from dams (0.05, CI_{95} 0.02–0.14) (p = 0.05, Pearson's χ^2). In the opportunistically collected samples MTC DNA was detected in 4/16 wild boar roots, 1/9 vulture feces and 1/19 soil from vulture feeding stations (Table 1).

In 4 environmental samples from the bTB-infected area the *M*. *bovis*/*caprae*-specific sequence was amplified being two sediments from dams, one from a river and one from a feeding site. Seven samples were positive for the *M*. *microti*/*tuberculosis*/*africanum*/*pinnipedii*-

Table 3. Environmental samples from which *M. bovis/caprae*- or *M. microti/tuberculosis/africanum/pinnipedii*-specific sequences were amplified.

Mycobacterial species	Type of sample	Date of collection	MTC DNA estimated concentration (MPN/g)	CI_{95} MTC DNA estimated concentration (MPN/g)
	Feeding site (soil)	March 2012	0.62	0.23–1.7
M. bovis/caprae	River (sediment/ water)	March 2012	0.93	0.34–2.5
	Dam (sediment)	March 2012	1.8	0.73–4.3
	Dam (sediment)	May 2012	0.45	0.14–1.4
	River (sediment/ water)	May 2012	2.6	0.83–8.4
	Dam (sediment)	December 2012	0.93	0.23–3.8
M. microti/tuberculosis/ africanum/ pinnipedii	Feeding site (soil)	January 2013	0.26	0.04–1.9
	Dam (water)	January 2013	1.2	0.37–3.8
	Dam (sediment)	January 2013	0.26	0.07–1.1
	Dam (sediment)	May 2013	2.8	0.96–8.4
	Dam (sediment)	April 2014	39.0	15.0–100.0

Details of the environmental samples from which the *M. bovis/caprae*-specific or the *M. microti/tuberculosis/africanum /pinnipedii*-specific sequences were amplified.

doi:10.1371/journal.pone.0142079.t003

specific sequence, all from the bTB-infected study area and spanning every type of environmental sample analyzed (Table 3).

Environmental MTCcontamination was detected mostly in spring

The proportion of positive samples for MTC DNA was significantly higher in spring than in the other seasons ($p<0.001$, Pearson's χ^2) (Table 4). This overall seasonal pattern was replicated in sediment samples from dams, in mixed sediment/water from rivers and soil from feeding points. In feeding points no MTC DNA was detected neither in summer nor in autumn, while in water from dams one positive result was obtained every season except in autumn

Table 4. Proportion of environmental samples where MTC DNA was amplified in the bTB-infected area by sample type and season.

Environmental sample	Spring		Summer		Autumn		Winter	
	no.	Proportion positive (CI_{95})	no.	Proportion positive (CI_{95})	no.	Proportion positive (CI_{95})	no.	Proportion positive (CI_{95})
Dam (sediment)	14	0.57 (0.33–0.79)**	15	0.07 (0.01–0.31)	15	0.13 (0.04–0.38)	14	0.14 (0.04–0.40)
Dam (water)	14	0.07 (0.01–0.31)	14	0.07 (0.01–0.31)	15	0.00 (0.00–0.20)	14	0.07 (0.01–0.31)
River	15	0.47 (0.25–0.70)**	15	0.13 (0.04–0.38)	16	0.13 (0.04–0.36)	15	0.00 (0.00–0.20)
Feeding site	7	0.57 (0.25–0.84)*	8	0.00 (0.00–0.32)	7	0.00 (0.00–0.35)	6	0.17 (0.03–0.56)
Total	53	0.40 (0.28–0.54)***	52	0.08 (0.03–0.18)	53	0.08 (0.03–0.18)	49	0.08 (0.03–0.19)

Proportion of samples with MTC DNA amplification in the bTB-infected area by sample type and season, with confidence intervals and statistically significant differences between seasons highlighted (Pearson's χ^2).

* p<0.05

** p<0.01

***p<0.001

doi:10.1371/journal.pone.0142079.t004

(Table 4). The proportion of MTC DNA positive samples in sediment from dams was not significantly different when comparing the spring of 2012 (8/15 positive samples), 2013 (4/12) and 2014 (6/15) (p = 0.56, Pearson's χ^2).

Estimated MTC DNA concentration showed a bimodal distribution

On the 56 samples positive for MTC DNA the average concentration was 0.82 MPN/g (CI_{95} 0.70–0.98 MPN/g). The highest concentration recorded was 39 MPN/g (CI_{95} 15–100 MPN/g), in a sediment/water sample collected from a dam in the bTB-infected area. The distribution of the MTC DNA concentrations followed a bimodal pattern with two modes in the classes <0.5 MPN/g and 2.51–3.0 MPN/g (Fig 3).

MTC DNA concentration was not significantly different across sample types, although a tendency was seen for higher concentration in feeding points and lower in water from dams (Fig 4A). Also, MTC DNA concentration tended to be higher in samples collected during autumn and lower in summer (Fig 4B), although no statistically significant influence of season was found.

Meteorological variables associated with the probability of MTC DNA detection

A Principal Components Analysis showed that temperature (air, soil 5 cm and soil 10 cm) and evapotranspiration are the variables most consistently positively associated with the probability

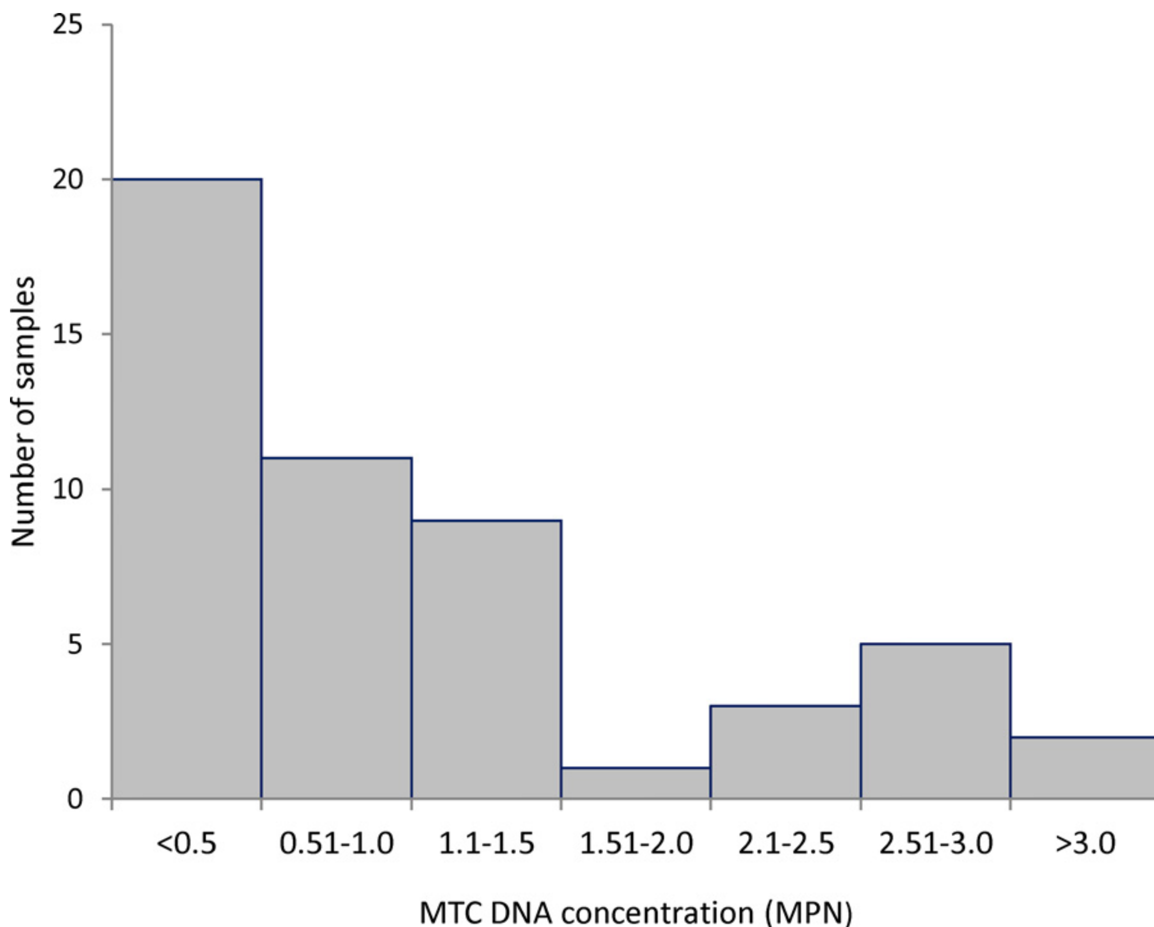


Fig 3. Distribution of MTC DNA concentrations. Histogram of MTC DNA concentrations estimated by the Most Probable Number method in the environmental samples from which MTC DNA was amplified (n = 56), both study areas combined.

doi:10.1371/journal.pone.0142079.g003

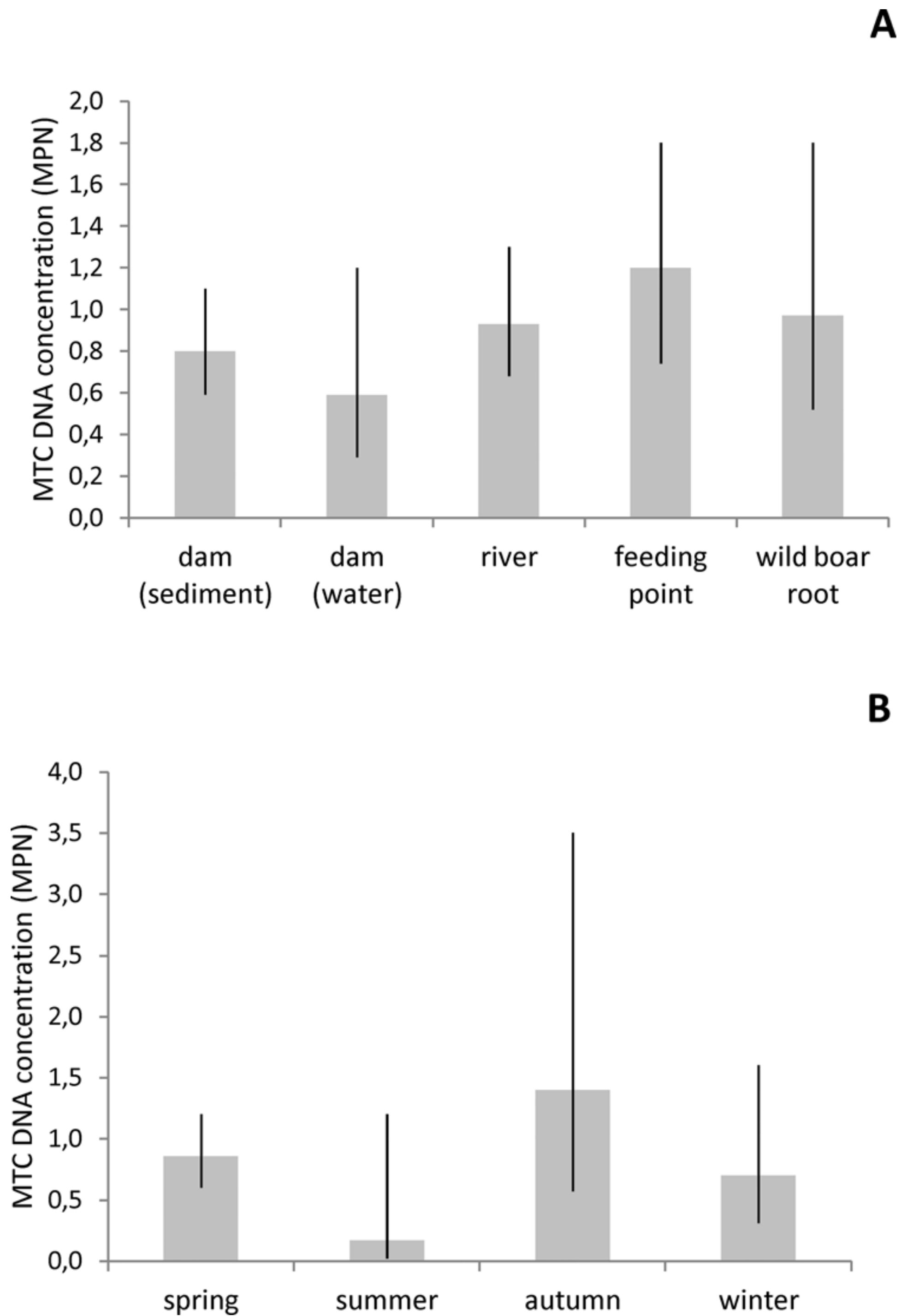


Fig 4. Estimated MTC DNA concentrations by season and sample type. Average MTC DNA concentration estimated by the Most Probable Number in environmental samples from the bTB-infected area, by sample type (A) and season (B), with 95% confidence intervals.

doi:10.1371/journal.pone.0142079.g004

of detection of MTC DNA in environmental samples when considering as time range for the meteorological data the 6 months, 1 month or 1 week previous to the collection of the samples (Table 5).

Table 5. Meteorological variables with the highest loadings on the Principal Components Analysis.

Meteorological variables	Previous 6 months	Previous 1 month	Previous 1 week
Air temperature (average)	0.982	0.985	0.938
Soil temperature (grass)	0.962	n.a.	n.a.
Soil temperature (depth 5 cm)	0.987	0.979	0.954
Soil temperature (depth 10 cm)	0.983	0.979	0.956
Evapotranspiration	0.977	0.996	0.957
Water content in soil	-0.990	n.a.	n.a.
Solar radiation	0.926	n.a.	0.953
Wind speed (average)	0.980	n.a.	n.a.
Fog-days	-0.989	n.a.	n.a.
Precipitation	0.900	0.947	n.a.
Rain-days		0.943	n.a.
Variation explained, 2 components combined	0.956	0.943	0.885

Meteorological variables with the highest loadings on the first two components of the Principal Components Analysis, with detection of MTC DNA as dependent variable. n.a.—not applicable.

doi:10.1371/journal.pone.0142079.t005

Discussion

Contamination of the environment with *M. bovis/caprae* is considered an important contribution to the persistence and interspecific spread of bTB, nevertheless methodological issues have impaired our knowledge on this matter [7,32]. The present study describes and applies an improved protocol for the molecular detection of MTC in environmental samples and reports for the first time the widespread occurrence of MTC DNA in the environment in areas where bTB is highly prevalent in wildlife. This contamination is detected in all types of *a priori* defined risk sites, where wild and domestic ungulates assemble, such as feeding and watering places. Spatial aggregation of wildlife at feeding or watering points was previously shown to be a risk factor for bTB prevalence [5]. Nevertheless, interspecific direct contact seems to be rare because of temporal segregation in their use [9]. Indirect transmission of bTB through environmental contamination with MTC provides a means to explain this risk effect, however mechanisms of infection from environmental sources still remain to be explained. In cattle, soil consumption when feeding in contaminated pasture has been proposed as a mechanism by which infection may occur [6]. Red deer have a mixed grazer and browser diet [33] which could theoretically put them at lower risk of infection thorough feeding. On the other hand, wild boar consistently root through soil when feeding [34] and so could be more exposed. Interestingly, MTC was detected in 4/16 wild boar roots, in an area where bTB prevalence in this species is 0.70 (unpublished data). Wild boar usually shows bTB prevalence much higher than sympatric red deer and their necrophagy habits have been proposed as a means to explain this difference [22]. Given the widespread environmental contamination we detected, their fossatorial habits could further explain this apparent increased exposure to infection.

The protocol we describe has the novelty of starting from a large volume of soil and sediment substrate, which we show to improve the detection rate. In fact, most published studies extract DNA from small volumes of substrate (0.1–1.0 g) [17,20,21]; by incorporating a homogenization step through the overnight agitation of approximately 1,000 g of substrate the detection rate increases considerably. We hypothesize that the agitation of the substrate in water homogenizes the MTC distribution in the substrate and so improves the detectability. In fact it was speculated that the uneven distribution of MTC in environmental samples hampers their molecular detection, together with the co-extraction of PCR inhibitors [20]. In our study

inhibition was detected in 18.8% of the environmental samples but could be managed by the dilution of the samples up to 1:4 in all but 1.3% of them. PCR inhibitors such as humic compounds concentrate in the organic matter [20,35], the content of which was average to high in our samples. Also clay adsorbs DNA, hampering its extraction from soil samples [17], nevertheless clay content was low in the environmental samples analyzed in the present study (Table 2).

The LD100 of the MTC molecular detection protocol we describe is approximately 10 times lower than the one reported by Pontirolli et al. [20] for soil samples. Our protocol detects MTC DNA in sediment from dams in the bTB high-prevalence study area at a rate almost double than that of an area where bTB has not been detected in wild and domestic ungulates despite active surveillance. Although the difference is not statistically significant, this suggests that environmental contamination with MTC is higher in areas where bTB is highly prevalent in wild ungulate populations. The low success in the specific identification precluded any conclusion on the MTC species responsible for the positive results from the presumed bTB-free study area, which could be caused by environmental contamination with MTC other than *M. bovis/ caprae*.

In fact, although our protocol represents a clear improvement from the previously published, it has limitations, the first of which is the low success rate in the specific identification of MTC. MTC includes several species, namely *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* [1,32]. The first three species are not known to have other maintenance host besides humans [1] and so are very unlikely to be widespread in the environment in semi-natural areas with low human density and low human TB prevalence such as our study areas. *M. pinnipedii* natural hosts are marine mammals [36] and so is also unlikely to be present in environmental samples from our study areas. On the other hand *M. bovis* and *M. caprae* are the etiological agents of bTB and have been isolated in wild and domestic hosts in our high-prevalence study area [4,22]. DNA from these two mycobacterial species was detected in 4 samples and they could account for a larger proportion of the MTC detected in environmental samples. Nevertheless, the 25 x higher LD100 of the *M. bovis/caprae*-specific molecular detection protocol in soil samples compared with the MTC molecular detection protocol precluded estimating their proportion in our sample. Although *M. microti* has not been reported in wildlife in the Iberian Peninsula, its natural hosts are rodents [37] and could plausibly be present in our study areas and account for an unknown proportion of the MTC DNA positive results from both study areas, but further work is needed on this subject.

MTC DNA concentrations in the environment follow a bi-modal pattern of two distributions roughly separated at 2 MPN/g (Fig 3). A possible explanation is that the lowest concentrations of MTC DNA could originate from standard excretion from infected animals, while the highest concentrations could come from occasional events leading to higher focal contamination, such as the location of carcasses of infected animals (Fig 1G) or mycobacterial excretion by "super-shedder" hosts, such as described for the badger [38]. Further work is needed to explain this result.

MTC presence in the environment is dependent on excretion rates from infected animals and also on the survival of mycobacteria. MTC DNA detection rates are significantly higher in spring in all types of samples except water from dams. The fact that no significant differences in detection rates are found between three consecutive springs suggests that this is a consistent seasonal phenomenon. In fact spring in areas of the Iberian Peninsula with Mediterranean climate is characterized by moderate air and soil temperatures (average 15.8 and 16.6°C respectively, spring 2012) and relatively high water content of soil (average water content of soil 49.8%, spring 2012) [29]. In our study, MTC DNA detection in environmental samples was positively associated with air and soil temperatures and evapotranspiration. *M. bovis* survival

in the environment was shown to be influenced by meteorological determinants; Fine et al. [15] reported that temperature (only air temperature was measured in that study) was significantly and positively associated with *M. bovis* persistence in the environment in Michigan. In the present study, the lowest average MTC concentration is found in summer, when climatic conditions are theoretically the worst for mycobacterial survival because of extremely high temperature (average maximum air temperature 32.6°C, average soil temperature 10 cm 26.4°C, summer 2012) and low water content of soil (average 2.0%, summer 2012) [15]. Soil dryness was expected to be an important limiting factor for MTC survival in feeding areas, where the only water content of soil is that of rainfall. In fact, it is noteworthy that no MTC DNA is detected in soil samples from feeding areas collected during summer, down from 0.57 positivity rate in the previous spring. In our study, MTC DNA detection rates and concentration are not significantly different between substrates (soil, sediment and water) as also reported by Fine et al. [15].

Summarizing, we describe an improved version of a protocol for the sensitive detection of MTC DNA that is simple, mass-scalable and applicable in several substrates of environmental samples. This protocol allowed for the first time the detection and description of overall spatio-temporal patterns of environmental contamination with MTC in areas where bTB is highly prevalent in wild ungulates. The data generated raises several questions which will need further study, such as the specific identification of MTC involved, assessment of its viability, quantification of the contribution of indirect transmission on bTB persistence in multi-host-pathogen systems and investigation of MTC excretion from infected hosts.

Supporting Information

S1 Dataset. MTC nested PCR dataset. Dates and results of the nested PCR protocols targeting IS6110 and RD12, including the negative controls. In brackets the initial date the nested PCR was performed, which was repeated whenever the negative control amplified a sequence. (XLSX)

Acknowledgments

We are grateful to those landowners who allowed us to access their estates for the collection of environmental samples.

Author Contributions

Conceived and designed the experiments: NS CG VA MCN. Performed the experiments: NS CS TV. Analyzed the data: NS CS TV CG VA MCN. Contributed reagents/materials/analysis tools: TV MCN. Wrote the paper: NS CS TV CG VA MCN.

References

1. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. PNAS. 2002; 99(6): 3684–3689. PMID: 11891304
2. Gortázar C, Delahay RJ, McDonald RA, Boadella M, Wilson GJ, Gavier-Widen D et al. The status of tuberculosis in European wild mammals. Mammal Rev. 2012; 42(3): 193–206.
3. Corner LAL. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Vet Microbiol. 2006; 112: 303–312. PMID: 16326039
4. Cunha MV, Matos F, Canto A, Albuquerque T, Alberto JR, Aranha JM, et al. Implications and challenges of tuberculosis in wildlife ungulates in Portugal: a molecular epidemiology perspective. Res Vet Sc. 2012; 92(2): 225–235.

5. Santos N, Correia-Neves M, Almeida V, Gortázar C. Wildlife tuberculosis: a systematic review of the epidemiology in Iberian Peninsula. In Cunha MLRS, editor. Epidemiology Insights. Rijeka: InTech; 2012. http://www.intechopen.com/books/epidemiology-insights/wildlife-tuberculosis-a-systematic-review-of-the-epidemiology-in-the-iberian-peninsula
6. Phillips CJC, Foster CRW, Morris PA, Teverson R. The transmission of *Mycobacterium bovis* infection to cattle. Res Vet Sci. 2003; 74: 1–15.
7. Humblet MF, Boschiroli ML, Saegerman C. Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach. Vet Res. 2009; 40(5): 1–24.
8. Courtenay O, Reilly LA, Sweeney FP, Hibberd V, Bryan S, Ul-Hassan A, et al. Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis? Biol Lett. 2006; 2(3): 460–462. PMID: 17148430
9. Kukielka E, Barasona JA, Cowie CE, Drewe JA, Gortázar C, Cotarelo I, et al. Spatial and temporal interactions between livestock and wildlife in South Central Spain assessed by camera traps. Prev Vet Med. 2013; 112(3): 213–221.
10. Morris RS, Pfeiffer DU, Jackson R. The epidemiology of *Mycobacterium bovis* infections. Vet Microbiol. 1994; 40: 153–177. PMID: 8073623
11. Palmer MV, Whipple DL, Waters WR. Experimental deer-to-deer transmission of *Mycobacterium bovis*. Am J Vet Res. 2001; 62(5): 692–696. PMID: 11341387
12. Palmer MV, Waters WR, Whipple DL. Shared feed as a means of deer-to-deer transmission of *Mycobacterium bovis*. J Wildl Dis. 2004a; 40(1): 87–91.
13. Palmer MV, Waters WR, Whipple DL. Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. Am J Vet Res. 2004b; 65(11): 1483–1489.
14. Tanner M, Michel AL. Investigation of the viability of *M*. *bovis* under different environmental conditions in the Kruger National Park. Onderstepoort J Vet Res. 1999; 66: 185–190. PMID: 10631708
15. Fine AE, Bolin CA, Gardiner JC, Kaneene JB. A study of the persistence of *Mycobacterium bovis* in the environment under natural weather conditions in Michigan, USA. Vet Med Int. 2011: 765430. doi: 10.4061/2011/765430 PMID: 21547222
16. Ghodbane R, Medie FM, Lepidi H, Nappez C, Drancourt M. Long-term survival of tuberculosis complex mycobacteria in soil. Microbiol. 2014; 160: 496–501.
17. Young JS, Gormley E, Wellington EMH. Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. Appl Environ Microbiol. 2005, 71: 1946–1952. PMID: 15812024
18. Adams AP, Bolin SR, Fine AE, Bolin CA, Kaneene JB. Comparison of PCR versus culture for detection of *Mycobacterium bovis* after experimental inoculation of various matrices held under environmental conditions for extended periods. Appl Environ Microbiol. 2013; 79(20): 6501–6506. doi: 10.1128/AEM.02032-13 PMID: 23956383
19. Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl Environ Microbiol. 2000; 66(12): 5488–5491. PMID: 11097934
20. Pontiroli A, Travis ER, Sweeney FP, Porter D, Gaze WH, Mason S, et al. Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. PLoS ONE. 2011; 6(3): e17916. doi: 10.1371/journal.pone.0017916 PMID: 21448453
21. Sweeney FP, Courtenay O, Ul-Hassan A, Hibberd V, Reilly LA, Wellington EMH. Immunomagnetic recovery of *Mycobacterium bovis* from naturally infected environmental samples. Lett Appl Microbiol. 2006; 43(4): 364–369. PMID: 16965365
22. Santos N, Correia-Neves M, Ghebremichael S, Källenius G, Svenson S, Almeida V. Epidemiology of *Mycobacterium bovis* infection in wild boar *Sus scrofa* from Portugal. J Wildl Dis. 2009; 45(4): 1048–1061. PMID: 19901381
23. Rivas-Martínez S. Les étages biaclimatiques de la végétation de la Peninsule Ibérique. Ann Jard Bot Madrid. 1981; 37: 251–268.
24. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinf. 2012; 13(1): 134.
25. Hiraishi A. Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett Appl Microbiol. 1992; 15(5): 210–213. PMID: 1280147
26. Soo P, Horng Y, Hsueh P, Shen B, Wang J, Tu H, et al. Direct and simultaneous identification of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium tuberculosis* (MTB) by rapid multiplex nested PCR-ICT assay. J Microbiol Methods. 2006, 66: 440–448. PMID: 16516314

27. Warren RM, van Pittius NCG, Barnard M, Hesseling A, Engelke E, De Kock M, et al. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. Int J Tuberc Lung Dis. 2006; 10(7): 818–822. PMID: 16850559
28. Peeler JT, Houghtby GA, Rainosek AP. The most probable number technique. In Vanderzant C, Splittstoesser DF, editors. Compendium methods for the microbiological examination of foods. Washington DC: American Public Health Association, 3rd ed.; 1992. pp. 105–120.
29. IPMA (2014) Agrometeorological bulletin. https://www.ipma.pt/pt/publicacoes/boletins, assessed 4th September 2014 [In Portuguese].
30. USDA (1987) Soil mecanics, USDA textural classification. United States Department of Agriculture, ftp://ftp.wcc.nrcs.usda.gov/wntsc/H&H/training/soilsOther/soil-USDA-textural-class.pdf
31. AWWA. Standard methods for the examination of water and wastewater. 18th edition. Washington DC: American Public Health Association; 1992.
32. Kaneene JB, Miller R, De Kantor IN, Thoen CO. Tuberculosis in wild animals. Int J Tuberc Lung Dis. 2010; 14(12): 1508–1512. PMID: 21144234
33. Bugalho MN, Milne JA. The composition of the diet of red deer *Cervus elaphus* in a Mediterranean environment: a case of summer nutritional constraint?. For Ecol Manag. 2003; 181(1): 23–29.
34. Welander J. Spatial and temporal dynamics of wild boar (*Sus scrofa*) rooting in a mosaic landscape. J Zool. 2000; 252(2): 263–271.
35. Young JM, Rawlence NJ, Weyrich LS, Cooper A. Limitations and recommendations for successful DNA extraction from forensic soil samples: a review. Sci Just. 2014; 54(3): 238–244.
36. Cousins DV, Bastida R, Cataldi A, Quse V, Redrobe S, Dow S, et al. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Int J Syst Evol Microbiol. 2003; 53(5): 1305–1314.
37. Burthe S, Bennett M, Kipar A, Lambin X, Smith A, Telfer S, et al. Tuberculosis (*Mycobacterium microti*) in wild field vole populations. Parasitol. 2008; 135(03): 309–317.
38. Delahay RJ, Langton S, Smith GC, Clifton-Hadley RS, Cheeseman CL. The spatio-temporal distribution of *Mycobacterium bovis* (bovine tuberculosis) infection in a high-density badger population. J Anim Ecol. 2000; 69(3): 428–441.

Chapter IV – Spatial analysis and modeling of bovine tuberculosis in wildlife in Portugal

Santos et al. (in prep) Spatial analysis and modeling of emerging wildlife tuberculosis in a multi-host pathogen system

Spatial analysis and modeling of emerging wildlife tuberculosis in a multi-host pathogen system

Abstract

We explore the real-life model of the multi-host pathogen system of Iberian Peninsula to assess the spatial epidemiology of bovine tuberculosis (bTB) in wildlife, using the wild boar as sentinel species. Wildlife bTB was assessed by serology from hunted wild boar at national scale from 2006-13, while at regional scale it was assessed by bacteriological culture from 2009-14. Disease mapping, cluster analysis and modeling at both geographical scales using sparse area generalized linear mixed model with conditional autoregressive priors were performed. We show that blood collected in absorbent papers is a valid substitute for serum in bTB serological surveys aimed at detecting antibodies against bovine purified protein derivative (bPPD), with a Kappa=0.805 between both types of samples. Antibodies against bPPD were detected in 3.7 % (CI_{95} 2.5–5.4 %) of 700 wild boar, with 2 geographical clusters identified in central- and south-eastern Portugal. The final conditional autoregressive model included the variables "historical refuge", "red deer hunting bag", "management intensity" and "red deer hunting bag × management". The risk map based on this model shows good agreement with published reports of *M. bovis* isolation from wildlife. At one of the clusters identified, we estimated an overall bTB prevalence of 15.6 % in wild boar. In one particular area bTB prevalence was 67.7 %, significantly higher than 46.2 % in 2005-06 (p=0.049, Fisher's exact test). The final conditional autoregressive model included the variables "historical refuge", "red deer hunting bag", "fallow deer hunting bag" and "red deer hunting bag × historical refuge". The risk map based on this model confirms the strong spatial structure of wildlife bTB and shows good agreement with independent reports of *M. bovis* isolation from wildlife. We demonstrate that serological tests coupled with blood collection in absorbent paper are a valid strategy for large-scale wildlife bTB surveys. Data reported here confirms that bTB is an emerging disease of wildlife at the Iberian Peninsula, stressing the need to implement control programs to prevent further geographical spread and increase in prevalence.

Introduction

Bovine tuberculosis (bTB) is a zoonosis caused by *Mycobacterium bovis* or *Mycobacterium caprae*, both members of the *Mycobacterium tuberculosis* complex (MTC), whose natural hosts are wild and domestic mammals (Brosch et al., 2002; Gortázar et al., 2012). Bovine tuberculosis is a disease of economic and public health relevance subjected to eradication programs in livestock in many countries. As a consequence, bTB has been eradicated in a few countries but in others the disease persists despite massive investment in prevention, control and surveillance. This scenario has been attributed to the existence of wildlife reservoirs, such as possums (*Trichosurus vulpecula*) in New Zealand, Eurasian badgers (*Meles meles*) in the United Kingdom and Ireland and cervids in North America (Corner, 2006). In several regions of Continental Europe, notably the Iberian Peninsula, bTB is maintained in a multi-host-pathogen system, with *M. bovis* and *M. caprae* circulating between sympatric wild ungulates (wild boar *Sus scrofa*, red deer *Cervus elaphus* and fallow deer *Dama dama*) and free-ranging domestic ungulates (Gortázar et al., 2012; Santos et al., 2012). In Portugal the control of bTB in livestock has led to very low incidence in 2014: 3.6 cases per 1,000 herds at risk and 6.0 cases per 10,000 bovines at risk (DGAV, 2015). Nevertheless, disease incidence has stabilized in the past years and the recent awareness of the existence of wildlife reservoirs has fueled the discussion over their role as bTB reservoirs (Cunha et al., 2012).

A systematic review of wildlife bTB in Iberian Peninsula showed a striking spatial structure of prevalence (Santos et al., 2012). This study highlighted a high-prevalence core area in central-southwestern Iberian Peninsula, where average prevalence is 52 % (Gortázar et al., 2011). To the periphery of this large core area prevalence decreases to the point where disease is not detected or at low prevalence in eastern, northern and westernmost regions of Iberian Peninsula (Santos et al., 2009; Boadella et al., 2011b; Muñoz-Mendoza et al., 2013). The Portuguese animal health directorate (*Direcção Geral de Alimentação e Veterinária* - DGAV) established in 2011 a special surveillance area for bTB in large game species, encompassing regions where the disease was known to be present in wild ungulates (Fig. 1A). Nevertheless no formal spatial analysis of bTB distribution has been conducted in Iberian wildlife.

Wildlife diseases tend to be geographically structured because of the variability in the environmental and biological conditions that sustain both pathogen and hosts. Spatial

epidemiology is a multidisciplinary scientific field aimed at describing and explaining the spatial heterogeneity in disease occurence (Ostfeld et al., 2005; Norman, 2008; Bergquist and Rinaldi, 2010), encompassing disease mapping, disease cluster detection and analysis and spatial regression (Norman, 2008; Bergquist and Rinaldi, 2010). The resulting spatial models of disease risk can inform management plans to control disease, namely on where to target interventions taking into account the underlying variables (Ostfeld et al., 2005).

Large-scale surveys for disease in wildlife require mass-scalable and inexpensive diagnostic tests, with serological methods being one of the most suitable techniques (Boadella et al., 2011b; Gilbert et al., 2013). An ELISA for detecting antibodies against MTC was described and validated for use in wild boar samples (Aurtenetxe et al., 2008; Boadella et al., 2011a). In this species the ELISA showed a moderately good estimated sensitivity (79.2 %) and excellent specificity (100 %) (Boadella et al., 2011a). Another improvement for large-scale surveys of disease in wildlife is a sampling protocol that can be conducted by non-specialized personnel (e.g. hunters). Blood collected in absorbent paper was originally developed for human sampling in remote locations (Nielsen et al., 1987), but has been increasingly used for wildlife disease surveys (e.g. Portejoie et al., 2009; Curry et al., 2014) and fulfills that prerequisite.

Wild boar was shown to be a maintenance host for bTB in Iberian Peninsula (Naranjo et al., 2008). Furthermore, wild boar has been used as sentinel for the presence of bTB in wildlife in New Zealand (Nugent et al., 2002; Yockney et al., 2013). The rationale for using this species as sentinel is its high susceptibility to chronic infection with *M. bovis* and *M. caprae*, together with a high exposure to these pathogens, either through its necrophagy or fossatorial habits (Nugent et al., 2002; Santos et al., 2009; 2015; Gortázar et al., 2011). Historically, hunting and habitat change reduced wild boar populations in Portugal to the point that in mid-XXth century the species was almost extinct, surviving only in 5 small, isolated populations, herein called historical refuges (Lopes and Borges, 2004; Ferreira et al., 2008) (Fig. 1A). Since the mid-XXth century wild boar populations in Portugal increased markedly (Acevedo et al., 2006) as to species being currently present all over the Portuguese continental territory, except the most heavily urbanized areas (Lopes and Borges, 2004; Bosh et al., 2012).

In the present study we explore the real-life model of the multi-host pathogen system of Iberian Peninsula to assess the spatial epidemiology of wildlife bTB, using the wild boar as sentinel

species. The main aims of this study were: i) to map wildlife bTB distribution in Portugal making use of serological methods; ii) to investigate the spatial clustering of wildlife bTB; iii) to model the distribution of wildlife bTB at 2 different geographical scales.

Methods

Collection of samples

From 700 wild boar hunted in 2006-2013 we obtained biological samples for serology, of which 116 were serum and 584 blood collected in absorbent paper, either Protein Saver (PS) 903 cards (Whatman™, n=315) or FTA paper (Whatman™, n=269). Additional paired samples of serum and blood collected in PS cards were collected from 22 wild boar with macroscopic lesions compatible with bTB.

Blood obtained from the thoracic or abdominal cavity of hunted wild boar and stored refrigerated for 24-48 h was centrifuged (1,430 g for 10 min) and serum separated and stored at -20 °C. Absorbent papers were distributed to hunters along with instructions for them to be soaked in blood available at the carcass, allowed to dry protected from sunlight and kept at room temperature stored in ziplock bags, together with information on the location of collection. Absorbent papers were gathered at the end of the hunting season and kept frozen at -20°C until processing. Elutes were obtained by cutting half a circle of PS card or a quarter of FTA card which, according to the manufacturer's instructions, absorb 40 and 31 μl of blood, respectively. These papers were further divided into 5 pieces, incubated overnight refrigerated in 200 μl phosphate buffered saline (PBS) and the elute immediately processed for serology.

In one bTB cluster identified by serology, samples from tissue showing bTB-compatible lesions or pooled lymph nodes when lesions were absent, were collected from 340 wild boar from 17 hunting areas from 2009-2014 and stored at -20 °C until bacteriological culture. Gender was recorded and age estimated by tooth eruption and wear patterns, according to Matschke (1967) and Buruaga et al. (2001).

Laboratorial analysis

Serum and elute samples were tested for anti-PPD antibodies by means of an ELISA using bovine tuberculin purified protein derivative (bPPD) as antigen and protein G horseradish peroxidase as

a conjugate, as previously described (Boadella et al., 2011a). Briefly, after coating the plates for 18 h at room temperature, wells were washed with PBS solution containing 0.05 % Tween 20 (PBST) and blocked for 1 h at 37 °C with 140 ml of 5 % skim milk in PBST. Serological samples were added (10 µl/well) at a dilution of 1:200 in PBS in the case of serum and 1:50 in the case of elutes and incubated for 1 h at 37 °C. Blanks and positive and negative controls were tested in duplicate in each plate. Samples were tested in triplicate on different plates. Protein G was added (100 µl/well) at a dilution of 2.5 mg/ml in PBST and incubated at 37 °C for 1 h. After revealing, the reaction was stopped with 50 µl/well of sulfuric acid (H2SO4; 3N), and optical density (OD) was measured in a spectrophotometer at 450 nm. Pooled anti-bPPD–positive serum was obtained from wild boar previously described as *M. bovis* culture positive and negative controls from bTB-free wild boar previously described as *M. bovis* culture negative from bTB-free areas. Sample results were expressed as: mean sample OD/(2 × mean negative control OD) × 100. The cutoff for positivity was set at 100, based on Boadella et al. (2011a).

Bacteriological culture was performed in a BSL3 facility at Life and Health Sciences Research Institute (ICVS), Braga, Portugal following a previously described protocol (Santos et al., 2010). Briefly, 3 g of tissue were homogenized and decontaminated for 2 h with hexa-decylpyridinium chloride 0.75 %, centrifuged at 2,566 g for 30 min and the supernatant collected. Two tubes with Coletsos medium were inoculated with 250 µl of the supernantant-sediment interface and incubated at 37 °C for 15 weeks. Isolates were identified by PCR for genes 16SRNA, IS1561 and Rv1510 (Huard *et al.*, 2003) after DNA extraction by standard phenol-chloroform method with bead-beating. This panel allows identifying isolates as *M. bovis*, *M. caprae*, *M. microti*, other MTC or other mycobacteria.

Data analysis

We chose administrative divisions (county) as geographical unit at the national scale as this was the smallest areal unit for which all data was available. Samples were obtained from 92 out of 278 counties in continental Portugal (Fig. 1B) nevertheless the low sample size in many counties (range 1-57) prompted us to analyze bTB presence as a binomial variable rather than prevalence. Choropleth maps of bTB presence were produced in QGIS 2.6.1 Brighton software (QGIS Development Team). The area considered for each county was “natural terrestrial area”, i.e. area not considered as “urban” or “water bodies” in the CORINE database (EEA, 2006).

Spatial-only cluster analysis was performed based on Kuldorff's spatial scan statistics, using Bernoulli distribution and setting maximum cluster size at 50 %, with software SatScan™ 9.3.1 (Kulldorff, 1997).

The association between bTB presence in wild boar in each county and independent variables related to wild and domestic host densities, bTB incidence in domestic cattle, historical population dynamics of wild boar and intensity of hunting management (Table 1) was assessed by spatial generalized linear mixed modeling of areal data with the conditional autoregressive (CAR) priors proposed by Leroux et al. (1999). The model was implemented with CARBayes 4.3 package (Lee, 2013) in R (R Development Core Team, 2015). Inference was based on 8,000 Markov chain Monte Carlo iterations (80,000 iterations with thin=10) after 20,000 iterations as burn-in. Taking into consideration the home ranges of wild boar (Bosch et al., 2012), counties up to 25 km apart were included in each other's neighborhood matrix.

Domestic host densities (animals/km^2) were calculated based on data from the national animal movement database (SNIRA) with the following constraints: only animals over 6 months of age; free-range pigs; extensively reared sheep and goats; and meat production cattle were considered. This was intended to provide more realistic estimates of the domestic population with potential epidemiologically-effective contacts with wildlife, excluding intensively reared animals. Pigs, sheep and goats are explicitly indicated as extensively- or intensively-reared in the SNIRA database, while meat-production cattle was selected as a proxy for extensive rearing as this is the dominant rearing system for such cattle in Portugal, while being almost absent for dairy cattle.

Hunting bag (as hunted animals/km^2) was selected as a proxy for wild host density, based on data from the hunting statistics provided by the national hunting authority (*Instituto de Conservação da Natureza e Florestas* - ICNF I.P.). Data from 2008-2012 was considered, as the annual average of the hunting bag from those hunting areas for which at least 2 years of data were available. These statistics were available for approximately 40 % of the hunting areas that exist in Portugal and were computed as: average number of animals hunted per county per year/area of all the hunting areas for which data was available per county, and extrapolated to the whole area of each county. Wild hosts considered in the analysis were wild boar, red deer and fallow deer. This data was available for 77% of continental Portuguese counties; in order to fill gaps in data we performed a cubic spline interpolation of these variables to obtain estimates

for the whole territory and then assigned the median of the county area to those counties with missing data (n=65).

The proportion of "touristic" and "national" hunting areas in each county was selected as a proxy for the intensity of management, as these types of areas are dedicated to commercial hunting (i.e. usually intensively managed for maximizing profit), while other types of hunting areas ("associative" and "municipal") are predominantly dedicated to the social components of hunting activity (i.e. usually no fencing, restocking or large-scale artificial feeding of large game species is performed). Historical population dynamics of wild boar were included as a binary variable computed as each county being included or not in one of the historical refuges of wild boar (Gerês, Montesinho, Malcata and São Mamede mountains and the left bank of Guadiana River) (Fig. 1A), as described by Lopes and Borges (2004) and Ferreira et al. (2008). Incidence of bTB in cattle was determined as the average of each county's annual incidence from 2008-2012. Incidence was calculated as the number of cattle reacting to comparative intradermal tuberculin testing/number of cattle tested, based on data from DGAV.

Type of variable	**Variable**	**Unit**
Wild host density	Wild boar hunting bag	Wild boar hunted/km^2
	Red deer hunting bag	Red deer hunted/km^2
	Fallow deer hunting bag	Fallow deer hunted/km^2
Intensity of management	Proportion of "touristic" and "national" hunting areas	% (national analysis) Binomial (regional analysis)
	Cattle density >6 month age, meat	Cattle/km^2
Domestic host density	Sheep density >6 month age, extensive	Sheep/km^2
	Goat density >6 month age, extensive	Goats/km^2
	Pig density >6 month age, free-range	Pigs/km^2
bTB in cattle	bTB incidence in cattle	%
Historical population dynamics	County within an historical refuge	Binomial

Table 1 – Independent variables entered in the models at national and regional levels.

The best fit model was chosen based on Akaike's Information Criterion (AIC, non-spatial model) and Deviance Information Criterion (DIC, spatial model) (Hooten and Hobbs, 2015) and was used to calculate the risk of bTB being present in wild boar populations in all mainland Portuguese counties.

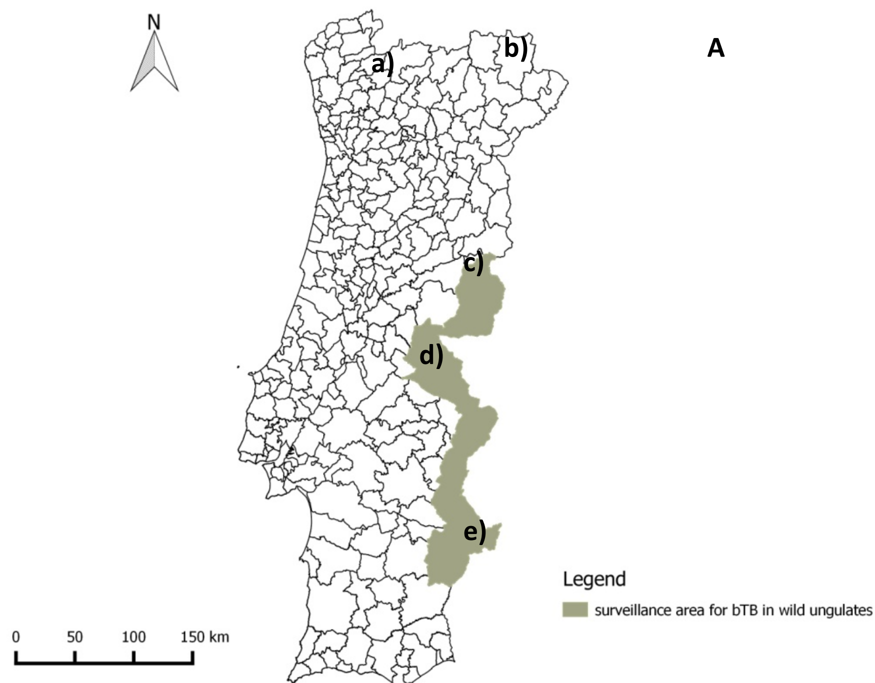
The same analysis was performed at the regional level, except that the geographical unit in this case was the hunting area and consequently the intensity of management was measured as a binomial variable (being or not a “touristic” or “national” hunting area). Also parish livestock densities were assigned to hunting areas from each parish, while county bTB incidence in cattle was assigned to hunting areas from each county.

Kappa statistic was computed to determine the agreement between ELISA results, expressed as a binary outcome (positive/negative), from paired serum and elute samples.

Results

Paired serum and PS elutes were tested by ELISA to assess the agreement between both types of samples. Thirteen paired samples were positive for anti-bPPD antibodies, 7 were negative and 2 were positive in serum and negative in elutes. This corresponds to an almost perfect agreement between serology results for both types of samples (Kappa = 0.805 ± 0.129).

In the serological survey at national scale, anti-bPPD antibodies were detected in 26/700 wild boar (3.7 %, CI_{95} 2.5–5.4 %). Positive wild boar originated from 4/92 counties tested (Fig. 1C).



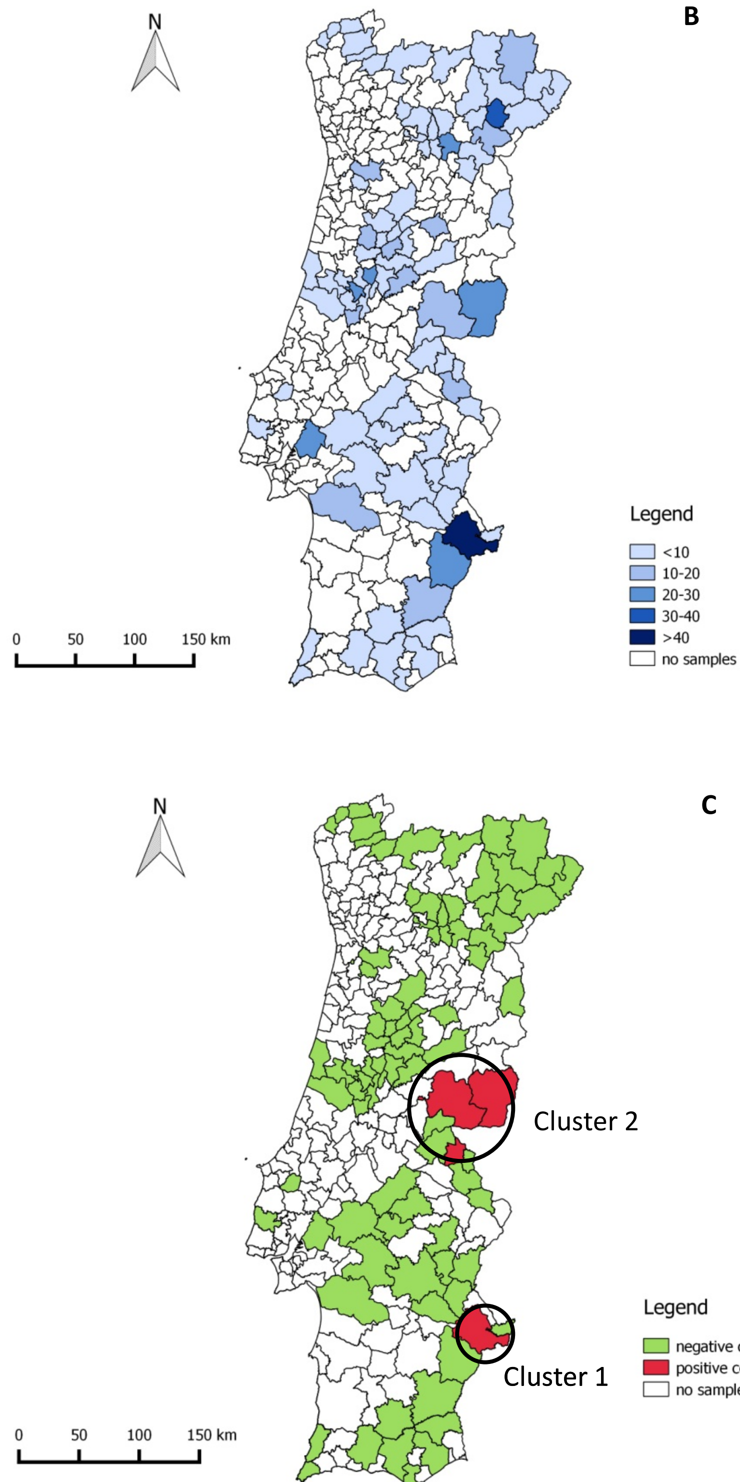


Fig 1 – Choropleth maps of the sample size and results of the serological survey, official surveillance area for bovine tuberculosis large game species and historical refuges of wild boar. (A) Official surveillance area for bTB in large game species established by DGAV (in grey) with the historical population refuges of wild ungulates identified: a) Gerês, b) Montesinho, c) Malcata, d) São Mamede, e) left bank of Guadiana river; (B) Map of the number of serological samples obtained per county; (C) Map of counties with positive results (in red), only negative results (green) and clusters identified (black circles).

Kulldorff's spatial scan statistics identified 2 wildlife bTB clusters, cluster 1 including the county of Moura (17/57 positive samples, $p<0.001$, RR=21.3) in the southeast; and cluster 2 including 8 counties in the central-east (Idanha a Nova, Castelo Branco, Castelo de Vide, Nisa, Vila Velha de Rodão, Fundão, Proença a Nova, Oleiros) (9/66 positive samples, $p=0.106$, RR=5.1) (Fig. 1).

Residuals from the non-spatial logistic regression model with bTB presence as dependent variable (AIC=25.762) showed significant spatial autocorrelation (Moran's I=0.062, $p=0.048$). The CAR model selected (DIC=26.625, p.d.=3.564) included the variables "historical refuge", "red deer hunting bag", "management" and "red deer hunting bag × management" (Table 2).

Variable	**Logistic regression model**			**Conditional Autoregressive model**	
	Coefficient	**CI_{95}**	**p**	**Median coefficient**	**CI_{95}**
County within historical refuge	5.741	2.095 - 12.657	0.020	4.971	1.858 - 10.203
Red deer hunting bag	3.299	0.406 - 7.550	0.045	2.615	-0.040 - 5.947
Management intensity	11.669	-0.031 - 29.861	0.087	8.411	-1.748 - 21.286
Red deer hunting bag × Management intensity	-8.112	-19.559 - -0.848	0.069	-6.426	-16.467 - -0.287

Table 2 – Logistic regression and conditional autoregressive models of bTB presence in wild boar at national scale.

Based on this model, probability of bTB being present in wildlife was computed for each county (Figure 2). The probability of wildlife bTB is significantly higher on the counties with independent reports of *M. bovis* isolation from wildlife than on the other counties ($p<0.001$, Mann-Whitney U test).

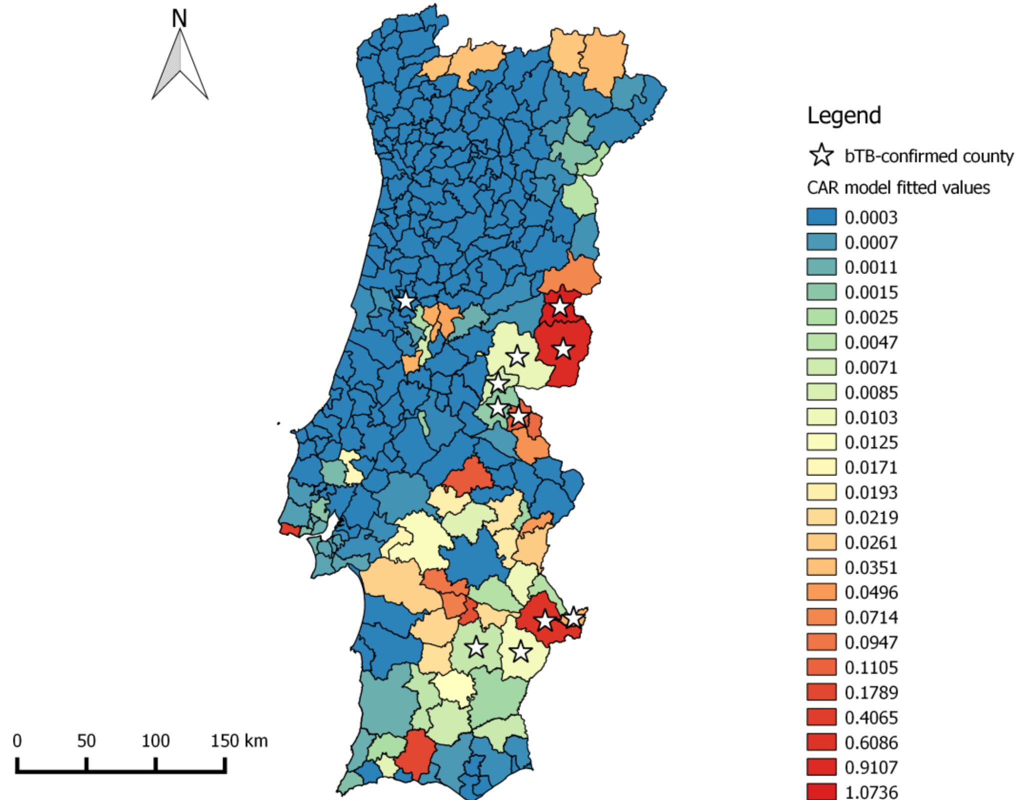


Fig 2 – Choropleth map of bTB risk in wildlife in continental Portugal. Probability of bTB being present in wildlife in mainland Portugal based on the CAR model fitted values. Counties with *M. bovis* isolation from wildlife highlighted (white star), from published data (Santos et al., 2009; Raposo et al., 2011; Vieira-Pinto et al., 2011; Cunha et al., 2012; Matos et al., 2014).

Bovine tuberculosis distribution was investigated in and around cluster 1, including the counties of Mourão, Moura, Barrancos, Serpa and part of Mértola. *M. bovis* (n=51) and *M. caprae* (n=2) were isolated from wild boar from 6 hunting areas, with an overall prevalence of 15.6 % (CI_{95} 12.1-19.8 %) (Fig 4 and Table 3).

Bovine tuberculosis prevalence was significantly higher in subadult wild boar (p=0.023, Fisher's exact test) (Fig. 3A), while no significant differences were found regarding gender. Wild boar from hunting area J showed a significant increase in prevalence (p=0.049, Fisher's exact test) since a previous study in 2005-06 (Santos et al., 2009) (Fig. 3B).

Hunting area	Positive/total tested	Prevalence (CI_{95})
A	1/36	2.8 (0.5-14.2)
B	0/16	0 (0-19.4)
C	0/23	0 (0-14.3)
D	0/17	0 (0-18.4)
E	0/3	0 (0-56.2)
F	3/14	21.4 (7.6-47.6)
G	0/16	0 (0-19.4)
H	0/6	0 (0-39.0)
I	0/3	0 (0-56.2)
J	42/62	67.7 (55.4-78.0)
K	0/23	0 (0-14.3)
L	3/16	18.8 (6.6-43.0)
M	1/63	1.6 (0.3-8.5)
N	3/12	25.0 (8.9-53.2)
O	0/9	0 (0-29.9)
P	0/11	0 (0-25.9)
Q	0/10	0 (0-27.8)
Total	53/340	15.6 (12.1-19.8)

Table 3 – Bacteriological culture results from 18 hunting areas in and around cluster 1. In hunting area I *M. bovis* was isolated from 3/12 red deer, although none from 3 wild boar tested.

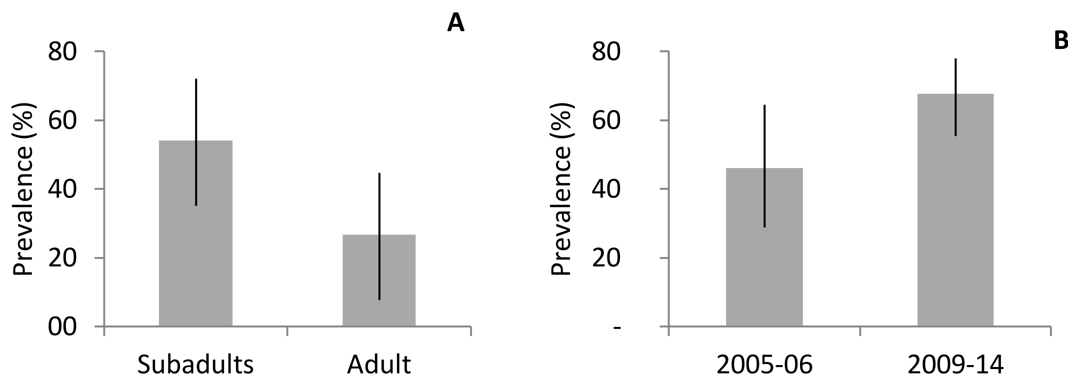


Fig 3 – Bovine tuberculosis prevalence in wild boar by bacteriological culture. (A) Prevalence and 95 % confidence intervals for wild boar from hunting areas where *M. bovis* or *M. caprae* were isolated, by age classes. (B) Wild boar with an estimated age less than 2 years old were considered subadults. Comparison of bTB prevalence by culture in hunting area J, previously sampled in 2005-06 (Santos et al., 2009).

Kulldorff's spatial scan statistics identified one wildlife bTB cluster including hunting area J (42 positive wild boar, p<0.001, RR=17.1) (Fig. 4).

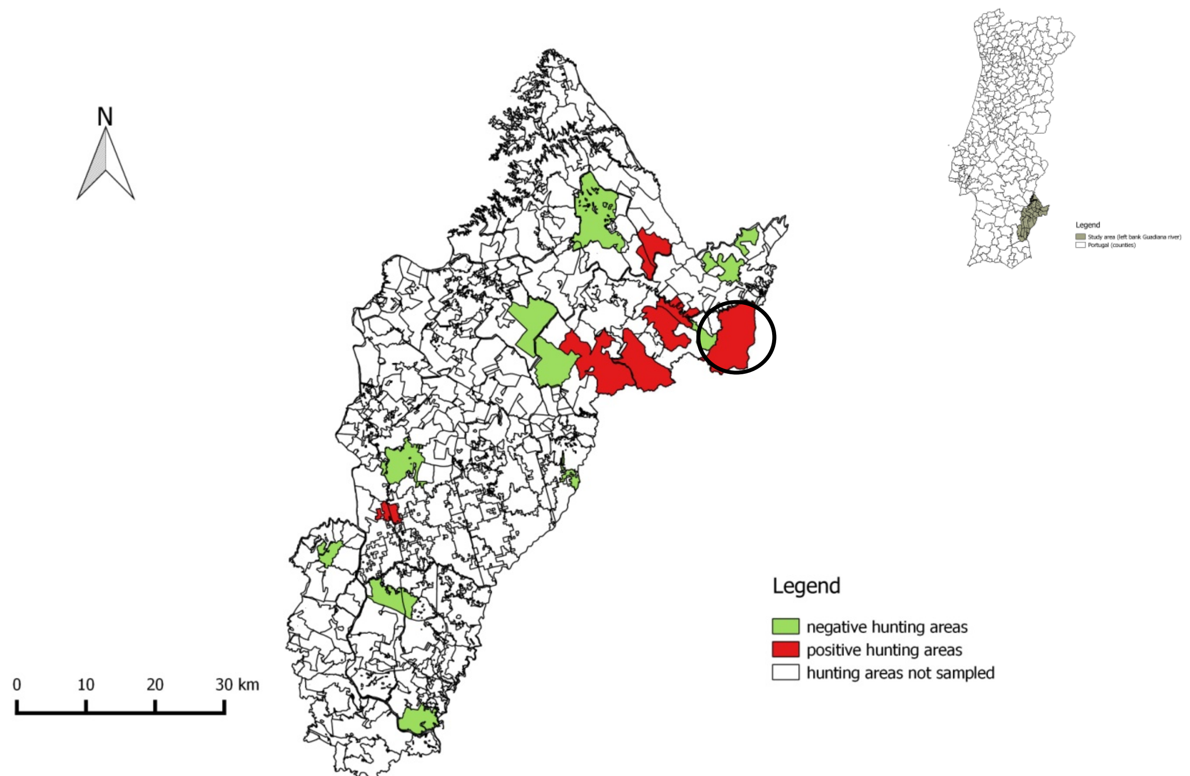


Fig 4 – Choropleth map of the results from the bacteriological survey on the left bank of the Guadiana River. Choropleth map of the hunting areas with positive samples (in red), only negative samples (green) and the cluster identified (black circle). Insert depicts the location of the study area in Portugal.

Only hunting areas with >10 wild boar sampled were considered for model inference. Hunting area I was considered infected because we isolated *M. bovis* from 3 red deer (data not shown), although none from 3 wild boar. Residuals from the non-spatial generalized linear model with bTB prevalence as dependent variable (AIC=96.281) showed no spatial autocorrelation (Moran's I=-0.158, p=0.850). The best CAR model (DIC=96.726, p.d.=4.771) included the variables "historical refuge", "red deer hunting bag", "fallow deer hunting bag" and "red deer hunting bag × historical refuge" (Table 4).

Variable	Logistic regression model			Conditional Autoregressive model	
	Coefficient	CI_{95}	p	Median coefficient	CI_{95}
Fallow deer hunting bag	-188.086	-264.597 - -111.576	0.002	-152.240	-222.529 - -54.535
Wild boar historical refuges	-4.603	-20.885 - 11.679	0.597	-0.875	-17.235 - 18.593
Red deer hunting bag	-0.678	-15.262 - 13.905	0.930	0.846	-12.569 - 15.454
Red deer hunting bag × Wild boar historical refuges	17.519	1.569 - 33.469	0.068	13.086	-3.774 - 27.428

Table 4 – Non-spatial generalized linear mixed and conditional autoregressive models of bTB presence in wild boar at regional scale.

Based on this model, the probability of bTB being present in wildlife was computed for each hunting area (Figure 5). The probability of wildlife bTB is significantly higher on the hunting areas with independent reports of *M. bovis* isolation from wildlife than on the other hunting areas (p=0.013, Mann-Whitney U test).

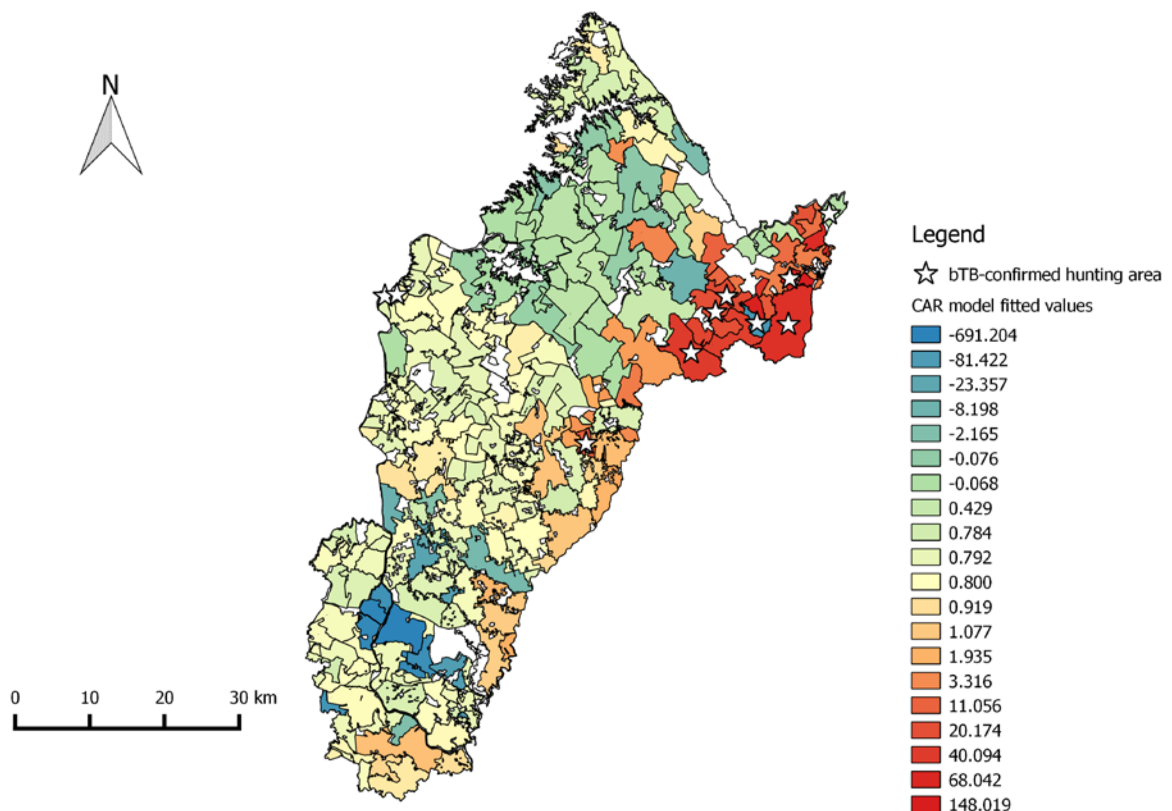


Fig 5 – Map of the risk of bTB in wildlife in the left bank of the Guadiana River. Probability of bTB being present in wildlife in the left bank of the Guadiana River based on the CAR model fitted values. Hunting areas with *M. bovis* isolation from wildlife highlighted (white star), based on official surveillance of large game species.

Discussion

We report here for the first time a spatial analysis of wildlife bTB in mainland Portugal at national and regional geographical scales. At national scale the analysis was based upon serological data from hunted wild boar. The serological method used was previously shown to present reasonably good sensitivity and excellent specificity (Boadella et al., 2011a), while wild boar was shown to be a suitable sentinel species for bTB in the ecosystem (Nugent et al., 2002). Also we relied on blood samples collect by non-specialized personnel and stored dried at room temperature in absorbent paper. This technique was previously validated for other hosts and pathogens (e.g. Curry et al., 2014), and we were able to show its suitability for bTB serological surveys, with an almost perfect agreement (Kappa=0.0805) between serology data from serum and elutes.

Our results confirm previous data suggesting a strong spatial structure of wildlife bTB in Iberian Peninsula (Santos et al., 2012). A cluster is a group of disease events located close together in space, which can be detected and the associated probability calculated using the spatial scan statistic (Kulldorff and Nagarwalla, 1995). In mainland Portugal 2 wildlife bTB clusters were identified in south- and central-easternmost regions, located at the periphery of the high-prevalence core area previously described in central-southwestern Iberian Peninsula (Santos et al., 2012).

At the regional level our survey for bTB in wild boar relied on bacteriological culture from tissues of hunted animals, a more specialized labour-intensive technique. Our data allowed for the confirmation of bTB as an emerging disease in wildlife, documenting a 47 % increase in prevalence from 2005-06 to 2009-14. An increase in prevalence and distribution area has also been reported in Spain (Vicente et al., 2013).

We were further able to obtain geographical models of bTB risk in wildlife, at national and regional scales. Both models generally agree with independent assessments of bTB distribution in wild hosts, with almost all reported isolates of *M. bovis* in Portugal falling inside moderate to high risk areas. One notable exception is the *M. bovis* isolated from a wild boar in Coimbra, central-western Portugal (Fig. 2), reported by Cunha et al. (2012). Interestingly, in this region red deer were reintroduced in 1995-99, with some of the founders coming from bTB-infected areas included in cluster 1 (Salazar, 2009). No more *M. bovis* isolates were reported from this region since then, suggesting that after introduction and initial spillover to local wild boar, the infection

faded out. This is consistent with threshold densities in wildlife disease (Lloyd-Smith et al., 2005) and suggests that relatively dense red deer populations are needed to maintain bTB in wild ungulate populations in the absence of intensive management. It is noteworthy that *M. bovis* has not been isolated in wildlife in Portugal from regions where red deer is absent, further strengthening the key role of this species in bTB maintenance in wildlife in Portugal. In south-central Spain the wild boar is considered the main driver of bTB (Gortázar et al., 2012). This difference could be caused by differences in hunting management between these countries. In fact, while intensive hunting management is rare and localized in Portugal, as also described in Atlantic Spain (Vingada et al., 2010; Gortázar et al., 2011), it is common in central southwestern Spain (Vicente et al., 2013).

Risk models are essential tools for risk-based disease surveillance, allowing targeting efforts to geographical areas where disease is most likely to be present and pose a risk to human and animal health (Ostfeld et al., 2005; Stärk et al., 2006). The ultimate goal of these models is to generate a value that represents the risk in any given area as a function of the risk variables. Spatial risk models are useful to describe diseases with low infection rates or slow epidemic fronts, such as bTB (Conner et al., 2007). Although the official high-risk area for bTB surveillance in large game species in Portugal (Fig. 1A) encompasses most of the predicted high-risk areas that our models identify, it misses some regions at the periphery and includes some areas where risk is predicted to be minimal. These minimal risk areas are support by very low wild boar hunting bags coinciding with open agricultural areas, so the models we describe could be used for better allocating resources on wildlife bTB surveillance and public health protection.

Another modeling goal is to link the spatial structure of wildlife populations and the spatial variability in abiotic and biotic attributes of their environment with disease transmission dynamics. Statistical approaches seek correlations between environmental conditions and the distribution of disease (Lawson, 2001). In this study we opted to include variables related to host population density, bTB incidence in cattle, historical population dynamics and intensity of management of wild ungulates, as these were previously shown to correlate with bTB presence (Santos et al., 2012).

At national scale, red deer density and its interaction with hunting management intensity were positively correlated with bTB presence in wild boar. Red deer density was previously shown to be

positively associated with bTB prevalence in wild boar (Santos et al., 2012). As maintenance host for bTB, it is expected that density-dependence exists between host density and disease presence (Lloyd-smith et al., 2005; Nugent, 2011). Nevertheless, it is noteworthy that red deer density is a better predictor of bTB presence in wild boar populations than wild boar density, further strengthening the role of red deer as a key reservoir of wildlife bTB in Portugal. Also intensification of management for hunting purposes, including the elimination of large predators, fencing, restocking and provision of food and water, all lead to artificially high densities and aggregation (Delibes-Mateos et al., 2009), which were previously shown to be risk factors for wildlife bTB (Santos et al., 2012).

Interestingly, the historical population dynamics of wild boar influences bTB distribution nowadays, which agrees with this disease being of slow spread (Conner et al., 2007). Starting in mid-XXth century, wild boar populations expanded from the abovementioned historical refuges and so wildlife bTB seems to be expanding but at a much slower pace. It is expected that as wild boar populations expanded, densities at the front of the expansion wave were low (Holland et al., 2007), so bTB could not be maintained even with the recruitment of infected animals from the high-density historical refuges (Santos et al., 2009). As expansion continued and wild boar densities became high at the periphery of the core area (Holland et al., 2007), the range of *M. bovis* also increased.

Interestingly, at regional scale fallow deer density seem to become a protective factor, as it is strongly negatively correlated with bTB prevalence in wild boar. This is compatible with fallow deer acting as a dilution host (Huang et al., 2013), but would imply that this species is not an efficient transmitter of *M. bovis*. Information available on the pathology of bTB in this species (e.g. Martín-Hernando et al., 2010) does not support such assumption; nevertheless MTC shedding as well as the ecological factors that influence disease transmission have not been studied in this species. Also this observed effect could be a sampling bias due to particular aspects of fallow deer distribution in this cluster. Further study on the role of fallow deer in bTB epidemiology is warranted.

At regional scale the intensity of management is not significantly correlated with bTB prevalence. This could be a scale effect as at larger scales bTB tends to be present where the management intensity is high, but on a particular bTB cluster the disease spreads to hunting areas in the

vicinity of the cluster regardless of the intensity of management of each particular hunting area. This stresses that it is critical to prevent further geographical spread of bTB in wild ungulate populations and the creation of new disease clusters, particularly by the translocation of infected ungulates.

Summarizing, we report a spatial analysis of wildlife bTB in Portugal using the wild boar as sentinel species and validate the use of blood collected in absorbent papers as a new tool for large-scale serological surveys of bTB. We confirm the strong spatial structuring of wildlife bTB and identify risk factors related to red deer density, intensity of management and historical population dynamics of wild boar. The risk maps obtained provide new tools for the targeted control of bTB in wild ungulate populations. As diseases do not recognize political borders, a spatial analysis encompassing the whole Iberian Peninsula would be of great interest for scientists and authorities involved in disease management.

Acknowledgements

The authors acknowledge the collaboration of the hunters who collected blood samples from wild boar. *Direcção Geral de Alimentação e Veterinária* (DGAV) kindly provided data on population of domestic hosts (thanks to Eng Abílio Dias), bTB incidence in cattle and isolation of MTC from large game species in the left bank of Guadiana river. *Instituto de Conservação da Natureza e Florestas* (ICNF I.P.) kindly provided data on hunting bag of wild ungulates. Nuno Santos benefited from PhD grant SFRH/BD/69390/2010 from *Fundação para a Ciência e Tecnologia* (FCT).

References

Acevedo P et al. (2006) Factors affecting wild boar abundance across an environmental gradient in Spain. Acta Theriol, 51:327–336.

Aurtenetxe O et al. (2008) Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium bovis* in European wild boar. BMC Vet Res, 4: 43. doi: 10.1186/1746-6148-4-43

Bergquist R and Rinaldi L (2010) Health research based on geospatial tools: a timely approach in a changing environment. J Helminthol, 84: 1–11.

Boadella M et al. (2011a) Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium* subspecies *paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). J Vet Diag Invest, 23: 77-83.

Boadella M et al. (2011b) Spatio-temporal trends of Iberian wild boar contact with *Mycobacterium tuberculosis* complex detected by ELISA. EcoHealth, 8(4): 478-484. doi: 10.1007/s10393-011-0713-y

Bosch J et al. (2012) Distribution, abundance and density of the wild boar on the Iberian Peninsula, based on the CORINE program and hunting statistics. Folia Zool, 61(2): 138–151.

Brosch R et al. (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Nat Acad Sci, 99(6): 3684–3689.

Buruaga M et al. (2001) Reconocimiento de sexo y edad en espécies cinegéticas. Edilesa, León, Spain, 127 pp.

Conner MM et al. (2007) Scale-dependent approaches to modeling spatial epidemiology of chronic wasting disease. Utah Division of Wildlife Resources, Special Report, p. 68.

Corner LAL (2006) The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Vet Microbiol, 112: 303–312.

Cunha MV et al. (2012) Implications and challenges of tuberculosis in wildlife ungulates in Portugal: a molecular epidemiology perspective. Res Vet Sci, 92(2): 225-235.

Curry PS et al. (2014) Blood collected on filter paper for wildlife serology: detecting antibodies to neospora caninum, west nile Virus, and five bovine viruses in reindeer. J Wildl Dis, 50(2): 297–307.

Delibes-Mateos M et al. (2009) Long-term changes in game species over a long period of transformation in the Iberian Mediterranean landscape. Environ Manage, 43(6): 1256-1268.

DGAV (2015). *Relatório técnico de sanidade animal: tuberculose bovina, 2014*. Lisboa, Accessed December 2015. http://www.dgv.min-agricultura.pt/portal/page/portal/DGV/genericos?actualmenu= 8961117&generico=166927&cboui=166927.

EEA (2006) NATLAN. Nature/Land Cover Information Package. European Environment Agency, Luxemburg.

Ferreira E et al. (2008) Genetic structure of the wild boar population in Portugal: evidence of a recent bottleneck. Mamm Biol, 74(4): 274-285. doi: 10.1016/j.mambio.2008.05.009.

Gilbert AT et al. (2013) Deciphering serology to understand the ecology of infectious diseases in wildlife. EcoHealth. doi: 10.1007/s10393-013-0856-0

Gortázar C et al. (2011) Progress in the control of bovine tuberculosis in Spanish wildlife. Vet Microbiol, 151: 170-178.

Gortázar C et al. (2012) The status of tuberculosis in European wild mammals. Mammal Rev, 42(3): 193-206.

Holland E et al. (2007) Spatial sensitivity of a generic population model, using wild boar (*Sus scrofa*) as a test case. Ecol Model, 205: 146–158.

Hooten MB and Hobbs NT (2015) A guide to bayesian model selection for ecologists. Ecol Monog, 85(1): 3-28.

Huang ZY, de Boer WF, van Langevelde F, Xu C, Jebara KB, Berlingieri F, Prins HH (2013) Dilution effect in bovine tuberculosis: risk factors for regional disease occurrence in Africa. Proc Royal Soc B: Biol Sci, 280(1765): 20130624.

Huard R et al. (2003) PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J Clin Microbiol, 41: 1637–1650.

Kulldorff M and Nagarwalla N (1995) Spatial disease clusters: detection and inference. Statist Med, 14: 799-810.

Kulldorff M (1997) A spatial scan statistic. Commun Statistics — Theory Meth, 26(6): 1481–1496.

Lawson AB (2001) Disease map reconstruction. Stat Med, 20(14): 2183-2204.

Lee D (2013) CARBayes: an R package for Bayesian spatial modeling with conditional autoregressive priors. J Stat Soft, 55(13): 1-24.

Leroux B, Lei X, Breslow N (1999). Estimation of disease rates in small areas: a new mixed model for spatial dependence. In Halloran M and Berry D (eds) Statistical models in epidemiology, the environment and clinical trials. Springer-Verlag, New York, pp. 135-178.

Lloyd-Smith J et al. (2005) Should we expect population thresholds for wildlife disease? Trends Ecol Evol, 20: 511–520.

Lopes F and Borges J (2004) Wild boar in Portugal. *Galemys*, 16(special number):243–251.

Martín-Hernando MP, Torres MJ, Aznar J, Negro JJ, Gandía A, Gortázar C (2010) Distribution of lesions in red and fallow deer naturally infected with *Mycobacterium bovis*. J Comp Pathol, 142(1): 43-50.

Matos AC et al. (2014) New insights into *Mycobacterium bovis* prevalence in wild mammals in Portugal. Transb Emerg Dis, . doi: 10.1111/tbed.12306

Matschke G (1967) Aging European wild hogs by dentition. J Wildl Manag, 31: 109–113.

Muñoz-Mendoza M et al. (2013) Wild boar tuberculosis in Iberian Atlantic Spain: a different picture from Mediterranean habitats. BMC Vet Res, 9:176. doi: http://www.biomedcentral.com/1746-6148/9/176

Naranjo V et al. (2008) Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex. Vet Microbiol, 127: 1–9.

Nielsen C et al. (1987) Detection of HIV antigens in eluates from whole blood collected on filter paper. Lancet, 329(8532): 566-567.

Norman SA (2008) Spatial Epidemiology and GIS in Marine Mammal Conservation Medicine and Disease Research. EcoHealth, 5: 257–267.

Nugent G et al. (2002) Use of released pigs as sentinels for *Mycobacterium bovis*. J Wildl Dis, 38(4): 665–677.

Nugent G (2011) Maintenance, spillover and spillback transmission of bovine tuberculosis in multi-host wildlife complexes: A New Zealand case study. Vet Microbiol, 151: 34-42.

Ostfeld RS et al. (2005) Spatial epidemiology: an emerging (or re-emerging) discipline. Trends Ecol Evol, 20(6): 328-336.

Portejoie Y et al. (2009) Investigation of specific European Brown Hare Syndrome antibodies in wild hares using blood samples dried on blotting paper. Eur J Wildl Res, 55: 53–58.

Raposo ASS (2011) Contributo para o estudo epidemiológico da tuberculose bovina em animais domésticos e silváticos na região de Portalegre. MSc Thesis, Faculty of Veterinary Medicine, Lisbon University, p. 56.

Salazar D (2009) Distribuição e estatuto do veado e corço em Portugal. MSc Thesis, University of Aveiro, 101 pp.

Santos N et al. (2009) Epidemiology of *Mycobacterium bovis* infection in wild boar *Sus scrofa* from Portugal. J Wildl Dis, 45(4): 1048-1061.

Santos N et al. (2010) Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): a comparison of methods applicable to hunter-harvested animals. PLoS ONE, 5(9): e12663.

Santos N et al. (2012) Wildlife tuberculosis: a systematic review of the epidemiology in Iberian Peninsula. In Cunha MLRS, editor. Epidemiology Insights. Rijeka: InTech; 2012. http://www.intechopen.com/books/epidemiology-insights/wildlife-tuberculosis-a-systematic-review-of-the-epidemiology-in-the-iberian-peninsula

Stärk KD et al. (2006) Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: review of current approaches. BMC Health Serv Res, 6(1): 20. doi: 10.1186/1472-6963-6-20

Vicente J et al. (2013) Temporal trend of tuberculosis in wild ungulates from Mediterranean Spain. Transbound Emerg Dis, 60(1):92–103.

Vieira-Pinto M et al. (2011) Combined evaluation of bovine tuberculosis in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) from Central-East Portugal. Eur J Wildl Res, 57: 1189–1201.

Vingada J et al. (2010) Ungulates and their management in Portugal. *In*: Appolonio M, Andersen R, Putman R (eds.) European ungulates and their management in the 21st century. Cambridge University Press, Cambridge, p. 392-418.

Yockney IJ et al. (2013) Comparison of ranging behaviour in a multi-species complex of free-ranging hosts of bovine tuberculosis in relation to their use as disease sentinels. Epid Infect, 141(7): 1407-1416.

Chapter V – General discussion and final conclusions

1. General discussion

Wildlife epidemiological research tends to deal with single-host single-pathogen systems, in which disease persistence depends solely on the intra-species transmission rate (Tompkins *et al.*, 2001). By contrast, in multi-host pathogens systems such as the bTB-wild-domestic ungulates system of Iberian Peninsula, disease persistence is dependent on both intra- and inter-species transmission rates (Renwick *et al.*, 2007; Nugent, 2011; Gortázar et al., 2012). Moreover, these rates depend on pathological, epidemiological, ecological and behavioral factors (Corner, 2006). In such a complex epidemiological setting, it is imperative to determine the role of each host species in pathogen persistence before comprehensive control measures are undertaken (Calley et al., 2009; Nugent, 2011).

1.1. Systematic review of the bibliography

With this thesis we aimed to contribute to elucidate the epidemiological role of wild ungulates and the mechanisms of bTB transmission in wildlife in Iberian Peninsula. As a first step, we performed a systematic bibliographic review on wildlife bTB epidemiology in Iberian Peninsula in order to understand the mechanisms underlying bTB persistence in multi-host ecosystems (presented in Chapter I).

From the above-mentioned review it is evident that the knowledge available on bTB in wildlife is considerable, particularly regarding pathology (e.g. Martín-Hernando et al., 2007; 2010; García-Jiménez et al., 2013b), descriptive epidemiology (e.g. Vicente et al., 2006a; Gortázar et al., 2008; Santos et al., 2009), molecular epidemiology (e.g. Cunha et al., 2011; Gortázar et al., 2011b) and identification of risk factors (e.g. Vicente et al., 2006b; Gortázar et al., 2008; Santos et al., 2009; Vicente et al., 2013). Briefly, published evidence suggests that bTB is a natural disease of autochthonous wild ungulates in Iberian Peninsula, where at least wild boar and red deer act as maintenance hosts. Several other species of ungulates and carnivores are affected by bTB, most probably as spillover hosts, although fallow deer and badger could also be maintenance hosts in particular conditions and locations. Bovine tuberculosis is an emergent disease in wild boar and red deer, expanding from a core high-prevalence area in south-western Iberian Peninsula, fuelled by high host densities. Such artificially high densities result from the

intensive management for hunting purposes, including interventions such as removal of predators, fencing, translocation, artificial provision of food and water and even medication. Although bTB is known to be an important emerging disease in wildlife, large gaps remain in the knowledge of its epidemiology. Among these stand out the intra- and inter-species transmission routes, geographical analysis of disease and effectiveness of control methods.

1.2. *Mycobacterium tuberculosis* complex excretion

Since this systematic review was performed, considerable efforts were directed to investigate management actions aimed at controlling bTB in wildlife populations, including vaccination (e.g. Béltran-Beck et al., 2012; Gortázar et al., 2014), culling (e.g. Boadella et al., 2011; García-Jiménez et al., 2013a; Mentaberre et al., 2014) and biosecurity strategies (e.g. Barasona et al., 2013; Kukielka et al., 2013). Nevertheless to progress from knowledge to bTB control in wildlife populations, it is essential to have a thorough understanding of host and environmental factors that influence disease transmission and maintenance in these populations (Corner, 2006; Nugent, 2011). Among this essential epidemiological information are the routes of infection, routes and doses of excretion and minimum infective doses (Corner, 2006; Nugent, 2011). Although the understanding of the bTB excretion routes and doses is critical for defining control strategies for wild reservoirs, few solid data is available on this subject (Calley et al., 2009). One of the aims of this thesis was thus to investigate MTC excretion routes from naturally-infected wild ungulates (Chapter II).

We report for the first time the detection of MTC excretion in 83.0 % (CI_{95} 70.8–90.8 %) of naturally-infected wild boar and red deer. MTC DNA was amplified in all types of excretion routes (oronasal, bronchial-alveolar, fecal and urinary). MTC concentrations greater than the minimum infective doses for cattle, red deer or wild boar were estimated in excretion routes from naturally-infected wild boar and red deer. Also for the first time we provide evidence for the existence of a proportion of super-shedders within the naturally-infected populations of these host species (28.2 % of wild boar, CI_{95} 16.6–43.8 %; and 35.7 % of red deer, CI_{95} 16.3–61.2 %). These super-shedders are responsible for a disproportionately large amount of MTC excretion from infected wild ungulates.

These results have implications for the design of control programs in these species. In fact, selective culling targeting super-shedder individuals would be a more effective way to reduce MTC transmission than indiscriminate reduction of host density (Carter et al., 2009). Nevertheless this approach requires the previous identification of super-shedder correlates, allowing targeting in a more or less specific way this subclass of the infected population (Carter et al., 2009).

1.3. Environmental contamination with *Mycobacterium tuberculosis* complex

Transmission of *M. bovis* can occur by direct or indirect routes (Phillips et al., 2003; Humblet et al., 2009). As for bTB, direct transmission requires close contact between infected excretors and susceptible hosts (Corner, 2006). Therefore, this route is expected to play a major role in intra-specific transmission of infection, as such close contact is common among individuals of the same species (Kukielka et al., 2013; Cowie et al., 2015). However, close contact between individuals of different species seems to be rare (Corner, 2006; Kukielka et al., 2013; Cowie et al., 2015) and so indirect routes are expected to play an essential role in inter-specific transmission. Indirect routes of transmission require the contamination of the environment with viable mycobacteria (Phillips et al., 2003; Adams et al., 2013). Although indirect transmission of *M. bovis* was shown to be rare in cattle grazing infected pasture (Humblet et al., 2009), it is strongly suspected to play a major role in the white-tailed deer-cattle system of North America, where it has been experimentally achieved through contaminated feed (Palmer et al., 2015). It is also suspected to occur in other wildlife-cattle systems, including the domestic-wild ungulates system of Iberian Peninsula (Kukielka et al., 2013).

The lack of clear data on environmental contamination with MTC is mainly due to the lack of sensitive and mass-scalable techniques to detect MTC in the environment (Humblet et al., 2009; Kaneene et al., 2010; Adams et al., 2013). Molecular techniques show a greater promise over bacteriological techniques to overcome these constraints (Adams et al., 2013), nevertheless available protocols have exceedingly high detection limits, possibly due to the uneven distribution of mycobacteria and the co-extraction of PCR inhibitors (Young et al., 2014).

We explore the real-life model of the multi-host pathogen system of Iberian Peninsula to assess the occurrence of environmental contamination with MTC at the interface between wild and domestic ungulates (Chapter III).

This study describes an improved protocol for the molecular detection of MTC in environmental samples, which provides a valuable new tool for the study of environmental contamination. We report for the first time the widespread occurrence of MTC DNA in the environment in areas with high bTB prevalence in wildlife. Seasonal rates of detection of MTC in environmental samples can be as high as 39.6 % (CI_{95} 27.6 –53.6 %) in the spring. This contamination is detected in all types of *a priori* defined risk sites, where wild and domestic ungulates assemble, such as feeding and watering places but also in wild boar root and vulture feeding stations. While spatial aggregation of wildlife at feeding or watering points was previously shown to be a risk factor for bTB (Vicente et al. 2006b, Gortázar et al, 2011a), inter-specific direct contact seems to be rare because of temporal segregation in their use (Kukielka et al., 2013, Cowie et al., 2015). Indirect transmission of bTB through environmental contamination with MTC provides a means to explain this risk effect.

These results also have implications for the design of control programs, as the identification of risk sites for the indirect transmission of MTC allows the targeted implementation of biosecurity measures that could reduce this type of transmission. As an example of simple biosecurity measures that could be used in such sites, Barasona and colleagues (2013) described a system of fences and gates that selectively allows either wild ungulates or cattle to access watering sites.

1.4. Spatial epidemiology of wildlife bovine tuberculosis

Wildlife diseases tend to be geographically structured because of the variability of the environmental and biological conditions that sustain both pathogen and hosts (Ward et al., 2009). We further explored the real-life model of the multi-host pathogen system of Iberian Peninsula to assess the spatial epidemiology of wildlife bTB in Portugal, using the wild boar as sentinel species (Chapter IV).

Initially we confirmed that elutes from absorbent paper soaked with blood are a suitable alternative for serum in bTB serological surveys, with an almost perfect agreement between serology data from serum and elutes. This provides a valuable new tool for bTB large-scale serological surveys in wild boar populations.

Our data allowed for the confirmation of bTB as an emerging disease in wildlife in Portugal, documenting a 47 % increase in prevalence in one hunting area from 2005-06 to 2009-14. Also we confirmed previous data suggesting a strong spatial structure of wildlife bTB in Iberian Peninsula, with 2 wildlife bTB clusters identified in south- and central-easternmost Portugal, in the periphery of the high-prevalence core area previously described in central-southwestern Iberian Peninsula (Santos et al., 2012).

Next we obtained 2 geographical models of bTB risk in wildlife, both generally agreeing with independent studies reporting MTC isolation from wild hosts. One notable exception is the *M. bovis* isolated in 2003-09 from a wild boar in Lousã mountain (Cunha et al., 2012) in a region where red deer were reintroduced in 1995-99, with some of the founders originating in bTB-infected areas included in cluster 1 (Salazar, 2009). No more *M. bovis* isolates were reported from this region since then, suggesting that after introduction and initial spillover to local wild boar, the infection faded out. This suggests that relatively dense red deer populations are needed to maintain bTB in wild ungulate populations in the absence of intensive management. Although the official area for bTB surveillance in large game species in Portugal encompasses some of the predicted high-risk areas that our models identify, it misses some regions at the periphery and includes some areas where risk is predicted to be minimal. The models we describe provide new tools for better allocating resources on wildlife bTB surveillance and public health protection.

Red deer density was significantly associated with bTB presence in wild boar at both geographical scales, while surprisingly wild boar density was not. This strongly highlights the critical role of red deer in bTB maintenance in wild ungulate populations in Portugal. Also the historical refuges of wild boar were identified as risk factors at both geographical scales. This agrees with bTB being a slow epidemic, as the distribution of wild boar more than 50 years ago is still reflected in the present distribution of the disease.

While at larger scale the intensity of management is significantly associated with bTB presence in wild boar populations, it is not so at a smaller scale. This could be a scale effect as at larger scales bTB tends to be present where the management intensity is high, but on a particular bTB cluster the disease spreads to those hunting areas in the vicinity of the cluster regardless of the intensity of management of each particular hunting area. This stresses that it is critical to prevent

further geographical spread of bTB in wild ungulate populations and the creation of new disease clusters, particularly by the translocation of infected ungulates.

2. Future perspectives

The results obtained in the scope of the present thesis allow gaining insight into several aspects of bTB epidemiology, previously poorly understood, but they also raise a number of questions needing further study.

Regarding MTC excretion from naturally-infected wild ungulates, deeper knowledge is required on routes not so thoroughly or not at all studied in the present thesis. Although we detected urinary excretion in 3/4 samples analyzed, it is puzzling that kidney lesions are seldom detected in these species (Martín-Hernando et al., 2007). Thorough surveys for bTB lesions in the kidneys coupled with detection of MTC urinary excretion in wild boar and red deer would provide valuable information on this subject. Also, although mammary lesions have been seldom reported in wild ungulates, they have been described in wild boar (Martín-Hernando et al., 2007) but excretion of viable mycobacteria in the milk has not been assessed.

Selective culling of super-shedders individuals is suggested to maximize the control of bTB transmission among wildlife populations, minimizing the ethical and socio-economical constraints of such actions (Cross et al., 2009). Nevertheless this selective culling requires the previous identification of super-shedder correlates, such as age, gender, physical condition or others that could be used to select that subset of the infected population in the field (Carter et al., 2009). Further work is needed to characterize the MTC super-shedder individuals both in wild boar and red deer.

Vaccine candidates reported in the literature have in common that they do not protect from infection but usually limit the pathology and severity of disease (Buddle et al., 2013). Consequently, their main advantage in controlling bTB in free-ranging populations would be to limit excretion from infected animals. As such, quantification of excretion in vaccination and experimental infections would be of utmost importance to gain insight into the beneficial effects of vaccination.

Indirect routes mediated by the environmental contamination with viable MTC seem to be important for inter-specific transmission of bTB. In this thesis we show that MTC DNA can be detected in feeding and watering sites, with seasonally high positivity rates. Furthermore, we show that MTC DNA is also present in other types of environmental samples, such as soil from wild boar roots, soil from vulture feeding stations and vulture feces. More surveys will allow a better characterization of the environmental contamination with MTC, namely identifying other types of high-risk locations of disease transmission by indirect routes.

Results obtained in this thesis also highlight that other MTC species, most probably *M. microti*, also contribute to the contamination of the environment with MTC. Further work is needed to identify which MTC species occur in the environment. *M. microti* infection has not been reported in the Iberian Peninsula, so surveys for this pathogen in its maintenance hosts, wild rodents, will provide interesting information on the epidemiology of MTC.

To promote bTB indirect transmission, mycobacteria need to be present in a viable state in the environment. Although capable of surviving for some time in the environment, MTC viability was not assessed in this thesis, requiring further study to prove its infectiousness. Also data on the precise mechanisms of infection from environmental sources would be valuable to fully understand the importance of such epidemiological mechanism of disease maintenance in free-ranging populations.

As the core area of wildlife bTB distribution in Iberian Peninsula is mostly located in Spain, spatial analysis at the Iberian scale shows promise to better highlight the spatial structure and obtain risk models at a larger scale. This would provide a valuable tool for disease management in both countries.

Incorporating all this information into mathematical spatially-explicit models of bTB transmission in free-ranging populations would allow assessing the effectiveness of interventions such as vaccination, widespread or selective culling and improved biosecurity of livestock farms in the control of the disease.

3. References

Adams AP et al. (2013) Comparison of PCR versus culture for detection of *Mycobacterium bovis* after experimental inoculation of various matrices held under environmental conditions for extended periods. *Appl Environ Microbiol*, 79(20):6501-6506.

Barasona JA et al. (2013) Effectiveness of cattle operated bump gates and exclusion fences in preventing ungulate multi-host sanitary interaction. *Prev Vet Med*, 111(1–2):42–50.

Beltrán-Beck B et al. (2012) Progress in oral vaccination against tuberculosis in its main wildlife reservoir in Iberia, the Eurasian wild boar. *Vet Med Int*, (2012):978501, doi: 10.1155/2012/978501

Boadella M et al. (2011) Effects of culling Eurasian wild boar on the prevalence of *Mycobacterium bovis* and Aujeszky's disease virus. *Prev Vet Med*, 107(3):214-221.

Bergquist R and Rinaldi L (2010) Health research based on geospatial tools: a timely approach in a changing environment. *J Helminthol*, 84:1–11.

Buddle BM et al. (2013) Overview of vaccination trials for control of tuberculosis in cattle, wildlife and humans. *Transbound Emerg Dis*, 60(1):136–146.

Caley P, Marion G, Hutchings MR (2009) Assessment of transmission rates and routes, and the implications for management. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 31–51.

Carter SP et al. (2009) Options for the control of disease 2: targeting hosts. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 121–146.

Corner LAL (2006) The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. *Vet Microbiol*, 112:303–312.

Courtenay O et al. (2006) Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis? *Biol Lett*, 2(3):460-462.

Cross PC, Drewe J, Patrek V, Pearce G, Samuel MD, Delahay RJ (2009) Wildlife population structure and parasite transmission: implications for disease management. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 9–29.

Cunha M et al. (2012) Implications and challenges of tuberculosis in wildlife ungulates in Portugal: a molecular epidemiology perspective. *Res Vet Sci*, 92(2): 225-235.

García-Jiménez WL et al. (2013a) Reducing Eurasian wild boar (*Sus scrofa*) population density as a measure for bovine tuberculosis control: effects in wild boar and a sympatric fallow deer (*Dama dama*) population in Central Spain. *Prev Vet Med*, 110(3):435-446.

García-Jiménez WL et al. (2013b) Immunopathology of granulomas produced by M*ycobacterium bovis* in naturally infected wild boar. *Vet Immunol Immunopathol*, 156:54–63.

Gortázar C et al. (2008) Bovine tuberculosis in Doñana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds. *PLoS ONE* 3(7):e2776. doi: 10.1371/journal.pone.0002776

Gortázar C et al. (2011a) Progress in the control of bovine tuberculosis in Spanish wildlife. *Vet Microbiol*, 151:170-178.

Gortázar C et al. (2011b) Fine-tuning the space, time, and host distribution of mycobacteria in wildlife. *BMC Microbiol*, 11:27. doi: www.biomedcentral.com/1471-2180/11/27

Gortázar C et al. (2012) The status of tuberculosis in European wild mammals. *Mamm Rev*, 42(3):193-206.

Gortázar C et al. (2014) Oral re-vaccination of Eurasian wild boar with *Mycobacterium bovis* BCG yields a strong protective response against challenge with a field strain. *BMC Vet Res*, 10(1):96.

Griffiths RI et al. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Appl Environ Microbiol*, 66(12):5488–5491.

Humblet MF et al. (2009) Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach. *Vet Res*, 40(5):1-24.

Kaneene JB et al. (2010) Tuberculosis in wild animals. *Int J Tuberc Lung Dis*, 14(12):1508-1512.

Kukielka E et al. (2013) Spatial and temporal interactions between livestock and wildlife in South Central Spain assessed by camera traps. *Prev Vet Med*, 112(3):213-221.

Lloyd-Smith J et al. (2005) Should we expect population thresholds for wildlife disease? *Trends Ecol Evol*, 20: 511–520.

Martín-Hernando MP et al. (2007) Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. *Tuberculosis*, 87:360-367.

Martín-Hernando MP et al. (2010) Distribution of lesions in red and fallow deer naturally infected with *Mycobacterium bovis*. *J Comp Pathol*, 142:43-50.

Mentaberre G et al. (2014) Long-term assessment of wild boar harvesting and cattle removal for bovine tuberculosis control in free ranging populations. *PloS ONE*, 9(2): e88824. doi: 10.1371/journal.pone.0088824

Norman SA (2008) Spatial epidemiology and GIS in marine mammal conservation medicine and disease research. *EcoHealth*, 5:257–267.

Nugent G (2011) Maintenance, spillover and spillback transmission of bovine tuberculosis in multi-host wildlife complexes: A New Zealand case study. *Vet Microbiol*, 151:34-42.

Ostfeld RS et al. (2005) Spatial epidemiology: an emerging (or re-emerging) discipline. *Trends Ecol Evol*, 20(6):328-336.

Palmer MV et al. (2015) Tuberculosis in wild and captive deer. *In* Mukundan H et al. (eds.) Tuberculosis, leprosy and mycobacterial diseases of man and animals: the many hosts of mycobacteria. CAB International, Oxfordshire, UK, pp. 334-364.

Phillips CJC et al. (2003) The transmission of *Mycobacterium bovis* infection to cattle. *Res Vet Sci*, 74:1–15.

Renwick AR et al. (2006) Bovine tuberculosis in southern African wildlife: a multi-species host–pathogen system. *Epid Infect*, 135(4):529-540.

Salazar D (2009) Distribuição e estatuto do veado e corço em Portugal. MSc Thesis, University of Aveiro, 101 pp.

Santos N et al. (2009) Epidemiology of *Mycobacterium bovis* infection in wild boar *Sus scrofa* from Portugal. *J Wildl Dis*, 45(4):1048-1061.

Santos N et al. (2012) Wildlife tuberculosis: a systematic review of the epidemiology in Iberian Peninsula. In Cunha MLRS, editor. Epidemiology Insights. Rijeka: InTech; 2012. http://www.intechopen.com/books/epidemiology-insights/wildlife-tuberculosis-a-systematic-review-of-the-epidemiology-in-the-iberian-peninsula

Tompkins D et al. (2001) Parasites and host population dynamics. *In* Hudson P et al. (eds.) The ecology of wildlife disease. Oxford University Press, Oxford, p 45-62.

Vicente J et al. (2006a) Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. *Vet Res*, 37:107–119.

Vicente J et al. (2006b) Risk factors associated with prevalence of tuberculosis-like lesions in wild boar and red deer in South Central Spain. *Vet Res*, 38:451–464.

Vicente J et al. (2013) Temporal trend of tuberculosis in wild ungulates from Mediterranean Spain. *Transbound Emerg Dis*, 60 (1):92–103.

Ward AI et al. (2009) Options for the control of disease 3: targeting the environment. *In* Delahay R et al. (eds.) Management of disease in wild mammals. Springer, Tokyo, p. 147–168.

Young JS et al. (2005) Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. *Appl Environ Microbiol*, 71:1946–1952.

Young JM et al. (2014) Limitations and recommendations for successful DNA extraction from forensic soil samples: a review. *Sci Just*, 54(3):238-244.